Investigations on cocoa by-products with a special emphasis on the cocoa fruit pulp

Cumulative Dissertation

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Sworn declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I consent to the use of an anti-plagiarism software to check my thesis. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ in carrying out the investigations described in the dissertation.

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Place and date Signature

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Abstract

This dissertation explores valorisation technologies for using cocoa fruit pulp and cocoa pod husks as food ingredients. Chapter 1 examined the aroma composition of fresh pulp from four countries of origin, identifying a wide range of aroma-active substances with qualitative variations across different pulps. Pasteurization and ultra-high temperature treatment were studied in Chapter 2 to prolong the shelf-life of cocoa pulp while aiming to maintain its sensory attributes. Enzyme-assisted hydrolyses were investigated in Chapter 3 for cocoa pulp processing, focusing on the influence of temperature, enzyme activity, and enzyme combinations on different physicochemical parameters. In Chapter 4, the fermentation of cocoa pod husks by *Pleurotus salmoneo-stramineus* was delved into to produce a protein- and fibre-rich ingredient, thereby focusing on its techno-functional properties. This dissertation provides valuable knowledge for optimising the utilization of cocoa by-products for food applications and promoting more sustainable practices in the cocoa supply chain.

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1 Summary

The cocoa supply chain is challenged by the production of large amounts of by-products, representing significant economic losses and missed opportunities for farmers and the food sector. The objective of this dissertation was to investigate valorisation technologies for using cocoa fruit pulp and cocoa pod husks as food ingredients.

In Chapter 1, the aroma composition of fresh cocoa pulp from different origins was investigated. The aroma-active substances in pulps from Indonesia, Vietnam, Cameroon, and Nicaragua were identified using aroma extract dilution analyses (AEDA) combined with gas chromatography-mass spectrometry/olfactometry (GC-MS/O). In total, 65 aroma-active substances were determined, of which 36 were found in all pulps. In higher dilutions, Indonesian cocoa pulp had fatty, cheesy, and phenolic notes, while Vietnamese pulp had fatty, green, and smoky notes. Cameroonian pulp was butter-like, popcorn-like, flowery, and fruity, and Nicaraguan pulp extract was fruity and flowery, with hints of honey, clove, and vanilla. This chapter identified significant variability in the aroma composition of cocoa pulps across diverse origins, presenting a broad spectrum of possibilities for food applications. Fresh cocoa pulp's high moisture and sugar contents make it an ideal growth medium for microorganisms, posing a high risk of spoilage. Hence, preservation processes are necessary while sustaining the quality of the pulp. Chapter 2 focused on the pasteurization and ultra-high temperature (UHT) treatment of cocoa pulp and their effects on quality parameters, i.e., sensory properties, dry matter content, water activity, total soluble solids, colour, and peroxidase activity. Both thermal treatments were successful in the inactivation of enzymes. The colour of pasteurized pulp was determined to be akin to fresh pulp, while the UHT-treatment resulted in brownish samples. Sensory properties of fresh, pasteurized, and UHT-treated pulps were characterised by descriptive analysis, AEDA, and GC-MS/O for identification. The fresh pulp exhibited 74 aroma-active substances, while UHT-treated and pasteurized pulp accounted for 66 and 60, respectively. The thermally treated pulps remained stable at 4 °C and 23 °C for 24 weeks without a significant growth of microorganisms. During storage, the rate of nonenzymatic browning was determined to be higher in samples stored at 23 °C compared to those stored at 4 °C. This study demonstrated pasteurization and ultra-high temperature treatment as effective methods for preserving cocoa fruit pulp, extending its shelf life with minimal alterations in sensory quality and offering valuable insights for the preservation of other side-streams. Chapter 3 focused on the use of enzymes to facilitate cocoa pulp processing, for instance in the de-pulping of beans and the subsequent preservation. This chapter describes the effect of temperature, enzyme activity and enzyme combinations on the viscosity, particle diameter ($d_{v,0.9}$), browning index

(BI) and total soluble solids of cocoa pulp by means of Response Surface Methodology and D-optimal design. The enzymes investigated were endo-polygalacturonase (P), endo-cellulase (C) and hemicellulase (H). The reduction in relative viscosity, the $d_{v,0.9}$ and the BI fitted a reduced quadratic model. When used alone, P led to the highest reduction in viscosity, while C was the least effective. Moreover, combinations of P with C and/or H increased the effect on the viscosity. Maximal viscosity reductions by 70% were predicted for 300 U P and 300 U H at 40 °C. The $d_{v,0.9}$ of fresh pulp was 613 µm. When combined, P, C and H reduced the $d_{v,0.9}$ to 418 µm (40 °C, 580 U). Moreover, only the temperature significantly influenced the BI, where higher temperatures led to higher indices. Lastly, the design was successfully validated for the reduction in viscosity and the $d_{v,0.9}$. The study results suggest that P plays the primordial role in the hydrolysis of cocoa pulp, while C and H play more supporting ones.

Chapter 4 studied the fermentation of cocoa pod husks (CPH) by *Pleurotus salmoneo-stramineus* (PSS) to produce a fibre- and protein-rich ingredient, which was investigated after its physicochemical composition and techno-functional properties. Fermentation resulted in a significant increase in protein content from 7.3 g/100 g DM in CPH to 18.9 g/100 g DM. The water and oil binding capacities of the fermented CPH (CPHF) were 3.5 mL/g and 2.1 mL/g, respectively. The particle diameter $d_{v,0,90}$ of CPHF was smaller compared to CPH. Regarding total dietary fibre content, CPHF contained 73.4 g/100 g DM compared to 63.6 g/100 g DM in CPH. The soluble fibre content was lower in CPHF, while the insoluble fibre fraction was higher in CPHF at 71.1 g/100 g DM as to CPH at 53.6 g/100 g DM. To investigate a possible use in food matrices, bread doughs with CPH or CPHF were characterised for texture, colour and farinographic properties. The dough hardness, consistency and browning index increased with the CPH concentration, whereas for CPHF, springiness and peak viscosities declined. This study confirmed the capability of PSS to grow on cocoa pod husks, yielding high-quality protein, conceivably providing cocoa farmers alternative income streams, and fostering a more sustainable cocoa production.

By focusing on the composition of cocoa fruit pulp and cocoa pod husks, this dissertation explored diverse technologies for their valorisation into food ingredients. The aroma analysis highlighted qualitative variations in cocoa pulps, offering versatility for creating a wide range of food products. Moreover, the investigation of stabilisation processes for cocoa pulp demonstrated the successful extension of its shelf life with minimal impact on sensory properties. The enzyme-assisted hydrolyses showcased great potential for the efficient separation and processing of the pulp. Finally, the fermentation of pod husks by a fungal strain revealed a promising approach to upcycle these into protein- and fibre-rich ingredients. Overall, this doctoral thesis contributes valuable knowledge for optimising cocoa by-product utilization, reducing economic losses, and promoting sustainable practices in the cocoa supply chain.

2 Zusammenfassung

In der Kakaolieferkette fallen viele Nebenprodukten an, die für die Bauern und den Lebensmittelsektor erhebliche wirtschaftliche Verluste und verpasste Chancen darstellen. Ziel dieser Doktorarbeit war daher die Untersuchung von Aufwertungstechnologien für die Verwendung von Kakaofruchtpulpe und Kakaofruchtschalen als Lebensmittelzutaten.

Kakaopulpe kann in Lebensmitteln wie Konfitüren, Fruchtzubereitungen und Getränken eingesetzt werden. Im ersten Kapitel dieser Arbeit wurde die Aromazusammensetzung von frischer Kakaopulpe verschiedener Herkunft untersucht. Die aromaaktiven Substanzen in Pulpen aus Indonesien, Vietnam, Kamerun und Nicaragua wurden mittels Aromaextraktverdünnungsanalysen (AEDA) in Kombination mit Gaschromatographie-Massenspektrometrie/Olfaktometrie identifiziert. Insgesamt wurden 65 Substanzen nachgewiesen, von denen 36 in allen Pulpen gefunden wurden. In den höheren Verdünnungen wurde die indonesische Kakaopulpe als überwiegend fettig, käsig und phenolisch wahrgenommen, während die vietnamesische Pulpe hohe FD-Faktoren mit fettigen, grünen und rauchigen Noten aufwies. Die kamerunische Kakaopulpe zeichnete sich durch butterartige, popcornartige, blumige und fruchtige Geruchsqualitäten aus, während der nicaraguanische Pulpenextrakt vor allem fruchtig, blumig mit honig-, nelken- und vanilleartigen Nuancen wirkte. Es konnte nachgewiesen werden, dass die Aromazusammensetzung von Kakaopulpen unterschiedlicher Herkunft stark variiert und somit eine große Vielfalt an Möglichkeiten für Lebensmittelanwendungen bietet. Der hohe Feuchtigkeits- und Zuckergehalt frischer Kakaopulpe macht sie zu einem idealen Wachstumsmedium für Mikroorganismen, was ein hohes Verderbsrisiko darstellen. Daher sind Konservierungsmaßnahmen erforderlich, die die Qualität der Pulpe erhalten. In Kapitel 2 wurden die Pasteurisierung und die UHT-Behandlung von Pulpe aus Kamerun erforscht und ihre Auswirkungen auf die Qualitätsparameter Sensorik, Trockensubstanzgehalt, Wasseraktivität, gesamte lösliche Feststoffe, Farbe und Peroxidase-Aktivität beschrieben. Beide Verfahren inaktivierten erfolgreich die Enzyme. Zudem entsprach die Farbe der pasteurisierten Pulpe derjenigen der frischen, während die UHT-Behandlung zu bräunlicheren Proben führte. Die sensorischen Eigenschaften der frischen, pasteurisierten und UHT-behandelten Pulpen wurden durch eine beschreibende Analyse und die Identifizierung der Aromastoffen charakterisiert. Frische Pulpe wies 74 aromaaktive Substanzen auf, während in UHT-behandelten und pasteurisierten Proben 66 bzw. 60 Aromastoffe identifiziert wurden. Thermisch behandelte Pulpen waren bei 4 °C und 23 °C 24 Wochen lang stabil, ohne dass es zu einem signifikanten Mikroorganismenwachstum kam. Während der Lagerung wurde eine höhere Rate der nicht-enzymatischen Bräunung bei den bei 23 °C gelagerten Proben im Vergleich zu den bei 4 °C

gelagerten Proben festgestellt. Diese Studie zeigte, dass Pasteurisierung und UHT-Behandlung wirksame Methoden zur Konservierung von Kakaopulpe sind, die die Haltbarkeit bei minimaler Veränderung der sensorischen Qualität verlängern und wertvolle Erkenntnisse für die Konservierung anderer Nebenprodukte liefern. Kapitel 3 befasste sich mit dem Einsatz von Enzymen als potenzielle Hilfsmittel für die Verarbeitung von Kakaopulpe, z. B. bei der Entpulpung der Bohnen. Dieses Kapitel beschreibt die Auswirkungen von Temperatur, Enzymkonzentration und Enzymkombinationen auf die relative Viskosität, den Partikeldurchmesser ($d_{v,0,9}$), den Bräunungsindex (BI) und die gesamten löslichen Feststoffe von Kakaopulpe mittels Response Surface Methodology und D-Optimal Design. Die untersuchten Enzyme waren Endo-Polygalakturonase (P), Endo-Cellulase (C) und Hemicellulase (H). Die Verringerung der Viskosität, des $d_{v,0,9}$ und des Bräunungsindex befolgten ein reduziertes quadratisches Modell. Bei der alleinigen Verwendung von P wurde die Viskosität am stärksten reduziert, während C am wenigsten wirksam war. Darüber hinaus verstärkte die Kombination von P mit C und/oder H die Wirkung auf die Viskosität. Maximale Viskositätssenkungen um 70 % wurden für 300 U P und 300 U H bei 40° C vorhergesagt. Der $d_{v,0.9}$ des frischen Materials betrug 613 µm. Bei der Kombination von P, C und H verringerte sich der $d_{v,0.9}$ auf 418 µm (40° C, 580 U). Hinsichtlich des BI der enzymatisch behandelten Proben erwies sich nur der Einfluss der Temperatur als signifikant, wobei höhere Temperaturen zu einem höheren BI führten. Das Design wurde erfolgreich für die Verringerung der Viskosität und des $d_{v,0,9}$ validiert. Die Ergebnisse deuten darauf hin, dass P die wichtigste Rolle bei der Hydrolyse von Kakaopulpe spielt, während C und H eher unterstützend wirken.

Kapitel 4 befasste sich mit der Fermentation von Kakaofruchtschalen (CPH) durch *Pleurotus salmoneo-stramineus* (PSS) zur Herstellung von einer ballaststoff- und proteinreichen Zutat. Die Fermentation führte zu einem signifikanten Anstieg des Proteingehalts von 7,3 g/100 g Trockensubstanz (TS) in CPH auf 18,9 g/100 g TS. Die Wasser- und Ölbindekapazität der fermentierten CPH (CPHF) betrug 3,5 mL/g bzw. 2,1 mL/g. Der Gesamtballaststoffgehalt betrug 73,4 g/100 g TS in CPHF und 63,6 g/100 g TS in CPH. Die Menge der löslichen Ballaststoffe betrug 2,3 g/100 g TS in CPHF und 10,1 g/100 g TS in CPH; die unlösliche Fraktion machte 71,1 g/100 g TS bzw. 53,6 g/100 g TS aus. Um eine mögliche Verwendung in Lebensmittel zu untersuchen, wurden Brotteige mit CPH oder CPHF hergestellt und hinsichtlich Textur, Farbe und farinographischer Eigenschaften charakterisiert. Die Teighärte, die Konsistenz und der BI nahmen mit der CPH-Konzentration zu, während bei CPHF die Elastizität und die Spitzenviskosität abnahmen. Diese Studie bestätigte die Fähigkeit von PSS, auf Kakaoschalen zu wachsen und dabei hochwertige Zutaten zu liefern, die den Kakaobauern alternative Einkommensquellen bieten und eine nachhaltigere Kakaoproduktion fördern könnten.

Diese Dissertation konzentrierte sich auf die Zusammensetzung von Kakaofruchtfleisch und Kakaofruchtschalen und untersuchte verschiedene Verarbeitungstechnologien für deren Aufwertung zu Lebensmittelzutaten. Die Analyse der Aromazusammensetzung zeigte qualitative Unterschiede in

Kakaopulpen auf, die sich für die Herstellung einer breiten Palette von Lebensmitteln eignen. Die Untersuchung von Stabilisierungsverfahren für Kakaopulpe belegte zudem die erfolgreiche Verlängerung ihrer Haltbarkeit bei minimaler Beeinträchtigung der organoleptischen Eigenschaften. Der Einsatz von enzymunterstützten Hydrolysen stellte ein großes Potenzial für die effiziente Trennung und Verarbeitung der Pulpe dar. Schließlich zeigte die Fermentation von Kakaofruchtschalen durch den Pilz *Pleurotus salmoneo-stramineus* einen vielversprechenden Ansatz, um diese in protein- und ballaststoffreiche Zutaten umzuwandeln. Insgesamt liefert diese Doktorarbeit wertvolle Erkenntnisse zur Optimierung der Verwertung von Kakao-Nebenprodukten, zur Verringerung wirtschaftlicher Verluste und zur Förderung nachhaltiger Praktiken in der Kakaolieferkette.

3 List of publications

The work presented in this doctoral thesis is a selection of papers published in international peer-reviewed journals listed below.

3.1 Full Papers

1. Bickel Haase, T.; Schweiggert-Weisz, U.; Ortner, E.; Zorn, H.; Naumann, S. (2021). Aroma Properties of Cocoa Fruit Pulp from Different Origins. *Molecules*, 26, 7618. doi:10.3390/molecules26247618
2. Bickel Haase, T.; Ortner, E.; Zorn, H.; Naumann-Gola, S.; Schweiggert-Weisz, U. (2023) Thermal stabilisation of cocoa fruit pulp — effects on sensory properties, colour and microbiological stability, *Current Research in Food Science*, 7, doi: 10.1016/j.crfs.2023.100549
3. Bickel Haase, T.; Huseini Babat, R.; Zorn, H.; Gola, S; Schweiggert-Weisz, U. (2024) Enzyme-assisted hydrolysis of *Theobroma cacao* L. pulp, *Journal of Agriculture and Food Research*, 18, doi: 10.1016/j.jafr.2024.101466.
4. Bickel Haase, T., Klis, V., Hammer, A. K., Pinto Lopez, C., Verheyen, C., Naumann-Gola, S., & Zorn, H. (2024). Fermentation of cocoa pod husks with *Pleurotus salmoneo-stramineus* for food applications. *Food Science & Nutrition*, 00, 1–16. doi: 10.1002/fsn3.3937

3.2 Further scientific contributions resulting from the same period

3.2.1 Oral presentations

1. Bickel Haase, T.; Schweiggert-Weisz, U.; Ortner, E.; Zorn, H.; Naumann, S.: Influence of Origin and Thermal Processing on the Aroma Quality of Cocoa Fruit Pulp for its Use as a Food Ingredient, 2nd International Symposium on Cocoa Research (ISCR), Book of Abstracts, p. 304, Montpellier, France, December 5th-7th, 2022.

3.2.2 Poster presentations

1. Bickel Haase, T.; Klis, V.: Complete utilization of cocoa fruits for innovative food products and ingredients: Fermentation substrate from cocoa pod husks for mushroom cultivation & Usage of the cocoa pulp as food, “Whistleblower CHOCO TEC”, ZDS Die Süßwaren-Akademie, online conference, February 9th, 2021.

2. Bickel Haase, T.; Schweiggert-Weisz, U.; Ortner, E.; Zorn, H.; Naumann, S.: Cocoa pulp, from by-product to versatile food ingredient, 10th Round Table Cocoa, Hamburg, Germany, June 2nd, and 3rd, 2021.
3. Bickel Haase, T.; Schweiggert-Weisz, U.; Ortner, E.; Zorn, H.; Naumann-Gola, S.: Effects of thermal stabilization and enzyme assisted hydrolysis on cocoa fruit pulp for food applications, 36th EFFoST International Conference 2022 "Shaping the Production of Sustainable, Healthy Foods for the Future", Dublin, Ireland, November 7th – 9th 2022.
4. Bickel Haase, T.; Schweiggert-Weisz, U.; Ortner, E.; Zorn, H.; Naumann-Gola, S.: Tasty Cocoa Pulp — Effect of thermal processing on the aroma composition "CHOCO TEC 2022", ZDS Die Süßwaren-Akademie, Cologne, Germany, December 13th – 15th 2022,

3.2.3 Presentations for science communication

1. Bickel Haase, T.: Project CocoaFruit: Separation, characterization and thermal stabilization of cocoa pulp, 56. IVLV Arbeitsgruppensitzung Schokoladentechnologie 2021, Online conference, June 29th and 30th, 2021,
2. Bickel Haase, T.: Multitalent Kakaofrucht — mehr als nur Schokolade!, 2. Freisinger Innovationstag, TUM Campus Weihenstephan, Freising, Germany, July 2nd 2022
3. Bickel Haase, T. Complete valorization of cocoa fruits – is it possible? 52nd AK Schoko, Hybrid conference, Zürich, Switzerland, March 23rd, 2023.

