

Review

Yeasts as Producers of Flavor Precursors during Cocoa Bean Fermentation and Their Relevance as Starter Cultures: A Review

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Abstract: During the fermentation of cocoa beans, the yeasts produce volatile organic compounds (VOCs). Through reactions associated with amino acid metabolism, yeasts generate important aroma precursors as acetate esters and fatty acid ethyl esters are essential in developing fruity flavors and aromas in the final product (usually chocolate). In addition, some yeasts may have pectinolytic and antifungal activity, which is desirable in the post-harvest process of cocoa. The main yeast species in cocoa fermentation are *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, and *Hanseniaspora opuntiae*. These produce higher alcohols and acetyl-CoA to make acetate-esters, compounds that produce floral and fruity notes. However, there are still controversies in scientific reports because some mention that there are no significant differences in the sensory characteristics of the final product. Others mention that the fermentation of cocoa by yeast has a significant influence on improving the sensory attributes of the final product. However, using yeasts as starter cultures for cocoa bean fermentation is recommended to homogenize sensory attributes such as notes and flavors in chocolate.

Keywords: yeast; cocoa bean; fermentation; flavor precursors; starter cultures

1. Introduction

Yeasts have been involved in the fermentation of products for thousands of years in the production of wine, bread, sake, chocolate, and other fermented foods. Some of them have been produced for more than 10,000 years [1,2]. It has been shown that increasing yeast diversity in food fermentations increases the sensory complexity and diversity of aroma compounds found in the final products. Aromatic compounds play many key roles for yeasts, as survival strategies, defense mechanisms, and cellular communication. Humans have used their production to enhance the flavor and sensory attributes of fermented foods [2]. One of the most relevant fermentations in which yeasts are involved is the fermentation of the cocoa bean [3].

Cocoa is the fruit of the *Theobroma cacao* L., which is a perennial tree native to the South American tropical region. The dry fermented cocoa bean is the raw material for chocolate production and is composed of two cotyledons and an embryo, enveloped in a sweet and white mucilaginous pulp [4,5]. Based on Statista data [6], the global cocoa production scenario for the period 2021–2022 is 4,955,000 metric tons. Leading cocoa-producing countries for the same period are the Ivory Coast, Ghana, Indonesia, Nigeria,

Cameroon, and Brazil [7]. There are three main cocoa varieties: *Forastero* (bulk or ordinary accounts for 95% of world cocoa production), *Criollo*, and *Trinitario* [4,5].

When the cocoa beans are removed from the pod, the pulp is degraded by a spontaneous fermentation conducted by yeast, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). Several authors have found that good quality in fermented dry cocoa beans was correlated with good on-farm agricultural and post-harvest practices, bean selection, placenta removal prior to fermentation, and blending of the cocoa bean pulp mass [8–10]. In addition, well-performed fermentation is a prerequisite for producing high-quality chocolate [11]. Several studies have shown that yeasts produce various aromatic precursor compounds, such as alcohols and esters, which positively contribute to the aromatic profile of the chocolate [11–16]. The ethanol produced by yeast strains during cocoa fermentation is used as a carbon source for acetic acid bacteria and triggers diverse biochemical reactions inside cocoa bean that drives the aroma and flavor precursors in cocoa cotyledons [17]. Cocoa aroma is the result of various reactions that occur during the processing of beans and is related to the cocoa genotype, as well as environmental conditions, microbial diversity during fermentation, and subsequent processing steps, mainly drying and roasting [18]. Furthermore, cocoa flavor comprises of non-volatile compounds (polyphenols, carbohydrates, alkaloids, and proteins) and volatile compounds (esters, phenols, alcohols, aldehydes, ketones, furanones, and pyrazines) [19]. This review aims to describe the contribution of yeasts as producers of flavor precursors and their utilization as starter cultures for cocoa bean fermentation to impact the sensory attributes of chocolate made with yeasts starter cultures.

2. Cocoa Bean Postharvest Stages

To obtain chocolate that fulfills the standards required by the market, producing flavor precursors such as free amino acids, peptides, and reducing sugars in the cocoa beans is necessary. These precursors are formed from specific cocoa bean components such as proteins and carbohydrates by reactions catalyzed by enzymes occurring inside the cocoa bean. For this, cocoa undergoes several post-harvest stages. The most important is the fermentation process [20]. The initial step is harvesting mature cocoa pods and opening of the pods either manually or sometimes using a simple tool such as a long-bladed knife, also known as “machete” in some countries (Figure 1A). After the pods have been opened, the most critical step begins, the fermentation. During fermentation, various autochthonous microorganisms deriving from the environment (tools, worker’s hands), pod surfaces, and fermentation containers utilize the pulp for growth [4,21]. This process is conducted by yeast, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) [22], and is performed in heaps, baskets, trays, or wooden boxes [21]. However, wooden boxes are most commonly used for the fermentation of cocoa beans worldwide [4]. The fermentation lasts about three to six days and can reach a temperature between 40 °C to 50 °C (Figure 1B) [23]. After three to six days, the fermentation is over, and the next step is drying. This operation is carried out until the beans reach a moisture content of $\leq 7\%$; the most used method in small cocoa farms is sun drying, and it is considered the best method to obtain the full flavor development [20].

