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Influence of Yeast Interactions on the Fermentation Process and Aroma Production in Synthetic Cocoa Pulp vs. Real Mucilage Media

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Abstract: Cocoa fermentation plays a key role in defining chocolate's flavor, with yeasts being central to this process. This study aimed to explore intraspecific genetic diversity of major indigenous yeasts (i.e., *Saccharomyces cerevisiae* and *Pichia kudriavzevii*), and their potential interaction in the cocoa pulp environment. Their metabolic intraspecific diversity was characterized in synthetic cocoa pulp medium. Then, *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, and other strains were introduced to each other to evaluate their potential negative interaction. Interesting strain associations were selected to further explore their interaction in synthetic cocoa pulp medium as well as real fresh cocoa pulp. From a fermentation campaign in Ivory Coast, a set of *Saccharomyces (S.) cerevisiae* and *Pichia (P.) kudriavzevii* strains were isolated from batches classified according to their chocolate quality (i.e., standard, intermediate, or premium chocolate). Less abundant species (i.e., *Torulaspora franciscae*, *Kluyveromyces marxianus*) were also isolated and tested for their potential negative interactions with *S. cerevisiae* and *P. kudriavzevii*. A set of strains were selected and cultured in single and in co-culture in a minimal cocoa pulp synthetic medium and in fresh cocoa pulp to highlight potential positive and/or negative interactions regarding fermentative aroma profile (i.e., higher alcohols, acetate esters, medium-chain fatty acids, and ethyl esters). The results highlighted the dominance of *S. cerevisiae* in fermentation kinetics and medium- to long-chain ester production, contrasted with *P. kudriavzevii*'s efficiency in short-chain ester synthesis. Intraspecific aroma profile variations can be pointed out. The co-cultures of *P. kudriavzevii* and *S. cerevisiae* strains isolated from the premium chocolate batch had a positive impact on the fermented pulp aroma profile. Negative interactions were observed with *Torulaspora franciscae*, which eliminated *P. kudriavzevii*'s aroma expression. Finally, the comparison of the data obtained for the minimal cocoa pulp synthetic medium compared to the cocoa pulp allowed us to draw conclusions about the use of synthetic media for studying cocoa fermentation. These findings emphasize the complex microbial interactions in cocoa fermentation that could shape future cocoa bean aroma.

Keywords: fermented cocoa pulp; higher alcohol; acetate and ethyl esters; *Saccharomyces cerevisiae*; *Pichia kudriavzevii*; co-culture; killer effect; *Theobroma cacao*

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1. Introduction

To achieve the desired sensory qualities of chocolate, raw cocoa beans must first go through processing steps, including fermentation, drying, and roasting. Among these, fermentation plays a key role in the transformation of cocoa beans from their initial bitter

and astringent state to a source of complex flavors and aromas [1–5]. The variability observed in cocoa quality can originate from other sources, including the initial composition of the cocoa bean and potentially, with regard to raw composition and varietal aromas, genetic and agronomic factors [1,6–10]. During this process, the microbial activity in the mucilaginous pulp surrounding the beans drives a cascade of biochemical reactions that result in the development of precursors essential for the characteristic flavors of chocolate [5,6,11–15].

Cocoa fermentation is a spontaneous process typically lasting 5–7 days, carried out directly on farms under spontaneous conditions. The cocoa pulp, also known as "mucilage", is a white, fleshy, and sugar-rich layer encasing the beans within the pod [16,17]. This pulp provides an ideal substrate for the growth of various microorganisms, including yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). The fermentation process involves the interaction of these three different types of microorganisms (i.e., yeasts, LAB, and AAB), contributing directly or indirectly to the final aroma quality of cocoa, with varying degrees of demonstrated effect [2–4,11–15,18–20]. The main fermentation metabolites and their roles in the structural and chemical evolution of beans have been studied in the literature, revealing a rather clear impact of ethanol's transformation to acetic acid during AAB fermentation on the release of specific cocoa aroma precursors into the cotyledon [21], and a temperature increase that kills the embryos [6].

Among all the microorganisms involved in cocoa bean fermentation, yeast populations are recognized as the first to develop. They dominate the initial stage of fermentation, during the first 24–48 h. The yeast population includes several genera, such as *Saccharomyces, Pichia, Hanseniaspora, Candida*, and *Debaryomyces* [2,12–15,19,20,22–26], which thrive initially due to the chemical composition of the cocoa pulp, particularly its acidity and high sugar content. The primary metabolic activity of yeasts is the alcoholic fermentation of pulp sugars, leading to the production of ethanol and carbon dioxide. In addition, their metabolic activity produces a range of secondary metabolites, including higher alcohols, carboxylic acids, esters, aldehydes, ketones, and volatile sulfur and nitrogen compounds. These secondary metabolites can have a significant impact on flavor [27,28]. Additional roles of yeast metabolism include accelerating carbohydrate consumption, exhibiting pectinolytic activity, and inhibiting the growth of spoilage microorganisms. These activities collectively contribute to optimizing the dynamics of the fermentation process [1,3,12,15]. Within the yeast species involved in cocoa fermentation, *Saccharomyces (S.) cerevisiae* and *Pichia (P.) kudriavzevii* are of particular interest due to their prevalence and their metabolic contributions [12,19,22–24,26,29,30]. Studies from Ghana and Ivory Coast [19,22,23] reported that these species constitute over 80% of yeast isolates, highlighting their dominance and adaptability to the challenging conditions of cocoa fermentation, such as high temperatures and ethanol concentrations and low pH. For instance, the strains *Pichia kudriavzevii* LPB06 and LPB07 demonstrated the ability to accelerate the cocoa bean fermentation process, characterized by efficient yeast growth, rapid sugar consumption, and ethanol and aroma production (including two families of esters and acetate compounds) compared to spontaneous fermentation [19]. In the literature, *P. kudriavzevii* has also been associated with the production of volatile compounds such as benzaldehyde (bitter/almond), 2,3-butanediol (creamy), 3-methylbutanal (chocolate), isoamyl alcohol (malty), and benzyl acetate (floral, jasmine). However, the yeast species most frequently linked to a broader range of volatile compounds is *S. cerevisiae* [12,13,31]. *S. cerevisiae* has been associated with key aroma compounds like acetaldehyde (green apple), 2-butanal (fruity, grassy), and 2-hexanal (fruity, grassy), as well as a variety of ethyl esters, including ethyl octanoate (fruity) and ethyl decanoate (fruity). In comparison, *Torulaspora delbrueckii* and *Kluyveromyces marxianus* are associated with the production of fewer volatile compounds. These species are primarily recognized for their high pectinolytic activity rather than their significant contributions to flavor production.

Yeasts have thus gained more interest due to their potentially more complex role in forming the final chocolate aroma profile. Their ability to produce various aromatic esters

and alcohol compounds, which are often found in premium chocolate and directly correlated with markers such as fruity and floral notes, has garnered significant attention from both research and industrial communities [4,12,13,15,18–20,32–35]. Due to these aspects, yeast starter cultures are an effective tool for conducting controlled cocoa fermentation. In fact, "starter industries" already exploit these specificities of yeast metabolism in various products such as wine and beer, and a considerable number of commercial yeast starters (mostly *S. cerevisiae*) with diverse aromatic expressions are already on the market for several applications. However, the main difference between cocoa and other fermentations using starters lies in the part of the fruit that is consumed, which is the seeds for cocoa, while in most cases, the fermented fruit (e.g., grape in wine) is the final product. Hence, an emerging area of research is the transfer of aroma compounds from the pulp to the cocoa beans during fermentation, and their preservation throughout the cocoa process [9,17,36,37]. These studies have shown that fermentative aroma compounds synthesized by yeasts in the pulp can be transferred to the beans, potentially affecting the final chocolate flavor. However, the question on the use of yeast starters remains poorly documented in the literature.

These findings have opened a route for precise investigations into yeast behavior within the cocoa pulp matrix. This enables a deeper understanding of yeast aromatic expression and allows for the exploration of intraspecific variability among the most promising species, as initiated by earlier studies [33,38]. Despite this potential, cocoa fermentation remains largely spontaneous, particularly in bulk-producing countries. The lack of controlled conditions also introduces variability in the microbial community and fermentation dynamics, resulting in inconsistencies in cocoa quality [4,11,18,19,34,35]. Additionally, while the role of individual yeast species in fermentation has been studied, the interactions between yeast strains and their collective impact on aroma production remain poorly understood. These interactions could significantly influence fermentation performance, either through synergistic effects that enhance aroma profiles or antagonistic behaviors, such as a "killer effect" that could suppress certain strains.

To address these gaps, this study focused on the interactions between *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, and other indigenous strains and their impact on aroma production. We adopted a progressive approach by selecting yeast strains isolated from fermentation batches classified as standard, intermediate, and premium quality by a cocoa manufacturer. A genetic study was conducted to identify intraspecific genetic variability within the groups of *S. cerevisiae* and *P. kudriavzevii* isolated from cocoa. Furthermore, non-*Saccharomyces cerevisiae* strains that exhibited potential antagonistic interactions with *S. cerevisiae* and *P. kudriavzevii* were included to evaluate their influence on aroma profiles. A synthetic minimal medium (MPS) based on synthetic must used in oenological studies early on [39] was adapted to better reflect the cocoa pulp composition. The use of a synthetic medium has largely demonstrated its added value for yeast metabolic studies under fermentation conditions, particularly for aroma production [40–43]. It has the great advantage of being fully modifiable in terms of carbon and nitrogen sources, and as well as other parameters, such as organic acids, pH, minerals, and vitamins. This medium was used to evaluate both the intra- and interspecific variability in yeast performance under controlled conditions. Additionally, fermentation experiments were conducted using freshly extracted cocoa pulp to assess the impact of natural biotic factors on yeast behavior and aroma outcomes.

This study aimed to provide a deeper understanding of how these interactions can influence the aromatic quality of cocoa beans used for chocolate processing. Hence, the purposes of this work were to (i) study the interactions between major indigenous yeasts involved in cocoa fermentation, with a focus on *S. cerevisiae* and *P. kudriavzevii* and their impact on fermentation and aroma production; (ii) assess the influence of non-*S. cerevisiae* strains with potential antagonistic effects on fermentation dynamics; and (iii) compare fermentation outcomes in synthetic and natural cocoa pulp media, highlighting the role of environmental factors in shaping yeast metabolic activity and aroma profile.