3.2.4 Publications for science communication

1. Klis, V.; Bickel Haase, T.: Konfitüre – Müsliriegel –Wurst: Ganzheitliche Nutzung der Kakaofrucht (2022). Rundschau für Fleischhygiene und Lebensmittelüberwachung, 5/2022, p. 150-152
2. Bickel Haase, T.: Interview: Was macht eigentlich…? Nebenströme der Kakaobohnenproduktion, Lebensmittelchemie 76, 161–204 (2022), p. 168-170 https://doi.org/10.1002/lemi.202200504
3. Vom Nebenstrom zu schmackhafter Lebensmittelzutat - Kakaopulpe, Food Lab 2022, 5/2022, p. 8-11 https://blmedien.aflip.in/Food-Lab_05_2022.html#page/
4. Bickel Haase, T.; Rothkopf, I.: *Theobroma cacao* L. (2023), Journal Culinaire, No. 37, ISBN 978-3941121-37-9, p.113 - 120

4 List of abbreviations

Abbreviation	Term
AEDA	Aroma extract dilution analysis
ANOVA	Analysis of variance
C	Endo-cellulase
cAEDA	Comparative aroma extract dilution analysis
CPH	Cocoa pod husks
CPHF	Cocoa pod husks fermented
DCM	Dichloromethane
DM	Dry matter
DoE	Design of experiments
EFSA	European Food Safety Authority
FD	Factor of dilution
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
GC-O	Gas chromatography olfactometry
H	Hemicellulase
Lb.	Lactobacillus
m/z	Mass-to-charge ratio
MS	Mass spectrometry
ODP	Odour detection port
OAV	Odour activity value
P	Endo-polygalacturonase
PSS	*Pleurotus salmoneo-stramineus*
RI	Retention index
SAFE	Solvent Assisted Flavour Evaporation
SBSE	Stir Bar Sorptive Extraction
SPME	Solid-phase microextraction
TF	Traditional food
TDF	Total dietary fibre
SDF	Soluble dietary fibre
IDF	Insoluble dietary fibre
TPA	Texture profile analysis
VOC	Volatile organic compound

5 General introduction

5.1 *Theobroma cacao* L.

Theobroma cacao L (theos gr. = god, broma gr. = food, "food of the gods") or cocoa is an evergreen tree from the family of *Malvaceae*. It originated in the Amazon basin, yet it is often believed to have been domesticated in Mesoamerica by the Mokaya people (Patiño 2002; Powis et al. 2007). In the 1520s, colonisers returning from the New World introduced cocoa as a bitter beverage into Europe. After the incorporation of sugar and milk, as well as the subsequent widespread of chocolate, the Spanish monopoly on cocoa production became uncertain, and cocoa cultivation soon expanded to the Italian, Dutch and Portuguese colonies. (Minifie 1981).

Cocoa is a shade-loving crop that grows up to a size of 10 – 15 m but is usually cut to 4 – 8 m to ease the harvest of cocoa fruits (also referred to as cocoa pods) (Figure 1). The cocoa flowers grow on the trunk and branches (cauliflory) and are pollinated artificially or by the *Forcipomyia* flies. After pollination, the flowers develop into fruits. Cocoa fruits need around 5–6 months to be fully developed and will most likely be 15 – 30 cm long and 300 – 500 g in weight. However, cocoa pods can vary considerably between varieties, origin, and season. Ripe cocoa pods contain approximately 20 – 60 seeds. Each cocoa tree carries 20 to 50 fruits per harvest, depending on the region, climate, variety, and age of the tree. These begin to bear fruits after 3 years and can produce fruits until the age of around 40 years, when they need to be replaced (Beckett et al. 2017). Tropical climates with temperatures around 18 to 32 °C, humidities between 70 – 90%, altitudes below 1000 m as well as sufficient rainfall throughout the year (1000 – 4000 mm) are necessary for the cultivation of cocoa. Therefore, cocoa plantations lie primarily between 20° north and 20° south of the Equator (Afoakwa 2010). In most producing countries, the cocoa harvest takes place twice a year. The main harvest season is around October and a smaller harvest is carried out around March (Beckett et al. 2017).

Figure 1 Cocoa tree with cocoa pods growing on the tree stem and the branches (Reproduced with permission of the Fraunhofer Institute for Process Engineering and Packaging IVV).

5.2 Cocoa production and trade

Cocoa is a crucial agricultural export and a significant part of the economies of various countries in West Africa, South America, and South-East Asia (Afoakwa 2010). The International Cocoa Organization reported a worldwide cocoa bean production of around 4.9 million tonnes in the 2021/2022 cocoa season (ICCO 2022). Currently, the seven largest producers are Côte d'Ivoire (Ivory Coast), Ghana, Ecuador, Cameroon, Nigeria, Brazil, and Indonesia. They account for more than 90% of the world's production (Figure 2) (ICCO 2022). In the last decades, the largest cocoa importers were the Netherlands, Germany, and the United States, importing 0.99 Mt, 0.44 Mt, and 0.37 Mt, respectively, in 2020. However, there has been an increase in the demand for cocoa beans from new emerging markets such as Malaysia (0.38 Mt in 2020) (Bermudez et al. 2022).

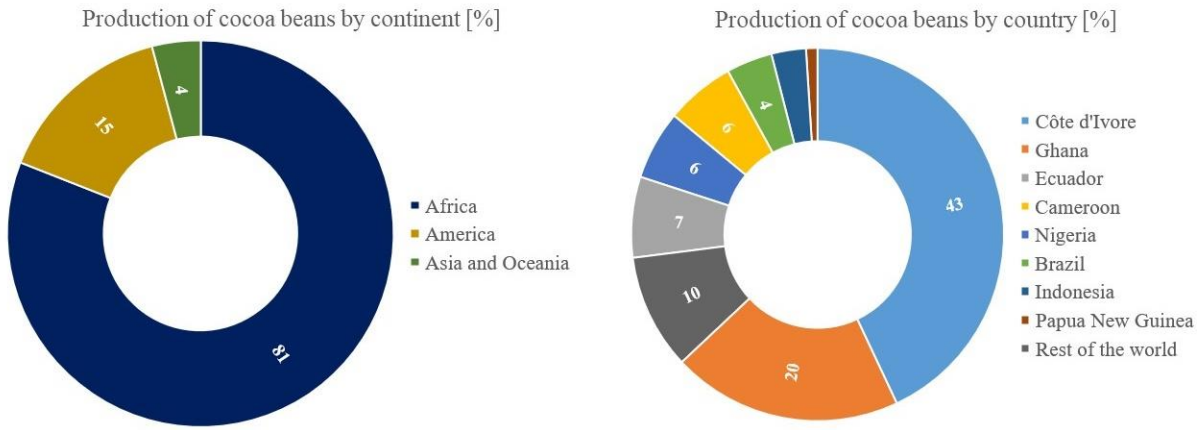

Figure 2 Production of cocoa beans in the cocoa season 2021/2022 as reported by ICCO (2022)

The cocoa sector provides livelihoods for approximately 50 million people worldwide. An estimate of 6 million farmers grow cocoa and 90% of cocoa farms comprise areas of five hectares or less. An estimated 70% of the grown cocoa is produced by smallholders, who often live on less than US$ 2 per day and depend on cocoa for up to 90% of their income (Bermudez et al. 2022). Farmers are the first actors in the cocoa supply chain, which ends with billions of consumers buying the finished chocolate and cocoa-containing products via retailers. The supply chain is primarily prevailed by the cocoa processors and traders, comprising a rather small number of companies with an entrenched influence on the whole supply chain (Voora et al. 2019; Fountain and Huetz-Adams 2020). Due to the large bargaining power of these companies, farmers may be reduced to the role of 'price takers', limiting their access to financial support, market information and agricultural aid (Gayi and Tsowou 2017).

The cocoa sector faces further challenges. One of them is the huge loss of biomass prior to harvest. An estimated 30% of the world's cocoa is lost to pests and diseases (Beckett et al. 2017). An additional challenge is the conversion of farms to other crops as a result of cocoa farmers' low incomes, making cocoa compete with commodities like palm oil, coffee, rubber, citrus and cloves (Gayi and Tsowou 2017; Beckett et al. 2017). Furthermore, climate change is predicted to negatively affect the cocoa value chain, as some regions will show lower cocoa productivity or even become unsuitable for its cultivation (Bunn et al. 2017). Further factors leading to changes in the cocoa supply chain can be of a political and social nature (Bermudez et al. 2022). For instance, international conflicts have led to reduced availability of fertilisers, sharply increasing their prices and making them unaffordable for many farmers (Confectionery Production 2022; Tridge 2021; Global Agriculture and Food Security Program 2021). Strong changes in cocoa prices create risks for all actors of the value chain. However, farmers are at the highest risk, as they cannot regulate the market prices, are at the mercy of weather events and increasing production costs, and are less flexible in their supply to varying price fluctuations (Hütz-Adams and Schneeweiß 2018). Therefore, valorisation technologies for the cocoa by-products are imperative to reduce economic losses, provide cocoa farmers alternative income streams and foment a more sustainable cocoa production, while offering interesting ingredients to the food sector.

5.3 Cocoa by-products

The transformation of cocoa beans into cocoa liquor for chocolate-making takes place in two successive stages: the pre-processing and the post-processing (Castro-Alayo et al. 2019). Along the chain, several side streams accumulate (Figure 3). Upon opening the cocoa fruits, the cocoa pod husks (CPH) and the cocoa placenta are the first fractions to be discarded. CPH, representing the major fraction of the fruit and making up to 80% of the total dry weight, are either discarded in the fields or burned by the farmers (Lu et al. 2018). Fungi are the main responsible for the breakdown of CPH due to their capacity to metabolise complex fibrous structures (Rahim et al. 2015). Nonetheless, the accumulation of larger amounts of CPH-biomass, often in combination with humid climatic conditions, can foment the

proliferation of phytopathogenic fungal strains (Watson et al. 2013). This poses a risk of infection to healthy cocoa trees and, ultimately, a reduction in the cocoa yield. Next in the processing chain, the wet cocoa beans are fermented. A spontaneous fermentation process begins immediately as cocoa fruits are opened (Chagas Junior et al. 2020). A sour-sweet juice exudes from the fresh beans during the processing in the field, transportation to the fermentation sites, and is principally drained during cocoa fermentation (Santos et al. 2014). This juice is known as cocoa honey and is sometimes referred to as cocoa sweating, exudado, or as miel de cacao or mel du cacau for its translation to Spanish and Portuguese (Santos et al. 2014; Balladares et al. 2016; Vuyst and Leroy 2020; Díaz-Muñoz et al. 2020). The term cocoa honey refers primarily to the liquid fraction of the cocoa mucilage, distinct from the cocoa pulp, which denotes the whole unfermented mucilaginous layer around fresh cocoa beans (Guirlanda et al. 2021b). The collection and valorisation of cocoa honey to foods was presumed to be economically viable. In estimation, a farm with an average productivity of 300 kg of dry cocoa seeds per ha could generate 0.59 kg of cocoa honey per kg of dry cocoa seeds (Anvoh et al. 2009; Kongor et al. 2018). Nonetheless, the collection of cocoa honey is challenged by its rapid quality decay due to the high microbial loads on the fruits and in the surroundings. Therefore, in rural cocoa-producing communities reliant on manual labour, scaling up the cocoa honey collection would demand advancements such as using stainless steel equipment to improve efficiency and minimize microbiological contaminations (Guirlanda et al. 2021a, 2021b). In addition, the ongoing fermentation of the fresh cocoa beans causes the pulp to be liquefied and lost on farms. Adding to the loss of a valuable raw material, its release can contaminate the soil and water, as well as increase the risk of pest infestation (Dwapanyin 1991). In contrast to cocoa honey, collecting cocoa pulp follows a more controlled and less artisanal process, frequently involving the use of industrial equipment and designated collection rooms for better hygiene management (Schwan, Lopez 1988; Guirlanda et al. 2021a). Additional steps, such as washing the cocoa fruits prior to opening and the subsequent preservation of the pulp, can be more easily incorporated, contributing to higher pulp's shelf-life. Further along the chain, the fermented and dry cocoa beans are cleaned to remove foreign materials. This is followed by a heat pre-treatment and/or a roasting step. Subsequently, the beans are processed to remove the shells, which also accrue as by-products. The cocoa bean shells are rich in dietary fibre and have gained attention for their potential in the chemical, food, and environmental industries, as well as for their health benefits (Soares and Oliveira 2022; Martínez et al. 2012). Among the cocoa by-products, the bean shells have been described to contain the highest protein content, making them a potential source of protein for animal and human nutrition (Soares and Oliveira 2022). Lastly, after de-shelling the beans, the obtained cocoa nibs are ground to cocoa mass for use in chocolate and confectionary (Beckett et al. 2017).

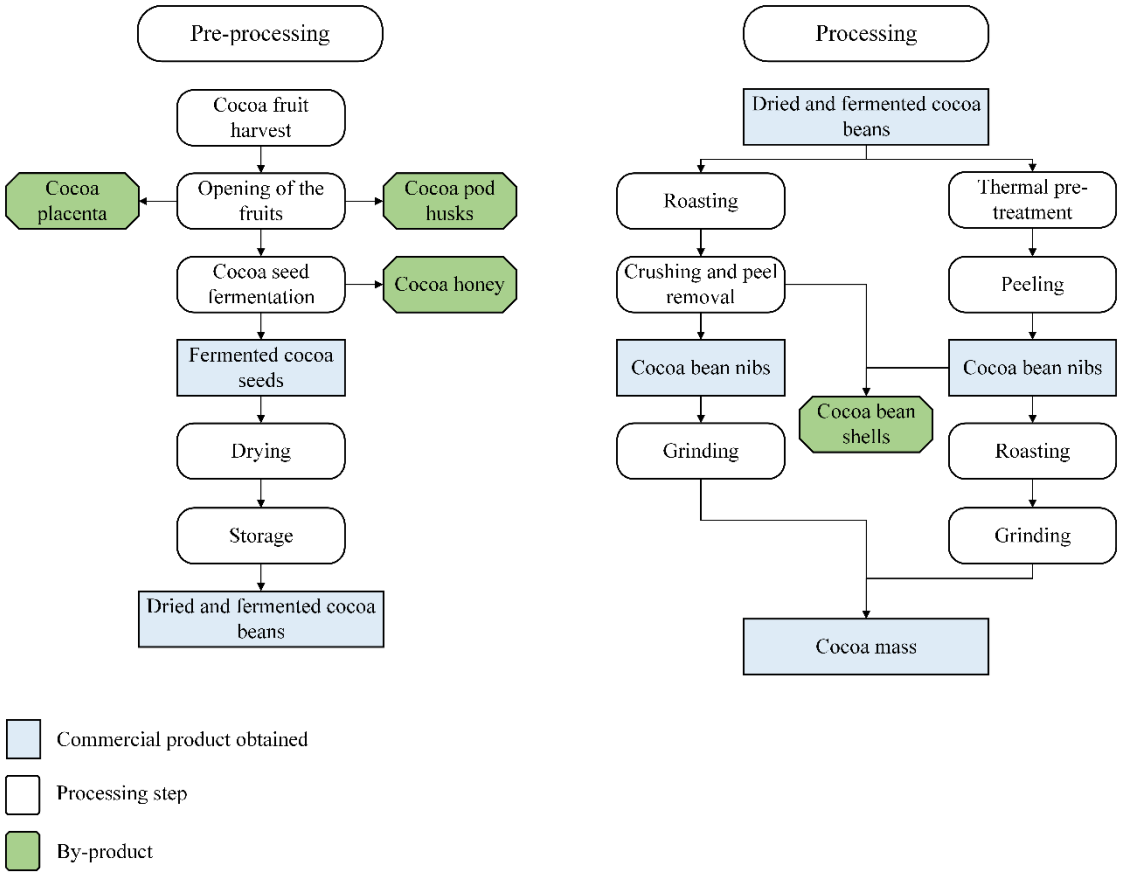

Figure 3 Pre-processing and processing of cocoa fruits from the harvest to finished products (adapted from Soares and Oliveira 2022)

5.4 Cocoa pulp

5.4.1 Chemical composition and possible uses

The mucilaginous pulp is made of spongy parenchymal cells, which contain sap cells rich in sugars, citric acid, and salts. Cocoa pulp is acidic and has been attributed pH-values below 3.9. It contains approx. 79 - 86% water, 11-13% sugars (D-glucose, D-fructose and sucrose), 0.77–2.50% non-volatile acids and 0.02–0.04% volatile acids (Roelofsen 1958; Pettipher 1986; Schwan and Wheals 2004; Igbinadolor and Onilude 2013). The pulp's major polysaccharides are pectin (3.7-6.6%), hemicellulose (1.5-2.8 %) and cellulose (4.7-5.1%) (Pettipher 1986). The pectin fraction comprises 62.5% (*w/w*) galacturonic acid, with 4.9% arabinose, 16.3% galactose, 4.1% glucose, 1.4% xylose, 2.6% mannose, and 8.2% rhamnose (Oloye et al. 2023). The total dietary fibre in cocoa pulp amounts to approximately 16.9 g per 100 g of dry matter and mainly consists of soluble dietary fibre (approximately 95%) (Martínez et al. 2012). The protein and ash contents are low in cocoa pulp and range between 0.42–0.50% and 0.40–0.50%, respectively (Igbinadolor and Onilude 2013). Cocoa pulp has been reported to

exhibit some antioxidant activity and possesses a content of phenolic compounds of 103.76 mg gallic acid equivalents in 100 g of pulp (Endraiyani et al. 2017).