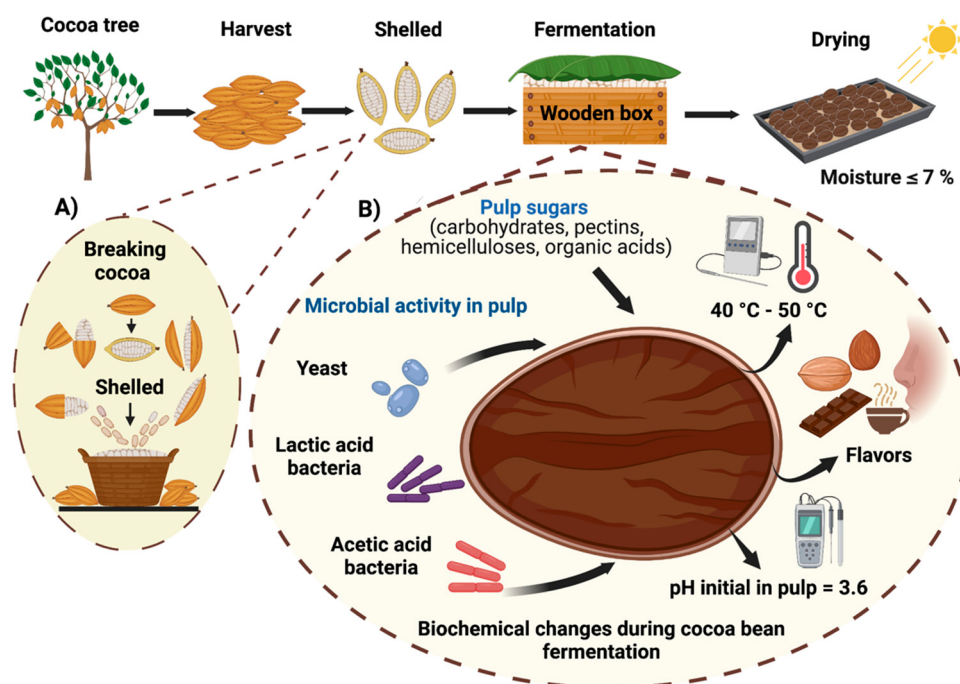


Figure 1. Cocoa bean post-harvest stages. (A) After harvest, the cocoa pod is broken and collected and placed in a wooden box for fermentation. (B) The cocoa beans are fermented in wooden boxes where autochthonous microorganisms (yeast, LAB, and AAB) produce various metabolites, increase the temperature, decrease the pH of the cotyledon, and trigger the formation of flavor precursors.

3. Cocoa Bean Fermentation and Biochemical Transformations on Cocoa Bean during Fermentation

Fermentation is essential for developing flavor and reaching the final acidity of cacao beans [5,23–25]. Four different methods are used to ferment cocoa beans: platform, box, heap, and basket fermentation. The selection of the fermenting method is related to the region of cocoa production [25]. The cocoa bean fermentation process involves the degradation of the mucilaginous pulp surrounding the beans by complex microbial interactions, mainly by yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (BAA). Other microorganisms such as spore-forming bacteria (*Bacillus* and *Paenibacillus*), enterobacteria, and filamentous fungi are also present; however, their role remains unclear [4,5,23,25–27]. Cocoa pulp is a rich medium for microbial growth. It consists of water (80–90%), sugars, mainly glucose, sucrose, and fructose (0–15%), citric acid (1–3%), and pectin (1–1.5%). Proteins (0.5–0.7%), amino acids, vitamins (mainly vitamin C), and minerals (K^+ , Na^+ , Ca^{+2} , Mg^{+2} , Fe^{+2} , and Zn^{+2}) are also present [4,18,23,25,26]. There are two important phases in the fermentation of cocoa beans, anaerobic, and aerobic. The anaerobic phase lasts about 48–72 h after cocoa pod breaking and involves yeast and LAB strains [28]. The aerobic phase occurs after approximately 48 h of fermentation with the growth of AAB strains [4,23].

3.1. Anaerobic Phase of Cocoa Bean Fermentation

3.1.1. Yeast

The first stage of cocoa bean fermentation involves the growth of yeasts mostly belonging to the genera *Hanseniaspora*, *Saccharomyces*, *Candida*, *Kluyveromyces*, *Kazachstania*, *Meyerozyma*, *Rhodotorula*, *Wickerhamomyces*, and *Pichia* [16,29,30]. Yeasts are the microorganisms that predominate this process during the first 24 h of fermentation, and subsequently, their population decreases [4,24]. Yeasts are favored by the initial acidity of the cocoa pulp (pH 3.6), the concentration of citric acid, the low oxygen levels, and environmental temperature ranging from 25–35 °C [4,23]. Yeast metabolizes glucose, fructose, and sucrose present in the cocoa pulp, yielding ethanol and carbon dioxide [31]. Yeast central metabolism begins with the basic conversion of sugars to pyruvate, producing ATP and

reduced NADH cofactors. Under aerobic conditions, pyruvate is converted to acetyl-CoA by pyruvate dehydrogenase and directed to the citric acid cycle.

The anaerobic conversion of pyruvate to ethanol is a two-step process. First, pyruvate is converted to acetaldehyde by pyruvate decarboxylase (PDC), releasing carbon dioxide. Next, acetaldehyde is converted to ethanol by alcohol dehydrogenase (ADH). This oxidoreductase type can catalyze the reversible interconversion of alcohols and the corresponding aldehydes or ketones (Figure 2A) [17]. Some yeast species can produce organic acids, including acetic, phosphoric, oxalic, malic, and succinic [30,32]. Yeasts also contribute to the development of the characteristic flavor of chocolate due to the generation of volatile compounds [33]. Furthermore, it has been reported that some yeast strains such as *Pichia kudriavzevii* can hydrolyze the pectin present in the mucilaginous pulp surrounding the cocoa bean since they can produce pectinolytic enzymes [28].

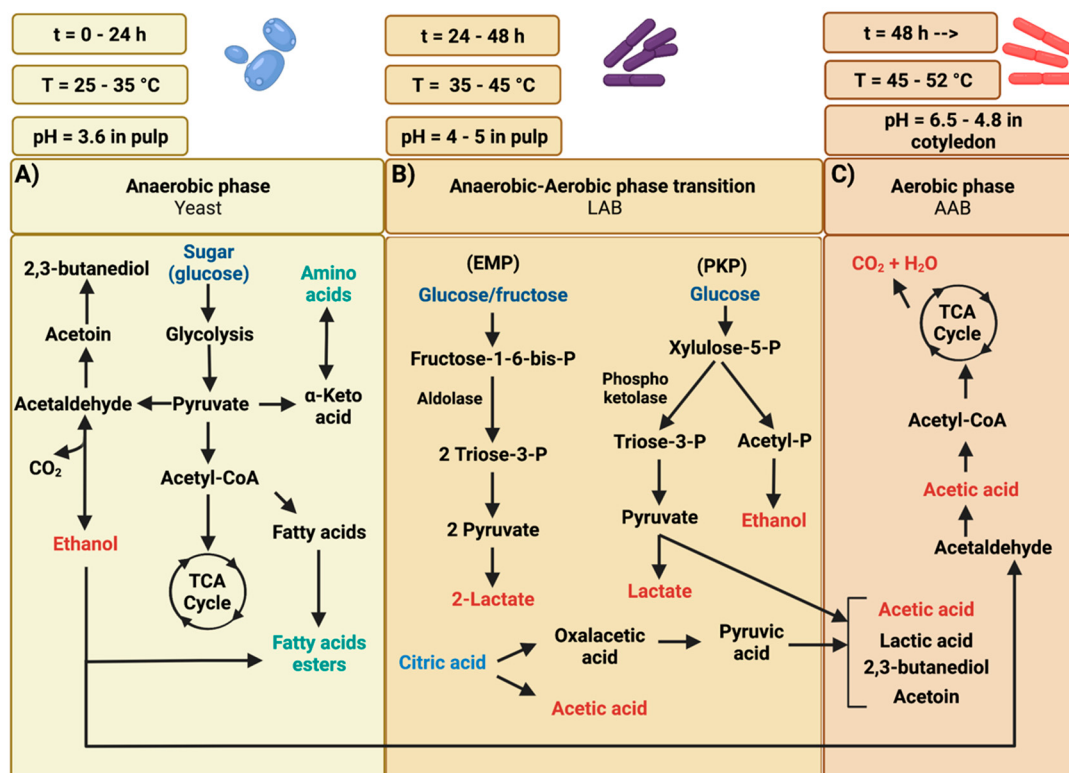


Figure 2. Main phases of cocoa fermentation. (A) Yeasts produce ethanol from sugar (glucose), fermenting it to pyruvate through glycolysis to obtain ATP, reduce equivalents production, and produce ethanol and carbon dioxide. (B) LAB strains utilize glucose through the Embden–Meyerhoff–Parnas EMP pathway (Homofermentative LAB) or phosphoketolase PKP pathway (Heterofermentative LAB). (C) Lastly, AAB strains oxidize ethanol produced by yeasts to acetic acid.

