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Fermentation of Cocoa (*Theobroma cacao* L.) Pulp by *Laetiporus persicinus* Yields a Novel Beverage with Tropical Aroma

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Abstract: Cocoa pulp represents an interesting by-product of cocoa production, with an appealing flavor. We developed a non-alcoholic beverage via the submerged fermentation of 10% pasteurized cocoa pulp in water with *Laetiporus persicinus* for 48 h; the product was characterized by tropical fruity notes such as coconut, mango, passion fruit and peach. The overall acceptance of the beverage compared to the non-fermented medium, as rated by a panel, increased from 2.9 to 3.7 (out of 5.0 points) for odor and from 2.1 to 4.2 for taste. (*R*)-Linalool (flowery, fruity), methyl benzoate (green, sweet), 2-phenylethanol (rose, sweet), 5-butyl-2(*5H*)-furanone (coconut, peach) and (*E*)-nerolidol (flowery, woody) contributed to the overall aroma with odor activity values of >1. During aroma dilution analysis, further substances with coconut, passion fruit and peach-like notes were perceived and structurally assigned to the group of sesquiterpenoids. The fermentation generated a highly interesting beverage using only 10% of the valuable cocoa pulp. The aroma formation via the fungus *L. persicinus* on cocoa pulp is of great interest for further research as an example of the formation of substances not yet described in the literature.

Keywords: cocoa pulp; basidiomycetes; beverage; *Laetiporus persicinus*; aroma dilution analysis

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1. Introduction

Cocoa represents a valuable natural resource with a steadily increasing annual level of production. The forecasted amount of cocoa beans produced in 2022/2023 was over 5.0 million tons [1]. The cocoa value chain offers much potential for improvement as low levels of value added and price fluctuations have major economic and environmental consequences for many smallholders. Several by-products are generated during the production of cocoa beans, which could contribute to more sustainable cocoa farming through upcycling [2]. In addition to cocoa pod husks and cocoa bean shells, by-products include cocoa pulp, which is traditionally used for the fermentation of the cocoa beans and is thus ordinarily lost [3]. However, it has been shown in various studies that a part of the cocoa pulp can be separated prior to fermentation without negatively affecting the flavor of the beans [4,5].

Cocoa pulp has become the focus of recent studies due to its chemical composition and interesting aroma. Depending on the origin, Bickel Haase et al. detected up to 65 different aroma-active substances in the pulp [6]. The substances that typically characterize the aroma include, besides others, 4-vinyl-2-methoxyphenol (clove), δ-decalactone (coconut), linalool (flowery), β-damascenone (fruity, grape) and γ-nonalactone (fruity, coconut). Because of its high sugar content, it represents a suitable starting material for the production of alcoholic beverages like fruit wine or beer via fermentation with yeasts [7,8]. Other fermented products have also been studied, such as the cocoa pulp-based kefir drink [9].

Fermentation employing higher fungi of the division Basidiomycota has been described in the literature, especially due to the potential of these fungi for use in the production of natural-aroma compounds [10]. Well-known examples are the production of vanillin

by *Phanerochaete chrysosporium* [11] and benzaldehyde by different *Pleurotus* species [12,13], or the release of a wild strawberry-like flavor caused by the formation of (*R*)-linalool, methyl anthranilate, 2-aminobenzaldehyde and geraniol during the fermentation of black current pomace by *Wolfiporia cocos* [14]. Fermentation by basidiomycetes in submerged cultures has the advantage of a direct use as a beverage after separation of the fungal mycelium [15,16]. A broad spectrum of aroma compounds can be formed by de novo synthesis or by biotransformation, thereby naturally flavoring the beverage. The production of flavoring substances by biotechnological processes is an important alternative to plant-based and chemical sources. It is also advantageous that biotechnologically obtained flavors can be marketed as natural flavors according to current European and US legislation [17–19].

Generating valuable products from cocoa pulp is an important approach for making cocoa farming more sustainable and adding value to the cocoa fruit [2]. Therefore, the aim of the present study was to develop a novel beverage through the fermentation of cocoa pulp with basidiomycetes.

2. Materials and Methods

2.1. Screening of Basidiomycetes

To select a suitable fungus for the fermentation of cocoa pulp, 20 different basidiomycetes were screened in surface cultures for 20 days on cocoa pulp agar plates and on malt extract agar plates. The latter were used as reference media for the comparison of substrate-specific aroma formation. The smell was described and rated every second day for intensity and overall rating by three panelists (all female, 25–27 years old, all non-smokers) as '- -' means very weak/very bad; '-' weak/bad; '0' medium/neutral; '+' intensive/good; and '++' very intensive/very good. Malt extract agar contained 15 g/L agar–agar (Carl Roth GmbH, Karlsruhe, Germany) and 20 g/L malt extract (Carl Roth GmbH, Karlsruhe, Germany). Cocoa pulp agar contained 15 g/L agar–agar and 100 g/L pasteurized cocoa pulp (origin: Ecuador; purchased from Carbosse Naturals AG, Zürich, Switzerland).

2.2. Sterile Control of Pasteurized Cocoa Pulp

Submerged fermentations were carried out with pasteurized cocoa pulp. Sterile controls were performed on LB agar (15 g/L agar–agar, 20 g/L LB-medium (Carl Roth GmbH, Karlsruhe, Germany)). Approximately 1 g cocoa pulp was dispersed in 10 mL sterile, demineralized water and 1 mL of the solution was inoculated on the agar plate and spread with a Drigalski spatula. The plates were incubated at 37 °C for 48 h.