In some cocoa-producing countries, e.g. Brazil, the consumption of cocoa pulp, cocoa honey and foods made therewith has been reported. Figueira et al. (1993) described the incorporation of cocoa pulp into jellies, alcoholic beverages, vinegar, and processed juices. Additionally, cocoa pulp has been integrated into fermented beverages, such as probiotic drinks (Puerari et al. 2012; dos Santos Filho et al. 2019), wine (Dias et al. 2007) and beer (Nunes et al. 2020). As in its report of October 10th 2019, under Article 14, the European Food Safety Authority (EFSA) approved cocoa pulp —subjected to pasteurization and freezing— as a traditional food (TF) for use within Europe as fruit pulp for final consumers or as fruit ingredient for fruit preparations, confectionary, juices and edible ice cream (European Food Safety Authority 2019). Furthermore, in Europe, the chocolate industry has used cocoa pulp to replace the added sugar in chocolate-like products (Vieira et al. 2018; Alfred Ritter GmbH & Co. KG 2021; Lindt und Sprüngli GmbH). Additionally, the company Hang zur Sonne GmbH launched a fizzy cocoa pulp drink, a cocoa pulp ice tea and a pulp sparkling wine to the German market (Hang zur Sonne GmbH 2023). In the Netherlands, a cocoa pulp-based beverage is commercialised under the name “Kumasi” (Kumasi Drinks 2023). Moreover, in Switzerland, the company Koa commercialises cocoa pulp juice produced in Ghana (Koa 2022).

5.4.2 Aroma composition of cocoa fruit pulp

Cocoa pulp has been described to have a pleasant fresh-fruity and floral flavour, making it highly interesting for food applications (Pino et al. 2010; Soares and Oliveira 2022). Nonetheless, the sum of literature focusing on the aroma composition of cocoa pulp, especially on the fresh material, remains scarce, as the focus has been mainly set on the cocoa beans and the role of the pulp during fermentation (Chetschik et al. 2018). Interestingly, the floral or fruity character of the cocoa fruit pulp has been linked to the fine flavour attributes of the beans. It was proposed that aroma-active substances migrate into the seeds during fermentation and are detectable in the beans post-fermentation and drying, thereby influencing the final chocolate flavour quality (Eskes et al. 2007; Eskes et al. 2018; Ali et al. 2014). Amongst the fine flavour attributes in cocoa beans, some substances can be traced back to the cocoa fruit pulp, which contains fluctuating concentrations of terpenes, alcohols and esters (Pino et al. 2010; Kadow et al. 2013).

Pino et al. (2010) first reported the volatile composition of fruit pulps from the *Theobroma* genus from Colombia, including cocoa pulp, by headspace solid-phase microextraction gas chromatography mass spectrometry (HS-SPME CG-MS). The authors identified sixty-six different volatile organic compounds (VOCs), with the aroma-active VOCs 2-heptyl acetate, 2-pentyl acetate and linalool detected in the highest concentrations. Kadow et al. (2013) quantified the VOCs of pulp and beans of

two fine flavour cocoa varieties (SCA6, EET62) and a bulk cocoa type (CCN51), linking also fine or flavour cocoa bean notes to the pulp's flavour characteristics. Monoterpenes found in SCA6 as well as methylketones, secondary alcohols and their respective esters, found in EET62, were shown to be prospective fine aroma substances originating in the pulp. Further flavour substances found in cocoa pulp are linalool and *β*-ocimene (Kadow 2020). Hegmann et al. (2020) investigated how the season and ripening stage affect the pulp aroma of five cocoa varieties from Costa Rica using HS-SPME GC-MS. The authors showed that the total aroma diversities and the aroma intensities of the pulps increased during ripening. Moreover, during the dry season, the aroma profiles of the pulps were more diverse, whereas the aroma intensity was higher in the wet season. The authors also reported a 10-fold increase in the concentration of linalool in cocoa pulp obtained during the rainy season.

In the aforementioned studies, the actual contribution of the identified volatiles to the overall aroma of cocoa pulp wasn't confirmed through gas chromatography-olfactometry (GC-O) combined with dilution to odour threshold techniques, such as aroma extract dilution analysis (AEDA). This helps identify aroma compounds with low odour thresholds that may be overlooked by the concentration-to-threshold ratio concept if the sensory aspect in the experimental setting is not taken into account (Blank 2002). To better understand the aroma quality of cocoa pulps and their influence on the beans during fermentation, Chetschik et al. (2018) investigated the aroma-active substances in three cocoa pulps by AEDA and GC-MS/O. Experiments were carried out with two cocoa pulp varieties from Costa Rica (UF654 and CCN51) and one from Colombia (FSV41). AEDA showed 37 aroma-active substances in the three cocoa pulps. Regarding the flavour dilution (FD) factors, the cocoa pulp of CCN51, often considered as bulk cocoa, was perceived with lower aroma intensities than the varieties FSV41 and UF564, which were perceived with more intense floral and fruity notes. In addition to linalool, 2-phenylethanol can contribute to the floral aroma of fresh cocoa fruit pulp. The authors described concentrations of 250 $\mu g \cdot kg^{-1}$ of 2-phenylethanol and 14 $\mu g \cdot kg^{-1}$ of linalool in fresh cocoa pulps. Even though linalool was determined in fresh pulp, the concentration did not surpass the threshold concentration and, seemingly, only 2-phenylethanol influenced the floral aroma. The substance 3-methylbutyl acetate (isoamyl acetate) was determined to be present above the threshold concentration, which added a fruity character to the pulp. Moreover, the same study confirmed that the concentration of aromatic alcohols, terpenes, and esters may vary during the fermentation process. Linalool was shown to be produced in the pulp and the beans, with concentrations increasing with the fermentation time (Chetschik et al., 2018). In a more recent study, Klis et al. (2023) reported a total of 32 aroma compounds in Ecuadorian cocoa pulp, which were detected olfactometrically by means of GC–MS/MS–O. While linalool and 2-phenylethanol were confirmed, the substances ethyl acetate, acetoin, 1-octanol, hexanoic acid and decanoic acid were described for the first time in cocoa pulp. Moreover, a panel described cocoa pulp with the attributes acidic, fruity, citrus and apple-like, highlighting its

potential for non-alcoholic beverages (Klis et al. 2023). Therefore, understanding the flavour of cocoa pulps, as well as how this is affected by the pulps' processing, is key to its success as a food ingredient.

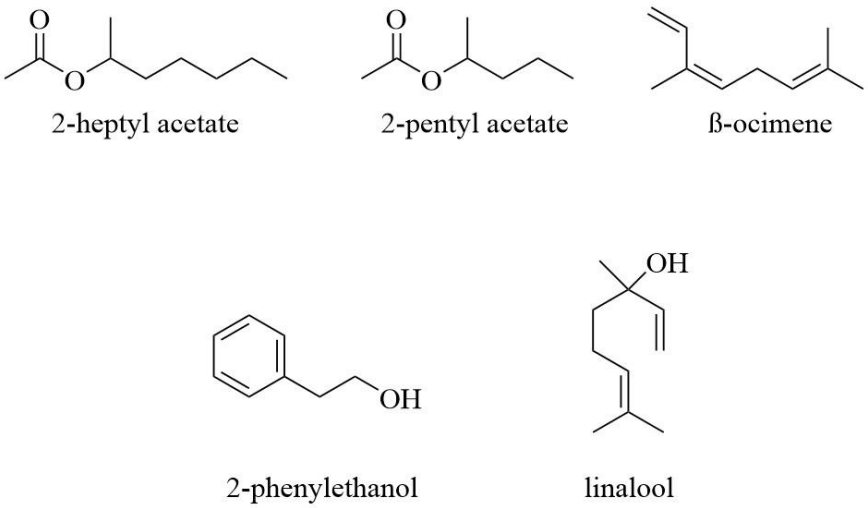

Figure 4 Aroma compounds found in fresh cocoa pulp from different origins.

5.4.3 Processing of cocoa fruit pulp

Although cocoa pulp is necessary for the fermentation of fresh cocoa beans, previous reports suggest that a fraction can be removed from these without having negative effects on their later quality (Schwan, Lopez 1988; Bangerter et al. 1991; Amanquah 2013). Particularly, excessive amounts of pulp can lead to over-acidification and a reduced bean quality. For this reason, researchers have previously studied various pulp preconditioning methods, including pod storage, bean spreading, and the partial de-pulping of fresh beans (Afoakwa et al. 2013; Rohan 1963; Schwan and Wheals 2004). Mechanical de-pulpers, commonly used in the processing of various fruits, make use of a rotating shaft pressing the fruit material against a cylinder with perforations, separating seeds and pulp based on size (Lozano 2006; Ridho et al. 2016). Commercial de-pulpers, as used in the cocoa juice industry, can remove 17-20% of the fresh seed weight (Passos et al. 1989). Nevertheless, mechanical de-pulpers present some disadvantages, as they can potentially damage the fruit seeds and result in incomplete pulp separation, resulting in pulps with inhomogeneous consistencies and broken seeds (Ridho et al. 2016). Therefore, in the processing of cocoa fruits, de-pulpers may leave loose pulp on the seeds, impairing the aeration of the fermenting cocoa bean mass, causing under-fermentation or leading to prolonged fermentation times (Schwan and Wheals 2004). A possible approach to overcome these challenges is the use of cell-wall degrading enzymes, a common practice in the processing of fruits (Aziz et al. 2009; Srivastava and Tyagi 2013). Pectinolytic, hemicellulolytic or cellulolytic enzymes may help break down the polysaccharides in cocoa pulp, reducing viscosity and facilitating its separation. Studies have reported that the addition of a technical pectinase in a 0.2% (*w/w*) solution increased the cocoa pulp yield by approximately 23% compared to mechanical de-pulping. This enzymatic treatment also reduced the

total fermentation time and the acidity of cocoa beans (Freire et al. 1990; Freire et al. 1996). The breakdown of pectin chains by enzymes may lead to a decrease in viscosity, making the pulp suitable for post-processing applications such as pasteurized juices, soft drink production, or the development of concentrates (Meersman et al. 2017; Nunes et al. 2020).

In spite of the cocoa pulp's inherent acidity limiting the growth of microorganisms to lactic and acetic bacteria, moulds, and yeasts, its shelf-life is short. (Vuyst and Weckx 2016). The preservation of cocoa fruit pulp remains a significant operational challenge due to its high moisture and sugar content, as well as the elevated microbial load, naturally found on cocoa fruits together with poor artisanal post-harvest conditions in farms (Vuyst and Weckx 2016; Soares and Oliveira 2022). Recent findings by Firdaus et al. (2022) highlighted the importance of preserving cocoa pulp, showing that untreated cocoa pulp syrups exhibited limited safety for consumption after five days. Therefore, mild processing technologies for preserving the pulp (and the derived products) with minimal effect on its characteristics are required. In light of this, the potential of thermal technologies like pasteurization, a method effective for products with a pH value of 4.5 or less (Ashurst et al. 2017), was described in the past. In cocoa-producing countries, to preserve fresh cocoa honey and prevent its degradation during transport before processing, it can be frozen at temperatures from -18 to -20 °C (Santos et al. 2014). Prior to freezing, cocoa honey may be pasteurized at 70 °C for 20 minutes (Leite et al. 2019). Alternatively, the processing and commercialisation of cocoa honey may involve a homogenization step and a moderate pasteurization at 77°C for 1 minute (Ortiz and Jaimes Jaimes 2005). Subsequently, preservatives sorbate and benzoate are added, followed by packaging and rapid cooling at 10 °C for 8 minutes. Moreover, Quimbita et al. (2013) suggested the use of pasteurization processes for the thermal stabilisation of cocoa honey under similar operational conditions (77 or 85 °C for 1 minute and 88 °C for 15 seconds). This process included an additional chemical stabilisation with sodium metabisulfite and ascorbic acid to prevent browning. Pasteurization was also investigated with a focus on its effect on the total phenolic content of cocoa pulp (Endraiyani et al. 2017). This study revealed minimal changes in the total phenolics during an 8 week storage at different temperatures, with colder storage conditions showing better preservation of the quality. However, the aforementioned studies did not assess the effects of thermal stabilisation on the organoleptic quality and aroma composition of cocoa pulps, highlighting an evident gap in knowledge and underscoring the necessity for subsequent studies.

5.5 Cocoa pod husks (CPH)

For every ton of cocoa beans, up to ten tons of cocoa pod husks (CPH) are generated (wet weight) (Vriesmann et al. 2011). CPH comprise the epicarp, mesocarp, endocarp, and a sclerotic portion (Figure 5) (Campos-Vega et al. 2018). Regarding their composition, dried CPH contain low amounts of fat and protein. Reports on CPH indicated 76.6 g/kg crude protein in the dry matter, 325 g/kg crude fibre and 101 g/kg ash (Sobamiwa and Longe 1994; Vriesmann et al. 2011; Martínez et al. 2012). The

carbohydrate and fibre fraction of CPH include 19.7-26.1% cellulose, 8.7-12.8% hemicelluloses, 14-28% lignin, and 6.0-12.6% pectin (Donkoh et al. 1991). The high amount of lignin in CPH leads to a woody texture, which makes them hardly digestible for humans and complicates their utilization in food products (Vásquez et al. 2019). Among the CPH fractions, the epicarp is the most lignified layer and contains the highest amounts of total ash, calcium, potassium, and phosphate. Moreover, the mesocarp exhibits the highest content (about 50%) of crude fibre and cellulose, whereas the endocarp contains about 60% of the pectins (Sobamiwa and Longe 1994). Pectin can be extracted from CPH through various methods, such as hot water or citric acid extraction (Vriesmann et al. 2011; Vriesmann et al. 2012). Depending on the extraction method, the degree of pectin esterification, and acetylation may vary, affecting its viscosity and gel-forming properties (Yapo and Koffi 2013). Diverse applications for the pectin extracted from CPH have been proposed, e.g. as an emulsifier, elastic coating for food, cosmetics, and pharmaceutical products, as well as a stabiliser, texturizer, or lecithin substitute in foods (Bernaert et al. 2020). Some more applications for the use of the pod husks exist. For instance, CPH have been used in animal feed (Sobamiwa and Longe 1994; Donkoh et al. 1991), as a feedstock for soap making (Gyedu-Akoto et al. 2015), and used as an alternative energy source (Agyeman and Oldham 1986). Other applications of CPH include the incorporation into food systems (Vriesmann et al., 2011) and the biofuel production (Vásquez et al., 2019). Nevertheless, the use of cocoa pods for human nutrition and as food ingredients has not yet been fully explored, urging the need for additional studies with a focus on the valorisation of CPH. This could provide cocoa farmers with new sources of income while encouraging better waste management practices on the farms.

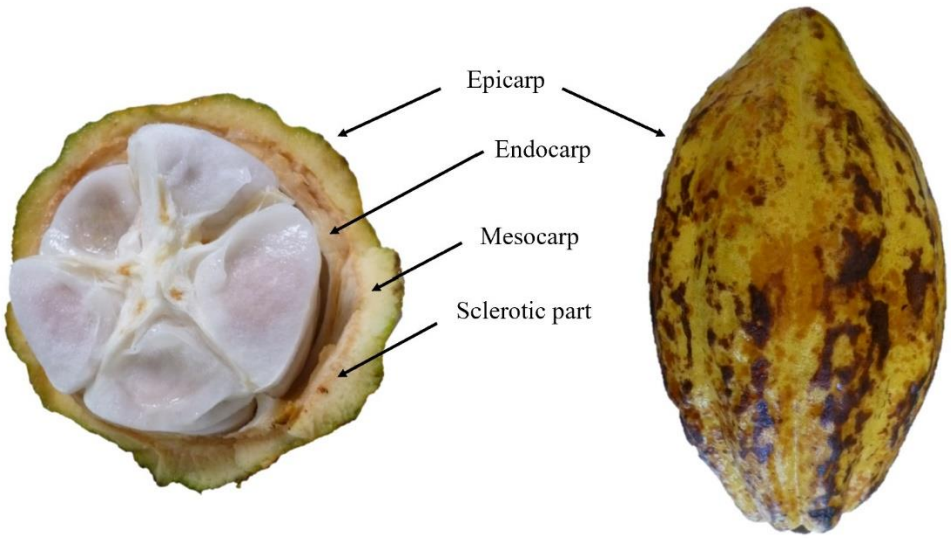

Figure 5 Cross-section of a fresh cocoa fruit, showing the individual parts of the cocoa fruit husk, including epicarp, mesocarp, sclerotic part, and endocarp (Reproduced with permission of the Fraunhofer Institute for Process Engineering and Packaging IVV).

6 Objective of the thesis

While cocoa beans possess commercial importance, the cocoa fruit pulp and the cocoa pod husks have traditionally been considered by-products. The principal objective of this doctoral thesis is to explore valorisation technologies for these and characterise them for possible uses as food ingredients, aiming to contribute to sustainable practices in the cocoa supply chain. This thesis is divided into four chapters, three focusing on the pulp and one on the pod husks.

A broad variety of pulp-based products in the producing countries emphasizes cocoa pulp's promising application potential in the European food sector, largely due to its appealing tropical flavour. However, the emerging cocoa pulp market misses a comprehensive understanding of the influence of origin on the pulp's quality. In the first chapter of this dissertation, the focus is set on investigating how the origin of cocoa fruits influences the aroma composition of the pulp. This chapter aims to identify the aroma-active compounds in cocoa pulps from different origins, laying a foundation for developing tailored food products for diverse markets and consumer preferences.

A significant challenge in bringing cocoa fruit pulp to the European market is its short shelf-life, making it susceptible to spoilage and a potential hazard for consumers. The impact of processing on cocoa pulp's sensory quality and microbial stability for food applications remains largely unknown. Addressing the challenge of the short shelf-life of cocoa pulp, Chapter 2 explores the effects of widespread stabilisation technologies, such as pasteurization and UHT-treatment, on the sensory quality, colour, and microbial stability of cocoa pulp, identifying their suitability in the preservation of the pulp for transport and commercialisation.

Investigating effective de-pulping and processing technologies for cocoa pulp is vital to optimise yields and minimize waste. However, conventional fruit de-pulpers often struggle to remove the tightly adhering mucilaginous layer without compromising bean quality. Recognising the need for efficient processing technologies for cocoa pulp, Chapter 3 investigates the influence of cell-wall degrading enzymes (endo-polygalacturonase, endo-cellulase, and hemicellulase), activities, and incubation temperature on the pulp's relative viscosity, particle diameter and distribution, colour, and total soluble solids content, as a mean to facilitate the pulp's recovery and later processing.

Fungi play a key role in degrading cocoa pod husks (CPH), but the uncontrolled accumulation of CPH-biomass, particularly in humid climates, can promote the proliferation of phytopathogenic fungal strains. Chapter 4 explores the potential transformation of cocoa pod husks, characterised by woody

structures, into protein- and fibre-rich ingredients through fungal fermentation, concentrating on the techno-functional properties of the resulting ingredients. This approach aims to make cocoa pod husks available for human nutrition and food products.

Concisely, this doctoral thesis endeavours to advance the understanding and application of valorisation technologies for cocoa by-products, encouraging sustainability, minimising waste, and creating new opportunities for farmers and food producers.