3.1.2. Lactic Acid Bacteria (LAB)

LAB is a group of Gram-positive bacteria whose main product of fermentable carbohydrate metabolism is lactic acid [34]. The LAB population increase when some of the pulp and lixiviate have drained mainly due to pectin degradation, and the yeast population decreases. Carbon dioxide production favors this increase in LAB populations by the yeasts and by the release of vitamins and other nutrients from the autolysis of yeast cells during cocoa fermentation [31]. The most abundant species after 24 h of fermentation are *Limosilactobacillus fermentum*, *Lactiplantibacillus plantarum*, *Leuconostoc mesenteroides*, and *Lactococcus lactis* [5,18,28,35,36]. During cocoa fermentation, LAB utilizes glucose via the Embden–Meyerhof–Parnas pathway. The homofermentative LAB strains use glycolysis or Embden–Meyerhof–Parnas pathway (EMP) and yield more than 85% lactic acid. However, other species utilize glucose via the known pentose phosphoketolase pathway (PKP), hexose

monophosphate shunt, or 6-phosphogluconate pathway producing only 50% lactic acid, and other metabolites such as ethanol, acetic acid, glycerol, mannitol, and CO₂, as shown in Figure 2B [23,37]. LAB strains can consume fructose and metabolize citric acid. In the case of fructose, it is metabolized homofermentative (glycolysis) or heterofermentative (phosphoketolase pathway) to pyruvate, while citric acid is metabolized to acetic acid and oxaloacetic acid [5]. Oxaloacetic acid is converted into pyruvate, which will yield either lactic acid, acetic acid, or pyruvate metabolites as 2,3-butanedione (diacetyl; buttery notes), 2,3-butanediol, and 2-butanone (acetone; buttery notes) [5]. Some LAB strains can metabolize citric acid yielding diacetyl, acetoin, and butanediol [38].

3.2. Aerobic Phase of Cocoa Bean Fermentation

On the third day of fermentation, when the pulp of the cocoa beans has been decreased, and both the temperature and the amount of air inside the fermentation mass have been increased, the environmental conditions are favorable for the proliferation of AAB. These bacteria metabolize the ethanol produced during yeast growth as their primary carbon source [18,39].

Acetic Acid Bacteria (AAB)

AAB dominates this phase of cocoa bean fermentation; in recent years, these bacteria have been extensively studied due to their significant contribution to cocoa bean fermentation [40–42]. AAB conducts ethanol and lactic acid oxidation to acetic acid. Acetic acid is considered one of the main metabolites produced by an exothermic reaction oxidizing ethanol to acetic acid (Figure 2C). The rise in temperature to 40–52 °C, decrease in pH from 6.5 to 4.8 in the cotyledon, and penetration of acetic acid and ethanol to the cocoa bean is the cause of the death of the embryo, promoting their inactivation and increasing the permeability of the cell wall of the grain and the release of precursor molecules of cocoa color and flavor precursors [4,18,28,41–43]. The diversity of AAB is practically limited to two genera: *Acetobacter* and *Gluconobacter* [28]. *Acetobacter pasteurianus* is the most identified AAB during cocoa bean fermentation in Ivory Coast [44,45], Cameroon [16,46], Honduras [47], and Brazil [48,49].

3.3. Biochemical Transformations on Cocoa Bean during Fermentation

The biochemical transformations that occur inside the cocoa bean are driven mainly by the production of ethanol, lactic acid, and acetic acid, and an increase in temperature during fermentation provoked by the oxidation of ethanol by AAB [5,20,23,24,50,51]. Acetic acid penetrates the bean and induces a drop in the pH of the cotyledons (approximately 6.5 to 4.8). This low pH of the cotyledons, combined with the presence of non-dissociated acetic acid and ethanol and the heat effect during fermentation, causes the embryo's death (Figure 3) [20,23,24,52] damages the cotyledon's internal structure to prevent the germination of cocoa beans. The physicochemical modifications result in desirable enzymatic and non-enzymatic conversions and the release of compounds from the cocoa bean.

Consequently, the different enzymes found inside the cocoa bean can be activated or inactivated gradually during the fermentation and drying processes [5,20,23,24,52,53]. The free amino acids and peptides are formed by proteolytic enzymatic reactions, while reducing sugars, such as fructose and glucose, are products of sucrose hydrolysis by invertase [54]. Peptides and hydrophobic-free amino acids, such as alanine, phenylalanine, leucine, and tyrosine, are precursors that contribute to the cocoa and chocolate flavor formation that develops through acetic acid and lactic acid-induced proteolysis of vicilin-class globulin (VCG). Strecker degradation of each specific amino acid produces a unique aldehyde with a unique aroma, e.g., from alanine; fruity notes (acetaldehyde), phenylalanine; sweet, bitter, and almond notes (benzaldehyde), leucine, malty/chocolate notes (3-methylbutanal), isoleucine; malty/chocolate notes (2-methylbutanal), valine; malty/nutty/chocolate notes (2-methyl propanal) and phenylalanine; floral/honey notes (phenylacetaldehyde) as shown in Figure 3 [5,55,56]. Cocoa bean's phenolic compounds impart astringency; however,

their concentrations decrease significantly during fermentation and drying. Anthocyanins are rapidly hydrolyzed to cyanidins and sugars (catalyzed by glycosidases). Polyphenol oxidases convert polyphenols (mainly catechins) to quinones. The complex of proteins and peptides with polyphenols gives rise to the brown coloration typical of fermented cocoa beans. Methylxanthines impart bitterness. However, their levels decrease by 30% during cocoa bean fermentation [57]. The invertase, optimally active at an acidic pH of 4.5, is active mainly at the beginning of the cocoa bean fermentation and hydrolyses sucrose into the reducing sugars glucose and fructose that serve as flavor precursors [5].