2. Materials and Methods

2.1. Yeast Isolates

A total of thirty-eight yeast strains of *Saccharomyces cerevisiae* (19) and *Pichia kudriavzevii* (19) were isolated from five different batches during the spontaneous fermentation of cocoa beans in Ivory Coast in the context of the Frenchoc Premium project [44]. These strains were identified by amplifying and sequencing ribosomal DNA (rDNA) internal transcribed spacer regions (ITS4 and ITS5 primers) and comparing sequences by homology with the NCBI database. Amplification and sequencing were performed by Eurofins (Eurofins Genomics France SAS, Nantes, France). The most predominant strains were *Saccharomyces cerevisiae* (28%) and *Pichia kudriavzevii* (25%). All strains were stored at −80 °C in a glycerol-based YPD containing bacterial peptone (2%), yeast extract (1%), glucose (2%), and glycerol (25%) until use. Table 1 provides an overview of the yeast strains isolated and used in this study, as well as the sensory qualities of the final chocolate from the fermentation boxes where they were isolated. The strains are thus grouped by their associated final chocolate sensory attributes: standard chocolate/cocoa, fruity flavor (yellow and dried fruits), and premium fruity/floral profile.

Table 1. Overview of yeast strains isolated from 5 different batches during the spontaneous fermentation of cocoa beans, along with their associated final chocolate sensory qualities. The yeast strains used in this study are bolded, and those studied in greater detail are marked with an asterisk (*).

Chocolate Main Sensorial Attributes	Yeast Species	Yeast Strains
Chocolate/cocoa Standard quality	*Saccharomyces cerevisiae*	**S4 *, S15, S16, S23 *, S25, S26, S27**
	Pichia kudriavzevii	**P1, P12 *, P13, P14, P18, P19, P21**
	Saturnispora diversa	N2
	Hanseniaspora meyeri	N3
	Torulaspora franciscae	**N5 *,** N6
	Candida boidinii	N7
	Hanseniaspora opuntiae	N8
	Candida pararugosa	N11
	Kluyveromyces marxianus	**N22 ***
Fruity, yellow fruits and dried fruits Intermediate quality	*Saccharomyces cerevisiae*	**S68 *, S69, S70, S77 *, S81**
	Pichia kudriavzevii	**P46, P65, P66, P76 *, P79 ***
	Schwanniomyces etchelsii	N61
	Candida tropicalis	N63
	Pichia kluyveri	N72
Fruity and floral high intensity Premium quality	*Saccharomyces cerevisiae*	**S34, S35 *, S38 *, S40, S56 *, S59, S60 ***
	Pichia kudriavzevii	**P28, P29, P30 *, P31, P32, P36 *, P37**
	Candida intermedia	N41
	Candida jarooni	N44
	Candida sarboxylosa	N45
	Wickerhamomyces pijperi	N48
	Candida aaseri	N49
	Torulaspora delbrueckii	N55
	Candida ethanolica	N58

2.2. GTG5 Fingerprinting

2.2.1. DNA Extraction

Yeast strains were grown on YPD medium at 30 °C for 48 h. A single colony was selected for DNA extraction, which involved lysis in a lithium acetate (LiOAc)-SDS solution, following the protocol of Lõoke et al. (2011) with modifications [45]. The yeast colony was suspended in 200 µL of lysis buffer (200 mM LiOAc, 1% SDS solution) and incubated at 70 °C for 5 min. To remove protein and lipid debris, 200 µL of phenol/chloroform was added. The mixture was centrifuged at 15,000× *g* for 3 min, and the supernatant was transferred to a tube containing 400 µL of 100% ethanol at −20 °C to precipitate the DNA. The sample was centrifuged again at 15,000× *g* for 3 min to remove the supernatant. The

DNA pellet was dried under vacuum in a SpeedVac (Eppendorf, Germany) at 60 °C for 5 min and resuspended in 50 µL of molecular biology-grade water (DNase and RNase-free). The extracted DNA was then subjected to rep-PCR and electrophoresis to compare the profiles of different strains.

2.2.2. Repetitive Element Sequence-Based PCR (rep-PCR) Fingerprinting

The rep-PCR fingerprinting protocol was adapted from Ramírez-Castrillón et al. (2014) with minor modifications [46]. The reaction was performed in a 25 µL volume containing 0.1 µg of purified DNA template (8.26–12.10 µL), 0.5 µL of 100 µM (GTG)5 primer, 12.5 µL of AmpliTaq Gold 360 PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), and qsf 25 µL of molecular-grade water. Amplification was conducted using a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: initial denaturation at 95 °C for 15 s, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 45 s, and extension at 72 °C for 90 s, with a final extension at 72 °C for 7 min. PCR products were separated by electrophoresis on 1.8% (*w*/*v*) agarose gels (Sigma Aldrich, St. Louis, MO, USA) prepared in 1X TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, pH 8.0). Electrophoresis was performed with an initial migration at 125 V for 4 min, followed by 75 V for 180 min. Gels were stained with ethidium bromide for visualization under UV light, and digital image capturing was carried out using the Geni2 gelDoc System. The fingerprints were analyzed using the software GeneTools (version 08-3d.3.SynGene). The 1 Kb plus or 1 Kb (Invitrogen) molecular weight marker was used to compare the sizes of the bands.

The dendrogram was developed in RStudio (v. 2024.09.0 "Cranberry Hibiscus" Release, © 2009–2024 RStudio, Inc., Boston, MA, USA) using the "dendextend" and "vegan" packages. A clustering analysis was performed using Bray–Curtis distance to construct the similarity matrix. The dendrogram was generated using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) aggregation method, allowing samples to be grouped based on their similarity.

2.3. Rich Medium for Screening of Fermentation Performance

To assess the fermentative capacities of *P. kudriavzevii* and *S. cerevisiae*, the fermentation was conducted using glucose-rich YPD (YPGlu) medium. Yeast pre-cultures were initially grown in 5 mL of 2% YPGlu (2% [*w*/*v*] glucose) medium and incubated under shaking conditions (200× *g* rpm) at 30 °C for 16 h. Subsequently, 100 µL of the pre-culture was used to inoculate 10 mL of 4% YPGlu medium, which was then incubated for another 16 h under the same conditions. This second pre-culture was used to inoculate 10% YPGlu medium at an initial optical density at 600 nm (OD600) of 0.5 for *S. cerevisiae* and 1.0 for *P. kudriavzevii*, corresponding to approximately 10^6 cells/mL. Fermentation was carried out in sterile 40 mL polypropylene pots containing 20 mL of 10% YPGlu medium and statically incubated at 30 °C for 7 days. CO_2 production was evaluated through weight loss measurement, which was performed daily to monitor the progress of fermentation [47].

Boxplots were used to visualize the distribution of data and developed in RStudio (v. 2024.09.0 "Cranberry Hibiscus" Release, © 2009–2024 RStudio, Inc., Boston, MA, USA) using the "ggplot2" package.

2.4. Interaction Between Yeast Strains: Killer Assay

To investigate the negative interactions between *S. cerevisiae* and *P. kudriavzevii* strains isolated from cocoa, as well as those among non-*S. cerevisiae* strains, a killer assay was performed according to Rowley et al. (2016) [48]. Briefly, the assay was conducted on YPD medium acidified with citrate–phosphate buffer (0.1 M citric acid solution and 0.2 M dibasic sodium phosphate solution) at pH 3.7 and pH 4.5, supplemented with 0.003% methylene blue as a viability indicator. pH 4.5 is commonly used in killer assays. pH 3.7 was used to approximate the average pH of cocoa pulp during fermentation. To perform the assay, approximately 10^5 cells/plate were spread on the surface of the agar plate, and the

confronted strains were streaked from isolated colonies. All *S. cerevisiae* and *P. kudriavzevii* were tested against each other. All *S. cerevisiae* and *P. kudriavzevii* were also tested against non-*S. cerevisiae* strains.

2.5. Aroma Production in the Minimal Cocoa Pulp Synthetic Medium and in the Cocoa Pulp

2.5.1. Minimal Cocoa Pulp Synthetic Medium (MPS) Preparation

One strain from each intraspecies group previously identified by GTG5 fingerprinting was evaluated for aroma production by using a minimal cocoa pulp synthetic (MPS) medium. Eight *S. cerevisiae* (S4, S23, S35, S38, S56, S60, S68, and S77); five *P. kudriavzevii* (P12, P30, P36, P76, and P79); and two non-*S. cerevisiae* strains (*Torulaspora (T.) franciscae* N5, and *Kluyveromyces (K.) marxianus* N22) were used in single and co-cultures. The MPS is a synthetic medium with a defined composition that is widely used in grape fermentation studies. In our study, the MPS was derived from a synthetic medium widely used in grapes fermentation studies and was prepared as described earlier by Bely et al. (1990) [39] with adaptations to match the pH and sugar content (pH 3.5, 8 g/L of citric acid, 60 g/L of glucose, and 60 g/L of fructose) of cocoa pulp. Other components were 3 g/L of malic acid; 1.14 g/L of potassium hydrogen phosphate; 0.44 g/L of magnesium sulfate heptahydrate; 1.23 g/L of calcium chloride dihydrate; vitamins (mg/L): myo-inositol (100), calcium pantothenate (1), thiamin hydrochloride (0.5), nicotinic acid (2), pyridoxine hydrochloride (2), biotin (0.125), PABA.K (para-aminobenzoate acid K; 0.2), riboflavin (0.2), and folic acid (0.2); and trace elements (µg/L): manganese(II) chloride tetrahydrate (200), zinc chloride (135), iron chloride (30), copper chloride (15), boric acid (5), cobalt nitrate hexahydrate (1), sodium molybdate dihydrate (25), and potassium iodate (10). Given the lack of literature on the variability in free amino acids' nitrogen content in cocoa pulp, we selected a total nitrogen concentration of 200 mg/L with an equal N content provided by all amino acids (9.5 mg N/L for each amino acid), aiming at maintaining a balanced nitrogen composition, as described by Rollero et al. (2018) [42]. All reagents used in the preparation of the medium composition, as well as those used throughout the experiments described, were sourced from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA).

Apart from its advantage regarding the ability to modulate all components, this medium contains no volatile compounds compared to classical microbiological media, minimizing contamination of the aroma fingerprints and ensuring a better understanding of the yeast specific metabolism (i.e., aroma production). The pre-cultures were prepared under the same conditions as described in Section 2.3. The second pre-culture was centrifuged at 2500× *g* for 3 min, and the pellet was washed with 25 mL of physiological water. Fermentation was carried out in 10 mL vials with 1 mL of MPS medium. The inoculum culture was added to obtain an initial concentration of 10^6 cells/mL of medium. Each vial was equipped with a Minisart® syringe filter (Sartorius, Göttingen, Germany) to release the CO_2 while minimizing evaporation. The vials were incubated at 30 °C for 5 days. The samples were stored at −80 °C before the aroma analysis. The trials were performed in triplicate.