Chapter 1: Aroma Properties of Cocoa Fruit Pulp from Different Origins

Summary: Various cocoa pulp-based products already exist in cocoa-producing countries, demonstrating great potential for its utilization in Europe. Nonetheless, information on the influence of origin on the pulp's quality for diverse food products is missing. In this chapter, the aroma-active substances of fresh cocoa fruit pulp from different origins, namely Indonesia, Cameroon, Vietnam, and Nicaragua, were studied by means of aroma extract dilution analyses in combination with gas chromatography-mass spectrometry/olfactometry for identification. The identified aroma-active compounds were corroborated using HS-SPME-GC-MS/O and SBSE-GC-MS/O. Overall, 65 different aroma-active substances could be determined with factors of dilution (FD) ranging between 2 and 1024. The odorants *trans*-4,5-epoxy-(*E*)-decenal, 2- and 3-methylbutanoic acid, 3-(methylthio)propanal, 2-isobutyl-3-methoxypyrazine, (*E*,*E*)-2,4-nonadienal, (*E*,*E*)-2,4-decadienal, 4-vinyl-2-methoxyphenol, *δ*-decalactone, 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one, dodecanoic acid, and linalool showed the overall highest FD factors in the pulp extracts. Cocoa pulp from Vietnam was perceived to have the highest aroma diversity, while Cameroonian pulp presented the lowest. Additionally, Cameroonian cocoa pulp exhibited the lowest FD factors, suggesting lower concentrations of aroma-active substances. This study demonstrated that the aroma composition of fresh cocoa pulp may vary between different origins, enabling a broad range of food applications.

Keywords: *Theobroma cacao* L.; by-product; aroma; aroma extract dilution analyses; gas chromatography olfactometry; mass spectrometry

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Aroma Properties of Cocoa Fruit Pulp from Different Origins

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Abstract: Cocoa pulp occurs as a by-product of cocoa bean production and can be repurposed to different food applications, such as jams, fruit preparations and beverages, improving the sustainability of cocoa production, as well as the livelihoods of cocoa farmers. In this work, aroma-active compounds of fresh cocoa fruit pulps from different origins were investigated by applying aroma extract dilution analyses in combination with gas chromatography-mass spectrometry/olfactometry for identification. In total, 65 aroma-active compounds were determined in four different pulps originating from Indonesia, Vietnam, Cameroon, and Nicaragua. Vietnamese pulp showed the highest number of aroma-active regions, while Cameroonian pulp accounted for the lowest. Moreover, Cameroonian cocoa pulp showed the lowest FD factors. Overall, the odorants with the highest FD factors were *trans*-4,5-epoxy-(*E*)-decenal, 2- and 3-methylbutanoic acid, 3-(methylthio)propanal, 2-isobutyl-3-methoxypyrazine, (*E*,*E*)-2,4-nonadienal, (*E*,*E*)-2,4-decadienal, 4-vinyl-2-methoxyphenol, *δ*-decalactone, 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one, dodecanoic acid, and linalool. This study provides insights into the aroma composition of fresh cocoa pulp from different origins for future food applications.

Keywords: *Theobroma cacao* L.; by-product; aroma; aroma extract dilution analyses; gas chromatography-olfactometry; mass spectrometry

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1. Introduction

The International Cocoa Organization [1] forecasted for 2020/2021 a production of over 4.8 million tons of cocoa beans mainly destined for the chocolate industry. Taking into account the by-products of the cocoa bean processing chain, which represent together about 70–80% of the dry weight of the fruit and comprise the cocoa pod husk, the bean shells, and the pulp [2]; approximately 19 million tons of residual biomass will be produced. As the disposal of these by-products causes several social and environmental concerns, some studies have focused on the use of cocoa by-products in the formulation of new and versatile ingredients for the food, pharmaceutical, and cosmetic industries. By adding value to these side fractions, cocoa farmers may profit from new sources of income and better livelihoods, allowing also a more sustainable cocoa production [3,4]. Due to its pleasant flavor, cocoa fruit pulp has recently gained increasing attention of the food sector.

Cocoa pulp is a moist white fibrous layer that covers the fresh cocoa beans. With a low pH, usually ranging between 3.3 and 3.9, fresh cocoa pulp contains 83–86% water, 11–13% sugars (D-glucose, D-fructose, and sucrose), 0.5–1.2% pectin, 0.2–3% hemicelluloses, 0.7–0.9% cellulose, 0.1–0.3% lignin, and 0.3–1.3% citric acid [5–7]. The cocoa pulp is hydrolyzed during the fermentation of cocoa beans and flows out of the fermentation boxes as the so-called cocoa sweatings and is, thereby, lost [8]. Previous reports suggest that a fraction of the pulp can be separated from the fresh cocoa beans without having a negative effect on the later fermentation of the beans [9,10].

Accordingly, alternative applications for the use of the aromatic pulp are gaining popularity. Possible applications for the cocoa pulp, such as the production of cocoa jelly, alcohol, vinegar, nata, and processed juices, were described by Figueira et al. [11]. Moreover, Puerari et al. [12] took advantage of the high sugar content of cocoa pulp and used kefir grains for the development of an alcohol-containing drink, while dos Santos Filho et al. [13] inoculated cocoa juice with *Lactobacillus casei* for the production of a probiotic beverage. Further approaches focused on the use of cocoa pulp for wine production [14] and as an adjunct for the production of beer [15]. Additionally, the chocolate industry has used cocoa pulp as a replacement for added sugar and released chocolate-like products to the European and Asian market [16–18]. This broad range of pulp-based products highlights the high application potential of cocoa pulp in foods, which can be mainly attributed to its pleasant tropical flavor.

To date, aroma research has primarily focused on the role of cocoa pulp for the later aroma quality of fermented cocoa beans. Pino et al. [19] studied the volatile composition of cocoa pulp from Colombia. The authors identified sixty-six different volatile organic compounds (VOCs) in the pulp, wherein the substances 2-heptyl acetate, 2-pentyl acetate, and linalool were present in the highest concentrations. Kadow et al. [20] quantified the VOCs of pulp and seeds of two fine flavor cocoas (SCA6, EET62) and a bulk cocoa (CCN51). Monoterpenes, present in SCA6, as well as methylketones, secondary alcohols, and their respective esters, present in EET62, were shown to be potential fine aroma components of cocoa beans originating in the pulp. Both studies investigated the cocoa pulp by means of headspace solid-phase microextraction gas chromatography mass spectrometry (HS-SPME CG-MS), yet did not investigate the aroma activity of the determined VOCs using olfactometric methods. Furthermore, to gain insights on how the cocoa pulp odorants affect the aroma quality of the beans during fermentation, Chetschik et al. [21] studied odor-active constituents in three cocoa pulp varieties by means of aroma extraction dilution analysis (AEDA) and gas chromatography-mass spectrometry/olfactometry (GC-MS/O). The experiments were performed with two cocoa varieties from Costa Rica (UF654 and CCN51) and one from Colombia (FSV41). In terms of flavor dilution (FD) factors, the CCN51 cocoa pulp presented lower aroma intensities than the varieties FSV41 and UF564, which showed more intense floral and fruity notes. By means of headspace solid-phase microextraction and GC-MS, Hegmann et al. [22] studied the influence of season and ripening stage on the cocoa pulp aroma of five cocoa varieties from Costa Rica. The total aroma diversity and intensity were shown to increase during ripening and aroma profiles were found to be more diverse when fruits ripened during the dry season, whereas the aroma intensity of the pulp was higher in the wet season.

Several authors showed the influence of background (e.g., cocoa genotype, growing and harvesting conditions, and origin) as well as processing on the flavor of cocoa beans (e.g., fermentation, drying, and roasting); and how these factors determine the later quality of the cocoa beans [23–28]. In particular, the origin plays a decisive role on their final quality, as different countries cultivate botanically different subspecies of the cocoa tree. The cocoa cultivars can be divided into Criollo, Forastero, Trinitario, and Nacional [29,30]. Cocoa pulp is still an emerging market and little is known on how the origin of cocoa fruits determine the pulp's flavor. In order to obtain cocoa pulps with constant and high qualities for the food sector, it is vital to study how the sourcing of cocoa pulp may affect its aroma composition and, thus, its suitability as a food ingredient. Investigations on the aroma-active compounds present in cocoa pulps from various origins and genetic backgrounds are a pre-requisite to identify possible application fields for the different cocoa pulps. Understanding the importance of certain aroma-active volatile organic compounds on the flavor of the cocoa pulps and elucidating the differences between the raw materials might help in the development of tailored food products for diverse markets and consumer groups. Therefore, the aim of this study was to investigate the differences in the main aroma-active compounds of four fresh cocoa pulps from different origins including comparative aroma extract dilution analysis (cAEDA) and GC-MS/O. In addition, the cocoa pulps

were analyzed by HS-SPME CG-MS and stir-bar sorptive extraction gas chromatography-olfactometry/mass spectrometry (SBSE-GC-MS/O). Fresh cocoa pulps from South East Asia (Indonesia and Vietnam), Central America (Nicaragua), and Africa (Cameroon) were investigated in this study.

2. Results and Discussion

The aroma-active volatile compounds responsible for the aroma impression of cocoa pulps originating from Indonesia, Vietnam, Cameroon, and Nicaragua were isolated by extraction with dichloromethane (DCM) and separated from non-volatiles lipids using the solvent-assisted flavor evaporation (SAFE)-distillation technique (c.f. 3.2). The distillates obtained exhibited the typical characteristic overall aroma of each kind of pulp, proving the successful extraction of all key aroma compounds. The distillates were subjected to cAEDA by means of GC-O analyses. The cAEDA revealed a total of 65 aroma-active regions with an FD factor range of 2 to 1024 (Table 1). Of the detected aroma-active regions, five could not be conclusively identified. Substances identified by SBSE- and HS-SPME-GC-MS/O in the fresh pulps were also found in cocoa pulp distillates by GC-MS/O or did not exhibit aroma-active regions with FD > 2 in the cAEDA. Therefore, the results obtained with SBSE- and HS-SPME-GC-MS/O measurements are provided as Supplementary Materials. The identified aroma-active volatiles belonged to various chemical groups. Aldehydes were the most predominant group, followed by carboxylic acids, lactones, phenols, and ketones. Additionally, terpenes, alcohols, esters, pyrazines, furans, sulfides, and thiazolines were found in the sample distillates. Overall, 36 odorants could be perceived in all cocoa pulps (compounds **1**–**4**, **6**, **9**–**21**, **23**, **24**, **26**, **27**, **29**, **30**, **32**, **33**, **35**, **37**, **42**, **44**, **47**, **49**, **51**, **58**, **64**, and **65**). Out of the 65 identified aroma-active regions, 24 odorants (No. 1, 2, 5, 10, 11, 17, 18, 19, 21, 23, 28, 29, 30, 31, 32, 35, 37, 42, 44, 45, 47, 51, 54, and 65) were previously reported in cocoa pulp by means of AEDA [21]. In total, the authors identified 37 aroma-active regions with FD factors below FD 128. Overall, higher FD factors (up to FD 1024) were determined in this work. This may result from the fact that distillates were concentrated to a final volume of 100 µL, compared to 300 µL in the previous study. Moreover, compared to Chetschik et al. [21], more odorants were identified in this work, which may also be explained by differences in the innate aroma compositions of the investigated cocoa pulp varieties.

Table 1. Aroma-active compounds identified in extracts of cocoa pulps grown in Indonesia, Vietnam, Cameroon, and Nicaragua.

			Retention Index on		FD Factor [d]			
No. [a]	Odorant [b]	Odor Quality [c]	DB-FFAP	DB-5	Indonesia	Vietnam	Cameroon	Nicaragua
1	2,3-butandione	butter-like	1008	731	64	16	256	16
2	methyl 2-methylbutanoate	fruity, banana-like	1017	776	4	32	4	4
3	2,3-pentanedione	butter	1056	706	64	16	256	16
4	hexanal	green	1089	802	4	128	32	32
5	3-methylbutyl acetate	fruity	1118	880	16	16	<2	128
6	*δ*-carene	green	1140	1014	2	32	8	64
7	3-methylbutanol	malty, roasty	1200	760	<2	32	<2	8
8	2-heptanone	fruity, flowery	1207	891	16	<2	32	<2
9	(*Z*)-4-heptenal [f]	fishy	1245	894	32	128	8	32
10	octanal	citrus-like, green	1280	1002	256	128	64	64
11	1-octen-3-one	mushroom-like	1285	978	32	128	16	128
12	(*E*)-2-heptenal	green, flowery	1311	951	16	32	16	128
13	1-hexanol	green, grassy	1338	n.d. [e]	2	128	128	32
14	2-acetyl-1-pyrroline [f]	popcorn-like	1342	930	64	128	128	32
15	nonanal	citrus-like, soapy	1376	1106	8	32	32	256
16	(*E*)-2-octenal	fatty, grassy, green	1417	1055	128	128	64	128
17	acetic acid	vinegar-like	1430	n.d. [e]	128	128	256	16
18	3-(methylthio)propanal	cooked potato-like	1455	903	64	128	64	512
19	(*E*,*E*)-2,4-heptadienal	fatty, roasty	1486	1020	4	32	128	32
20	(*Z*)-2-nonenal	green, fatty	1494	1140	256	256	128	128

Table 1. *Cont.*

			Retention Index on		FD Factor [d]			
No. [a]	Odorant [b]	Odor Quality [c]	DB-FFAP	DB-5	Indonesia	Vietnam	Cameroon	Nicaragua
21	2-isobutyl-3-methoxypyrazine	bell pepper -like, earthy	1510	1090	2	512	32	4
22	*(E)*-2-nonenal	fatty, cardboard-like	1524	1164	<2	128	128	<2
23	linalool	flowery	1539	1103	32	512	512	512
24	2-methylpropanoic acid	cheesy	1562	782	256	128	256	64
25	(*E*,*Z*)-2,6-nonadienal	cucumber-like, fatty	1574	1158	<2	64	<2	<2
26	*(E)*-2-decenal	green, fatty	1616	1252	16	128	64	128
27	butanoic acid	cheesy	1626	804	128	256	32	4
28	phenylacetaldehyde	flowery, honey-like	1634	1040	<2	<2	<2	32
29 a/b	2- and 3-methylbutanoic acid	rancid, cheesy	1662	860	1024	128	128	128
30	(*E*,*E*)-2,4-nonadienal	fatty, deep fried	1696	1216	512	256	64	512
31	2-acetyl-2-thiazoline [f]	popcorn-like, roasty	1747	n.d. [e]	16	256	32	<2
32	(*E*,*E*)-2,4-decadienal	fatty	1800	1325	64	128	512	256
33	*ß*-damascenone [f]	fruity, grape-like	1808	1374	2	256	64	128
34	geraniol	flowery, earthy	1841	1428	64	64	<2	16
35	2-methoxyphenol	smoky, ham-like	1848	1096	16	64	128	128
36	ethyl (*E*,*E*)-2,4-decadienoate [f]	metallic, pear-like	1890	n.d. [e]	<2	128	<2	<2
37	2-phenylethanol	rose-like, flowery	1897	1110	32	128	128	32
38	*γ*-octalactone	fruity, coconut-like	1908	1154	<2	4	<2	<2
39	unknown	metallic	1920	n.d. [e]	<2	128	128	<2
40	unknown	metallic	1947	1545	<2	16	<2	<2
41	2-methoxy-4-methylphenol	clove-like, vanilla-like	1962	1198	<2	<2	<2	128
42	*trans*-4,5-epoxy-(*E*)-2-decenal	metallic	1994	1379	1024	1024	512	1024
43	4-methylhexanoic acid [f]	sweaty, fishy	2011	n.d. [e]	<2	8	<2	<2
44	*γ*-nonalactone	fruity, coconut-like	2014	1364	16	64	256	8
45	4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	caramel-like	2026	1080	<2	64	<2	<2
46	octanoic acid	green, soapy	2043	1180	<2	32	4	<2
47	4-methylphenol	fecal	2073	1085	64	32	256	8
48	*δ*-nonalactone	fruity, coconut-like	2084	1380	32	64	<2	128
49	unknown	flowery, earthy	2103	n.d. [e]	4	64	32	2
50	2,3-dimethylphenol	phenolic	2109	1200	256	<2	<2	<2
51	4-vinyl-2-methoxyphenol	smoky, clove-like	2128	1326	256	128	512	256
52	*γ*-decalactone	fruity, peach-like	2133	1474	<2	16	<2	<2
53	*δ*-decalactone	coconut-like	2188	1507	<2	<2	128	512
54	3-hydroxy-4,5-dimethylfuran-2(5*H*)-one	maggi-like, celery-like	2194	1106	16	512	<2	128
55	3-propylphenol	medical	2247	1285	64	128	<2	256
56	undecanoic acid	soapy, coriander-like	2323	1475	<2	64	<2	<2
57	unknown	smoky, phenolic	2341	1345	<2	1024	<2	<2
58	*γ*-dodecalactone	caramel-like, flowery	2371	1667	256	128	32	64
59	4-methoxyphenol	phenolic	2388	1071	<2	<2	<2	16
60	*δ*-dodecalactone	peach-like	2393	1700	<2	16	<2	<2
61	coumarin	cinnamon-like	2435	1440	256	<2	<2	<2
62	indole	fecal	2485	1320	16	<2	<2	<2
63	dodecanoic acid	fatty, wax–like	2496	1574	<2	1024	<2	256
64	unknown	smoky, flowery	2516	n.d. [e]	64	256	128	16
65	phenylacetic acid	honey-like	2545	1256	16	128	64	256

[a] Consecutive numbering of odorants according to their retention indices on capillary column DB-FFAP. [b] Odorant was identified by comparison of its odor quality and intensity and retention indices on capillaries DB-FFAP and DB-5 as well as mass spectra (EI mode) with data of reference compounds. [c] Odor quality perceived at the odor detection port by four trained panelists. [d] Flavor dilution factor determined on DB-FFAP. [e] n.d. not detected. [f] No unequivocal mass spectrum was obtained; identification is based on the remaining criteria given in footnote b.