2.3. Fermentation of Cocoa Pulp with Laetiporus persicinus in Submerged Cultures

L. persicinus (CBS 274.92) was obtained from the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands). For strain maintenance, the fungus was kept on malt extract agar plates and transferred to a new plate every nine days using a spatula by cutting a ~1 cm^2 piece of overgrown agar. For the pre-cultures, 100 mL sterilized malt extract media (20 g/L malt extract in drinking water) were placed in an Erlenmeyer flask (250 mL) and inoculated with 1 cm^2 of overgrown agar. Homogenization was performed with an ULTRA-TURRAX (IKA Works Inc., Staufen, Germany) at 10,000 rpm for 30 s. Cultivation took place on a horizontal shaker at 150 rpm at 24 °C in the dark for nine days. Main cultures with cocoa pulp medium (CP-M) were prepared by autoclaving 185 mL tap water in 500 mL Erlenmeyer flasks. After cooling to room temperature, 20 g pasteurized cocoa pulp was added under sterile conditions. The pre-culture was homogenized using an ULTRA-TURRAX as described above and centrifuged (10 min, 3500× *g*, 20 °C). The supernatant was discarded, and the tube was filled with sterile water. This procedure was repeated three times. The washed pre-culture was inoculated with 20 mL (10%) to the main culture medium. Fermentation took place on a horizontal shaker at 150 rpm at 24 °C in the dark for 72 h. The fermentates were harvested after fermentation times of 12, 24, 36, 48, 60

and 72 h by centrifugation (10 min, 3500× *g*, 20 °C). The supernatant was either stored at −20 °C and used for further analysis, or directly subjected to sensory evaluation.

2.4. Sensory Evaluation over the Cultivation Period

For the sensory evaluation of CP-M and fermented beverages, the samples were first examined by a panel in a simple descriptive test (DIN 10964) in order to establish their attributes for odor and taste. Subsequently, a conventional profile test with quantitative descriptions of the intensities of the respective attribute was performed. Therefore, the panelists rated the attributes from 0 (not recognizable) to 5 (very strongly recognizable) (DIN 10967-1). The attributes used were sweet/sweetish, acidic, tropical, passion fruit-, peach-, mango-, coconut-, citrus-, honey-, rhubarb-, pineapple-, apricot-, apple-, and tea-like. The test was followed by an overall evaluation of the acceptability of the respective sample. The beverages were analyzed after 12, 24, 36, 48, 60 and 72 h of fermentation. The cocoa pulp medium (CP–M) served as blank. The panel comprised ten trained panelists (two male, eight female, 21–29 years old, all non-smokers). All sensory descriptions were carried out in a test laboratory according to DIN 10962.

2.5. Aroma Analysis Using Direct Immersion Stir Bar Sorptive Extraction (diSBSE)

A total of 5 mL of the respective fermentate or CP–M was added to a 20 mL GC vial. The stir bars (10 mm with 0.5 mm PDMS coating) were conditioned prior to every analysis in a TubeConditioner TC 2 (GERSTEL, Mülheim an der Ruhr, Germany). diSBSE was carried out at room temperature on a multimagnetic stirring plate (MIXdrive 12, 2 mag, Munich, Germany) at 150 rpm for 30 min. After extraction, the stir bars were rinsed with *dd*H_2O, dried with lint-free tissues and placed back in a conditioned tube.

Gas chromatographic analyses were performed using an Agilent 8890 GC-system (Agilent Technologies, Santa Clara, CA, USA) connected to a 7010B GC/TQ mass spectrometric detector (Agilent Technologies). The system was equipped with a Thermal Desorption Unit 2 (TDU) (GERSTEL), an Olfactory Detection Port 4 (ODP 4) (GERSTEL) and a VF-WAXms column (30 m, i.d. 250 µm, film thickness 0.25 µm) or a DB-5ms column (30 m, i.d. 250 µm, film thickness 0.25 µm; both Agilent Technologies). Desorption started with an initial temperature of 40 °C in the TDU (0.5 min). This temperature increased from 40 °C with 120 °C per min to 250 °C, and this level was maintained for 12 min. Cryogenic focusing started at −70 °C in the CIS (0.5 min), increased from12 °C/s to 250 °C, and this was maintained for 5 min. Helium 5.0 (Nippon Gases GmbH, Hürth, Germany) served as carrier gas with a constant flow of 1.56 mL/min. The gas flow was split 1:1 between the MSD and the ODP (transferline temperature 250 °C both). The ODP mixing chamber was heated to 150 °C, and N_2 was used as make-up gas. The oven temperature program started at 40 °C (3 min), increased from 5 °C/min to 240 °C, and this level was maintained for 12 min. The MS source temperature was 230 °C; detection was conducted in scan mode with 70 eV (m/z 33–300). Splitless measurements were performed with 30 mL/min purge flow to split vent at 2 min in CIS, and a splitless mode was used in TDU.

2.5.1. Aroma Dilution Analysis (ADA)

ADA started at 6.24 mL/min purge flow to the split vent at 0 min in CIS, whereas it began in a splitless mode in TDU. Different split ratios for ADA in CIS and TDU were adapted from Trapp et al. to divide the split ratio in every step by two [20]. ADA was carried out by three trained panelists (all females, 25–27 years old, all non-smokers). In order to determine the flavor dilution factors (FD), the median of the lowest dilution level was chosen at the point at which the odorant could still be perceived by the panelists.

2.5.2. Identification and Quantitation of Selected Aroma Compounds via Standard Addition

Compound identification was carried out via a comparison of their retention indices (RIs) according to the specifications of van den Dool and Kratz [21] and a comparison of

their mass spectra (MS), as well as of their odors, with those of authentic standards and/or with literature data. Enantioselective analyses of linalool were performed according to the recommendations of Brescia et al. [22].