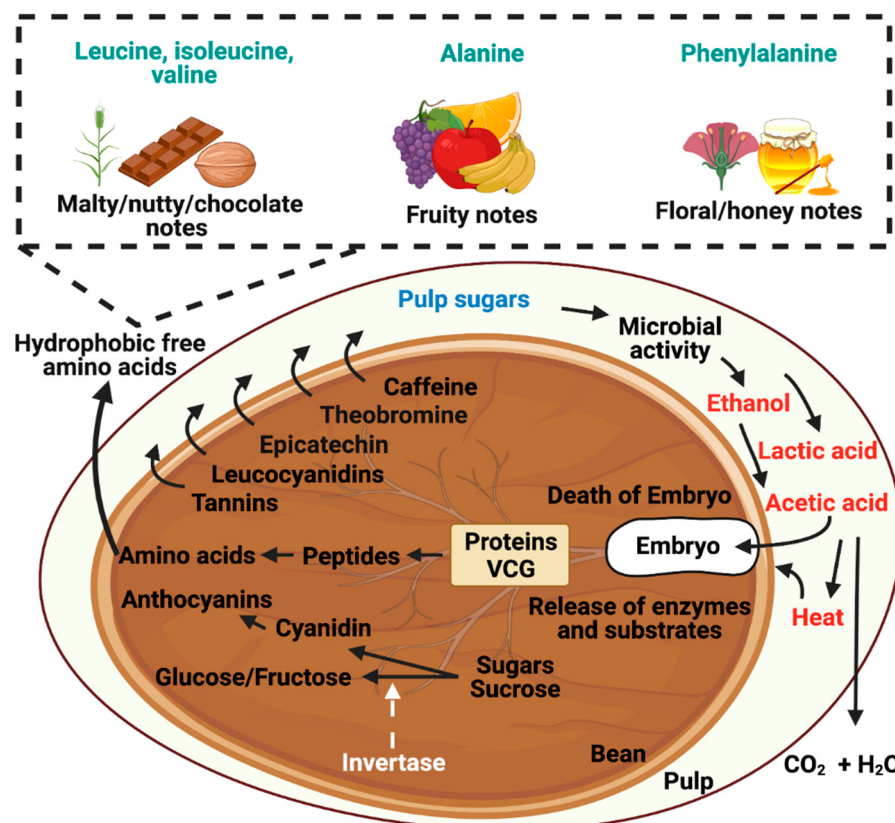


Figure 3. Formation of flavor precursors from hydrophobic-free amino acids. Microorganisms utilize the available substrates present in the cocoa pulp, such as carbohydrates, pectin, and organic acids, to produce the main metabolites of the process, such as ethanol, lactic acid, and acetic acid. Acetic acid penetrates the beans' interior, causing the embryo's death and the release of enzymes and endogenous substrates that, through proteolytic reactions, generate the flavor precursors (amino acids).

4. Contribution of Yeasts during Cocoa Fermentation

The fresh cocoa pulp is favorable for yeast growth since it consists of an anaerobic environment rich in sugars and a low pH that inhibits the development of other microorganisms [5,58]. Many studies have demonstrated a great diversity of yeast species during cocoa fermentation. The main yeast genera involved in the fermentation process of spontaneous cocoa are *Pichia*, *Saccharomyces*, *Hanseniaspora*, and *Candida*. Other genera found in lower abundance are *Wickerhamomyces*, *Torulaspora*, *Kluyveromyces* and *Rhodotolura* [9,11,13,15,16,29–32,36,44,48,49,59–82]. Concerning the yeast species found in this process, several authors have highlighted that the most frequent are, in decreasing order, *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *Hanseniaspora opuntiae*, *Hanseniaspora uvarum*, *Hanseniaspora guilliermondii*, *Pichia manshurica*, *Pichia kluyveri*, and *Candida tropicalis* [28,58]. The main activities performed by yeasts during the cocoa fermentation process are the production of volatile organic compounds (VOCs), pectin hydrolysis, and carbohy-

drate fermentation [58]. Some species may have some antifungal effect [47,83,84] and can metabolize citric acid [32,77].

4.1. Flavor Precursor Formation by Yeast during Fermentation

Yeasts are involved in the production of VOCs, which are essential in developing fruity flavors and aromas. These compounds are also determinants in developing fruity, caramel, or chocolate flavors and aromas [18]. Ho et al. [15] demonstrated that the absence of yeast during cocoa bean fermentation caused the absence of higher alcohols and esters in the fermented cocoa beans. This suggests that yeasts are the leading producers of these compounds. They concluded that yeasts were essential to the cocoa fermentation process. Koné et al. [13] identified 33 VOCs produced by yeasts. The species *P. kudriavzevii*, *S. cerevisiae*, *C. tropicalis*, and *Wickerhamomyces anomalus* were found to produce higher alcohols (isobutanol and isoamyl alcohol), acids (acetic acid and isovaleric acid) and esters (ethyl acetate, isobutyl acetate, and isovaleric acid). Table 1 shows the main VOCs produced by yeasts during the fermentation process and their associated sensory descriptor.

In the metabolism of yeasts, a fraction of the carbon is shuttled to the Krebs cycle, which forms important aroma precursors through reactions associated with amino acid metabolism [17]. Some yeast species such as *Saccharomyces kudriavzevii* produce higher alcohols, either catabolically or anabolically. The catabolic formation by the Ehrlich pathway involves consecutive transamination, decarboxylation, and dehydrogenation of amino acids. The anabolic production is by side products of amino acid biosynthesis starting from pyruvate. Some yeasts produce acetoin from acetaldehyde (green apple notes), which can be further reduced to 2,3-butanediol; similarly, diacetyl can be reduced to acetoin and 2,3-butanediol forming higher alcohol. Additionally, yeasts produce higher alcohols such as 3-methylbutanol and 2-phenylethanol and esters such as ethyl acetate, ethylphenyl acetate, and 2-phenylethyl acetate, contributing to the floral and fruity notes of the cocoa beans (Figure 4A) [5,17]. Esters are formed by a condensation reaction between acetyl/acyl-CoA and alcohol. The use of acetyl-CoA or acyl-CoA divides esters into acetate esters and fatty acid ethyl esters (Figure 4B). Acetate esters have significantly more influence over flavor and fragrance than the fatty acid counterparts due to their contribution of fruity and floral notes [17].

Table 1. Main VOCs and sensory descriptors produced by yeasts in cocoa bean fermentation.