In the investigated cocoa pulps, Vietnamese pulp displayed the highest number of aroma-active regions with a total of 57 odorants. It was followed by Nicaraguan cocoa pulp with 47, and Indonesian pulp with 46 aroma-active regions. Cocoa pulp originating from Cameroon accounted for a total of 43 aroma-active volatiles. Among others, all investigated cocoa pulps exhibited *fatty* (e.g., No. 19, 20, 26, 30, 32); *fatty*, *cheesy* (e.g., No. 24, 27, 29); *green*, *grassy* (e.g., No. 4, 6, 10, 13, 16); *flowery* (e.g., No. 12, 23, 37, 49, 58); *fruity* (e.g., 2, 33, 44); and *smoky* (e.g., No. 35, 51, 64) smelling compounds. The highest FD factors of 1024 and 512 were determined for 3-(methylthio)propanal (No. 18, *cooked potato-like*, RI 1455), 2-isobutyl-3-methoxypyrazine (No. 21, *bell pepper-like*, *earthy* RI 1510), linalool (No. 23; *flowery*, RI 1539), 2- & 3-methylbutanoic acid (No. 29 a/b, *rancid*, *cheesy*, RI 1662),

(*E*,*E*)-2,4-nonadienal (No. 30, *fatty*, *deep fried*, RI 1696), (*E*,*E*)-2,4-decadienal (No. 32, *fatty*, RI 1800), *trans*-4,5-epoxy-(*E*)-2-decenal (No. 42, *metallic*, RI 1994), 4-vinyl-2-methoxyphenol (No. 51, *smoky*, *clove-like*, RI 2128), δ-decalactone (No. 53, *coconut-like*, RI 2188), 3-hydroxy-4,5-dimethylfuran-2(5H)-one (No. 54, *maggi-like*, *celery-like*, RI 2194), and dodecanoic acid (No. 63, *fatty*, *wax-like*, RI 2494). The aldehyde *trans*-4,5-epoxy-(*E*)-2-decenal was determined in all pulp samples with the highest FD factor (FD 1024). Correspondingly, Chetschik et al. [21] reported *trans*-4,5-epoxy-(*E*)-2-decenal with the highest FD factor in cocoa pulp from the varieties CCN51, FSV41, and UF654, which suggests an ubiquitous occurrence of this odorant in cocoa pulp. Due to the low odor threshold of *trans*-4,5-epoxy-(*E*)-2-decenal (6×10^{-7} µg/kg), very low concentrations can be determined by means of olfactometric methods [31]. Although cocoa pulp contains low amounts of fat (<0.8 g 100 g^{-1} fresh pulp) [6] and its fatty acid composition has not been yet elucidated, the presence of unsaturated fatty acids in cocoa pulp is probable, as these compounds have been reported in unroasted cocoa beans [32]. This may also explain the presence of the *fatty* and *wax-like* smelling odorant dodecanoic acid (No. 63, RI 2496) in cocoa pulp original from Nicaragua and Vietnam, as this fatty acid has been reported in cocoa butter [32]. Furthermore, as typical fatty acid degradation products, *peach-like* and *coconut-like* smelling lactones were perceived in all sample distillates (e.g., No. 44, 48, 52, 53). Lactones are formed from unsaturated fatty acids through several pathways, and often impart tropical aromas, whereas δ-lactones are less odor-active than γ-lactones [33,34]. While the *coconut-like* compound γ-nonalactone (No. 44, RI 2014) exhibited the highest intensity in pulp from Cameroon (FD 256), the related compound δ-nonalactone (No.48, *fruity*, *coconut-like*, RI 2084), which was identified in pulps of the other origins, was not detected. In addition, the odorants geraniol (No. 34, *flowery*, *earthy*; RI 1841), 3-hydroxy-4,5-dimethylfuran-2(5H)-one (No. 54, *maggi-like*, *celery-like*, RI 2194) and 3-propylphenol (No. 55, *medical*, RI 2247) were present in all but the Cameroonian cocoa pulp. The *butter-like* odorants 2,3-butandione (No. 1, RI 1008) and 2,3-pentandione (No. 3, RI 1056) were also determined in all samples (FD < 64), yet with higher intensities in Cameroonian pulp (FD 256). Similarly, the phenolic compound 4-methylphenol (No. 47, *fecal*, RI 2073) displayed the highest FD factor in pulp from Cameroon (FD 256). The *fruity* and *flowery* odorant 2-heptanone (No. 8, RI 1207) was shared only by samples from Indonesia and Cameroon with FD 16 and FD 32, respectively. This odorant has been described as more prevalent in cocoa pulp from ripe pods harvested during the rainy season [22]. Cameroonian cocoa pulp did not exhibit any exclusive odorants. It is of note that West Africa is responsible for about 70% of the total world production of cocoa and the largest producers are Côte d'Ivoire and Ghana, followed by Nigeria and Cameroon [35]. In West Africa, several subvarieties of the Forastero cocoa type are produced. While the Amelonado cocoa, a subvariety of the Forastero type, is extensively cultivated in most West African countries, the hybrid Trinitario, a mix of Criollo with Forastero, is the predominant variety in Cameroon [36,37]. Moreover, even if the terms fine or flavor cocoas are often used to describe the cocoa qualities, there is no agreed definition of the terms, except that these cocoas are sold at a premium price for their flavor. Fine or flavor cocoas can exhibit attributes that are often described as *fruity*, *raisin*, *brown fruit*, *floral*, *spicy*, *aromatic*, *nutty*, *molasses-like*, and *caramel* [38]. Generally, these cocoas come from Criollo, Trinitario, or Nacional-type trees. Nonetheless, not all cocoas of these varieties can be considered as fine or flavor [36]. For instance, Nacional trees in Ecuador, considered to be Forastero type trees, produce fine or flavor cocoa, yet cocoa beans from Cameroon, produced by Trinitario type trees have, hitherto, been classified as bulk cocoa beans [39]. It is therefore reasonable to assume that the Cameroonian cocoa pulp analyzed in this work, would be considered as bulk cocoa.

With regard to aroma diversity, the intensity of the *flowery* smelling linalool showed a lower FD in Indonesian pulp (FD 32) compared to the other samples (FD 512). This odorant is thought to be a *fine flavor* aroma compound in cocoa beans, where it is present in higher concentrations compared to bulk cocoa [40]. It has been also proposed that certain aroma compounds present in the cocoa pulp, including the odorant linalool, may originate in the

pulp and migrate to the beans during cocoa bean fermentation [41]. In addition, odorants of the group of phenols were identified in this work. The compound 4-vinyl-2-methoxyphenol (No. 51, *smoky*, *clove-like*, RI 2128) was found in all cocoa pulp samples with similar FD factors. The odorant 2,3-dimethylphenol (No. 50, *phenolic*, RI 2109) was exclusively found in Indonesian pulp with factor FD 256. On the other hand, the unknown substance No. 57 (RI 2341), which exhibited a *smoky* and *phenolic* odor quality, was only found in pulp from Vietnam. Phenols can be released by the degradation of polyphenols and lignin by enzymes and microbes [42]. Phenols and their derivatives can be potent odor-active compounds. Methylphenols usually impart *phenolic* and *smoky* odor qualities, while methoxyphenols are often described with a broad range of aroma qualities such as *smoky*, *vanilla*, *leather*, *meat-*, and *ham-like*. Terpene-derived phenols can be *herbal* and *spice-like* odorants [34]. Further odorants present only in Indonesian cocoa pulp were the lactone coumarin (No. 61, *cinnamon-like*, RI 2435, FD 256) and the heterocyclic compound indole (No. 62, *fecal*, RI 2485, FD 16). Coumarin can be found naturally in many food products such as fruits, oils, nuts, and spices [42], and was identified by Balladares et al. [43] in cocoa pulp sweatings from Ecuador. Additionally, Mashuni et al. [44] found coumarin derivatives in the polyphenol fraction obtained from cocoa pod husks by means of a microwave-assisted extraction method. The compound indole is a nitrogen-containing odorant that imparts a floral taint with a fecal note to food products [24] and is often produced by bacteria [45]. Indonesia is mainly a bulk cocoa producer and only 1% of its total cocoa exports is classified as fine and flavor cocoa [46]. Therefore, it is likely that the analyzed SUL2 variety, categorized as a Trinitario cocoa [47,48], would be also considered to have a bulk cocoa quality. Altogether, the low FD factor of linalool in Indonesian cocoa pulp and the *fatty*, *cheesy*, and *phenolic* notes suggest that the pulp from this origin, specifically of the variety SUL2, is less suitable for products in which predominant attributes such as *fresh*, *fruity*, and *floral* are desired (e.g., juices, jams, fruit snacks).

Cocoa pulp from Vietnam also showed unique aroma compounds. The substances (*E*,*Z*)-2,6-nonadienal (No. 25, *cucumber-like*, *fatty*, RI 1574, FD 64), ethyl (*E*,*E*)-2,4-decadienoate (No. 36, *metallic*, *pear-like*, RI 1890, FD 128), γ-octalactone (No. 38, *fruity*, *coconut-like*; RI 1908, FD 4), 4-methylhexanoic acid (No. 43, *sweaty*, *fishy*, RI 2011, FD 8), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (No. 45, *caramel-like*, RI 2026, FD 64), γ-decalacton (No. 52, *fruity*, *peach-like*, RI 2133, FD 16), undecanoic acid (No. 56, *soapy*, *coriander-like*, RI 2323, FD 64), substance No. 57 (unknown, *smoky*, *phenolic*, RI 2341, FD 1024), and δ-dodecalactone (No. 60, *peach-like*, RI 2393, FD 16) could not be determined in Indonesian, Cameroonian, and Nicaraguan cocoa pulp. The odorant 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone was first identified as a Maillard reaction product [49]. In addition, it has been determined in strawberries [50], pineapples [51], tomatoes [52], grapes [53], and raspberries [54]. Chetschik et al. [21] identified 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (furaneol) in cocoa pulp from Colombia and Costa Rica. Furthermore, Vietnamese pulp displayed the most perceivable odorants from the group of lactones ($n = 7$) compared to the three counterparts ($n < 5$). However, lactones were determined with low FD factors, accentuating the predominance of odorants with *green*, *fatty*, and *smoky* notes. Moreover, compared to other varieties, the odorant 2-isobutyl-3-methoxypyrazine (No. 21, *bell pepper-like*, *earthy*, RI 1510) was more intense (FD 512) in the South East Asian pulp. This pyrazine is characteristic for bell peppers [55] and certain types of grapes [56]. Vietnamese pulp also shared odorants with other single samples. The odorant 3-methylbutanol (No. 7, RI 1200), which exhibits *malty* and *roasty* odor qualities, was only perceived in Vietnamese and Nicaraguan pulp.

Odorants (*E*)-2-nonenal (No. 22, *fatty*, *cardboard-like*, RI 1524), compound No. 39 (*metallic*, RI 1920), and octanoic acid (No. 46, *green*, *soapy*, RI 2043) were found only in pulp from Vietnam and Cameroon. Although cocoa pulps from Vietnam and Indonesia came both from South East Asia and were expected to show strong resemblances in their aroma compositions, they did not share odorants exclusively. ICCO recognized that 40% of the total cocoa exports of Vietnam can be classified as fine and flavor cocoa [46]. Along with

this, the predominance of *green*, *fatty*, and *smoky* notes suggests that Vietnamese cocoa pulp investigated in this work originated from a bulk cocoa variety.

The cocoa variety TSH 565, short for Trinidad Selected Hybrid 565, was obtained from the crossing of varieties ICS1 (Imperial College Selection 1) and SCA6 (Scavina 6). While the variety ICS1 has been reported to be original to Trinidad as well as to produce superior beans [57], Scavina cocoa varieties originated from the Ucayali River basin [58]. Scavina cocoa is known for its floral aroma notes in the fruit pulp and raw cocoa [20]. The resulting hybrid TSH 565 can be associated to fine flavor cocoa due to its high concentrations of terpenes such as β-myrcene and β-cis-ocimene [59]. Compared to other origins investigated in this work, cocoa pulp from the TSH 565 variety grown in Nicaragua exhibited higher FD factors in aroma compounds with the odor qualities *fruity* and *flowery*. For instance, the odorant 3-methylbutyl acetate (No. 5, *fruity*, RI 1118) was determined with FD 128 in Nicaraguan, FD 16 in both South East Asian samples, and FD < 2 in Cameroonian pulp. In addition, the odorant (*E*)-2-heptenal (No. 12, *green*, *flowery*, RI 1311) displayed an FD factor of 128 in Nicaraguan, 16 in Indonesian and Cameroonian, and 32 in Vietnamese cocoa pulp. The aldehyde nonanal (No. 15, *citrus-like*, *soapy*, RI 1376) showed FD factors of 256, 8, 32, and 32, respectively. The odorants (*E*)-2-heptenal and nonanal have been reported previously in cocoa pulp from Colombia [19], but could not be found in five cocoa pulp varieties from Costa Rica [22]. Furthermore, cocoa pulp from Nicaragua stood out for having the lower intensity of acetic acid (FD 16) compared to the Indonesian (FD 128), Vietnamese (FD 128), and Cameroonian (FD 256) counterparts. Odorant No. 53, *δ*-decalactone (*coconut-like*, RI 2188), was only perceived in Cameroonian and Nicaraguan cocoa pulp (FD 128 and FD 512, respectively). The odorants phenylacetaldehyde (No. 28, *flowery*, *honey-like*, RI 1634, FD 32) as well as 2-methoxy-4-methylphenol (No. 41, *clove-like*, *vanilla-like*, RI 1962; FD 128) were also exclusive for pulp from Nicaragua (FD 32). Together with the *honey-like* phenylacetic acid (No. 65, RI 2545, FD 256), the odorants may have contributed to the overall *honey-like* impression of the Nicaraguan extracts. The combination of *fruity, flowery, honey-like,* and *vanilla-like* odor qualities of Nicaraguan cocoa pulp make it interesting for the development of food products. The results may possibly relate to the fact that Nicaragua is solely a fine flavor cocoa exporter [46]. An industrial implementation as well as the existing infrastructure in the country would have to be revised, as Nicaragua is a small cocoa producer compared to countries such as Indonesia and Cameroon [60,61].

3. Materials and Methods

3.1. Separation and Storage of the Fresh Cocoa Pulp

Cocoa pulps from Indonesia (SUL2), Cameroon (unknown variety), Vietnam (unknown variety), and Nicaragua (TSH 565) were investigated. Cameroonian, Vietnamese, and Nicaraguan fresh cocoa fruits, harvested in 2019, were imported to Germany in a cool shipment directly after harvest. At Fraunhofer IVV, the fruits were washed, cut open and the cocoa pulp was separated mechanically immediately upon arrival. After de-pulping, the pulp was vacuum-sealed in odorless plastic bags (PA/PE 90/130 × 280 mm, Dagema eG, Willich, Germany) and immediately frozen at −50 °C. Indonesian cocoa pulp was supplied by the Indonesian Cocoa and Coffee Research Centre (ICCRI) in Jember, East Java. The cocoa pulp was separated mechanically on-site (Indonesia) directly after cocoa pod harvest (wet season 2019) and frozen immediately. The material was shipped in a frozen state to Germany, where it was stored at −50 °C in the same manner as its counterparts.

3.2. Isolation of Volatile Organic Compounds (VOC)

To isolate the volatile organic compounds, 50 g (±0.1) fresh cocoa pulp was extracted by stirring vigorously with 200 mL dichloromethane (DCM) for one hour at room temperature in a closed vessel. Dichloromethane (DCM) was purchased from Merck KGaA (Darmstadt, Germany) and distilled for purification prior to use. After decanting of 150 mL DCM, the volatiles were separated from the non-volatiles by means of the Solvent Assisted

Flavor Evaporation (SAFE) technique [62]. The distillation was carried out under high vacuum, maintaining the round flask in a water bath at 50 °C and thermostating the SAFE apparatus to 55 °C. The obtained distillates were dried over anhydrous sodium sulfate (Merck KGaA, Darmstadt, Germany), filtered, and concentrated at 50 °C to ~3 mL by a Vigreux column (50 cm × 1 cm i.d.) and finally to a volume of ~100 µL by microdistillation [63]. To enable a comparison between the pulps, the same amounts were extracted, subjected to SAFE distillation, concentrated to the same final volume, and, finally, the same volume was used for GC-O.

3.3. Comparative Aroma Extract Dilution Analysis (cAEDA)

To enable a comparison between all pulps, the same volumes of the extracts were used for gas chromatography-olfactometry (GC-O). To avoid a potential overlooking of aroma-active areas, the original distillates were evaluated by four trained panelists of the Fraunhofer IVV sensory panel using GC-O. The panelists undergo trainings once a week on an in-house established odor-language with selected reference compounds, corresponding to about 150 different odorants. All panelists exhibited normal olfactory function and had no known illnesses at the time of examination. A blank was performed for each sample by applying the same work-up procedure (c.f 3.2). The flavor dilution (FD) factors of the odorants were determined by diluting the distillate stepwise (1 + 1, $v + v$) with dichloromethane up to factor 1024 and analyzing the dilutions with GC-O [64]. For each aroma-active area, the respective FD factor, correlating to the highest dilution in which the compound was perceived by the panelists at the odor detection port for the last time, was assigned.

3.4. Gas Chromatography-Olfactometry (GC-O)

GC-O was carried out by means of a Trace GC Ultra (Thermo Fisher Scientific GmbH, Dreieich, Germany) equipped with either a DB-FFAP or DB-5 capillary column (both 30 m × 0.32 mm, 0.25 µm film thickness) (J&W Scientific, Agilent Technologies GmbH, Waldbronn, Germany). Aliquots (2 µL) of the sample distillates were injected manually by the cold on-column technique at 40 °C, using helium as carrier gas at a constant flow mode (2.2 mL/min). The initial temperature of 40 °C was held for 2 min, raised at 6.0 °C/min to 235 °C (DB-FFAP) or 250 °C (DB-5), respectively, and held for 5 min. At the end of the column, the effluent was split 1:1 by volume through two deactivated fused silica capillaries of the same length (70 cm × 0.2 mm) leading to a flame ionization detector (FID) held at 250 °C and to an odor detection port (ODP) held at 235 °C. Linear retention indices (RI) were calculated using a homologous series of n-alkanes ranging from C_6 to C_{26} for the DB-FFAP and C_6 to C_{18} for the DB-5 column [65].

3.5. Gas Chromatography-Mass Spectrometry/Olfactometry (GC-MS/O)

For identification of the VOCs present in the distillates, GC-MS/O was performed using a Trace GC Ultra and a Trace dual stage quadrupole (DSQ) mass spectrometer (both Thermo Fisher Scientific GmbH) equipped with a DB-FFAP column (30 m × 0.32 mm, 0.25 µm film thickness, J&W Scientific, Waldbronn, Germany). The distillates (2 µL) were injected automatically by a multipurpose autosampler MPS 2 (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) using the cold on-column technique. The initial temperature of 40 °C was held for 2 min, raised at 6.0 °C/min to 235 °C, and held for 5 min. The flow rate of the helium carrier gas was 2.2 mL/min. At the end of the capillary column, the effluent was split between an ODP and the MS using deactivated fused silica capillaries (50 cm × 0.2 mm). Mass spectra were recorded in positive electron ionization (EI) mode at 70 eV (m/z range 35–250).