2-Nonanone (≥99%, Thermo Fisher Scientific, Waltham, MA, USA), (*R*)-linalool (≥95%, Thermo Fisher Scientific, Waltham, MA, USA), methyl benzoate (99%, Alfa Aesar, Karlsruhe, Germany), 1-phenylethyl acetate (≥99%, Sigma Aldrich, St. Louis, MO, USA), 2-phenylethanol (99%, Acros Organics, Waltham, MA, USA), 5-butyl-2(*5H*)-furanone (76% c.f. Figure S1, synthesized in-house by S.Y. [23]) and (*E*)-nerolidol (100%, Sigma Aldrich, Steinheim, Germany) were quantitated in the final beverage via standard addition in duplicate experiments. A mixed-stock solution was prepared in *dd*H_2O, from which four standard solutions (K1–K4) were prepared. For standard addition, 100 µL of each standard solution was added to the sample (5 mL), respectively (cf. Section 2.5) (S1–S4). For S0, 100 µL *dd*H_2O was added. The concentrations of the stock solution as well as the dilution levels are presented in the Supplementary Material (Table S1). Extraction and measurement were carried out as described above. Quantifier ions were chosen as follows: 2-nonanone (*m/z* 58), (*R*)-linalool (*m/z* 93), methyl benzoate (*m/z* 105), 1-phenylethyl acetate (*m/z* 122), 2-phenylethanol (*m/z* 91), 5-butyl-2(*5H*)-furanone (*m/z* 84) and (*E*)-nerolidol (*m/z* 93).

2.5.3. Odor Threshold and Odor Activity Values (OAV)

Odor thresholds were taken from the literature. For 5-butyl-2(*5H*)-furanone, to the best of our knowledge, no odor threshold in water has been published yet. The determination was thus carried out according to the methods of Czerny et al., 2008 [24]. Starting from a stock-solution of 1 mg/mL in *dd*H_2O, the sample was diluted seven times in 1:3 steps. A total of 10 mL of each dilution was filled into a 35 mL snap lid glass, covered with a small watch glass and equilibrated for 30 min. The odor threshold was tested as a triangle test to determine the difference between descending concentrations. The panel consisted of 21 trained panelists (7 male, 14 female, 24–33 years old, all non-smokers). The evaluation was carried out according to DIN EN ISO 4120:2007 at a significance level of $\alpha = 0.05$. The odor threshold in water was the mean value between the lowest distinguishable concentration from the references and the highest indistinguishable concentration of the substance. Odor activity values were calculated by dividing the quantitated concentrations by the respective odor threshold [25].

2.5.4. Dynamic Changes of Aroma Compound Formation during Cultivation

In order to investigate the development of selected aroma compounds over the cultivation period, the peak area of selected *m/z* ratios was used. The selected peak areas were 2-pentanone (*m/z* 86), 2-pentanol (*m/z* 45), 1-heptanol (*m/z* 70), 1-octanal (*m/z* 84), **18** (*m/z* 203), **21** (*m/z* 160), **23** (*m/z* 179), **25** (*m/z* 95) and **26** (*m/z* 123). For 2-nonanone, (*R*)-linalool, methyl benzoate, 1-phenylethyl acetate, 2-phenylethanol, 5-butyl-2(*5H*)-furanone and (*E*)-nerolidol, the same mass fragments were chosen as for quantitation via standard addition (cf. Section 2.5.2).

3. Results and Discussion

The results of the screening in surface cultures are summarized in the Supplementary Material (Table S2). *L. persicinus* showed an outstanding aroma formation on cocoa pulp agar plates and was therefore selected for further investigation. In recent years, the fermentation of various substrates with edible fungi of the division Basidiomycota has gained attention from different research groups due to the potential of the method to form a broad spectrum of aroma compounds. However, to the best of our knowledge, *L. persicinus* has thus far not been subjected to in-depth aroma analyses [14–16,26–29], and no data are available on the aroma composition of *L. persicinus*, whether grown in solid-state or in submerged cultures. The present study shows the formation of a highly delicious beverage via the fermentation of cocoa pulp with *L. persicinus* for the first time.

3.1. Sensory Evaluation of Cocoa Pulp Fermented with L. persicinus over the Cultivation Time

The evaluation of the fermented beverage, observed by the sensory panel, revealed differences between the respective cultivation times (Figure 1). Non-fermented CP-M served as a reference and was described mainly by the attributes of acidic, fruity, citrus- and apple-like. During the fermentation process, the highest values for the attributes sweet, tropical, passionfruit, peach, mango, coconut, and apricot were reached after 48 h. The acidic taste was decreased by fermentation and the lowest value was reached after 48 h, while the sweet taste reached its maximum value at this point of time. Longer cultivation periods resulted in the attainment similar sensory profiles, but these displayed lower intensities. The overall acceptance of the beverage in terms of smell and taste showed a maximum after 48 h. The acceptance of smell reached 2.9 out of 5.0 points for CP-M and increased up to 3.7 until a fermentation time of 48 h. Afterwards, the acceptance values decreased gradually. The acceptance of taste started with 2.1 out of 5.0 points for the CP-M and increased up to 4.2 points until 48 h and decreased again afterwards.