Yeasts	VOC	Sensory Descriptor	References
Aldehydes and ketones			
<i>S. cerevisiae</i>	Acetaldehyde	Green apple	[5]
<i>C. metapsilosis</i>	Benzene acetaldehyde	Green	[85]
<i>S. cerevisiae</i> , <i>K. marxianus</i> , <i>P. kudriavzevii</i>	Phenylacetaldehyde	Floral, honey	[5,86,87]
<i>S. cerevisiae</i>	2-butanal	Fruity, grassy	[5]
<i>S. cerevisiae</i>	2-hexanal	Fruity, grassy	[5]
<i>S. cerevisiae</i> , <i>C. metapsilosis</i> , <i>Galactomyces geotrichum</i> , <i>P. pastoris</i> ; <i>S. carlsbergensi</i> , <i>P. kudriavzevii</i>	Benzaldehyde	Almond, hazelnut, candy, burnt sugar	[13,85–87]
<i>S. cerevisiae</i>	Butanal, 2-methyl-	Malty, chocolate	[5,87]
<i>S. cerevisiae</i> , <i>C. metapsilosis</i>	Butanal, 3-methyl-	Malty, chocolate	[85]
<i>S. cerevisiae</i>	2-Methylpropanal	malty/nutty/chocolate	[5]
<i>S. cerevisiae</i> , <i>P. kudriavzevii</i>	2-Phenylbut-2-enal	Floral, honey, powdery, cocoa	[86]
<i>S. cerevisiae</i> , <i>P. kudriavzevii</i>	5-Methyl-2-phenyl-2-hexenal	Cocoa	[86]
<i>S. cerevisiae</i> , <i>P. kudriavzevii</i>	Acetophenone	Floral, fruity, almond, pungent, sweet	[5,85,86]

Table 1. Cont.

Yeasts	VOC	Sensory Descriptor	References
<i>S. cerevisiae</i>	2-heptanone	Floral, fruity	[5]
<i>P. kudriavzevii</i>	2-nonanone	Fruity, sweet, waxy, green herbaceous	[86]
Alcohols			
<i>S. cerevisiae</i>	Glycerol	Sweet	[5,85]
<i>S. cerevisiae</i>	2,3-butanediol	Fruity, creamy, buttery	[5,85]
<i>S. cerevisiae</i>	2-Propyldecan-1-ol	Floral	[85]
<i>S. cerevisiae</i>	Benzene ethanol	Floral	[85]
<i>S. cerevisiae</i>	1-butanol–3 methyl	Fruity, malty, bitter, chocolate	[5,85]
<i>S. cerevisiae</i> , <i>C. tropicalis</i> , <i>G. geotrichum</i> , <i>H. guilliermondii</i> , <i>H. uvarum</i> , <i>K. lactis</i> , <i>K. marxianus</i> , <i>P. anomala</i> , <i>P. farinosa</i> , <i>P. kudriavzevii</i> , <i>W. anomalus</i> , <i>P. kudriavzevii</i>	2-phenylethanol	Fruity, floral, honey, rummy	[5,13,23,86–89]
<i>S. cerevisiae</i> ; <i>P. kudriavzevii</i>	2-heptanol	Fruity, floral, citrus, herbal	[5,86,87]
<i>P. kudriavzevii</i>	2-nonanol	Fat, green	[86]
Acids			
<i>S. cerevisiae</i>	Acetic acid	Sour, vinegar	[86]
<i>C. metapsilosis</i>	Butanoic acid	Chessy	[85]
<i>S. cerevisiae</i>	2-methylbutanoic acid	Sweaty	[5]
<i>S. cerevisiae</i>	3-methylbutanoic acid	Sweaty, rancid	[5,86]
<i>P. kudriavzevii</i>	Octanoic acid	Sweat, fatty	[86]
Esters			
<i>S. cerevisiae</i> , <i>C. tropicalis</i> , <i>C. utilis</i> , <i>H. guilliermondii</i> , <i>H. uvarum</i> , <i>K. apiculate</i> , <i>P. anomala</i> , <i>P. farinosa</i> , <i>P. kudriavzevii</i> , <i>W. anomalus</i> , <i>K. lactis</i>	Ethyl acetate	Floral	[5,12,13,23,87,90]
<i>S. cerevisiae</i>	Acetic acid, ethyl ester	Fruity, sweet	[85]
<i>P. kudriavzevii</i>	Benzyl acetate	Floral, jasmine	[86]
<i>S. cerevisiae</i>	Ethyl octanoate	Fruity, floral	[86]
<i>S. cerevisiae</i> , <i>P. kudriavzevii</i>	Isoamyl benzoate	Balsam, sweet	[86]
<i>P. kudriavzevii</i>	Ethyl dodecanoate	Sweet, floral	[86]
<i>S. cerevisiae</i> , <i>C. metapsilosis</i>	Ethylphenyl acetate	Floral	[5,85]
<i>S. cerevisiae</i> , <i>H. guilliermondii</i> , <i>H. uvarum</i> , <i>K. marxianus</i> , <i>P. anomala</i> , <i>P. farinosa</i> , <i>P. kudriavzevii</i>	2-Phenylethyl acetate	Fruity, sweet, roses honey, floral	[5,13,86,87,89,90]
Other			
<i>S. cerevisiae</i>	2-acethyl-1-pyrrole	Caramel/chocolate/roasty	[5]
<i>C. metapsilosis</i>	2-Phenylethyl formate	Floral	[85]
<i>S. cerevisiae</i> , <i>P. kudriavzevii</i>	Tetramethylpyrazine	Roasted cocoa, chocolate	[86]
<i>S. cerevisiae</i>	Linalool	Floral	[86]