3.6. Stir-Bar Sorptive Extraction Gas Chromatography-Olfactometry/Mass Spectrometry (SBSE-GC-MS/O)

The SBSE method was applied to confirm the presence of VOCs and to compensate for possible losses and changes in the aroma composition of the pulp during the isolation

step (c.f. 3.2). Fresh cocoa pulp samples (2.0 g ± 0.01) were diluted individually 1:1 (*w*/*w*) in distilled water in headspace vials (volume 20 mL), sealed airtight, and the suspension was stirred at room temperature for 15 min with a preconditioned SBSE Twister® (polydimethylsiloxane sorbent (PDMS), 20 mm length, 0.5 mm coating thickness; Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany). Preconditioning was performed at 280° C for five hours. After extraction, the Twister® was automatically transferred to the Thermal Desorption Unit at 40 °C (TDU, Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany). Desorption started at a temperature of 40 °C (initial time: 0.1 min), then increased to 250 °C at a rate of 12.0 °C/s, before being held at 250 °C for 5 min. The desorbed volatiles were transferred to the cold-injection-system (CIS, Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany), cooled at −70 °C using liquid nitrogen, and then transferred onto the GC-MS/O system (cf. Section 3.5) by thermal desorption. After an initial time of 2 min at 40 °C, the oven temperature was raised at 6 °C/min to 235 °C and held for 5 min. The column flow of the helium carrier gas was adjusted to 50 mL/min. Mass spectra were generated in full scan mode (*m*/*z* range 35–400, EI 70 eV) on the same GC-MS system as described in Section 3.5.

3.7. Headspace Solid-Phase Microextraction Gas Chromatography-Olfactometry/Mass Spectrometry (HS-SPME GC-O/MS)

Highly volatile aroma-active compounds were additionally analyzed using HS-SPME-GC-O/MS to rule out any losses during the concentration steps by the liquid extraction technique. Fresh cocoa pulp samples (1.0 g ± 0.01) were diluted individually 1:1 (*w*/*w*) in distilled water and air tightly sealed in a 20 mL headspace glass vial. By means of an orbital shaker (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany), each vial was agitated for 10 min at 50 °C and 250 rpm, changing the direction every 90 s. Prior to use, the SPME fiber (PDMS, 100 µm, Supelco, Bellefonte, PA, USA) was conditioned at 250 °C for 5 min. The SPME fiber was introduced automatically into the vial for volatile adsorption, exposed to the fresh cocoa pulp for 10 min at 50 °C during HS equilibration, and analyzed using the MPS autosampler and GC-O/MS system equipped with a DB-FFAP capillary column as described in Section 3.5. Thermal desorption was performed using a PTV (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) for 180 s at 250 °C. After an initial time of 2 min at 40 °C, the GC-oven temperature was raised at 6 °C/min to 150 °C, then raised at 20 °C/min to 235 °C and held for 5 min. The column flow of the helium carrier gas was adjusted to 50 mL/min. Mass spectra were generated in full scan mode (*m*/*z* range 35–250, EI 70 eV).

4. Conclusions

Our study shows that cacao fruit pulp may present great qualitative differences in the composition of volatiles depending on their origin, which then may determine their suitability for diverse types of food products. By means of cAEDA, 65 aroma-active areas were detected in cocoa pulps from various origins, 60 of which were unequivocally identified by GC analysis on two columns of different polarity and comparison to authentic reference standards. Of the identified compounds, 36 were found in all four cocoa pulps. The highest odorant diversity was found in Vietnamese pulp. Cocoa pulp from Cameroon exhibited the lowest flavor intensities in terms of FD factors. In the higher dilutions, the Indonesian cocoa pulp extract was predominantly *fatty*, *cheesy*, and *phenolic*. The Vietnamese cocoa pulp extract displayed high FD factors with *fatty*, *green*, and *smoky* notes. Cameroonian cocoa pulp presented *butter-like*, *popcorn-like*, *flowery*, and *fruity* odor qualities in the higher extract dilutions, whereas the Nicaraguan cocoa pulp extract was primarily *fruity*, *flowery*, but also possessed *honey*, *clove*, and *vanilla-like* traits. Given that odor impressions result from complex interactions between many aroma-active substances, correlations between a single volatile and an olfactory perception are often not conclusive [66]. Synergistic, additively or suppressive effects between substances present in the sample may affect its overall aroma impression unpredictably [66,67]. In order to understand the final aroma as well as the aroma compound diversity in cocoa pulp better, a quantification of the VOCs

would be needed. Furthermore, the influence of genetic background on the aroma quality of the cocoa pulp should be investigated, as the genetic backgrounds of samples analyzed in this work were unclear. Studies with bigger cocoa pulp cohorts could provide important insights on how cocoa genetics determine the aroma composition of cocoa pulps. Finally, investigations using one commercial cocoa genotype, e.g., CCN51, grown in different regions of the world could help us understand better how other factors, such as the harvesting season, age of the cocoa tree, and growing conditions, may affect the aroma quality of cocoa pulp and its later application as a food ingredient.

Supplementary Materials: The following are available online: results obtained by HS-SPME-GC-MS/O and SBSE-GC-MS/O.

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Chapter 2: Thermal stabilisation of cocoa fruit pulp — Effects on sensory properties, colour and microbiological stability

Summary: Stabilisation is crucial for preserving fresh cocoa pulp, as the high water-activity and sugar content of fresh pulp pose high risks for spoilage. To extend the cocoa pulp's shelf-life while preserving its quality, pasteurization and ultra-high temperature (UHT) treatment were employed. Various quality parameters were investigated, including sensory properties, dry matter content, water activity, total soluble solids, colour, and peroxidase activity. Both technologies effectively deactivated enzymes, as indicated by the absence of peroxidase activity. Pasteurized pulp retained a colour like the fresh pulp, whereas UHT-treated pulp exhibited a more brownish tone. Sensory analysis and identification of aroma-active volatile organic compounds by GC-MS/O revealed distinct aroma profiles. Fresh pulp had the highest intensity in an *unripe banana-like* attribute, while UHT-treated pulp was more intense in a *tropical fruit-like* attribute. Pasteurized pulp closely resembled the fresh pulp. In fresh cocoa pulp, 74 aroma-active regions were determined, while UHT-treated and pasteurized pulps exhibited 66 and 60, respectively. Thermally treated pulps, stored at 4 °C and 23 °C for 24 weeks, showed no significant microbial growth. Non-enzymatic browning was more pronounced at 23 °C, resulting in higher browning indices. Pasteurization and UHT treatment proved effective for stabilising cocoa pulp, extending shelf-life, and inducing minimal changes in sensory properties. This chapter provides valuable insights into the thermal stabilisation of cocoa by-products, contributing to sustainability and the development of innovative food products. Considering the microbiological inactivation, the retention of aroma-active regions, and the sensory attributes, UHT treatment followed by cold storage was selected as the recommended process for cocoa pulp preservation.

Keywords: Cocoa pulp, Thermal treatment, Sensory quality, Aroma extract dilution analysis, Gas chromatography-olfactometry/mass spectrometry

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Thermal stabilisation of cocoa fruit pulp — Effects on sensory properties, colour and microbiological stability

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ABSTRACT

To improve cocoa pulp's shelf-life, preservation processes are necessary while maintaining the quality of the pulp. We applied pasteurisation and UHT-treatment and investigated different quality parameters: dry matter content, water activity, total soluble solids, colour and peroxidase activity. Both technologies inactivated peroxidase successfully. The colour of the pasteurised pulp was similar to the fresh, while UHT-treated pulp was more brownish. The sensory properties were investigated in detail by descriptive analysis and the identification of aroma-active volatile organic compounds. Fresh pulp revealed the highest aroma intensity for attribute *unripe banana-like*, whereas UHT-treated pulp scored highest in the intensity of attribute *tropical fruit-like*. Pasteurised pulp showed strong similarities to the fresh pulp. Fresh cocoa pulp exhibited 74 aroma-active regions identified by GC-MS/O. UHT-treated and pasteurised pulp accounted for 66 and 60 aroma-active regions, respectively. Five identified substances were only found in the fresh and pasteurised pulp, namely: δ-carene, 1-pentanol, 3-(methylthio)propanol, phenol and δ-undecalactone. Similarly, fresh and UHT-treated pulp shared ten exclusive odorants, such as decanal, geraniol, and δ-nonalactone. The pasteurised and UHT-treated pulp shared two compounds, δ–decalactone and 5-(hydroxymethyl)furfural. Furthermore, the thermally treated pulps could be stored at 4 °C and 23 °C for 24 weeks without observing a significant growth of microorganisms. The rate of non-enzymatic browning was higher in samples stored at 23 °C compared to those stored at 4 °C, leading to higher browning indices. We demonstrated that pasteurisation and ultra-high temperature treatment are suitable technologies for the stabilisation of cocoa fruit pulp. These resulted in prolonged shelf-lifes and minimal changes in the sensory prorperties of the treated pulps, characterised by a reduction in the aroma diversities. This work provides important insights for the thermal stabilisation of further side-streams.

1. Introduction

The fruits of the cocoa tree (*Theobroma cacao* L.), are composed of the cocoa pod husk, the beans and the pulp, representing about 70%, 20% and 10% of the whole fruit, respectively. Up to now, the cocoa beans have the main commercial importance, while the husks and the pulp have been considered as by-products (Figueroa et al., 2020). To increase the sustainability along the cocoa supply chain and to create new sources of income for cocoa farmers, concepts for a complete valorisation of cocoa fruits should be developed. This paper explores opportunities to increase the shelf-life of cocoa pulp for its transport from cocoa producing countries and its use in food applications.

The cocoa pulp is the moist layer embedding the cocoa beans. It contains approximately 83–86% water, 11–13% sugars, 0.5–1.2% pectin, 0.2–3% hemicelluloses, 0.7–0.9% cellulose, 0.1–0.3% lignin and 0.3–1.3% citric acid. The presence of citric acid and other organic acids make the cocoa pulp acidic, so its pH-value usually ranges between 3.3 and 3.9 (Pettipher 1986; Santos et al., 2014; Figueroa et al., 2020). The pulp is important for the fermentation of the cocoa beans, as it serves as a substrate for yeasts and bacteria. During fermentation, cocoa pulp is liquefied by the enzymes released by the microorganisms and is lost as so-called cocoa sweatings (van Ho et al., 2014). Fermentation is an

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important step for the aroma quality of the beans (Schwan and Fleet 2014), but previous studies suggest a partial de-pulping of the cocoa beans without negatively affecting the fermented beans' quality (Lopez 1979; Amanquah 2013). The separated pulp could then be used as a food ingredient, adding value to the cocoa fruit. Hitherto, some examples of foods produced with cocoa pulp exist in the cocoa-growing countries (dos Santos Filho et al., 2019; Firdaus et al., 2022).

A huge challenge in making cocoa fruit pulp available on the European market is its short shelf-life, caused by its high moisture and sugar content, making it prone to spontaneous fermentation (Vuyst and Weckx 2016). Thus, preserving the cocoa pulp through mild processing with minimal effect on the pulp's sensory quality needs to be thoroughly investigated. Pasteurisation can be used for products with a pH value of 4.5 or less (preferably below 4.0), in which the acidic conditions effectively prevent the growth of pathogens. Certain pathogenic bacteria (e.g. some *E. coli* strains) can still thrive in these conditions, hence some vegetable and freshly pressed juices are processed by ultra-high temperature (UHT) treatment (Ashurst et al., 2017). In a previous study, Endraiyani et al. (2017) studied the effect of single and double pasteurisation treatments on the total phenolic content of cocoa pulp. Throughout an 8-week storage at 4 °C, 25 °C, and 37 °C, single and double pasteurised pulps showed only minimal changes in phenolics when stored at 4 °C and 25 °C, suggesting colder storage temperatures are better suitable for maintaining the pulps' quality. The effects of thermal treatment on the organoleptic quality and aroma composition of cocoa pulp were disregarded in this study.

The pleasant flavour of cocoa pulp makes it highly attractive for the international food sector, especially for the development of beverages (Klis et al., 2023). Only a few studies focused on the aroma composition of cocoa pulp. However, they mainly investigated the role of cocoa pulp in the fermentation of cocoa beans and its influence on the chocolate quality (Chetschik et al., 2018; Hegmann et al., 2020; Schlüter et al., 2022). In our previous study, we identified the aroma-active regions of fresh cocoa pulp from Indonesia, Vietnam, Cameroon and Nicaragua. We reported substantial differences in the aroma compositions of the four pulps, providing key insights for future food applications (Bickel Haase et al., 2021). To the moment, the effect of processing on the sensory quality and microbial stability of cocoa pulp for food applications remains largely unexplored.

To obtain shelf-life-stable cocoa pulps with pleasant aroma attributes, it is crucial to understand how the stabilisation of cocoa pulp with well-established technologies such as pasteurisation and UHT-treatment may affect its sensory properties, colour and microbiological stability. This study focuses on the compositional changes of Cameroonian cocoa pulp after thermal treatment. Additionally, the microbiological and colour stabilities of thermally treated pulps were investigated in a 24-week storage test at 4 °C and 23 °C to predict the shelf-life of cocoa pulp. We describe for the first time the effects of pasteurisation and UHT-treatment on cocoa pulp, providing suitable processing protocols for future applications.

2. Material and methods

2.1. Separation of the fresh cocoa pulp

Fresh cocoa pods (*Theobroma cacao* L.), harvested in 2020, were imported from Cameroon to Germany in a cool shipment directly after harvest. Upon arrival, the fruits were washed, cut open and the cocoa pulp was immediately separated by means of a straining machine with a 2.8 mm sieve mesh (Fructmas P006, Karl Bockmeyer Kellereitechnik GmbH, Germany). An aliquot of the fresh pulp was vacuum-sealed in odorless plastic bags (PA/PE 90/130 × 280 mm, Dagema eG, Germany) and immediately frozen at −50 °C until analysis, while the residual fresh pulp was preserved by thermal treatment (2.2) immediately after de-pulping.

2.2. Thermal treatment of cocoa pulp

The fresh pulp was thermally treated in an ultra-high temperature System (HT220 Lab UHT/HTST; OMVE Nederland B.V., Netherlands). In a first run, the pulp was heated to 80 °C core temperature and held for 30 s. The hot pulp was filled into sterile 1 L flasks (Schott AG, Germany). After 20 min, the flasks were submerged in ice water (0 °C) to decrease the temperature rapidly (= **pasteurised samples**). In a second experimental setup, the pulp was heated to 135 °C and held for 30 s. The hot pulp was also filled into the sterile flasks and immediately cooled down in a bath with ice water (0 °C) (= **UHT-treated samples**).

2.3. Characterisation of fresh, pasteurised and UHT-treated cocoa pulp

2.3.1. Dry matter content, total soluble solids and water activity

The dry matter content was determined gravimetrically by drying the sample at 105 °C with a moisture analyser MA100 (Sartorius Lab Instruments GmbH & Co. KG, Göttingen, Germany) until constant weight was achieved. The total soluble solids (° Bx) were determined at room temperature (~21 °C) using a digital refractometer DR301-95 (A. Krüss Optronic GmbH, Hamburg, Germany) against distilled water. For this, approximately 1 mL of cocoa pulp was placed in the measuring cell for the reading. The water activity was determined by means of an AQUALAB 4 TE system (Meter Group Europe, Munich, Germany) by filling a sample cup (Aqualab, Meter Group, USA) until the cup's bottom was completely covered. Measurements were performed at room temperature (~21 °C). The equipment was calibrated using standards provided by the system producer with a_w-values of 0.984 (0.5 M KCl) and 0.760 (6 M NaCl), as well as distilled water (a_w-value of 1.0). All experiments were carried out in triplicate.

2.3.2. Colour measurement

The DigiEye colour imaging system (DigiEye V2.62, VeriVide, Leicester, UK) was used for colour measurements, comprising an illumination box with diffuse illuminant D65 and a Nikon D90 digital camera. Digitizer calibration charts were used to calibrate the system. For the colour measurements of the fresh, thermally treated and stored samples (2.5), pulp was evenly distributed in a white sample cup (Aqualab, Meter Group, USA) and the average surface colour was expressed as CIE L*a*b*-values. The browning index (BI) was calculated using the L*a*b* values and following the formula described by Bal et al. (2011):

$$BI = \frac{100(x - 0,31)}{0,17} \quad (1)$$

$$\text{Where x}: x = \frac{(a^* + 1.75L^*)}{(5.645L^* + a^* - 3.012b^*)} \quad (2)$$

Analyses were performed immediately after thermal treatment as well as after 2, 4, 6, 12 and 24 weeks of storage at 4 °C and 23 °C. Analyses were performed in triplicate to obtain three browning indices for each sample, which were used to obtain mean values and standard deviations (2.6).

2.3.3. Peroxidase activity

Peroxidase activity (POD) was determined in fresh, pasteurised and UHT-treated cocoa pulp adapted according to Ghamsari et al. (2007) using guaiacol as substrate. To extract the POD, 0.2 g of fresh pulp was weighed in a beaker and dispersed in 15 mL 0.1 M citrate-phosphate buffer (citric acid-Na_2HPO_4·12 H_2O) at pH 7.0. The mixture was stirred at 4 °C for 30 min and centrifuged at 2000 *g* at 4 °C for 10 min. The extract was kept in refrigeration until use. To determine the optimal pH for the enzymatic reaction, the enzymatic assay was carried out at different pH values (3.0–7.0). Therefore, the citrate-phosphate buffer solutions with 15 mM guaiacol (Merck KGaA, Darmstadt, Germany) were set to different pH values (Gomori 1955). The highest enzyme

activity was determined at pH 6 in the fresh pulp. Therefore, POD activity in the fresh and thermally treated samples was determined at pH 6. The reaction mixture contained 90 μL of the enzyme extract, 2.9 mL of a citrate-phosphate buffer with 15 mM guaiacol and 10 μL of 3.3 mM H_2O_2. Blanks of the reagents and of the sample were also prepared and analysed. The sample was placed in a 1 cm cuvette and measured immediately by means of a spectrophotometer (Specord 210 Plus, Analytik Jena GmbH, Germany) at 470 nm in intervals of 30 s for 10 min. The formula described by Chance and Maehly (1972) was used to calculate the enzymatic activity. Taking the samples' weight into consideration, the peroxidase activity was described as E_A (μmol s^{-1} g^{-1}).