2.5.2. Cocoa Pulp Medium Preparation

Cocoa pulp was manually separated from cocoa beans after opening the cocoa pods and stored at −80 °C. Frozen cocoa pulp containers were freeze-dried for 3 days to dehydrate the pulp completely. The cocoa pulp powder was then rehydrated with distilled water to obtain approximately 105 g of carbohydrates per kg of fresh cocoa pulp. The rehydrated cocoa pulp was pasteurized for 5 min at 105 °C before being used for fermentation experiments, as proposed by Meersman et al., (2016) [33].

To study the aroma production in cocoa pulp, three yeast strains, one of each species, *S. cerevisiae* S35, *P. kudriavzevii* P36, and *Torulaspora franciscae* N5, were chosen as inocula in single and co-cultures because of their fermentative and aromatic properties, or killer interactions. N5 was selected due to its potential killer effect on strain P36, which could negatively influence P36's capacity for aroma production. S35 and P36's interaction is of

relevance because these yeasts frequently predominate in the indigenous fermentation of cocoa. The inoculum pre-cultures were prepared by adding a single colony to 10 mL of 2% YPGlu (2% [*w*/*v*] glucose) medium in the 50 mL Erlenmeyer flasks and incubated in shaking conditions (180× *g* rpm) at 30 °C. After 24 h of growth, 500 µL of the pre-culture was used to inoculate 50 mL of 4% YPGlu in 500 mL Erlenmeyer flasks and incubated for 24 h in the same conditions. The second pre-culture was centrifuged at 2500× *g* for 3 min, and the pellet was washed with 20 mL of physiological water. This procedure was repeated twice. The pellet was eventually mixed with 20 mL of physiological water to create the yeast inoculum.

Fermentation was carried out in 10 mL vials equipped with a Minisart® syringe filter with 1 mL of cocoa pulp in triplicate. The inoculum was added to the vial at the initial optical density at 600 nm (OD600) between 0.6 and 0.8, roughly equivalent to ~10^6 cells/mL for single cultures and $5{\cdot}10^5$ cells/mL each for co-cultures (S35+P36 and N5+P36). The cocoa pulp vial was then vigorously mixed to ensure a homogenous distribution of the inoculum. The vials were statically incubated at 30 °C for 5 days. The vials were weighted every 24 h to estimate fermentation progress. Samples devoted to aroma analysis were stored at −20 °C. Yeasts were enumerated using a differential medium based on their carbon source assimilation profiles, determined using the API 20C AUX gallery (Biomérieux, Marcy-l'Étoile, France). Yeast Nitrogen Base with amino acids (Sigma-Aldrich, St. Louis, MO, USA) with 15 g/L agar was supplemented with either sucrose for *Torulaspora pretoriensis* and *Saccharomyces cerevisiae*, or N-acetyl glucosamine for *Pichia kudriavzevii*, to group yeasts species for the co-culture experiments.

2.6. MPS and Pulp Media Aroma Compound Analysis by DHS-GC/MS

2.6.1. Volatile Compound Extraction (DHS)

Volatile compounds were extracted using a GERSTEL dynamic headspace (DHS) coupled with a GERSTEL Robotic autosampler (GERSTEL, Mülheim an der Ruhr, Germany). A total of 1 mL of medium, already contained in a 10 mL glass vial with a screw cap and a hermetic seal, was first equilibrated at 50 °C for 10 min. The headspace was then purged for 16 min with 400 mL of nitrogen, at a flow rate of 25 mL/min, with agitation at 500× *g* rpm. Volatile compounds were trapped on a GERSTEL Tenax TA trap at 50 °C. To remove excess alcohol and water, the trap underwent a drying phase with an additional 600 mL of nitrogen at a flow rate of 100 mL/min and a purge flow at 50 °C.

2.6.2. Volatile Compound Separation and Extraction (GC/MS)

Desorption and analysis were performed on a GC 7890B/MS 5977 system (Agilent, Agilent Technologies, Santa Clara, CA, USA). Thermal desorption of the Tenax trap and injection were carried out using a GERSTEL automatic thermal desorption unit (TDU2). The Tenax trap was desorbed in splitless mode, starting at 30 °C, ramping this up to 300 °C at a rate of 120 °C/min, and holding for 5 min. The cold Tenax trap was initially cooled to 2 °C. Injection was performed in low-split mode by heating up the cold trap from 2 °C to 300 °C at a ramp rate of 12 °C/s.

Volatile compounds were separated on a HEAVY-WAX column (60 m × 250 µm i.d. × 0.25 µm, Agilent Technologies, Palo Alto, CA, USA). The oven temperature was programmed to increase from 40 °C (held for 5 min) to 140 °C at a rate of 2 °C/min, followed by an increase to 250 °C at a rate of 10 °C/min. Hydrogen was used as the carrier gas at a flow rate of 2.2 mL/min. The column effluent was ionized by electron impact at 70 eV. The source of ionization was heated at 230 °C. The molecules were separated based on their mass/charge (m/z) ratio, using a quadrupole analyzer maintained at 150 °C, sweeping an interval mass range of [40 to 300] Da in SCAN mode.

Peak identification was performed with Agilent MassHunter software (qualitative analysis v. 10.0 and quantitative analysis v. 12.0), and the spectra were compared to those of the National Institute of Standards and Technology database (NIST 20 spectral library, v. 2.4, Gaithersburg, MD, USA). The expression of results is based on the integration of surfaces.

2.6.3. Statistical Volatile Compound Data Analysis

MPS and pulp fermentation analysis was performed in independent triplicate. The statistical analyses were developed in RStudio (v. 2024.09.0 "Cranberry Hibiscus" Release, © 2009–2024 RStudio, Inc., Boston, MA, USA). All variables were centered by mean and scaled, with z-score = $(X - \mu)/\sigma$, where X is the value measurement, μ is the mean value, and σ is the standard deviation. The principal component analysis, radar chart, and their visualization were made with the "stat", "Factoextra", "ggplot2", and "fmsb" packages. The heatmap, boxplot, and visualization were made using the "ComplexHeatmap" package. The hierarchical clustering was performed using Ward's method.

3. Results

3.1. Intraspecific Diversity of S. cerevisiae and P. kudriavzevii Isolates and Fermentation Capacity in Glucose-Rich YPD Medium

This study included 38 yeast strains, with *Saccharomyces cerevisiae* and *Pichia kudriavzevii* accounting for 28% and 25% of the total isolates, respectively. These strains were isolated from different batches of cocoa beans, categorized as standard-quality, intermediate-quality, and premium-quality chocolate batches (Table 1). Using GTG5 fingerprinting, intraspecific diversity was analyzed, revealing seven distinct groups for *S. cerevisiae* and five for *P. kudriavzevii* (Figure 1a,b), with much more variability for *S. cerevisiae* strains (0.4 maximum distance) than for *P. kudriavzevii* strains (0.12 maximum distance). Among the *S. cerevisiae* strains (Figure 1a), the largest group (S15, S16, S23, S25, S26, S27, S40, S60, S69, S70, S81) was represented in each batch (standard, intermediate, and premium). *S. cerevisiae* S4 was the only strain that was only isolated from the standard batches and absent from the intermediate and premium batches. Other groups were composed of strains exclusively found in the intermediate (S77) and premium batches (S34, S35, and S38). The remaining strains were found in either the intermediate (S68) or the premium (S56, S59) batch. For *P. kudriavzevii* (Figure 1b), two groups (P79, P21, P19, P13, P18 and P12, P65) were found in both the standard and intermediate batches; however, the group composed of P79, P21, P19, P13, and P18 was mostly representative of isolates from the standard-quality chocolate. Another group (P66, P37, P36, P32, P1, P14) was found in the standard, intermediate, and premium batches, but it was mostly representative of isolates from the premium-quality chocolate (P37, P36, P32). The group consisting of *P. kudriavzevii* P46 and P76 was only present in the intermediate batch. The last group (P31, P30, P28, P29) represented strains exclusively found in the premium batch.

The screening of the fermentation performance of *S. cerevisiae* and *P. kudriavzevii* strains is presented in Figure 1c. The results showed that these strains presented a greater maximum CO_2 release rate than all *P. kudriavzevii* strains, demonstrating a higher level of fermentation efficiency for *S. cerevisiae*. *S. cerevisiae* S35, S34, S56, S59, and S60 exhibited the highest fermentation performance out of 19 *S. cerevisiae* strains due to their maximum CO_2 rate of about 2.0 $g/L.h^{-1}$ (see Figure S1 for details). The *P. kudriavzevii* P32, P30, and P79 strains showed the highest fermentation performance out of 19 *P. kudriavzevii* strains because of their maximum CO_2 rate of roughly 0.5 $g/L.h^{-1}$.

One strain from each intraspecific group was chosen to examine their aromatic potential: eight *S. cerevisiae* (S4, S23, S35, S38, S56, S60, S68, and S77) and five *P. kudriavzevii* (P12, P30, P36, P76, and P79). Two *S. cerevisiae* strains were selected in the larger genetic group but isolated from the premium (S60) and standard quality (S23) batches.