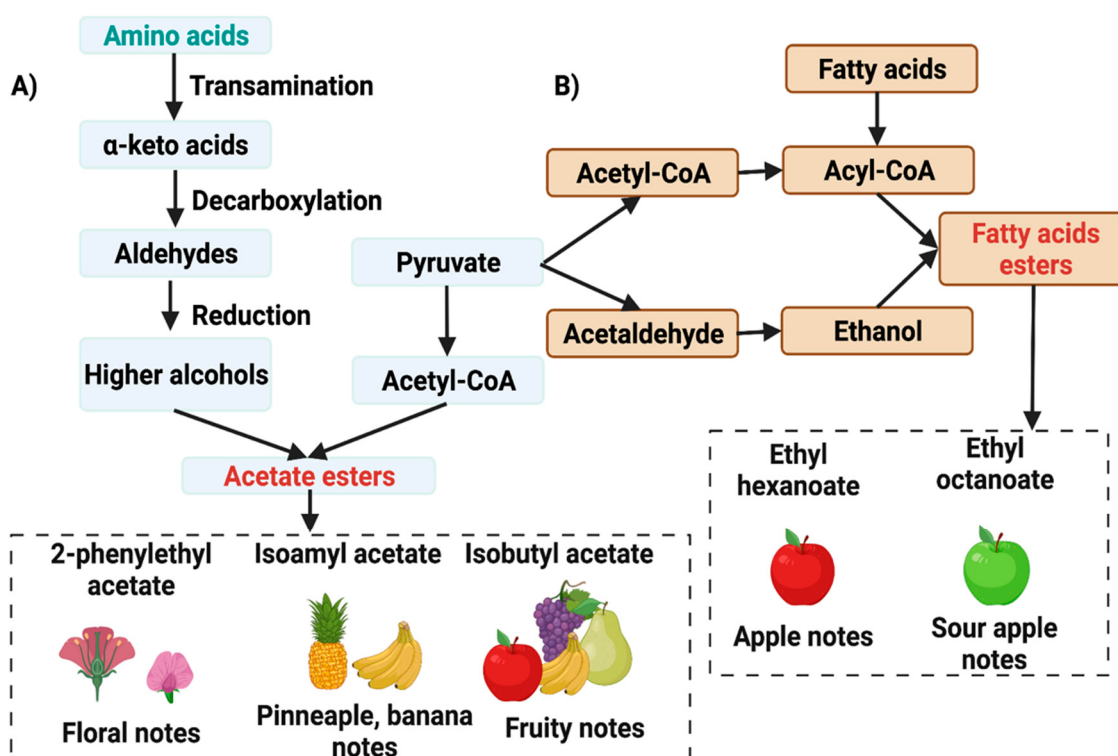


Figure 4. Formation of flavor precursors by yeast during fermentation via (A) Amino acids and (B) Fatty acids substrates. Yeast autochthonous to the cocoa fermentation process can produce higher alcohols either catabolically, through the Ehrlich pathway involving transamination, decarboxylation, and dehydrogenation of amino acids; or anabolically, as by-products of amino acid biosynthesis from pyruvate during the cocoa fermentation process.

4.2. Other Important Functions Performed by Yeast during Cocoa Fermentation

4.2.1. Pectinolytic Activity

Cocoa fermentation removes the mucilaginous pulp that surrounds the cocoa beans. This pulp, which has a high viscosity due to its pectin content, is liquefied during fermentation by the action of endogenous pectinases and microbial pectinolytic enzymes [5,58]. Several studies have reported the pectinolytic activity of various yeast species such as *Candida norvegensis*, *C. zeylanoides*, *C. nitrativorans*, *Kluyveromyces fragilis*, *K. marxianus*, *P. kudriavzevii*, *P. kluyveri* and *S. cerevisiae* in vitro or during in situ cocoa fermentations [11,29,67,77,91,92]. The pectinolytic activity of yeasts is mainly regulated by the expression of (endo) polygalacturonase genes [91,93]. Nevertheless, the presence of these genes is not always associated with pectin degradation, because both expression and activity depend on the physicochemical conditions of the cocoa fermentation process [94]. Yeast polygalacturonase enzyme hydrolyzes the α -1,4-glycosidic bonds of the pectin chain. This hydrolysis causes the loss of most of the fibrous and elastic consistency of the cocoa pulp [93,94]. The production of this enzyme is a constitutive characteristic of most yeasts and is suppressed by both glucose concentration and aeration [94]. It has been reported that the physicochemical conditions under which the enzyme can be active are species-dependent and even strain-dependent. The activity was found to range from 3.5 to 6.0 for pH and from 30–50 °C in the case of temperature. They generally act in anaerobic environments, such as that found at the beginning of cocoa fermentation [94,95]. The hydrolysis of pectin during cocoa fermentation allows oxygen to enter the fermenting mass, causing the AAB to grow more rapidly and thus produce the acetic acid necessary to penetrate the cocoa bean and conduct the biochemical transformations necessary for the generation of flavor precursor molecules [93].

4.2.2. Citric Acid Metabolism

The assimilation of citric acid during cocoa fermentation is a characteristic generally associated with the metabolism of LAB. However, it has been reported that some yeasts can metabolize this acid under in vitro conditions [32,77]. Some yeast species reported with this activity are *P. kudriavzevii*, *P. kluyveri*, and *C. tropicalis* [58]. Furthermore, some yeasts can assimilate citrate through the tricarboxylic acid (TCA) or glyoxylate cycle [96]. However, to achieve this, aerobic conditions are required [5].

4.2.3. Antifungal Activity

The growth of filamentous fungi during the spontaneous fermentation of cocoa beans results in deterioration of fermented cocoa bean quality and could represent a potential health risk to the consumer due to the possible accumulation of mycotoxins [83]. During the post-harvest stages of cocoa, several studies have reported the presence of aflatoxin B1-producing fungi, such as *Aspergillus flavus* and *A. parasiticus*, and ochratoxin A (OTA)-producing fungi, such as *Penicillium spp.* and *A. ochraceus* [97,98]. Romanens et al. [47] screened several cocoa autochthonous LAB and yeast strains to select antifungal co-cultures for starters for cocoa bean fermentation. They selected *Limosibacillus fermentum* M017, *L. fermentum* 223, *Hanseniaspora opuntiae* H17, and *S. cerevisiae* H290 due to their high fungal growth inhibition activity against seven filamentous fungal strains of the genera *Aspergillus*, *Penicillium*, and *Gibberella*. They also tested the antifungal activity of co-cultures consisting of the microorganisms mentioned above, using a LAB strain and a yeast. These co-cultures were found to limit the growth of filamentous fungi and the production of mycotoxins during cocoa fermentation. Romanens et al. [83] studied the antifungal activity of cocoa fermentation autochthonous microorganisms (LAB and yeast) and the reduction in ochratoxin A production. The microorganisms showed the highest antifungal activity against *Aspergillus candidus*, *A. flavus*, *A. nidulans*, *A. tamarii*, *A. versicolor*, *Gibberella moniliformis*, and *Penicillium citrinum* were *Limosibacillus fermentum*, *Lactiplantibacillus plantarum*, *S. cerevisiae*, and *C. ethanolica*. Moreover, their antifungal effect was mainly associated with the production of organic acids in the case of LAB strains and protein compounds in the case of yeast strains. Finally, in 2020 Romanens et al. [83] evaluated the effect of using two co-cultures with antifungal activity containing *L. fermentum* M017 and *S. cerevisiae* H290 (co-culture A) and *L. fermentum* 223 and *S. cerevisiae* H290 (B) as inoculum for 180 kg box cocoa fermentations. The comparison of inoculated and spontaneous fermentation processes revealed that the co-cultures only minimally affected the fermentation process and the quality of the fermented cocoa. However, co-culture B presented a higher capacity to limit fungal growth and mycotoxin production and is therefore recommended for use as inoculum for industrial-scale cocoa fermentations.