2.4. Sensory evaluation

2.4.1. Panelists

Sensory evaluation was performed by seven trained experts (2 male, 5 female, aged 27 to 42, all non-smokers) from the Fraunhofer Institute for Process Engineering and Packaging IVV (Freising, Germany) sensory panel. Panellists were trained to correctly identify and name odors during weekly training sessions with selected in-house made odorant pens, corresponding to a total of over 250 different odorants. The correctness of the individual answers was evaluated following DIN EN ISO 8586:2014–05. Panellists were trained for at least six weeks and were required to achieve a minimum average correctness score of 65% prior to inclusion in the panel. Furthermore, the panelists were trained regularly to accurately perceive and describe the five basic taste qualities. They were selected based on their extensive experience in sensory evaluation of different fruit pulps. The panelists reported no known illnesses at the time of the examination.

2.4.2. Aroma profile analysis

For the sensory evaluation following DIN 10969:2001–05 (Sensory analysis - Descriptive analysis with following quality evaluation), an aliquot of 20 ± 1 g of the fresh and the thermally treated pulps, respectively, was presented in 140 mL covered glass vessels in a sensory room at 21 °C. Aroma qualities were determined in consensus by the expert panel (2.4.1). The quality attributes were systematically elaborated by the trained experts based on their long time sensory experience on fruit pulps and their training in describing different odor qualities based on single aroma-active compounds. It was ensured that all testers understood the terms in the same way. Afterwards, the intensity of the selected attributes was directly evaluated on a scale from 0 (no perception) to 5 (strong perception). The results were averaged and the means were plotted in a spider-web diagram. Results were evaluated by Tukey's multiple comparison tests (2.6).

2.4.3. Comparative aroma extract dilution analysis (cAEDA)

The isolation of the volatile organic compounds was carried out as described in detail by Bickel Haase et al. (2021). For this, 50 g pulp was vigorously stirred with 200 mL of dichloromethane (DCM) (Merck KgaA, Darmstadt, Germany) for 1 h at room temperature in a closed vessel. After decanting 150 mL of DCM, the volatile compounds were separated from the non-volatile components using the Solvent Assisted Flavour Evaporation (SAFE) technique (Engel et al., 1999) under high vacuum, while the SAFE apparatus was maintained at a temperature of 55 °C. The resulting distillates were dried using anhydrous sodium sulfate (Merck KgaA, Darmstadt, Germany), filtered, concentrated to approximately 3 mL at 50 °C utilizing a Vigreux column (50 cm × 1 cm i.d.), and further reduced to a volume of approximately 100 μL through microdistillation (Bemelmans 1978). The same workup procedure was used for the fresh, pasteurised and UHT-treated pulps. To avoid a potential overlooking of aroma-active areas, the original distillates were evaluated by three trained panellists of the Fraunhofer IVV sensory panel using GC-O. A blank was performed for each sample by applying the same work-up procedure omitting the pulp sample. The flavour dilution (FD) factors of the odorants were determined by diluting the distillate stepwise (1 + 1, *v* + *v*) with dichloromethane up to factor 1024 and analysing the dilutions with GC-O (Grosch 1993). GC-O was performed as described in subsection 2.4.4. For each aroma-active area, the respective FD factor was assigned, correlating to the highest dilution in which the compound was perceivable at the odor detection port for the last time.

2.4.4. Gas chromatography-olfactometry (GC-O)

GC-O was carried out as described by Bickel Haase et al. (2021) using a Trace GC Ultra (Thermo Fisher Scientific GmbH, Dreieich, Germany) equipped with either a DB-FFAP or DB-5 capillary column (both 30 m × 0.32 mm, 0.25 μm film thickness) (J&W Scientific, Agilent Technologies GmbH, Waldbronn, Germany). The initial temperature of 40 °C was held for 2 min, raised at 6.0 °C/min to 235 °C (DB-FFAP) or 250 °C (DB-5), respectively, and held for 5 min. The effluent was split 1:1 leading to a flame ionization detector (FID) held at 250 °C and to an odor detection port (ODP) held at 235 °C. Linear retention indices (RI) were calculated using a homologous series of n-alkanes ranging from C_6 to C_{26} for the DB-FFAP and C_6 to C_{18} for the DB-5 column (van den Dool and Dec. Kratz, 1963).

2.4.5. Gas chromatography-mass spectrometry/olfactometry (GC-MS/O)

For identification of the volatile organic compounds (VOCs) present in the distillates, GC-MS/O was performed using a Trace GC Ultra and a Trace dual stage quadrupole (DSQ) mass spectrometer (both Thermo Fisher Scientific GmbH) equipped with a DB-FFAP column (30 m × 0.32 mm, 0.25 μm film thickness, J&W Scientific, Waldbronn, Germany). Measurements were performed following the method described in detail by Bickel Haase et al. (2021). Samples were evaluated by three trained panellists.

Further GC-MS/O determinations were performed using an Agilent GC-MSD (Agilent Technologies, Waldbronn DE). Aliquots (2 μL) of the samples were injected in pulsed splitless mode at 250 °C by means of an autosampler PAL RSI 85 (CTC Analytics AG, Zwingen, Switzerland). At the end of the column (DB-FFAP, 30 m × 0.25 mm, 0.25 μm film thickness, J&W Scientific), the effluent was split 1:1 by a Y-splitter. Two deactivated fused silica capillaries led either to an odor detection port (235 °C) or a mass spectrometer. The oven temperature was 40 °C at the beginning, raised at 6.0 °C/min after 2 min to 235 °C, and held for 5 min. The flow rate of the helium carrier gas was 2.0 mL/min in constant flow. Mass spectra were generated in the positive electron impact (EI) ionization mode at 70 eV (Scan 35–400 *m/z*).

2.4.6. Stir-bar sorptive extraction gas chromatography-olfactometry/mass spectrometry (SBSE-GC-MS/O)

SBSE was performed as described in detail by Bickel Haase et al. (2021) to confirm VOCs and to evaluate possible changes in the aroma composition of the pulp during the isolation step. Cocoa pulp samples (2.0 g ± 0.01) were diluted with distilled water in a 1:1 ratio (*w/w*) and placed in 20 mL headspace vials. The vials were sealed tightly, and the suspension was stirred at room temperature for 15 min using a pre-conditioned (280 °C, 5 h) SBSE Twister® (polydimethylsiloxane sorbent (PDMS), 20 mm length and 0.5 mm coating thickness, Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany). Following the extraction, the Twister® was automatically transferred to the Thermal Desorption Unit (TDU) at 40 °C (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany). Desorption commenced at 40 °C (initial time: 0.1 min) and ramped up to 250 °C at a rate of 12.0 °C/s, where it was held for 5 min. The desorbed volatile compounds were then transferred to the cold-injection system (CIS) (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany), cooled to −70 °C using liquid nitrogen, and subsequently introduced into the GC-MS/O system via thermal desorption. Mass spectra were generated in full scan mode (*m/z* range 35–400, EI 70 eV) on the same GC-MS system as described in 2.4.5. Samples were evaluated by two trained panellists.

2.4.7. Headspace solid-phase microextraction gas chromatography-olfactometry/mass spectrometry (HS-SPME GC-O/MS)

Highly volatile aroma-active compounds were identified by means of HS-SPME-GC-O/MS to exclude any losses during the concentration steps by liquid extraction (2.4.3). The same workup procedure as described by Bickel Haase et al. (2021) was followed. Cocoa pulp samples (1.0 g ± 0.01) were individually diluted 1:1 (*w/w*) with distilled water and sealed in 20 mL headspace glass vials. The vials were agitated using an orbital shaker (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) at 50 °C and 250 rpm for 10 min, with a change in direction every 90 s. Prior to use, a SPME fiber (PDMS, 100 µm, Supelco, USA) was conditioned at 250 °C for 5 min. The fiber was automatically introduced into the vial for volatile adsorption, and the cocoa pulp was exposed to it for 10 min at 50 °C during the headspace equilibration. The analysis was performed using the MPS autosampler and GC-O/MS system with a DB-FFAP capillary column, as described in section 2.4.5. The aroma-active regions of the fresh and thermally treated samples were evaluated by two trained panellists.

2.5. Storage test with the thermally treated cocoa pulps

Under a sterile bench, 30 g of thermally treated cocoa pulp was filled in sterile 50 mL tubes (PP, 30/115 mm, Greiner Bio-One GmbH, Germany). Samples were stored for 24 weeks at ambient temperature (23 °C) and at 4 °C. Three sample tubes each were taken after 0, 2, 4, 6, 12 and 24 weeks of storage and evaluated for their colour properties (2.3.2). The microbiological load was determined after 0, 6, 12 and 24 weeks of storage.

2.5.1. Microbiological load

Sample preparation was performed following the international guidance for preparation of test samples, initial suspension and decimal dilutions for the microbiological examination of the food chain (DIN EN ISO 6887–1:2017–07). The count of aerobic mesophilic bacteria [CfU/g] present in the fresh and thermally treated cocoa pulp samples was determined following DIN EN ISO 4833–2:2014–05. The total yeast and moulds' count [CfU/g] was determined following DIN 10186:2005–10. Analyses were performed after processing as well as after 0, 6, 12 and 24 weeks of storage at 4 °C and 23 °C. Analyses were performed in duplicate and the results were averaged.

2.6. Statistical analysis

The results of the pulp characterization were expressed as mean average ± standard deviation (SD). Statistical analysis was performed using Tukey's multiple comparison test ($p < 0.05$) to determine significant differences among two groups using Origin 2022b (OriginLab Corporation, Massachusetts, USA). Tukey's multiple comparisons tests were applied to following results: dry matter content, total soluble solids, pH-values, water activities, colour and browning index, peroxidase activity and descriptive sensory evaluation (taste and aroma profile).

3. Results and discussion

3.1. Characterization of fresh, pasteurised and UHT-treated cocoa fruit pulp

To understand how thermal processing affected the cocoa fruit pulp, fresh, pasteurised and UHT-treated cocoa pulps were investigated right after processing for their dry matter contents, total soluble solids (° Bx), pH, water activity (a_W), peroxidase activity, L*a*b* and the browning index (BI). Overall, the differences in the chemical composition of the fresh pulp were statistically significant from the pasteurised and the UHT-treated pulps, but the colour properties were similar (Table 1). The fresh material showed a dry matter content of 16.71%, a pH of 3.85 and total soluble solids of 16.1° Bx. The values correspond to those reported in the literature, accounting for a pH of 3.90 and total soluble solids of 16.2° Bx (Escalante et al., 2015). The dry matter content, pH and soluble solids of pasteurised pulp were 17.2%, 3.95 and 15.5° Bx, respectively. In the UHT-treated pulp, the dry matter content was 16.2%, the pH 3.88 and the soluble solids content corresponded to 14.30° Bx. Even though the dry matter contents, pH-values and soluble solids of the fresh, pasteurised and UHT-treated pulps were significantly different, all values ranged within common values for cocoa pulp. No clear effect of the treatments could be observed, so differences might be attributed to slight variations in the raw material.

Table 1
Characterisation of fresh, pasteurised and UHT-treated cocoa pulp.

	Fresh	Pasteurised	UHT-treated
Dry matter [%]	16.7 ± 0.1 ^{b}	17.2 ± 0.1 ^{a}	16.2 ± 0.2 ^{c}
pH [-]	3.85 ± 0.01 ^{b}	3.95 ± 0.01 ^{a}	3.88 ± 0.01 ^{b}
Total soluble solids [°Bx]	16.1 ± 0.1^{a}	15.5 ± 0.1 ^{b}	14.3 ± 0.1 ^{b}
a_w [-]	0.980 ± 0.02 ^{c}	0.981 ± 0.00 ^{b}	0.984 ± 0.00 ^{a}
L* [-]	68.25 ± 0.12 ^{c}	70.30 ± 0.19 ^{b}	71.25 ± 0.09 ^{a}
a* [-]	2.02 ± 0.12 ^{b}	1.65 ± 0,11 ^{c}	2.63 ± 0.17 ^{a}
b* [-]	11.03 ± 0.32 ^{b}	12.01 ± 0.67 ^{b}	14.37 ± 0.61 ^{a}
Browning index [-]	19.43 ± 0.61 ^{b}	20.07 ± 1.17 ^{b}	24.79 ± 1.20 ^{a}
Peroxidase activity [µmol/s g]	24.7 ± 0.3	n.d.	n.d.

Values are expressed as mean ± standard deviation of three experiments. Means in the same row with different letters indicate significant differences. n.d. not detected.

The water activity of fresh cocoa pulp accounted for 0.980. Pasteurised and UHT-treated samples showed similar values, with 0.981 and 0.984, respectively (Table 1). Due to the fresh cocoa pulp's high water activity, a stabilisation step is recommendable to diminish possible spoiling reactions and increase the shelf-life of the pulp.

We investigated the activity of guaiacol peroxidase (POD) as an indicator for the success of the thermal treatments in inactivating enzymes (Table 1). The POD activity of fresh cocoa pulp accounted for 24.7 ± 0.3 µmol $s^{-1}g^{-1}$. After pasteurisation and UHT-treatment, no POD activities could be determined. As POD is a relatively thermostable enzyme, it can be assumed that both thermal processing technologies were sufficient to inactivate other enzymes in the samples as well.

The L*a*b* values of the fresh and the thermally treated pulps were determined (Table 1). The fresh pulp showed L*, a* and b* values of 68.25, 2.02 and 11.03, respectively. In pasteurised pulp, the L*a*b* values were 70.30, 1.65 and 12.01. The b* value of pasteurised pulp presented no statistical difference compared with the fresh pulp's b* value. In contrast, the L*a*b* values of UHT-treated pulp were significant different from those of the fresh material. The L* value accounted for 71.25, while the a*value was 2.63 and the b* value corresponded to 14.37. Furthermore, the browning index was calculated (Table 1). The browning index of pasteurised pulp (20.07) was not statistically different from fresh pulp (19.43), whereas UHT-treated pulp (24.79) differed significantly from the fresh and the pasteurised materials. The higher browning index of the UHT-treated material may be explained by a higher processing temperature (135 °C compared to 80 °C), increasing the rate of non-enzymatic browning reactions.

3.2. Sensory evaluation and aroma composition of fresh and thermally treated cocoa pulp

3.2.1. Descriptive sensory analysis

The orthonasal aroma and the taste characteristics of the fresh, pasteurised and UHT-treated cocoa pulps were assessed according to simple descriptive testing (DIN 10969:2001–05) by rating various

impressions on a linear scale from 0 (not present) to 5 (strongly present). Fig. 1 displays the taste profiles (A) and the orthonasal aroma profile (B) of the three pulps. The means and standard deviations are listed in the supplementary material (Supplementary Table 1 and Supplementary Table 2). Even though the differences in the various taste perceptions (Fig. 1 A) between the pulps were not statistically significant, the pulps were rated differently to some extent. The fresh cocoa pulp scored highest in the attribute sour (3.7), followed by the pasteurised (3.2) and UHT-treated samples (3.1). The lower score in the sour attribute of the UHT-treated sample was also accompanied by a lower astringency, perceived with an intensity of 0.9. In contrast, this attribute scored similarly in the fresh and the pasteurised samples with 2.0 and 2.1, respectively. The UHT-treated sample was rated higher in the attribute sweet (3.0) compared to the pasteurised (2.4) and the fresh (2.8) pulps, possibly because the sample was perceived as less sour and less astringent than the other ones. As the differences were not significant, it can be assumed that both thermal processes do not alter the taste quality of the treated pulps compared to the fresh pulp. Therefore, we describe the effects of thermal processing on the aroma profiles and aroma composition of the treated cocoa pulps in the following.

The following orthonasal aroma properties were described for the fresh cocoa pulp (Fig. 1 B): the *malty* impression was rated with the highest intensity (2.8), followed by *unripe banana-like* and *lactic* (both with 2.6), *metallic* (2.4), *pungent* (2.3), *apple/pear-like* (2.1) and the *tropical fruit-like* (1.3) impressions. The attribute *unripe banana* was chosen by the panel as the note reminded of green banana peels. On the other hand, the panel agreed on the attribute *tropical fruit-like*, which encompassed notes perceived as *passionfruit-* and *peach-like*. In comparison, the pasteurised pulp scored higher in the impressions *apple/pear*-like (3.1), *lactic* (3.1) and *tropical fruit-like* (2.6). Interestingly, the intensity of the *tropical fruit-like* attribute was significantly higher in the UHT-treated sample (4.4) compared with the other samples, suggesting the higher thermal input during the UHT process promoted the formation of *tropical fruit-like* notes. Furthermore, the intensity of the *unripe banana-like* impression, present in the fresh sample, tended to decrease with an increase of the treatment temperature and accounted for 1.3 in the pasteurised pulp and for 0.7 in the UHT-treated material. The differences in the aroma profiles of the fresh and thermally treated samples could be described in more detail by identifying the aroma-active substances.

3.2.2. Identification of aroma-active volatiles in fresh, pasteurised and UHT-treated cocoa pulp

The cAEDA showed a total of 82 aroma-active regions within a FD factor range of 1 and ≥ 1024, of which 79 were successfully identified and corroborated by SBSE- and HS-SPME-GC-MS/O (Table 2). The identified aroma-active regions comprised substances from various chemical groups. Aldehydes predominated and were followed by carboxylic acids, ketones, alcohols, lactones and terpenes. Additionally, the distillates exhibited esters, aliphatic compounds, furans, thiazolines, pyrrolidines and pyrazines. The presence of these chemical groups in cocoa pulp was previously described (Chetschik et al., 2018; Bickel Haase et al., 2021). The fresh and thermally treated pulps shared 50 common aroma-active substances (No. 3–5, 10, 13–26, 29–33, 35, 36, 39, 40, 42, 45, 46, 48, 49, 51, 53, 55, 56, 58, 60, 61, 63, 64, 67, 71–74, 76, 78, 79 and 82). Of all aroma-active regions determined, 48 substances (No. 2–6, 10, 11, 13, 15, 16, 18–21, 23–26, 28–30, 32, 34–37, 39, 40, 42, 47–50, 52, 54, 55, 58, 61, 63, 65–67, 69–71, 76, 80 and 82) were previously reported in cocoa pulp by means of AEDA (Chetschik et al., 2018; Bickel Haase et al., 2021). In Bickel Haase et al. (2021), we identified 43 aroma-active regions in Cameroonian cocoa pulp with FD factors above 2. In this study, we also included aroma-active regions with FD factors of 1, resulting in 74 aroma-active regions in fresh, 66 in UHT-treated and 60 in the pasteurised cocoa pulp.