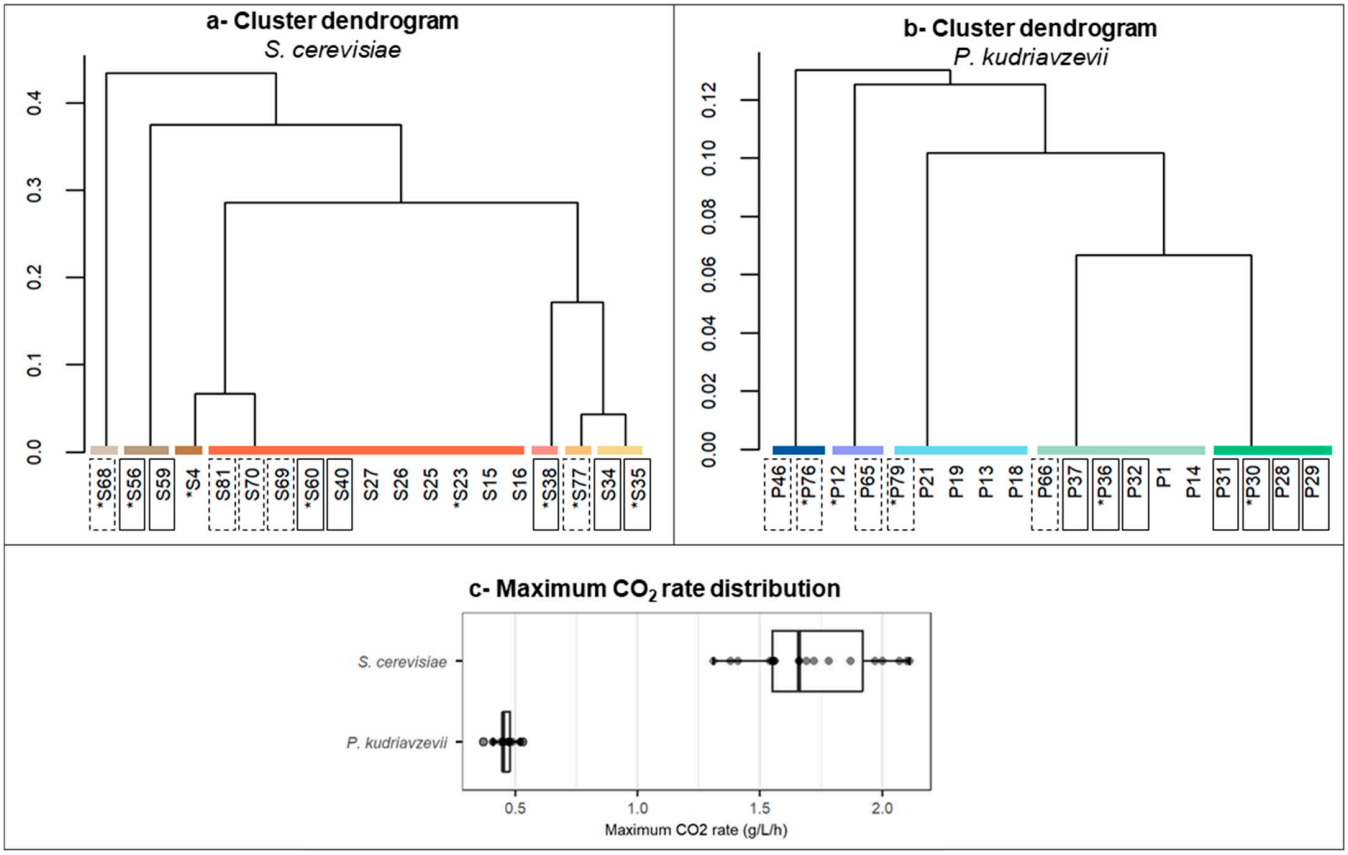

Figure 1. Cluster analysis showing the intraspecies relationship of the isolates of the 19 strains of *S. cerevisiae* (**a**) and the 19 strains of *P. kudriavzevii* (**b**) by (GTG)5-rep-PCR fingerprinting. The strains selected for the next steps of analysis are indicated by an *. Solid-line frames (—) indicate the premium-quality chocolate batch, dotted-line frames (- -) indicate the intermediate-quality batch, and unframed isolates indicate the standard-quality batch. (**c**) Maximum CO_2 release rate ($g/L.h^{-1}$) distribution between the 19 strains of *S. cerevisiae* and the 19 strains of *P. kudriavzevii* in glucose-rich YPD medium in static conditions at 30 °C for 7 days.

3.2. Negative Interactions Between Yeast Strains: Killer Assay

In order to identify potential negative interactions among all the strains (Table 1), all non-*S. cerevisiae* strains were tested for their potential negative interactions with every representative strain of the *S. cerevisiae* and *P. kudriavzevii* groups. All *S. cerevisiae* and *P. kudriavzevii* strains were also introduced to one another. No killer interaction or inhibition was observed between *S. cerevisiae* and *P. kudriavzevii* strains. Regarding non-*S. cerevisiae* strains, weak negative interactions reflecting potential killer effects were observed for *K. marxianus* (N22) against *S. cerevisiae* strains S56, S59, S60, and S77 at pH 3.7 and 4.5 (Figure 2a,b). Regarding *P. kudriavzevii*, only *T. franciscae* (N5) exhibited a very strong inhibitory effect at pH 4.5 (Figure 2c,d) against all strains.

Thus, two strains of non-*S. cerevisiae* (*Torulaspora franciscae* N5, *Kluyveromyces marxianus* N22) were selected for their potential influence on aroma production in interactions with *S. cerevisiae* and *P. kudriavzevii* strains. Interestingly, N5 was only isolated in the standard-quality batch.

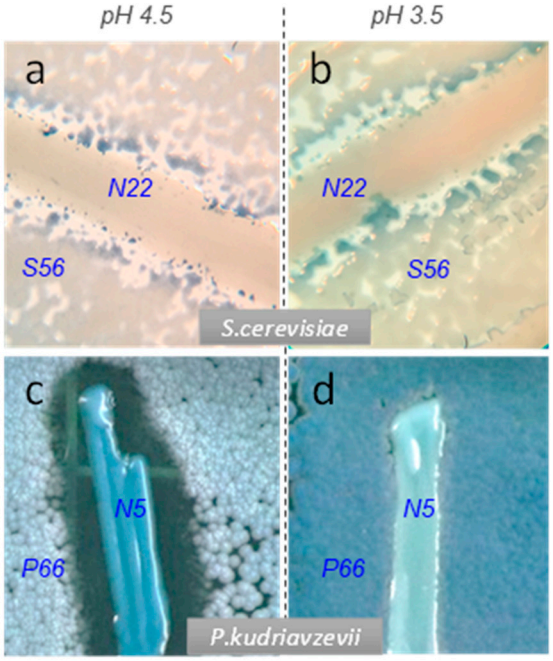

Figure 2. Illustration of the inhibitory effect observed during killer assay between (**a**,**b**) *S. cerevisiae* S56 and *K. marxianus* N22, and (**c**,**d**) *P.kudriavzevii* P66 and *T. franciscea* N5. YPD medium acidified with citrate phosphate buffer at pH 3.7 (**b**,**d**) and pH 4.5 (**a**,**c**) with methylene blue. A similar behaviour (**c**,**d**) was observed between N5 and all *P. kudriavzevii* strains. For *S. cerevisiae*, only strains S56, S59, S60, and S77 showed negative interactions with N22. No other interactions were observed between all the strains.

3.3. Aroma Profile in the Minimal Cocoa Pulp Synthetic Medium (MPS)

3.3.1. Comparative Analysis of Aroma Profile of *S. cerevisiae* and *P. kudriavzevii*

A total of 21 volatile compounds were identified and selected by DHS-GC/MS in the minimal cocoa pulp synthetic medium (MPS) after five days of fermentation (static 30 °C), including esters and higher alcohols. Using standardized values (z-scores), aroma production by *S. cerevisiae* strains S4, S23, S35, S38, S56, S60, S68, and S77 and *P. kudriavzevii* strains P12, P30, P36, P76, and P79 can be directly compared with their combined average, allowing for a direct comparison of each species' contributions to aroma production. The resulting data, shown in Figure 3, are presented using both heatmap and boxplot formats.

The heatmap and boxplot analysis clearly discriminated *P. kudriavzevii* strains, which had a much higher relative contribution to acetate esters and short-chain esters (C4–C7) (e.g., ethyl acetate, isoamyl acetate, isobutyl acetate, ethyl propionate) from *S. cerevisiae*, which was better characterized by its relative contribution to medium- to long-chain ester biosynthesis (C8–C18) (e.g., ethyl decanoate, ethyl octanoate, phenylethyl acetate) (Figures 3 and S2). Most *P. kudriavzevii* strains (e.g., P76, P36, P12, and P79) showed high z-scores for short-chain esters, with the exception of P30, which exhibited intermediate values. *P. kudriavzevii* showed greater variability for the latter, as indicated by a broader interquartile range, whereas *S. cerevisiae* presented a narrower range, implying more consistent but lower production levels for these compounds. On the other hand, medium- to long-chain esters had z-scores above 0 for *S. cerevisiae* and below 0 for *P. kudriavzevii*. This suggests that *S. cerevisiae* was more efficient in the generation of these medium- to long-chain esters, but also exhibited greater intraspecific variability, as observed through the interquartile range. The strains S35 and S38, which are associated with the premium chocolate batch, had elevated z-scores, while S60 exhibited much lower z-score values. There are some compounds, such as ethyl hexadecanoate, for which the median values were similar between the two yeast species. However, the larger boxplot size for *S. cerevisiae* clearly indicates more variability in production compared to *P. kudriavzevii*. For the higher alcohols

isobutanol, isoamyl alcohol, and phenylethyl alcohol, production varied depending on the yeast species. Isobutanol was produced in significantly higher amounts by *P. kudriavzevii*, but without major intraspecific variability, as indicated by the small size of the whiskers and boxes. In contrast, 2-phenylethanol was produced in greater quantities by *S. cerevisiae*, though with relatively more intraspecific variability compared to *P. kudriavzevii*. Isoamyl alcohol was produced in similar amounts by both groups. Finally, certain species stood out as outliers, with z-scores that were significantly higher (or weaker) than the overall average: P76, S38, and S35 vs. P30 and S60. Non-*S. cerevisiae* N22 and N5 strains generally did not exhibit strong or distinctive aromatic profiles, although some, like N22 (*K. marxianus*), produced unique high relative quantities of phenylethyl acetate.