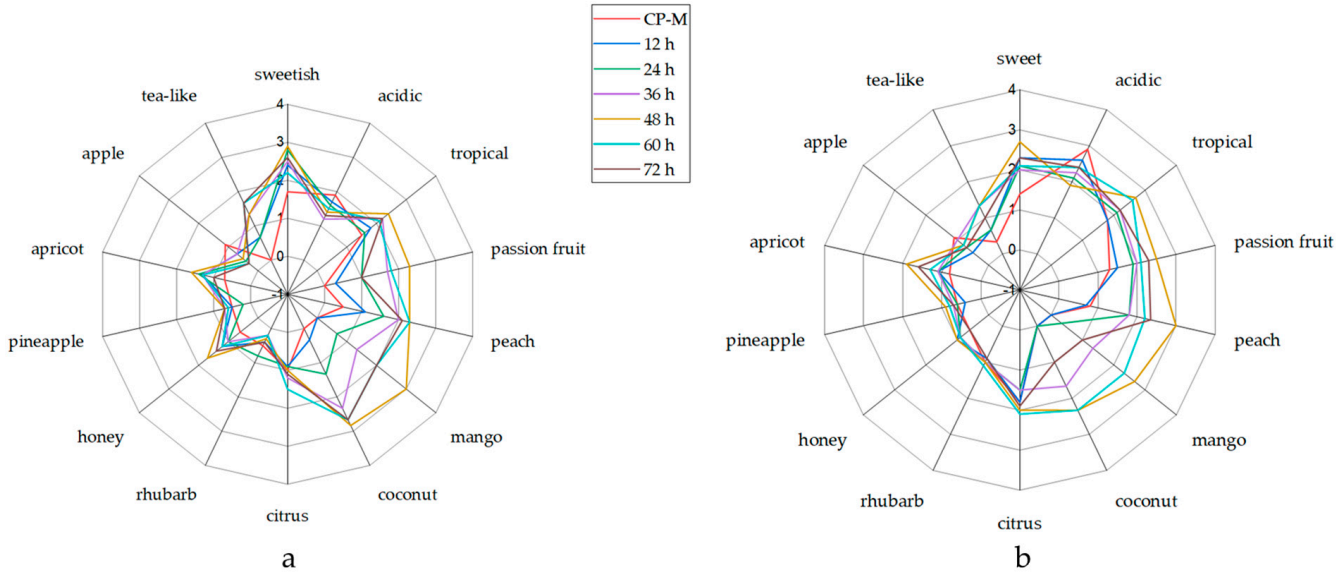

Figure 1. (**a**) Odor evaluation of the cocoa pulp medium (CP-M) and of the fermented beverage at specified cultivation periods; (**b**) taste evaluation (n = 10).

The sensory description of CP-M was consistent with those of cocoa pulp in the literature, where it was described as floral, fruity, honey, citrus-like and tropical [6]. An improvement in the overall acceptance of a beverage fermented by basidiomycetes has been shown for other substrates previously. For example, Sommer et al. produced a beverage via submerged fermentation of black current pomace with *Wolfiporia cocos* and the results showed an increase in the overall acceptance of 2.5 to 8.0 out of 10.0 points [27]. Wang et al. reported that the fermentation of okara with edible fungi can improve the flavor quality by decreasing the amount of off-flavor compounds and by forming new aromatic compounds [28]. Different from the fermentation of okara, no off-flavor contents had to be masked in the present study. The beverage was characterized by highly appealing tropical-fruity notes. Cocoa pulp thus represents a side-stream with enormous potential for use in new food products. The proportion of fresh cocoa pulp in cocoa fruits depends on various factors and ranges, according to the literature, from 10.0 to 26.4% [8,30]. Considering that only a part of the fresh cocoa pulp may be used due to the necessity of the fermentation of the beans, the amount of available fresh cocoa pulp is limited. Nevertheless, the development of novel products from cocoa side-streams can significantly contribute to the increased sustainability of the cocoa sector and generate added value for farmers [2].

The use of cocoa pulp in beer and fruit wine production has been reported in the literature. One study reported that, for the development of these alcoholic beverages,

30% cocoa pulp was used in beer production to obtain a high acceptance, and a medium starting from 100% cocoa pulp was used for fruit wine production, whereby the °Brix was subsequently adjusted with sucrose solution [7,8]. Compared to these examples, the beverage fermented by *L. persicinus* required a medium containing only 10% cocoa pulp. In addition, the beverage developed in this study was free of alcohol. The per capita alcohol consumption in Germany is steadily declining, which may be attributed to increased health awareness and a changing age structure [31]. At the same time, the market for non-alcoholic beverages is expanding to a great extent. Fermented non-alcoholic beverages are also increasingly gaining attention of consumers due to advantages regarding enhanced shelf life, improved flavor and the association between fermentation and health benefits [32]. Prior to bringing the novel beverage onto the market, a comprehensive evaluation of the chemical composition, including, e.g., sugars, acids and secondary metabolites potentially formed by the fungus will be required.

3.2. Aroma Compounds in CP-M

The occurrence of aroma substances in cocoa pulp depends on various factors, such as their variety and the origin [6]. In total, 32 aroma compounds were detected olfactometrically by means of GC–MS/MS–O in the CP–M, and seven non-odor-active compounds could be identified additionally (Table 1).

A total 27 of the 32 substances identified here have been described before by Bickel Haase et al., Chetschick et al., Hegmann et al. and Pino et al., who investigated cocoa pulps from different origins and cultivars [6,33–35]. Compounds which have not been described before in the literature on fresh cocoa pulp directly after opening the fruit were ethyl acetate, acetoin, 1-octanol, hexanoic acid and decanoic acid. Ethyl acetate and acetoin are typical flavoring substances produced by yeasts and lactic acid bacteria during fermentation [36]. In contrast to the studies mentioned above, cocoa pulp treated by pasteurization was used in this work. Prior contact with microorganisms could not be excluded and thus may have explained the occurrence of these two aroma compounds. Furthermore, other flavoring substances have been described for cocoa pulp in the literature that could not be detected here, such as β-damascenone (fruity, grape-like), γ- and δ-decalactone (coconut, peach) and *trans*-4,5-epoxy-(*E*)-2-decenal (metallic) [6,33,34].