Ruggirello et al. [84] studied the antifungal activity of cocoa fermentation autochthonous microorganisms (LAB and yeast). The autochthonous microorganisms that showed the highest antifungal activity against *A. flavus*, *A. niger*, *A. fumigatus*, *P. citrinum*, and *P. griseofulvum* were strains belonging to the species *L. fermentum*, *L. plantarum*, *S. cerevisiae*, and *C. ethanolica*. Rahayu et al. [99] tested cocoa fermentation autochthonous microorganisms (*C. famata* HY-37, *L. plantarum* HL-15, and *Acetobacter spp.* HA-37) as starter cultures for the same process. They found that using LAB strain (*L. plantarum*) alone or together with the other microorganisms (*C. famata*, and *Acetobacter spp.*) as an inoculum for cocoa bean fermentation can reduce the development of *A. niger* YAC-9, and the ochratoxin A synthesis during fermentation and drying.

5. Yeast as Starter Cultures and Their Effect on the Flavor and Sensorial Attributes of Chocolate

Yeasts have a significant role in developing the characteristic sensory features of some fermented foods and participating in the production of multiple metabolites and enzymes and metabolites during this process [3,100]. Sometimes these fermentations occur spontaneously (with a higher microbial diversity); in other cases, yeast strains are used

as starter cultures; thus, fermentation can be shortened and standardized [101]. A starter culture can be defined as a preparation of live microorganisms used deliberately to facilitate fermentation, causing specific changes in the food substrate's composition and sensory properties to obtain a more homogeneous product [102]. De Vuyst and Leroy [5] highlighted that using starter cultures could contribute to a faster and more efficient cocoa fermentation process and the production of high-quality and homogeneous fermented cocoa beans. It could positively impact farmers, cocoa traders, and chocolate manufacturers. There has been increasing interest in using yeasts as starter cultures for cocoa fermentation in recent years, especially those belonging to the *Saccharomyces*, *Pichia*, *Kluyveromyces*, *Candida*, and *Torulaspora* genera [12,14,16,33,103–106]. Table 2 shows the yeasts used as starter cultures during cocoa fermentation and their effect on VOC production and sensory attributes on chocolate made with fermented cocoa beans inoculated with yeast strains.

In some studies, mixed starter cultures consist of yeast and bacteria (LAB and AAB) [10,11,70,107–109], whereas, in others, only yeasts were found [14,58,110–112]. These cultures have only been applied in some cocoa-producing countries such as Brazil, Malaysia, Ivory Coast, Ghana, Costa Rica, and Cameroon [10,11,14,16,33,36,48,70,86,103,106–108,110–116]. The yeast species most used as starter cultures for cocoa fermentation are *S. cerevisiae*, *P. kluyveri*, *P. kudriavzevii*, *K. marxianus*, and *T. delbrueckii*. *S. cerevisiae* strains were selected as a starter culture for cocoa fermentation because of their capacity to assimilate citric acid and reducing sugars, killer toxins production, VOC production, and their high pectinolytic activity [23,32,66,72,76,77]. Besides using *Saccharomyces* as starter culture for the cocoa fermentation process, non-*Saccharomyces* yeasts (*Kluyveromyces*, *Hanseniaspora*, *Pichia* and *Torulaspora*) have also been used [11,14,16,22,33,36,86,107,111,112,116]. These yeasts have relevant pectinolytic activity and are associated with increased aroma complexity in wine [117,118]. They are usually inoculated together with *S. cerevisiae* and in some cases with one more non-*Saccharomyces* yeast [11,14,16,22,33,36,86,105,107,112,116]. However, using mixed starter cultures with two non-*Saccharomyces* yeast strains has a contradictory effect on sensory attributes in chocolate [14,111].

Table 2. Yeasts are used as starter cultures during cocoa bean fermentation and their effects on VOC production and sensory attributes on chocolates. Comparisons are made with spontaneous fermentation processes.

Country	Fermentation Method/Cocoa Variety	Yeast Species as Starter Culture (SC)	VOC Production	Effect on Chocolate Sensory Attributes	References
Brazil	Wooden boxes/common hybrids	<i>S. cerevisiae</i>	N.D.	No significant differences were observed.	[113]
Brazil	Plastic baskets/not mentioned	<i>K. marxianus</i>	N.D.	Chocolate made with inoculated fermentation has better flavor attributes and global acceptability.	[33]
Ivory Coast and Malaysia	Heaps and wooden boxes/Forastero hybrids	<i>S. cerevisiae</i> mixed with <i>L. fermentum</i> and <i>A. pasteurianus</i>	N.D.	Dark chocolates produced with fermented beans with mixed SC develop all necessary characteristics.	[10]
Ghana	Plastics trays/Forastero	<i>K. marxianus</i> mixed with <i>L. fermentum</i> and <i>A. pasteurianus</i>	N.D.	Chocolates with higher bitter, sour, and astringent notes. Lowest sweetness and general liking.	[11]
		<i>P. kluyveri</i> mixed with <i>L. fermentum</i> and <i>A. pasteurianus</i>		Chocolates with the highest intensity of sweetness, fruitiness, and cocoa aroma. Significantly higher general liking.	
Ghana	Plastics trays/Forastero	<i>K. marxianus</i> mixed with <i>L. fermentum</i> and <i>A. pasteurianus</i>	Higher amounts of benzyl alcohol, phenethyl alcohol, benzyl acetate, and phenethyl acetate	The inoculated chocolates were characterized as fruity, acid, and bitter with berry, yogurt, and balsamic notes.	[107]
		<i>P. kluyveri</i> mixed with <i>L. fermentum</i> and <i>A. pasteurianus</i>	Significantly higher concentration of phenylacetaldehyde		

Table 2. Cont.