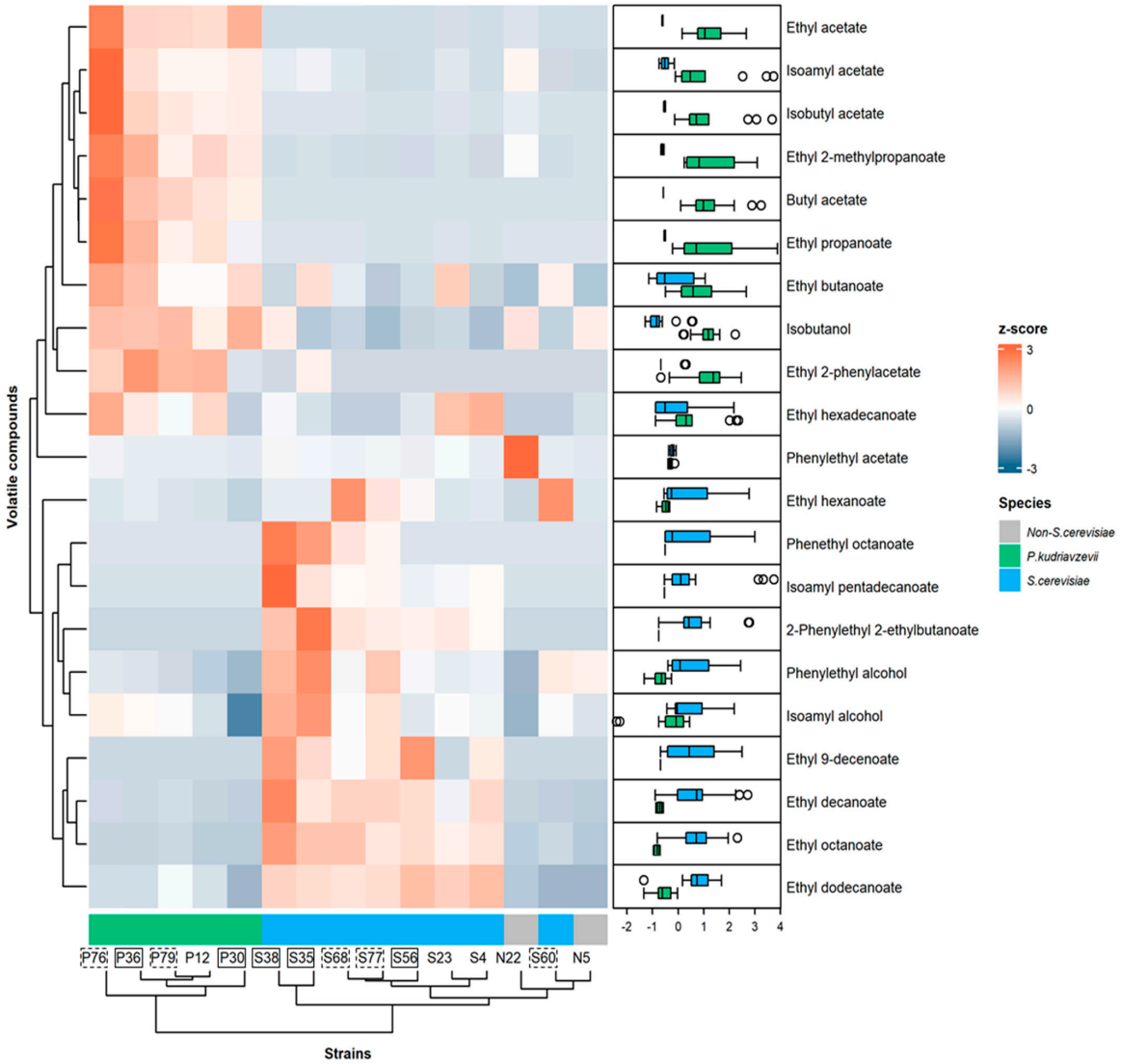

Figure 3. Heatmap (z-scores, with blue indicating lower values and red indicating higher values) and boxplot summary (z-scores) of the aroma profile of minimal cocoa pulp synthetic medium (MPS) fermented by single *S. cerevisiae* (S), *P. kudriavzevii* (P), and non-*S. cerevisiae* (N) strains after 5 days of fermentation (30 °C). Clustering was performed using the Euclidean distance metric for both yeast strains (columns) and aromatic compounds (row), grouping similar profiles together. Abbreviations: S77, single culture of *S. cerevisiae* S77; P76, single culture of *P. kudriavzevii* P76; N22, single culture of *K. marxianus* N22. Solid-line frames (—) indicate the premium-quality chocolate batch, dotted-line frames (- -) indicate the intermediate-quality batch, and unframed match indicate the standard-quality batch.

3.3.2. Interaction Between Selected *S. cerevisiae*, *P. kudriavzevii*, and Non-*S. cerevisiae* Strains in Minimal Cocoa Pulp Synthetic Medium (MPS): P36, S35, S4, S77, N22, and N5

The strains P36, S35, S4, S77, N22, and N5 were selected for co-culturing because of their contrasting performance of fermentation and their aroma production profiles. P36 was the top *P. kudriavzevii* strain producer from the premium batch and was notably effective in generating short-chain esters. S35 (*S. cerevisiae*), which was also from the premium batch, showed a high fermentation performance and strong aroma potential of medium- and long-chain esters. S4 (*S. cerevisiae*) was selected as a control with an intermediate fermentation performance and low aroma potential of both short- and medium- to long-chain esters and was isolated from a standard batch. N5 and N22 (non-*S. cerevisiae*) were selected for their demonstrative inhibitory impact. S77 (*S. cerevisiae)* was chosen for its sensitive profile to N22's potential killer effect. Aroma compounds were analyzed by distinguishing the aroma characteristic profile of *P. kudriavzevii* strains (Figure 4a–d), which primarily produce short-chain esters and acetate esters, and those of *S. cerevisiae* strains (Figure 4e-h), which are more closely associated with medium- and long-chain esters (Table 2). Figure 4 illustrates these profiles in radar charts, displaying standardized z-scores for individual strains (P36, S35, S4, S77, N22, and N5) as well as their co-cultures (S77+N22, S4+P36, S35+P36, and N5+P36) in the MPS medium.

Co-culturing often resulted in noticeable shifts in the aroma profile. When co-cultured with S4 or N5, *P. kudriavzevii* P36's profile was drastically affected (Figure 4b,d,f,h). In the P36+S4 co-culture, the overall aroma profile shifted towards S4's profile (Figure 4b), indicating a loss of P36's characteristic short-chain ester production. Even the *S. cerevisiae* aroma profile (Figure 4f) was negatively affected by the interaction for compounds such as ethyl hexanoate, ethyl octanoate, and isoamyl alcohol. Similarly, co-culturing P36 with the inhibitory strain N5 led to a sharp reduction in short-chain ester levels, suggesting that competitive interactions with N5 severely degrade P36's aroma production. Neither N22 nor S77 produced significant levels of short-chain esters (Figure 4a,e). However, S77 had a more robust profile of medium- to long-chain esters compared to N22. When co-cultured, the S77+N22 profile showed an intermediate aroma composition between both strains, indicating that the interaction did not drastically alter the profile but balanced the ester contributions of each strain. When *S. cerevisiae* S35 was co-cultured with *P. kudriavzevii* P36 (Figure 4c,g), the co-culture profile aligned more closely with S35's profile than P36's profile. Interestingly, for specific long-chain esters (e.g., ethyl dodecanoate, ethyl decanoate, and isoamyl pentadecanoate), the z-scores for the S35+P36 co-culture were even higher than those for S35 alone, indicating a positive effect that enhanced *S. cerevisiae*'s profile aroma output for these aromas, but a decrease for other volatiles (i.e., phenethyl octanoate, ethyl 9-decenoate, 2-phenylethyl 2-ethylbutanoate).

Table 2. Distinct aroma profiles of *Pichia kudriavzevii* and *Saccharomyces cerevisiae* strains based on volatile compound analysis in MPS medium after 5 days of fermentation (30 °C). Aroma descriptions are from The Good Scents database: https://www.thegoodscentscompany.com/ (accessed on 15 November 2024).

***P. kudriavzevii* Characteristic Aroma Profile**		***S. cerevisiae* Characteristic Aroma Profile**	
Ethyl acetate	Ethereal, fruity, sweet	Isoamyl alcohol	Fusel, fruity
Isoamyl acetate	Sweet, fruity, banana-like	Ethyl hexanoate	Sweet, pineapple
Isobutyl acetate	Sweet, fruity, tropical	Ethyl octanoate	Fruity, pineapple
Ethyl 2-methylpropanoate	Fruity	Ethyl decanoate	Waxy, fruity
Butyl acetate	Sweet, tropical	Ethyl 9-decenoate	Fruity
Ethyl propanoate	Ethereal, fruity, sweet	Ethyl dodecanoate	Waxy, soapy, floral
Ethyl butanoate	Fruity, sweet, tutti frutti	Isoamyl pentadecanoate	Fatty, creamy
Isobutanol	Fusel whiskey	Phenylethyl alcohol	Floral, sweet, rose
Ethyl 2-phenylacetate	Sweet, rosy, honey	2-Phenylethyl 2-ethylbutanoate	/
Ethyl hexadecanoate	Waxy, creamy	Phenethyl octanoate	Fruity, creamy
		Phenylethyl acetate	Honey, floral, cocoa