3.3. Aroma Dilution Analysis of the Beverage after 48 h Fermentation

Aroma dilution analysis (ADA) was performed for the aroma analysis of fermented beverages. A total of 37 substances were olfactometrically perceived with FD factors between 8 and 2048 (Table 2). Linalool (**13**) with sweet, fruity, flowery, and citrus notes, showed the highest FD factor of 2048. The enantioselective analysis showed that (*R*)-linalool was present in the sample, with an *ee* = 98.4%. 5-Butyl-2(*5H*)-furanone (**24**), which has a strong coconut and peach-like odor, was present, as was (*E*)-nerolidol (**27**) with sweet, popcorn, flowery and woody notes, and they had FD factors of 1024. 2-Nonanone (**8**) showed an FD factor of 512 with a fruity, musty, herbaceous, spicy, but also cheesy, odor. Methyl benzoate (**14**) with green, herbaceous, sweet and popcorn notes, as well as 2-phenylethanol (**22**) with sweet, rose, fruity and refreshing notes, showed an FD factor of 128. 1-Phenylethyl acetate (**15**) had an FD factor of 64 and a lavender, flowery, fruity, and tropical odor. Nine other substances with FD factors < 64 were identified: 2-pentanone (**1**), 2-pentanol (**3**), 2-hexanol (**4**), octanal (**6**), 1-octen-3-one (**7**), 1-heptanol (**11**), 2-acetylfuran (**12**) and τ-muurolol (**31**). However, these substances were not quantified due to their low FD factors.

The seven identified substances with FD factors $\geq$ 64 were quantified by means of standard addition. Using odor thresholds extracted from the literature, odor activity values (OAVs) were calculated (Table 3). As no odor threshold has been published so far for 5-butyl-2(*5H*)-furanone, its odor threshold in water was determined for the first time with 62 µg/L. Of the compounds identified and quantified, (*R*)-linalool (**13**), 2-phenylethanol (**22**), 5-butyl-2(*5H*)-furanone (**24**) and (*E*)-nerolidol (**27**) showed OAVs > 1 and thus most

likely contributed to the characteristic aroma of the beverage. 2-Nonanone (**8**) and 1-phenylethyl acetate (**15**) had OAVs << 1, indicating no or only a minor contribution to the overall aroma. Methyl benzoate (**14**) had an OAV of 0.8. It is thus difficult to issue a concluding statement on the contribution to the overall aroma. The linear regressions used for quantitation of the aroma compounds by means of standard addition are presented in the Supplementary Material (Table S3).

Table 1. Identified and olfactometrically perceived substances in CP–M with odor impressions and RI indices according to van den Dool and Kratz [21]; n.i. = not identified.

Compound	Odor Impression	$RI_{VF\text{-}Wax}$	$RI_{DB\text{-}5}$	Identification
ethyl acetate	fruity	877 [a]	-	RI, odor, $MS_{VF\text{-}Wax}$
2-pentanone	fruity, sweetish	972 [a]	<700 [a]	RI, odor, MS
2-methyl-3-buten-2-ol	fruity, green	1036 [a]	<700 [a]	RI, odor, MS
2-pentyl acetate	fruity, sweetish	1071 [b]	850 [b]	RI, odor, MS
hexanal	sweetish, caramel, fresh	1080 [a]	801 [a]	RI, odor, MS
n.i.	green, herbaceous, fruity	1103	-	-
2-pentanol	organic solvent, herbaceous	1121 [a]	709 [a]	RI, odor, MS
2-heptanone	-	1182 [a]	892 [a]	RI, MS
2-methyl-1-butanol	-	1216 [a]	735 [a]	RI, MS
2-heptyl acetate	fruity, flowery	1264 [b]	1039 [b]	RI, odor, MS
n.i.	sweetish	1278	-	-
acetoin	sweetish, fatty	1279 [a]	720 [a]	RI, odor, MS
octanal	sweetish, citrus	1290 [a]	1005 [a]	RI, odor, MS
1-octen-3-one	mushroom	1303 [a]	976 [a,c]	RI, odor, MS
2-heptanol	sweetish, coconut	1320 [a]	903 [a]	RI, odor, MS
6-methyl-5-hepten-2-one	-	1339 [a]	986 [a]	RI, MS
1-hexanol	-	1350 [a]	869 [a]	RI, MS
2-nonanone	fruity, herbaceous, cheesy	1390 [a]	1092 [a]	RI, odor, MS
1-heptanol	fruity	1455 [a]	972 [a]	RI, odor, MS
linalool-oxid (isomers)	sweetish, flowery, spicy	1443 [a] / 1471 [a]	1074 [a] / 1089 [a]	RI, odor, MS
acetic acid	acetic acid	1450 [a]	<700 [a]	RI, odor, MS
linalool	sweetish, flowery, citrus	1548 [a]	1101 [a]	RI, odor, MS
1-octanol	sweetish, flowery	1558 [b]	-	RI, odor, $MS_{VF\text{-}Wax}$
acetophenone	sweetish, fruity	1655 [a]	1070 [a]	RI, odor, MS
3-methylbutanoic acid	moldy, cheesy, banana	1681 [a]	842 [a]	RI, odor, MS
α-terpineol	citrus, woody	1698 [a]	1199 [a]	RI, odor, MS
1-phenylethyl acetate	sweetish, fruity, acidic	1704 [a]	1191 [a]	RI, odor, MS
ethylphenyl acetate	sweetish, fruity, flowery	1789 [a]	1245 [a]	RI, odor, MS
hexanoic acid	-	1857 [a]	-	RI, $MS_{VF\text{-}Wax}$
benzyl alcohol	sweetish, flowery	1868 [a]	1036 [a]	RI, odor, MS
2-phenylethanol	sweetish, rose, fruity, coconut	1901 [a]	1116 [a]	RI, odor, MS
γ-nonalactone	coconut	2039 [a]	1361 [a]	RI, odor, MS
octanoic acid	-	2069 [b]	1174 [b]	RI, MS
n.i.	fruity, tropical	2278	-	-
decanoic acid	-	2281 [a]	1370 [a]	RI, MS
n.i.	fruity, acidic	2452	-	-
n.i.	sweetish	2461	-	-
n.i.	fruity, cocoa pulp	2882	-	-

[a] = identified by authentic standard. [b] = identified by comparison with literature data (NIST chemistry webbook 2022). [c] = identified by odor at given RI.