Country	Fermentation Method/Cocoa Variety	Yeast Species as Starter Culture (SC)	VOC Production	Effect on Chocolate Sensory Attributes	References
Brazil	Wooden boxes/hybrids PH 16, PS1030, FA13, and PS1319	<i>S. cerevisiae</i>	Esters and alcohols were the most important groups of VOCs	N.D.	[106]
Brazil	Wooden boxes /hybrid PS1319	<i>S. cerevisiae</i> + <i>H. uvarum</i> + <i>P. kluyveri</i>	N.D.	Chocolates made with inoculated fermented cocoa beans have strong coffee and sour notes. No significant difference in overall acceptance	[14]
Malaysia	Baskets/local hybrid	<i>S. cerevisiae</i>	Higher production of ethyl acetate and acetate esters in cocoa liquors	Preference for the chocolate produced with inoculated cocoa beans.	[114]
Brazil	Wooden boxes/local hybrid PS1319	<i>S. cerevisiae</i> + <i>H. uvarum</i> + <i>P. kluyveri</i>	Higher isoamyl acetate and ethyl acetate	Differences in the sensory analysis. More intense fruity note in chocolates produced with inoculated cocoa.	[113]
Malaysia	Baskets/local hybrids	<i>S. cerevisiae</i>	Higher production of ethyl acetate and acetate esters in cocoa liquors; higher VOC concentrations in chocolates	Significant differences in the sensory analysis of chocolates.	[103]
		<i>P. kluyveri</i>	Lower VOC concentrations in chocolates	Significant differences in the sensory analysis of chocolates.	
		<i>H. uvarum</i>	Lower VOC concentrations in chocolates	Significant differences in the sensory analysis of chocolates.	
India	Wooden boxes/Forastero	<i>S. cerevisiae</i> mixed with <i>L. plantarum</i> and <i>A. aceti</i>	N.D.	Chocolates with intense cocoa flavor (10% inoculum). Chocolates with more acidic, astringent, and fruity flavor (30–60% inoculum)	[109]
Brazil	Wooden boxes/local hybrid PH16	<i>S. cerevisiae</i> mixed with <i>L. fermentum</i>	N.D.	Inoculation of cocoa with mixed SC affected the sensory chocolate attributes. A lower dominance of cocoa flavor and sensorial characteristics such as bitterness, astringency, and acidity were observed.	[48]
Brazil	Wooden boxes/local hybrid PH15	<i>S. cerevisiae</i> mixed with <i>L. fermentum</i> and <i>A. pasteurianus</i>	2,3-butanediol (cocoa butter notes) and 2,3-dimethylpyrazine (caramel and cocoa notes) were detected only in chocolates produced with inoculated cocoa beans	Chocolate made with inoculated beans showed bitter, sweet, and cocoa tastes.	[70]
Brazil	Wooden boxes/local hybrids PS1319 and SJ02	<i>S. cerevisiae</i>	Higher amounts of aldehydes and ketones in chocolate made from cocoa hybrid PS1319 inoculated with <i>T. delbrueckii</i>	Inoculated fermentation resulted in chocolate with higher values of desirable bitter taste, sweet, coffee flavor, fruity and roast. Moreover, chocolates made with PS1319 inoculated with <i>S. cerevisiae</i> , and <i>T. delbrueckii</i> showed a reduction in an astringent, woody, undesirable taste of bitter and hearty flavor.	[110]
		<i>T. delbrueckii</i>			
		<i>S. cerevisiae</i> + <i>T. delbrueckii</i>			
Cameroon	Heaps and Wooden boxes/Forastero hybrids	<i>S. cerevisiae</i>	No significant differences were observed between the inoculated and non-inoculated fermentations	N.D.	[16]
		<i>T. delbrueckii</i>			
Ivory Coast	Plastic boxes/Forastero x Trinitario	<i>S. cerevisiae</i> A	Higher producer of chocolate key aroma compounds such as esters (ethyl acetate) and several pyrazines such as di-, tri-, and tetramethyl pyrazines	Chocolate made with inoculated <i>S. cerevisiae</i> B cocoa the lowest scores for desirable sensory characterize beans attributes, compared obtained with fermented cocoa from spontaneous and inoculated <i>S. cerevisiae</i> A.	[115]
		<i>S. cerevisiae</i> B	Higher effect on VOCs profile		
		<i>S. cerevisiae</i>	Enhanced production of VOCs		
Costa Rica	Plastic buckets/Trinitario	<i>P. kudriavzevii</i>	Enhanced production of isoamyl acetate 3-methyl butanal, 2-phenyl ethanol, and ethyl decanoate	N.D.	[36]
		<i>S. cerevisiae</i> + <i>P. kudriavzevii</i>	Enhanced production of VOCs		

Table 2. Cont.

Country	Fermentation Method/Cocoa Variety	Yeast Species as Starter Culture (SC)	VOC Production	Effect on Chocolate Sensory Attributes	References
Brazil	Wooden boxes/Forastero	<i>S. cerevisiae</i>	Higher pyrazine Concentrations	N.D.	[86]
		<i>P. kudriavzevii</i>	Higher alcohol and aldehyde concentrations		
		<i>S. cerevisiae</i> + <i>P. kudriavzevii</i>	Higher concentrations of Esters and pyrazines concentrations		
Brazil	Wooden boxes/local hybrids CEPEC2002, FA13	<i>S. cerevisiae</i>	<i>S. cerevisiae</i> VOCs: 3-methyl-1-butanol, 2-phenylethanol, 2-pentanone	Chocolates are described as sourer, fruitier, sweeter, and less astringent.	[112]
		<i>S. cerevisiae</i> + <i>P. kluyveri</i>	<i>S. cerevisiae</i> VOCs: 3-methyl-1-butanol, 2-phenylethanol, 2-pentanone <i>P. kluyveri</i> metabolites: benzaldehyde, 1-butanol, phenylethyl alcohol	Chocolates are described as bitter and sweeter but less sour.	
Brazil	20-L plastic buckets/Forastero	<i>P. fermentans</i> mixed with <i>L. plantarum</i>	Enhanced production of VOCs	N.D.	[108]
Brazil	wooden boxes/hybrids CCN-51, FEC-2, FLE-2, and ICS-1	<i>H. thailandica</i>	Production of ethyl acetate, isoamyl acetate, and 2-phenylethyl acetate	High-intensity levels of fruity notes.	[116]

N.D. Not Determined. SC Starter Culture.

6. Conclusions

Yeasts are one of the most relevant microbial groups in cocoa bean fermentation. During this process, yeasts produce a variety of flavor precursors that are required to produce high-quality chocolate. The main yeasts in the cocoa fermentation process are *S. cerevisiae*, *P. kudriavzevii*, *H. opuntiae*, *H. uvarum*, *H. guilliermondii*, *P. manshurica*, and *P. kluyveri*. Some yeasts can produce 3-methylbutanol, 2-phenylethanol, and esters such as ethyl acetate, ethylphenyl acetate, and 2-phenylethyl acetate, which contribute to the floral and fruity notes of fermented cocoa beans.

Therefore, VOC production is a relevant criterion for adequate yeast selection to formulate a starter culture for cocoa fermentation. In this sense, *S. cerevisiae* has been the yeast most used as a starter culture for this process. However, this yeast species can also have a pectinolytic or citric acid metabolizing activity, giving some advantages to this microorganism during fermentation. Other yeasts widely used as starter cultures are non-*Saccharomyces* yeasts such as *Kluyveromyces*, *Hanseniaspora*, *Pichia*, and *Torulaspora*, some of which showed a positive effect on the aromatic profile or sensory attributes of chocolate produced. In addition, mixed yeast cultures have been tested, but they have shown contradictory results on chocolate sensory attributes, so further studies are needed. Nevertheless, using yeasts as starter cultures for cocoa fermentation is recommended to shorten and homogenize the process.

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