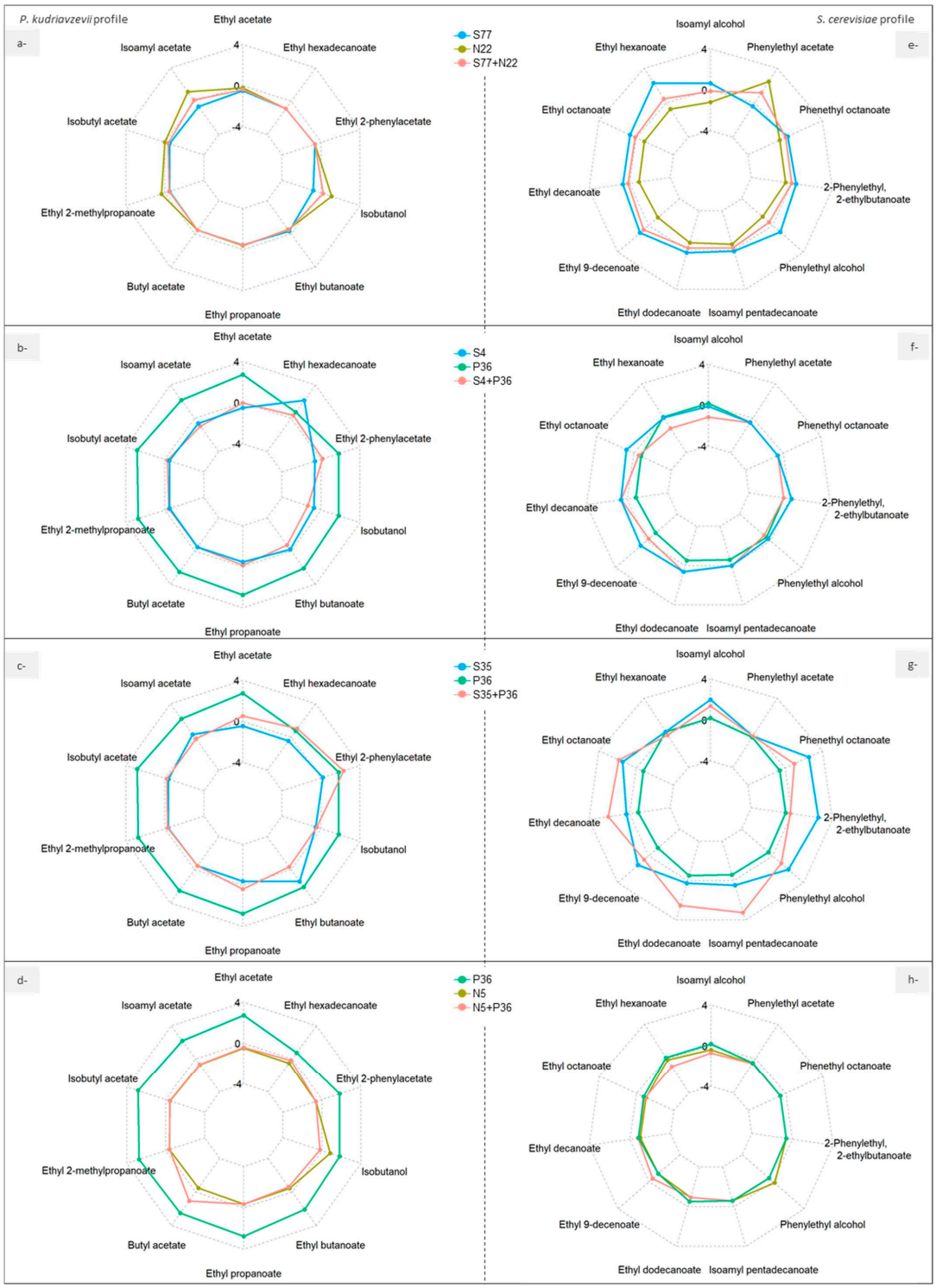

Figure 4. Radar chart representation of standardized aroma z-scores for individual yeast strains and their respective co-cultures in the MPS medium after 5 days of fermentation (30 °C). Each line represents the aroma profile of a particular yeast strain or co-culture (P36, S4, S35, S77, N5, N22, S77+N22, N5+P36, S35+P36, and P36+S4) based on the distinct aroma profiles of (**a**–**d**) *P. kudriavzevii* and (**e**–**h**) *S. cerevisiae.* The strains selected illustrate a range of fermentation and aroma production capabilities: P36, the top *P. kudriavzevii* strain aroma producer from the premium batch; S35, highly active *S. cerevisiae* strain with strong aroma potential from the premium batch; S4, the control with intermediate fermentation performance and low aroma potential; N5 and N22, non-*S. cerevisiae* strains with inhibitory impact; and S77, a *S. cerevisiae* strain known for its interaction with N22.

3.4. Kinetic Parameters and Aroma Production in Cocoa Pulp of Selected Strains' (S35, P36, N5) Co-Cultures (S35+P36 and N5+P36)

3.4.1. Kinetic Parameters in Cocoa Pulp Medium

Based on the previous results, we selected the most interesting strains and co-culture interactions for further investigations (i.e., S35, P36, N5, S35+P36, N5+P36). These strains were evaluated on a real cocoa pulp medium, extracted from freshly opened pods, in order to compare the observed fermentation behavior and aroma profile on a synthetic medium to a similar experiment on real cocoa mucilage.

Yeast growth and fermentation kinetics were monitored for single cultures (S35, P36, and N5) and co-cultures (S35+P36, N5+P36). Fermentation kinetics and yeast viability during cocoa pulp fermentation are presented in Figure 5. In single-culture fermentations, the maximum population of *S. cerevisiae* S35, *P. kudriavzevii* P36, and *T. franciscae* N5 was reached within 24 h, with cell counts ranging between $1.1 \cdot 10^8$ and $1.6 \cdot 10^8$ CFU/mL pulp (Figure 5a). S35, P36, and N5 achieved similar maximum average populations. In co-culture S35+P36, the maximum population ($1.3 \cdot 10^8$ CFU/mL pulp) was also reached at 24 h. However, the population dynamics were driven mostly by *S. cerevisiae*, which consistently represented the majority (79%, 77%, 85%, and 86% from 24 to 96 h of fermentation, Figure 5b). During this time, the population of *P. kudriavzevii* decreased from $2.8 \cdot 10^7$ CFU/mL to $1.6 \cdot 10^7$ CFU/mL pulp, particularly in the later stages of fermentation, suggesting competitive inhibition by *S. cerevisiae*. In the N5+P36 co-culture (Figure 5c), the total population increased progressively from $4.4 \cdot 10^7$ CFU/mL to $6.9 \cdot 10^7$ CFU/mL over the fermentation time. During the first 72 h, N5 dominated (67–73% of the population). Interestingly, in the final stage of fermentation, P36 overtook N5, reaching 57% of the total population compared to 43% for N5 (populations of $4.0 \cdot 10^7$ CFU/mL vs. $2.9 \cdot 10^7$ CFU/mL, respectively).

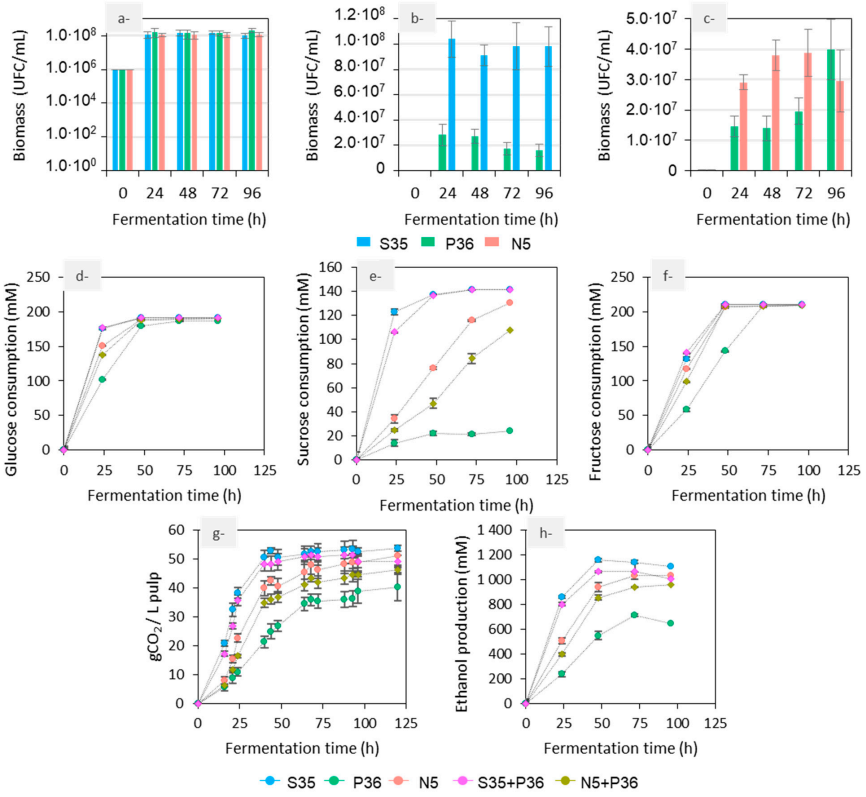

Figure 5. Fermentation kinetics and yeast viability during cocoa pulp fermentation (30 °C): (**a**) total yeast biomass for *S. cerevisiae* (S35), *P. kudriavzevii* (P36), and *T. franciscae* (N5) in single cultures; (**b**) yeast biomass distribution in co-cultures (S35+P36); (**c**) yeast biomass distribution in co-cultures (N5+P36); (**d**–**f**) sugar consumption (sucrose, glucose, and fructose); (**g**) CO_2 release; (**h**) ethanol production throughout fermentation.

The *S. cerevisiae* S35 strain completed its fermentation within 24 h, consuming 100% of available sucrose and glucose in this time frame. Fructose was also entirely assimilated, but within 48 h (Figure 5d,e), *T. franciscae* N5 had a similar behavior regarding glucose and fructose consumption, but its sucrose assimilation was much slower than that of S35, even if it almost fully degraded at 96 h. The *P. kudriavzevii* P36 strain exhibited slower glucose and fructose consumption, likely due to its preference for interfacial development and aerobic metabolism, which are more favorable at the beginning of fermentation for its growth. Sucrose utilization by P36 was minimal, as expected for this species, and fructose was fully consumed by P36, albeit more slowly than the other strains (72 h). In co-cultures, sugar and ethanol consumption and production kinetics reflected the behavior of the dominant population considered (S35 or N5). For instance, the sugar consumption and ethanol production profiles in the S35+P36 co-culture look similar to those of the S35 single culture, whereas N5+P36 displayed patterns similar to the N5 single culture. This further highlights the dominance of *S. cerevisiae* S35 and *T. franciscae* N5 over *P. kudriavzevii* P36 during co-culture fermentations of cocoa pulp.

3.4.2. Kinetic Aroma Production in Cocoa Pulp Medium

Figure 6 presents a principal component analysis (PCA) based on the volatile compounds (i.e., esters and higher alcohols) produced by *S. cerevisiae* (S35), *P. kudriavzevii* (P36), and non-*S. cerevisiae* (N5) strains in both single and co-cultures (N5+P36; S35+P36) at different times of fermentation. The first two dimensions explained 66% of the total variability, indicating that most of the relevant aroma production differences could be captured by these two axes.

Dimension 1 (42% of variance) primarily distinguished strains S35 and S35+P36 from the other strains (P36, N5, and N5+P36) after 48 h of fermentation. For S35 and the S35+P36 co-culture, dimension 1 also separated samples in the early fermentation stage (24 h) from those at later stages (72–120 h), highlighting the evolution of aroma profiles over time. The co-culture of S35+P36 retained much of the S35 aroma profile, with slight shifts indicating some mutual influence in aroma production.

Dimension 2 (24% of variance) differentiated strains based on their production of acetate and short-chain esters (C4–C7) vs. medium- to-long-chain esters (C8–C18), consistent with earlier observations in the minimal cocoa pulp synthetic medium (MPS). Compounds such as ethyl acetate, isobutyl acetate, ethyl propanoate, and ethyl 2-methylpropanoate had high cos^2 values (>0.70), indicating a strong association with this dimension (Figure 6a). This axis contrasted P36 against the other strains, including S35, N5, and their co-cultures, emphasizing P36's unique contribution to short-chain ester production. N5 displayed an aroma profile characterized by high production of higher alcohols, such as phenylethyl alcohol (Figure S3), and was clustered separately from S35 and P36. Over time, N5's aroma profile in the co-culture with P36 appeared to be influenced by P36, particularly at longer fermentation times (more than 24 h), even though the combined profile was diminished compared to when P36 was cultured alone, suggesting some competitive inhibition by N5.