Table 2. Olfactometrically perceived substances in the fermented sample used for ADA with FD factors, odor impressions and RI according to van den Dool and Kratz [21]; n.i. = not identified.

	Compound	FD	Odor Impression	$RI_{VF\text{-}Wax}$	$RI_{DB\text{-}5}$	Identification
1	2-pentanone	32	herbaceous, green, sweetish, flowery	972 [a]	<700 [a]	RI, odor, MS
2	n.i.	32	herbaceous, green	1079	-	-
3	2-pentanol	32	sweetish, flowery	1122 [a]	700 [b]	RI, odor, MS
4	2-hexanol	32	green, herbaceous, fruity, berry, spicy	1220 [a]	800 [a,c]	RI, odor, MS
5	n.i.	16	sweetish, herbaceous	1271	-	-
6	1-octanal	32	sweetish, flowery, citrus	1290 [a]	1000 [a,c]	RI, odor, $MS_{VF\text{-}Wax}$
7	1-octen-3-one	32	mushroom	1303 [a]	976 [a,c]	RI, odor, $MS_{VF\text{-}Wax}$
8	2-nonanone	512	fruity, musty, herbaceous, spicy, cheesy	1390 [a]	1097 [a]	RI, odor, MS
9	n.i.	16	fruity, flowery, citrus, fresh, mushroom	1402	-	-
10	(*E*)-2-octental	32	herbaceous, green, chocolate, earthy	1431 [a]	-	RI, odor, MS
11	1-heptanol	8	fruity, organic solvent, spicy	1455 [a]	972 [a,c]	RI, odor, $MS_{VF\text{-}Wax}$
12	2-acetylfuran	16	sweetish, citrus, flowery, caramel	1509 [b]	-	RI, odor, MS
13	(*R*)-linalool	2048	sweetish, fruity, flowery, citrus	1548 [a]	1101 [a]	RI, odor, MS
14	methyl benzoate	128	green, herbaceous, sweetish, popcorn	1626 [a]	1092 [a,c]	RI, odor, $MS_{VF\text{-}Wax}$
15	1-phenylethyl acetate	64	lavender, flowery, fruity, tropical	1704 [a]	1191 [a]	RI, odor, MS
16	n.i.	16	sweetish, popcorn, coconut, fruity, peach	1720	-	-
17	n.i.	64	green, herbaceous, spicy	1750	-	-
18	n.i. (sesquiterpenoid) *	64	fruity, coconut, sweet, passion fruit,	1803	1468	-
19	n.i. (sesquiterpenoid)	32	sweetish, fruity	1842	-	-
20	n.i. (sesquiterpenoid)	64	spicy, herbaceous, sweetish, flowery, fruity, green	1858	-	-
21	n.i. (sesquiterpenoid)	32	fruity, citrus, coconut	1902	1701	-
22	2-phenylethanol	128	sweetish, rose, fruity, refreshing	1910 [a]	1116 [a]	RI, odor, MS
23	n.i. (sesquiterpenoid)	64	sweetish, mushroom-like, fruity, peach	1951	-	-
24	5-butyl-2(*5 H*)-furanone	1024	coconut, peach	1970 [a]	1239 [a]	RI, odor, MS
25	n.i. (sesquiterpenoid)	64	sweetish, coconut, peach	1995	-	-
26	n.i. (sesquiterpenoid)	256	spicy, herbaceous, metallic	2003	-	-
27	(*E*)-nerolidol	1024	sweetish, popcorn, flowery, woody	2039 [a]	1563 [a]	RI, odor, MS
28	n.i. (sesquiterpenoid)	8	sweetish, fruity, spicy	2056	1603	-
29	n.i. (sesquiterpenoid)	32	fruity, sweetish, caramel	2078	-	-
30	n.i. (sesquiterpenoid)	8	burned, plastic, spicy	2110	1572	-

Table 2. *Cont.*

	Compound	FD	Odor Impression	RI_{VF-Wax}	RI_{DB-5}	Identification
31	τ-muurolol	16	sweetish, Maggi	2199 [b]	1662 [b]	RI, odor, MS
32	n.i. (sesquiterpenoid)	8	sweetish, caramel, peach	2230	1589	-
33	n.i. (sesquiterpenoid)	32	sweetish, citrus, fruity, caramel	2265	1630	-
34	n.i. (sesquiterpenoid)	64	citrus, fruity, popcorn, sweetish	2289	-	-
35	n.i. (sesquiterpenoid)	32	sweetish, fruity	2461	-	-
36	n.i. (sesquiterpenoid)	64	fruity, herbaceous, sweetish, pungent	2491	-	-
37	n.i. (sesquiterpenoid)	32	sweetish, coconut, flowery	2582	1756	-

[a] = identified by authentic standard. [b] = identified by comparison with literature data (NIST chemistry webbook 2022). [c] = identified by odor at given RI. * = assigned to the sesquiterpenes group on the basis of the mass spectrum.

Table 3. Quantitated amounts and calculated OAVs of selected compounds.