The PCA results further highlighted the impact of fermentation time on overall aroma profiles. For *S. cerevisiae* S35 strains, a slight modification in aroma profile was observed after 48 h, with profiles from 72 to 120 h remaining similar, indicating a plateau in aroma production. These trends align well with the fermentation kinetic parameters of S35 (CO_2 release, Figure 5g, and aroma compound production, see Figure S3 for details). For the *P. kudriavzevii* P36 strain alone, aroma production began to decline after 96 h of fermentation. The non-*S. cerevisiae* N5 strain exhibited a more stable profile throughout fermentation, with minimal changes from 0 to 120 h, reflecting a more limited contribution to dynamic aroma shifts compared to S35 and P36. The S35+P36 co-culture showed a similar trend to S35, with aroma production declining after 72 h. When N5 was co-cultured with P36, a decline in aroma profile was noted after 96 h, matching the behavior of P36 alone. These results suggest that both yeasts' interactions and fermentation time significantly impacted

aroma profiles, with some strains demonstrating a stabilizing or competitive influence when in a co-culture.

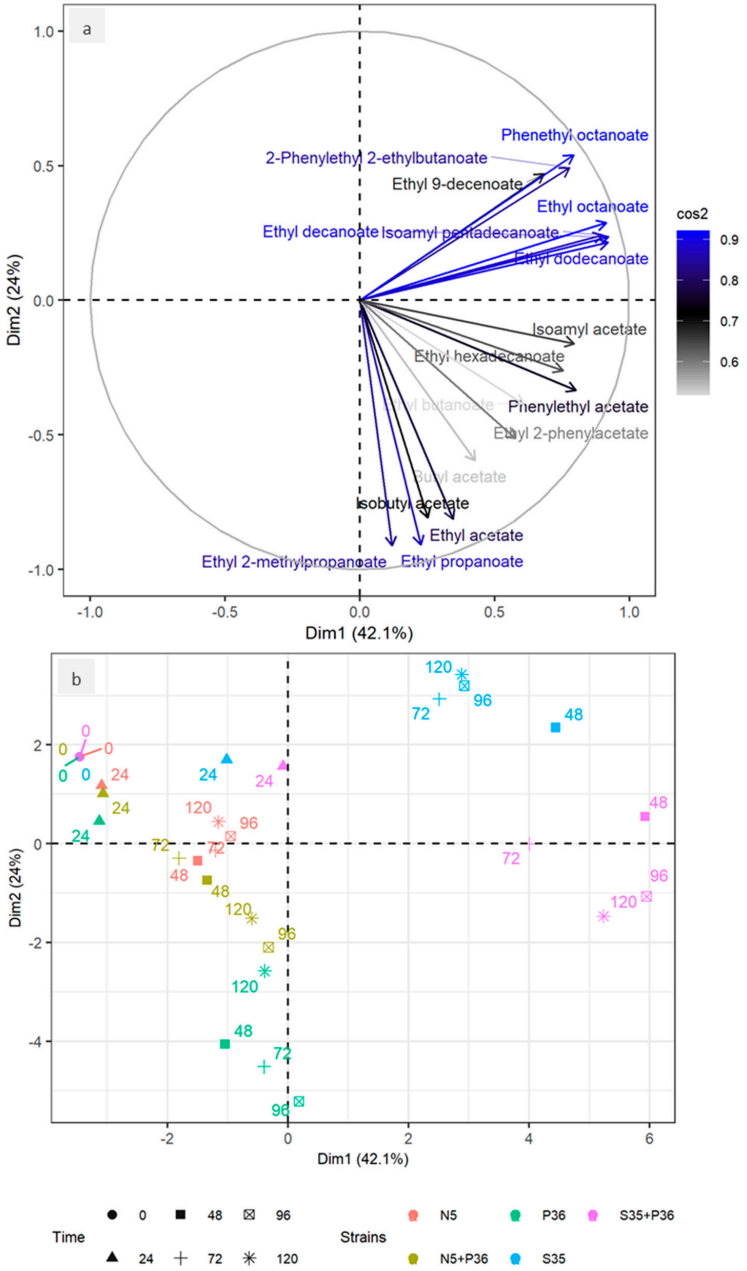

Figure 6. Principal component analysis (PCA) based on the main volatile compounds (esters and higher alcohols) produced by single and co-cultures of *S. cerevisiae* S35, *P. kudriavzevii* P36, and non-*S. cerevisiae* N5 in the cocoa pulp medium at different times of fermentation, from 0 h to 120 h. (**a**) Loading plot and (**b**) score plot showing the influence of the strains (colors) and the fermentation time in hours (shapes). Single and co-culture strains and species details are explained in the legend of the figure. Abbreviations: S35, single culture of *S. cerevisiae* S35; P36, single culture of *P. kudriavzevii* P36; N5, single culture of *T. franciscae* N5; S35+P36, co-culture of S35 and P36; N5+P36, co-culture of N5 and P36.

4. Discussion

The variability in the quality of fermented cocoa beans originates from several sources, including genetic complexity, agronomic and climatic factors, and interactions with indigenous and diverse microbiota [1,3,6–9,12,15,22–24,49]. Cocoa beans result from the

hybridization of two parental genotypes and therefore are highly exposed to genetic crossover and variability [7,8], in contrast with the pulp, which is a maternal tissue carrying a stable genetic background derived from a single parent. Combining cocoa and microbial genetic diversities is thus challenging, as illustrated by the varying findings, and sometimes contradictory, as seen in the impact of starters on the aromatic quality of cocoa [4,11,14,18–20,32–35]. As the pulp is the main substrate involved in the metabolic activity of microorganisms, de novo synthesis of a fermentative aroma is likely derived from pulp precursors. Therefore, separating the pulp from the cotyledon to study this microorganism's metabolism seems to be a good strategy.

To investigate yeast's metabolic expression and potential interactions in a controlled environment, two strategies were adopted: natural cocoa pulp and a synthetic medium. For the cocoa pulp fermentation experiments, a single homogeneous batch was used across all experiments. A "synthetic grape juice medium" [39] was adapted to cocoa pulp's composition (i.e., MPS medium) in terms of sugar concentration (120 g/L glucose et fructose), citric acid (12 g/L), and pH (3.5). The nitrogen content of our synthetic medium was adjusted at 200 mg N/L of Yeast-Assimilable Nitrogen, with all amino acids providing an equal nitrogen amount. This ensured a controlled exploration of yeast strain phenotypes, overcoming potential variability due to strains' nitrogen preferences. Indeed, amino acid uptake by yeasts can vary significantly, and the relative concentration of each amino acid might drastically impact aroma production, especially in co-culture conditions [40–42,50–52].

After studying the inter- and intraspecific diversity of aroma production among a set of *S.cerevisiae* and *P.kudriavzevii* strains isolated from cocoa fermentation batches in Ivory Coast (Figure 3, Table 2), we investigated the potential role of metabolic interactions in the expression of their respective aromatic profiles in the MPS medium (Figure 4). Two non-*S. cerevisiae* strains, *Kluyveromyces marxianus* (N22) and *Torulaspora franciscae* (N5), were included due to their potential inhibitory or killer interactions with *S. cerevisiae* and *P. kudriavzevii*, respectively (Figure 2). Moreover, N5 and N22 generally produced fewer aroma compounds, often lacking the diversity of esters produced by the *S. cerevisiae* and *P. kudriavzevii* (Figure 3). Then, the co-cultures that had the highest impact on the aroma profile in the MPS (i.e., S35+P36 and N5+P36) were selected and further studied in real cocoa pulp to explore their metabolisms alone and in interaction (Figures 5 and 6).

The aromatic profiles of the MPS and cocoa pulp fermentations media were clustered consistently for each yeast combination (Figure S4), demonstrating that the MPS medium was a suitable approach for profiling yeast aroma metabolism under cocoa-like conditions. Correlation analyses (Figure S5) of aroma production kinetics for single and co-culture of S35, P36, N5 in both media revealed strong correlation (>0.7) for some compounds (e.g., 2-phenylethyl 2-ethyl butanoate, ethyl 2-phenylacetate, ethyl acetate, ethyl octanoate, ethyl dodecanoate, isoamyl pentadecanoate, isobutyl acetate, phenethyl octanoate), while others showed significant differences (e.g., butyl acetate, ethyl butanoate, isoamyl acetate, isobutanol, phenylethyl acetate, phenylethyl alcohol). The difference in amino acid content between the MPS and cocoa pulp media likely contributed to the variation in the aroma production kinetics, especially for aromas derived from the Ehrlich pathways, which can be influenced by the concentration of specific amino acid precursors [40–42,50–52]. Other compounds, such as sterols and fatty acids (absent from the MPS medium), could be at play, along with physical parameters such as temperature [27,43,53]. The fine modulation of nutrients in synthetic media, such as nitrogen content, based on the analysis of cocoa pulp composition diversity, will open new avenues of research in designing cocoa synthetic media. The wide use of reproductible media by the research community involved in cocoa fermentation would enable better comparability of findings and foster collaborative advancements.

S. cerevisiae and *P. kudriavzevii* strains were accurately separated based on their aroma profiles in the MPS medium, highlighting their relative contributions to some typical fermentative aromas (Figure 3 and Figure S2), as already observed by several authors [27,28,54–56].

S. cerevisiae was a larger contributor to medium- and long-chain fatty acid esters, while *P. kudriavzevii* exhibited better potential for acetate ester and short-chain fatty acid ester production. *S. cerevisiae* strains also tended to produce higher levels of higher alcohols, such as phenylethyl alcohol (contributing to a floral and sweet note). This allowed us to define a distinct *P. kudriavzevii* aroma profile and a distinct *S. cerevisiae* aroma profile (Table 2), providing insight into the impact of co-cultures on their aromatic expression (Figure 4).

The fermentation abilities of *P. kudriavzevii* strains were clearly lower in O_2-limited conditions (Vmax ~0.5 g CO_2/L.h^{-1}) when compared with *S. cerevisiae* (Vmax > 2 g CO_2/L.h^{-1} for the fittest strains) (Figures 1c and S1). This emphasizes that *P. kudriavzevii's* capacity to settle in cocoa fermentation likely stems from attributes such as its resistance to harsh conditions, rather than its high fermentation kinetic performance. Its resistance to elevated temperatures and low pH, as reported in the literature [28,54,55], could enable this species to contribute to the generation of aroma during the fermentation process. Experiments in cocoa pulp with the P36 strain confirmed *P. kudriavzevii*'s inability to degrade sucrose (Figure 5d), as already described [28,54,55]. Sucrose, which depends on pod maturity and is in equilibrium with glucose and fructose content [57,58], might have a non-negligible influence on *P. kudriavzevii*'s metabolic activity and dominance. In our study, incomplete fermentation in pulp was observed, with a plateau at 72 h corresponding to the full consumption of glucose and fructose, and a stop in ethanol production (Figure 5). However, while most *P. kudriavzevii*-specific aromatic markers (Figure S3) reached their plateau in the first 48 to 72 h (associated with glucose and fructose depletion) and then decreased (e.g., ethyl acetate, ethyl propanoate, isobutyl acetate, ethyl butanoate), other compounds increased until 120 h (e.g., phenyl ethyl acetate, phenylethyl ethanol, Figure S3). Of note, even if sucrose was not consumed by P36, whereas it was a substantial part of carbon sources in cocoa pulp (roughly a third), *P. kudriavzevii*-specific aromatic marker contributions were still higher than *S. cerevisiae*-specific ones (e.g., ethyl acetate, ethyl propanoate, isobutyl acetate, butyl acetate, ethyl 2-phenyl acetate, phenylethyl acetate, Figure S3). The increase in some aroma compounds observed after the completion of sugar assimilation probably reflects metabolic activity based on other substrates such as amino acids, or metabolic turnover from compounds that have already been accumulated.

In the co-culture in the MPS medium, *S. cerevisiae* S35 and S4 largely overwhelmed P36 regarding its specific aromatic expression, reflecting their higher fermentative capacities (Figure 4b,c). However, while the S4 and P36 co-culture only resulted in a global depletion of all *P. kudriavzevii*-specific aroma compound profiles, and a similar or lower expression of the *S. cerevisiae* profile (Figure 4f), the S35+P36 co-culture displayed a different behavior. Indeed, the *P. kudriavzevii* aroma profile of S35+P36 was also mostly depleted, as observed for S4, but the *S. cerevisiae* profile was clearly modified (Figure 4g), with some compounds decreasing (e.g., phenethyl octanoate, 2phenylethyl 2-ethylbutanoate, phenylethyl alcohol) and others clearly increasing (e.g., ethyl dodecanoate, isoamyl pentadecanoate, and ethyl decanoate).

When comparing these results to those obtained for the S35+P36 co-culture in real cocoa pulp medium, the dominance of S35 was evident throughout the fermentation process. Its population consistently exceeded 75% of the total population, and the co-culture fermentation kinetics regarding sugar consumption and ethanol production/CO_2 release was very similar to those of S35 alone (Figure 5). Despite its relatively low proportion, P36 influenced S35 *S. cerevisiae*'s aroma profile until the end of fermentation through an increase in some acetate esters' production, as illustrated by the score plot (Figure 6b) and the kinetics of aroma formation (Figure S3). Some aroma compounds reached a maximum associated with sugar exhaustion in most situations and could even decrease after alcoholic fermentation arrest (e.g., ethyl butanoate, isoamyl acetate), while others increased even after sugar assimilation, mostly for medium- to long-chain ethyl esters (Figure S3). This phenomenon allowed the S35+P36 co-culture to modify the final aromatic profile and clearly outperform *S. cerevisiae* and *P. kudriavzevii* alone in the production of fine aromas such as ethyl butanoate, ethyl decanoate, and ethyl 2-phenyl acetate.

Though *S. cerevisiae* and *P. kudriavzevii* have been frequently studied together [59,60], recent findings raise interesting questions regarding their synergistic positive impact on final chocolate quality, influenced by the metabolic activity of other microorganisms, such as LAB. Recently, Deng and co-workers (2020) [60] demonstrated the cooperative behavior of *S. cerevisiae* and *P. kudriavzevii* when exposed to lactic acid stress under fermentation conditions, which leads to a modification of gene expression and an increase in the fermentation performance of *S. cerevisiae*. Moreover, the central role of the nitrogen metabolism of yeasts in developing mutualism with LAB has already been described and suggested as a positive attribute of non-*S. cerevisiae* species for LAB that is responsible for malolactic fermentation in wine [61,62]. In cocoa fermentation, co-cultures of *S. cerevisiae* and *P. kudriavzevii* have also been shown to exhibit a synergistic effect on polyphenol enzymatic degradation and inhibit decarboxylase activities involved in forming biogenic amines (putrescin and cadaverin) [52,63], revealing that, again, a complex interaction network between these two strains and the dynamic environment (e.g., sugar composition, lactic and acetic acid, temperature) likely occurs.

The *S. cerevisiae* S77 aromatic profile was almost unaffected by the addition of *K. marxianus* (N22). A decrease in ethyl hexanoate and phenylethyl alcohol was observed, but this was counterbalanced by an increase in phenylethyl acetate and, to a lesser extent, isoamyl acetate and isobutanol (Figure 4). The increase in acetate esters during mixed fermentation inoculated with *K. marxianus* were recently observed in cocoa fermentation but also in a cheese ecosystem [64]. Thus, its presence in cocoa fermentation along with *S. cerevisiae* representatives might be beneficial, even if it is not the best provider of aromatic complexity for the strain considered in our study. The exploration of intraspecific diversity among this group might shed light on the potential of this species to improve ester production in cocoa-like conditions.

In contrast, in the N5+P36 co-culture, we observed a clear extinction of the P36 aroma profile, whether considering the MPS or cocoa pulp media, showing strong negative interactions. The N5+P36 co-culture showed a significant reduction in short-chain esters, which are typically produced by *P. kudriavzevii* (Figure 4d). The fermentation kinetics in the cocoa pulp medium were very similar to the N5 kinetics, even if an increase in P36 was observed at the later stages (96–120 h) (Figure 5). This reversal may reflect a change in substrate consumption after the completion of alcoholic fermentation occurring at ~72 h. Thus, N5 might easily outperform P36 and affect nutrient availability, introducing competition for important nutrient resources. Even though the inhibition observed in the agar plate killer assay did not occur at the pH of the pulp (the inhibitory activity was observed at pH 4.5), the expression of the N5 inhibition phenotype under cocoa fermentation conditions remains to be investigated. Other killer strains were already isolated from cocoa fermentation batches, such as *Candida nitrativorans,* with an inhibition of *P. kudriavzevii* strains in vitro [24]. Their co-inoculation in fermentation heaps did not impede *P. kudriavzevii* colonization, but no attention was paid to the aromatic expressions and strain interactions specifically [19]. The mechanisms behind the significant impact of N5 on P36's aroma metabolic expression were not addressed specifically here, but could involve metabolic repression, inhibitory compounds, or competition for nutrients. These findings highlight the potential negative influence of some strains on the metabolic expression of aromatic species such as *P. kudriavzevii*, which has already been described to have a positive influence on cocoa quality.

Understanding how the dynamic and variable microbiome affects cocoa pulp composition evolution during fermentation should be explored in more details to uncover the role of microbiota in potential synergistic effects between *S. cerevisiae* and *P. kudriavzevii*. Their cooperative effects should be explored in relation to environmental parameters at different fermentation stages (e.g., pH, acetic acid, ethanol, nitrogen sources). Applying sequential inoculation strategies could also be an interesting strategy to explore how various ratios of *S. cerevisiae* and *P. kudriavzevii* affect their metabolic expression phenotype. Again, the development of a synthetic medium will offer opportunities to finely modulate conditions

to assess metabolic potential and regulatory mechanisms for further implementation in real process parameters. Thus, the accurate characterization of metabolic preferences, especially regarding nitrogen sources, but also lipid sources, as already proposed by authors regarding wine fermentation [42,43,65], could shed light on predicting potential positive or negative interactions between strains of various species as regards yeast diversity. A wide exploration of pulp composition variability will greatly help in the understanding of the influence of matrix parameters on yeasts as well as LAB and AAB metabolism, and of course how it affects their metabolic interactions and expression.

5. Conclusions

This study provides a comprehensive analysis of the interactions between *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, and other indigenous yeast strains (*Torulaspora franciscae*, *Kluyveromyces marxianus*) during cocoa fermentation, focusing on their contributions to some fermentative aromas. By isolating and characterizing yeast strains from various fermentation batches, we demonstrated significant intra- and interspecific variability in aroma profiles. Our results underscore the complexity of microbial interactions in cocoa fermentation and their critical role in determining the final aroma quality of cocoa beans. Additionally, yeast's aroma metabolism specificities, both alone and in interactions, were well reflected in the synthetic minimal cocoa pulp medium when compared to real cocoa pulp. However, the use of standardized nitrogen content does not fully reflect the cocoa pulp composition. Further research on the diversity of cocoa pulp composition is needed to design synthetic media that accurately mimic component variability, such as free nitrogen content, which impacts aroma metabolic expression.

Moreover, the interaction between yeast and bacteria (LAB, AAB), and how it affects the final aromatic profile, also remains to be elucidated. Taking into consideration abiotic parameters potentially influenced by cocoa fermentation practices, such as temperature or oxygen content, will also help in elaborating different lab-scale scenarios to expand on the results.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10120662/s1, Figure S1: kinetic parameter of maximum CO_2 production rate (VmaxCO_2 in g/L.h^{-1}) in YPD rich in glucose medium in static conditions at 30 °C for 7 days of *S. cerevisiae* (a) and *P. kludriavzevii* (b) strains. (c) Typical fermentation profile of *S. cerevisiae* (S35) and *P. kudriavzevii* (P36, P76) in minimal pulp medium and effect of initial oxygen concentration on the rate of CO_2 release during 120 h of fermentation; Figure S2: principal component analysis (PCA) based on the main volatile compounds (esters and higher alcohols) produced in the minimal cocoa pulp synthetic medium (MPS) after 5 days of fermentation by single and co-cultures of *S. cerevisiae*, *P. kudriavzevii*, and non-*S. cerevisiae*; Figure S3: evolution of a selection of volatile compound levels (expressed as area) over the fermentation time in the pulp medium for *P. kudriavzevii* (P36), *S. cerevisiae* (S35), and non-*S. cerevisiae* (N5) in both single and co-cultures; Figure S4: heatmap comparison of z-scores of the aroma profile of cocoa pulp medium (pulp) vs. MPS medium (synthetic) fermented by single *S. cerevisiae* (S35), *P. kudriavzevii* (P36), and non-*S. cerevisiae* (N5) strains and co-cultured (S35+P36; N5+P36) at 5 days of fermentation; Figure S5: correlation graphs comparing the area mean values of volatile compounds in the synthetic medium against the pulp medium for single S35 kinetics, P36 kinetics, N5 (120 h) and co-cultures (120 h). Each plot shows the correlation coefficient, indicating the weight of the relationship between the two media for each compound. $R^2 > 0.7$ show a strong positive correlation between the synthetic and pulp media, indicating that their production is similar in both environments.

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