Compound	Concentration [µg/L]	Odor Threshold in Water [µg/L]	OAV
2-nonanone (**8**)	1.5 ± 0.1	5.0 [37]	<1
(*R*)-linalool (**13**)	165.0 ± 1.6	0.087 [24]	1897
methyl benzoate (**14**)	0.4 ± 0.1	0.52 [38]	0.8
1-phenylethyl acetate (**15**)	0.6 ± 0.1	19.0 [39]	<1
2-phenylethanol (**22**)	192.8 ± 0.8	140 [24]	1.4
5-butyl-2(*5H*)-furanone (**24**)	457.4 ± 30.6	62	7.4
(*E*)-nerolidol (**27**)	42.4 ± 5.0	0.25 [40]	170

Linalool (**13**) is a well-known monoterpene alcohol that has been detected in many plants and fungi [41]. In other studies, on beverages fermented by different basidiomycetes, linalool almost always had an OAV of >1 [15,16,27,42]. Methyl benzoate (**14**) has been described for a variety of basidiomycetes, such as *Lentinula edodes* and *Grifola frondosa* with concentrations of up to 10 µg/L [10]. It is considered to be a key component of the flavor of mango [38]. Despite its OAV of 0.8, this substance may contribute to the mango-like impression of the fermented beverage. The formation of 5-butyl-2(*5H*)-furanone (**24**) by basidiomycetes of the genus *Laetiporus* was demonstrated by Yalman et al. [23]. They detected 5-butyl-2(*5H*)-furanone in liquid cultures of *Laetiporus montanus* with the highest FD factor of 4096. Little is known about the biosynthesis of this substance. Berger et al. suggested a pathway for biosynthesis, starting from octanoic acid and decanoic acid [23,43]. Both fatty acids were found in CP-M. With an OAV of 7.4, 5-butyl-2(*5H*)-furanone (**24**) contributed to the coconut and peach-like aroma impression of the beverage. (*E*)-Nerolidol (**27**) is a sesquiterpene alcohol that naturally occurs in various plants and is also known to be formed by basidiomycetes like *Polyporus* sp., with up to 260 µg/L [10,44]. Sommer et al., 2023 quantitated a concentration of 0.1 µg/L in submerged cultures of *Wolfiporia cocos* grown on black current pomace where it did not contribute to the overall aroma (OAV < 0.01) [27]. In the present study, (*E*)-nerolidol (**27**) showed the second highest OAV with 170.

3.4. Dynamic Changes of Aroma Compound Formation during Cultivation

The aroma profile of the beverage is composed of aroma compounds already present in CP-M, as well as of new aroma compounds formed during fermentation. Based on the peak areas of a characteristic *m/z* ratio, the concentrations of selected aroma compounds were investigated over a cultivation period of 72 h (Figure 2). The peak areas of the respective substance were related to the highest peak area, which was set to 100%.

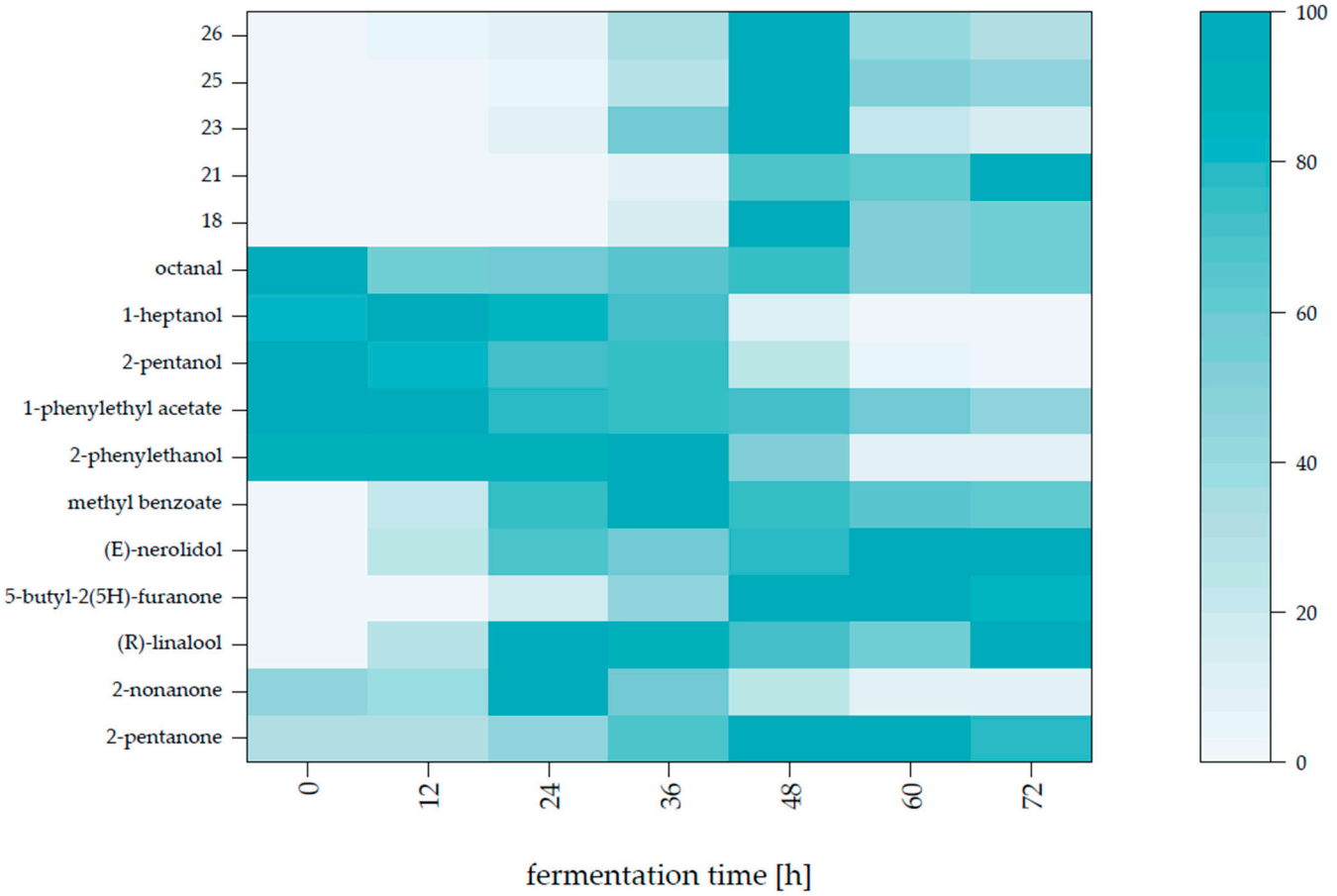

Figure 2. Heat map plot of peak areas of selected aroma compounds in the course of fermentation.

Octanal (**6**), 1-heptanol (**11**), 2-pentanol (**3**), 1-phenylethyl acetate (**15**) and 2-phenylethanol (22) were already present in the CP-M and showed decreasing intensities during fermentation. It is known that basidiomycetes can also form 2-phenylethanol (**22**) de novo or by biotransformation from asparagine or L-phenylalanine, although this occurs in much lower concentrations than yeasts, for example [18,45]. Özdemir et al. showed the production of 2-phenylethanol by *Lentinula edodes* in wort with an OAV of 1.3 [46]. However, based on the peak area change over time, the contribution to the overall aroma of 2-phenylethanol (**22**) might be attributed to its occurrence in the CP-M. Methyl benzoate (**14**), (*E*)-nerolidol (*27*), 5-butyl-2(*5H*)-furanone (**24**), (*R*)-linalool (**13**), as well as the unidentified substances **18**, **21**, **23**, **25** and **26,** were formed by fermentation. Linalool was also detected in trace amounts in CP-M. However, the content quantified in the beverage could mainly be attributed to the biosynthesis by the fungus. 2-Nonanone (**8**), as well as 2-pentanone (**1**), were already present in CP-M, but the concentrations were increased by fermentation, which indicated that these aroma compounds were formed by the fungus. However, as discussed in Section 3.3, 2-nonanone (**8**) did not contribute to the overall aroma. At the harvest time of 48 h, the highest intensities were detected for 2 pentanone (**1**), 5-butyl-2(*5H*)-furanone (**24**), **18**, **23**, **25** and **26**. For octanal (**6**), 1-phenylethyl acetate (**15**), 2-phenylethanol (**22**), methyl benzoate (**14**), (*E*)-nerolidol (**27**) and (*R*)-linalool (**13**), the intensities were not at the highest level after 48 h, but still at a high level. The formation of methyl benzoate, nerolidol, linalool, 2-nonanone and 2-pentanone by basidiomycetes in submerged fermentations has been described in the literature previously [10,47].

In addition to the identified aroma compounds discussed above, the thus-far-unidentified substances **18**, **21**, **23**, **25** and **26** were also listed in the heat map (Figure 2; related mass spectra cf. Figure S2). These compounds imparted coconut-, passion fruit- and peach-like odor impressions and were assigned to the group of sesquiterpenoids, but they could

not be conclusively identified. The supposed sesquiterpenoids showed their maximum concentrations after 48 h and decreased afterwards (except **21**). Compounds **18**, **23** and **25** (all FD 64) exhibited fruity, coconut-, peach- or passion fruit-like notes. The decrease in intensities in the sensory evaluation after 48 h (Figure 1) was accompanied by the decrease in peak intensities. This may indicate that these substances contribute to the coconut, passion fruit and peach notes. Compound **26** showed an FD factor of 256, indicating a contribution to the overall aroma with spicy, herbaceous and metallic notes. Unfortunately, a final identification of these substances was not possible. To the best of our knowledge, no sesquiterpenoids with these aroma notes from *L. persicinus* have been described in the literature to date, which offers an interesting field of research for the future.

The aroma of submerged cultures of *Laetiporus sulphureus* and *Laetiporus montanus*, close relatives of *L. persicinus*, has been described as seasoning-like and meaty, mainly due to the formation of sotolone, (*E*,*E*)-2,4-decadienal, (*E*,*Z*)-2,4-decadienal as well as some sulfur-containing aroma compounds [23,48]. Therefore, fruiting bodies of *Laetipores* are also called chicken of the woods. The aroma achieved here thus offers great research potential, especially as the aroma formation is dependent on the culture medium (cf. Table S2).

4. Conclusions

This study investigated aroma formation during the fermentation of 10% cocoa pulp in water with *L. persicinus* and the development of a non-alcoholic beverage with an outstanding aroma reminiscent of tropical-fruity notes like passion fruit, mango, peach, and coconut. After 48 h of fermentation, the acceptance of the beverage clearly increased. The main contributors to the overall aroma were (*R*)-linalool with an OAV of 1897, (*E*)-nerolidol with an OAV of 170, 5-butyl-2(*5H*)-furanone with an OAV of 7.4 and 2-phenylethanol with an OAV of 1.4. The generation of novel products from cocoa side streams may contribute to increasing the sustainability of the cocoa sector and generate added value for farmers. Aroma formation by *L. persicinus* in submerged cultures has not been described previously and offers an interesting field of research for the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9060533/s1, Table S1. Concentrations of stock solution and dilution levels of standard addition. Table S2. Odor impressions during the screening in surface cultures on cocoa pulp agar (CPA) and malt extract agar (MEA), as well as the overall rating R and the intensity of the odor I (–very weak/very bad; - weak/bad; 0 medium/neutral; + intensive/good; ++ very intensive/very good). Table S3. Regression curves and regression coefficient R2 of standard additions (n = 2). Figure S1. Chromatogram for determining the chromatographic purity of 5-butyl-2(5H)-furanone (30.333 min), minus blank measurement of the solvent. Purity = 76%. Figure S2. Mass spectra of 18 (a), 21 (b), **23** (c), **25** (d) and **26** (e).

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