3.2.3. Aroma-active volatiles with high FD factors in fresh and thermally treated pulps

In this study, the following odorants showed the highest intensities in fresh Cameroonian pulp: 1-pentanol (*pungent, cheesy,* FD 512), linalool (*flowery,* FD 512), (*E,E*)-2,4-decadienal (*deep fried, fatty,* FD 512), *trans*-4,5-epoxy-(*E*)-2-decenal (*metallic*, FD 512) and 2,5-dimethylphenol (*smoked ham-like, medical*, FD 512). Pasteurised pulp also exhibited the *deep fried-like* and *fatty* odorant (*E,E*)-2,4-decadienal. This, together with (*E,E*)-2,4-nonadienal, were determined with FD ≥ 1024. The aroma-active regions 3-(methylthio)propanal (*cooked potato-like*, FD 256), 2-methylphenol (*medical, ink-like, phenolic*, FD 256), 4-vinylphenol (*phenolic,* FD 256), 5-(hydroxymethyl)furfural (*butter-like, caramel-like, fatty*, FD 256) and phenylacetic acid (*honey-like,* FD 256) had lower FD factors. The aroma-active compound (*E,E*)-2,4-decadienal scored also high in the UHT-treated sample (FD ≥ 1024). (*E,E*)-2,4-Decadienal can be formed when decanal is dehydrogenized and *trans*-double bonds are introduced at the 2–3 and 4–5 positions. This aroma-active compound is also a product of lipid degradation (Belitz et al., 2004). (*E,E*)-2,4-Decadienal imparts a characteristic *fried* note, although some describe the same note as *lemon-* or *citrus-like* (Parker et al., 2015). This odorant is more occurrent in frying oils and oil fumes (Boskou et al., 2006) but it has been found naturally in several food products like orange and mandarin juices (Feng et al., 2018). Further aroma-active compounds perceived with high intensities in the UHT-treated pulp were 2,3-pentanedione (*butter-like,* FD 256), 2-methylbutanol (*solvent-like, fruity,* FD

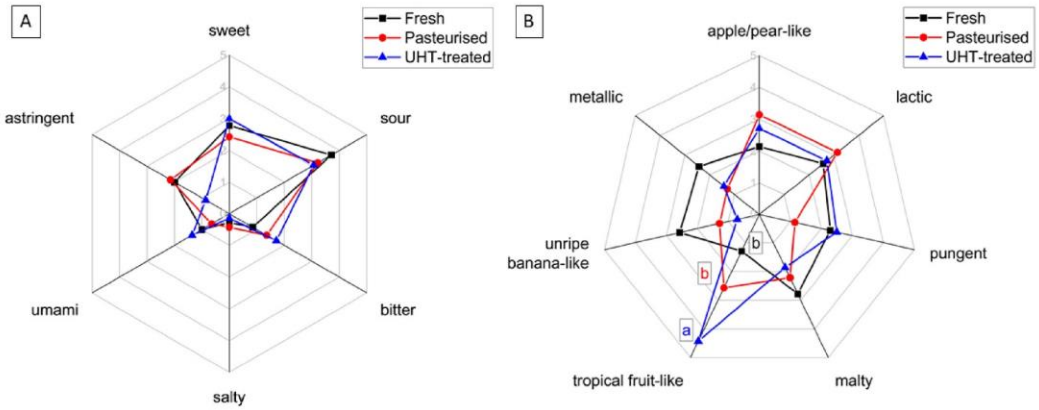

Fig. 1. Taste (A) and aroma profile analyses (B) by simple descriptive analysis of fresh, pasteurised and UHT-treated cocoa pulp. Different letters (c.f. attribute tropical fruit-like) indicate significant differences ($p < 0.05$) between the samples.

Table 2
Aroma-active regions identified in distillates obtained from fresh, pasteurised and UHT-treated cocoa pulps.

No.[a]	Odorant [b]	Odor Quality [c]	Retention Index on		FD Factor [d]		
			DB-FFAP	DB-5	Fresh	Past	UHT
1	α-pinene [f]	green, woody	1008	n.d.	16	<1	<1
2	methyl 2-methylbutanoate	fruity, banana-like	1017	776	<1	<1	64
3	2,3-pentanedione	butter-like	1056	706	64	128	256
4	hexanal	green	1089	802	32	32	16
5	3-methylbutyl acetate	fruity	1118	880	4	8	128
6	δ-carene	green	1140	1014	8	1	<1
7	1-penten-3-ol	pungent	1153	695	64	<1	4
8	myrcene	earthy, metallic, geranium-like	1158	991	8	<1	4
9	2-methylbutanol	solvent-like, fruity	1201	738	<1	<1	256
10	3-methylbutanol	malty, roasty	1206	745	1	32	2
11	2-heptanone	fruity, flowery	1207	891	<1	32	<1
12	1-pentanol	pungent, cheesy	1235	769	512	4	<1
13	(*Z*)-4-heptenal	fishy	1245	894	8	1	1
14	hexyl acetate	fruity, green apple-like	1275	1014	16	8	2
15	octanal	citrus-like, green	1280	1002	64	8	64
16	1-octen-3-one	mushroom-like	1285	978	16	32	4
17	2-methyl-3-furanthiol [f]	broth-like	1304	n.d.	128	128	4
18	(*E*)-2-heptenal	green, flowery	1311	951	32	32	64
19	2-acetyl-1-pyrroline [f]	popcorn-like	1342	930	32	32	64
20	(*Z*)-3-hexen-1-ol	green, grassy	1376	861	64	32	64
21	nonanal	citrus-like, soapy	1386	1106	32	4	64
22	2-nonanone	cheesy, fatty, green	1398	1094	32	16	64
23	(*E*)-2-octenal	fatty, grassy, green	1417	1055	64	32	16
24	acetic acid	vinegar-like	1430	619	256	64	16
25	3-(methylthio)propanal	cooked potato-like	1455	903	64	256	256
26	(*E,E*)-2,4-heptadienal	fatty, roasty	1480	1020	128	64	64
27	decanal	soapy	1489	1203	64	<1	64
28	2-isobutyl-3-methoxypyrazine	bell pepper-like, earthy	1510	1090	32	<1	2
29	benzaldehyde	almond-like	1515	965	2	4	1
30	(*E*)-2-nonenal	fatty, cardboard-like	1524	1164	128	128	16
31	propanoic acid	pungent, sweaty, fruity	1535	924	256	16	2
32	linalool	flowery	1539	1103	512	128	256
33	1-octanol	soapy, citrus-like, fatty	1550	1077	2	32	64
34	2-methylpropanoic acid	cheesy	1562	782	32	<1	<1
35	(*E,Z*)-2,6-nonadienal	cucumber-like, fatty	1574	1158	128	32	16
36	butanoic acid	cheesy	1626	804	1	1	4
37	(*E*)-2-decenal	green, fatty	1627	1252	64	<1	<1
38	acetophenone	almond-like, solvent-like	1634	n.d.	1	<1	4
39	phenylacetaldehyde	flowery, honey-like	1634	1040	4	1	256
40	3-methylbutanoic acid	cheesy, sweaty, banana-like	1650	859	128	32	256
41	unknown	musty, smoky	1665	n.d.	4	8	<1
42	(*E,E*)-2,4-nonadienal	deep fried, fatty	1696	1216	32	≥1024	64
43	3-(methylthio)propanol	cooked potato-like	1707	982	64	4	<1
44	farnesene	earthy, flowery	1712	1459	16	<1	2
45	pentanoic acid	fruity, sweaty, pungent	1725	892	16	16	16
46	(*E*)-b-farnesene	pumpkin-like, cucumber-like	1740	1508	64	16	16
47	2-acetyl-2-thiazoline [f]	popcorn-like, roasty	1747	n.d.	64	<1	<1
48	(*E,E*)-2,4-decadienal	deep fried, fatty	1800	1325	512	≥1024	≥1024
49	β-damascenone [f]	fruity, grape-like	1808	1374	128	8	16
50	geraniol	flowery, earthy	1841	1428	32	<1	64
51	1-undecanol	soapy, green	1851	1370	64	2	2
52	2-methoxyphenol	smoky, ham-like, vanilla-like	1853	1096	256	<1	4
53	butyl benzoate	fruity	1862	n.d.	4	4	32
54	2-phenylethanol	rose-like, flowery	1897	1110	128	<1	<1
55	δ-octalactone	fruity, coconut-like	1908	1152	2	8	16
56	5-methylguaiacol	smoky, vanilla-like	1920	1191	256	64	128
57	unknown	metallic	1947	1543	128	<1	<1
58	*trans*-4,5-epoxy-(*E*)-2-decenal [f]	metallic	1994	1379	512	64	64
59	2-methylphenol	medical, ink-like, phenolic	1997	n.d.	<1	256	<1
60	methyl cyclopentenolone	maggi-like, spicy	2003	1030	2	64	4
61	γ-nonalactone	fruity, flowery	2014	1360	32	16	32
62	phenol	phenolic	2017	959	16	16	<1
63	octanoic acid	green, soapy	2043	118	4	16	64
64	2,5-dimethylphenol	smoked ham-like, medical	2072	n.d.	512	32	64
65	4-methylphenol	horse stable-like, faecal	2078	1089	4	<1	<1
66	δ-nonalactone	fruity, coconut-like	2084	1380	256	<1	1
67	γ-decalactone	fruity, peach-like	2134	1474	128	8	1
68	unknown	honey-like, flowery	2158	n.d.	<1	<1	32
69	2-methoxy-4-vinylphenol	clove-like, smoked ham-like, vanilla-like	2182	1326	1	<1	1
70	δ-decalactone	coconut-like	2188	1498	<1	4	2
71	3-hydroxy-4,5-dimethylfuran-2(5*H*)-one	maggi-like, celery-like	2194	1002	256	128	256
72	3,4-dimethylphenol	horse stable-like, leather-like	2212	1216	16	8	16
73	decanoic acid	coriander-like, plastic-like, soapy	2254	1374	128	32	32

(*continued on next page*)

Table 2 (*continued*)

No.[a]	Odorant [b]	Odor Quality [c]	Retention Index on		FD Factor [d]		
			DB-FFAP	DB-5	Fresh	Past	UHT
74	3-phenyl-2-propen-1-ol	honey-like, vanilla-like	2269	1313	64	64	32
75	δ-undecalactone	fruity, peach-like	2278	n.d.	64	4	<1
76	4-vinylphenol [f]	phenolic	2388	1231	16	256	32
77	δ -dodecalactone	fruity, peach-like	2395	1700	<1	<1	4
78	2-(methylthio)benzothiazole	medical, smoky	2420	n.d.	128	64	16
79	benzophenone	flowery	2477	n.d.	16	128	32
80	indole	faecal	2485	1320	16	<1	<1
81	5-(hydroxymethyl)furfural	butter, caramel-like, fatty	2511	1228	<1	256	16
82	phenylacetic acid	honey-like	2545	1256	128	258	64

[e] n.d. not detected.

[a] Consecutive numbering of odorants according to their retention indices on capillary column DB-FFAP.

[b] Odorant was identified by comparison of its odor quality and intensity and retention indices on capillaries DB-FFAP and DB-5 as well as mass spectra (EI mode) with data of reference compounds.

[c] Odor quality perceived at the odor detection port by four trained panellists.

[d] Flavour dilution factor determined on DB-FFAP.

[f] No unequivocal mass spectrum was obtained; identification is based on the remaining criteria given in footnote b.

256), 3-(methylthio)propanal (*cooked potato-like,* FD 256), linalool (*flowery,* FD 256), phenylacetaldehyde (*flowery, honey-like*, FD 256), 3-methylbutanoic acid (*cheesy, sweaty, banana-like*, FD 256) and 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one (*maggi-like, celery-like*, FD 256).

3.2.4. Aroma-active volatiles found exclusively in the fresh and the thermally treated pulps

Fresh pulp. The aroma-active compounds α-pinene (*green, woody,* FD 16), 2-methylpropanoic acid (*cheesy,* FD 32), (*E*)-2-decenal, (*green, fatty,* FD 64), 2-acetyl-2-thiazoline (*popcorn-like, roasty,* FD 64), odorant no. 57 (*metallic,* FD 128), 4-methylphenol (*horse stable-like, faecal*, FD 4), 2-phenylethanol (*rose-like, flowery,* FD 128) and indole (*faecal,* FD 16) were only perceived in the fresh pulp (FD > 1). As a flavour molecule, 2-phenylethanol is of high commercial importance (Welsh et al., 1989). It is often added to ice cream, candy, non-alcoholic beverages, gelatines, puddings and chewing gum (Furia and Bellanca 1971). Many authors reported the presence of 2-phenylethanol in cocoa pulp from different origins (Pino et al., 2010; Chetschik et al., 2018; Bickel Haase et al., 2021). Several thermal degradation processes were proposed for this substance, yet further research is needed (Sakai et al., 2013). Moreover, indole was only perceived in the fresh material. Indole is often formed by bacteria (Lee and Lee 2010) and it imparts a floral taint with a faecal note to foods and beverages (Parker et al., 2015). Due to its off-flavour character, its absence in the thermally treated samples may be advantageous for the overall cocoa pulp's aroma profile.

Pasteurised pulp. The aroma-active compounds 2-heptanone (*fruity, flowery,* FD 32) and 2-methylphenol (*medical, ink-like, phenolic,* FD 256) were exclusively detected in pasteurised pulp. Methyl ketones such as 2-heptanone, 2-nonanone and 2-undecanone are important aroma constituents of blue cheese and are formed from triglycerides by thermally induced β-oxidation followed by a decarboxylation reaction (Belitz et al., 2004). Pino et al. (2010) identified the methylketone 2-heptanone in pulp of an undefined cocoa genotype from Colombia, while Rottiers et al. (2019) described this compound to be abundant in raw cocoa. Accordingly, Akoa et al. (2023) described the presence of 2-heptanone in cocoa beans from different varieties grown in Cameroon. Furthermore, in the degradation of polyphenols and lignin, enzymes and microorganisms release phenols (Zaprometov 1989). Phenols and their derivatives can be strong aroma-active substances. Methylphenols are often described as *phenolic* and *smoky*, whereas methoxyphenols can have a broad range of odor qualities reaching from *smoky* to *vanilla-like* (Lentz 2018; Genovese et al., 2018; Issa-Issa et al., 2020).

UHT-treated pulp. The exclusive substances perceived in UHT-treated pulp were methyl 2-methylbutanoate (*fruity, banana-like,* FD 64), 2-methylbutanol (*solvent-like, fruity,* FD 256), an unidentified compound with *honey-like* and *flowery* attributes (no. 68, FD 32) and δ–dodecalactone (*fruity, peach-like,* FD 4). It is to note that these compounds impart *fruity* and *flowery* odor qualities. Additioanlly, the UHT-treated pulp was considered more *tropical fruit-like* than the fresh and the pasteurised cocoa pulps during the sensory evaluation (Fig. 1). Even though these substances do not directly fit the description *tropical fruit-like,* they may have contributed to the UHT-treated pulp to be perceived as such. Due to the intricate interplay of multiple aroma-active compounds, it is often difficult to draw definitive conclusions regarding the correlation between a single volatile compound and olfactory perception (Brattoli et al., 2013). For instance, Strube et al. (2009) showed the interaction between several odorants, predominantly *fatty-smelling* aldehydes, resulting in a *plastic-like* off-odor in mineral water. Moreover, AEDA serves as a screening method, but it does not provide an absolute indication of which compounds have the greatest impact on the overall aroma of food. This limitation arises partly because the odor compounds in dilution extracts are volatilized and evaluated, whereas in actual foods, the volatility of aroma compounds depends on their solubility, vapour pressure and specific thresholds in the food matrix as well as their interactions with non-volatile constituents. Additionally, the FD factors ascribed in AEDA do not take into account the physiological impressions that influence our perception of odor character or the sensory thresholds of odor-active compounds in the food matrix (Marsili 2020). New studies indicate that odorants can undergo diverse biotransformation processes in the olfactory and/or respiratory nasal epithelium and mucus, influencing overall odor perception if their concentrationis lie above the detection thresholds (Kornbausch et al., 2022). In this sense, cAEDA, as applied in this study, only enables to determine qualitative changes in the cocoa pulp with thermal processing. To fully understand the influence of these *fruity* and *floral* odorants to the total aroma profile of thermally treated cocoa pulp a calculation of odor activity values and the implementation of recombination and omission experiments are required.

3.2.5. Comparison of aroma-active volatiles in the fresh and thermally treated pulps

Fresh and pasteurised pulp. Both cocoa pulps shared six common aroma-active compounds not perceived in the UHT-treated pulp. These aroma-active VOCs were δ-carene (*green*, RI 1140), 1-pentanol (*pungent, cheesy*, RI 1235), odorant no. 41 (*musty, smoky*, RI 1665), 3-(methylthio) propanol (*cooked potato-like*, RI 1707), phenol (*phenolic,* RI, 2017) and δ-undecalactone (*fruity, peach-like*, RI 2278). In the pasteurised material, δ-undecalactone showed a FD factor of 4 compared to FD 64 in fresh pulp. Furthermore, its absence in UHT-treated pulp hints at increased losses with higher processing temperatures. Moreover, while 3-(methylthio)propanol showed the highest FD factor in fresh pulp, its oxidised form 3-(methylthio)propanal exhibited a higher FD factor after thermal

treatment. It changed from 64 in the fresh pulp to FD 256 in the pasteurised and UHT-treated samples. 3-(Methylthio)propanal is a thermally induced volatile flavour compound and the result of the Maillard reactions of α-dicarbonyl compounds with the amino acid methionine, followed by Strecker degradation reactions (Havkin-Frenkel and Belanger 2008). Various compounds of the fresh pulp such as 2-methyl-3-furanthiol (*broth-like,* RI 1304), acetic acid (*vinegar-like*, RI 1430) and (*E*)-2-nonenal (*fatty, cardboard-like, green*, RI 1524) were better preserved with pasteurisation, showing lower reductions in the FD factors compared with the UHT-treatment. An explanation could be a lower stability of these aroma-active compounds to higher temperatures (135 °C), and/or a higher stability to longer heat exposures (approximately 80 °C for 20 min).

Fresh and UHT-treated pulp. They shared a larger number of exclusive aroma-active regions (n = 10) than the fresh and pasteurised pulp. These were myrcene (*earthy, metallic geranium-like*, RI 1158), 1-penten-3-ol (*pungent,* RI 113), decanal (*soapy,* RI 1489), 2-isobutyl-3-methoxypyrazine (*bell pepper-like, earthy,* R 1510), acetophenone (*almond, solvent-like,* RI 1634), farnesene (*earthy, flowery,* RI 1712), geraniol (*flowery, earthy*, RI, 1841), 2-methoxyphenol (*smoky, ham-like, vanilla-like*, RI, 1853), δ-nonalactone (*fruity, coconut-like*, RI, 2084) and 2-methoxy-4-vinylphenol (*clove-like, smoked ham-like, vanilla-like*, RI 2182). The FD factor of decanal remained the same after UHT-treatment. Likewise, UHT-treatment affected geraniol's FD factor minimally, changing it from FD 32 in the fresh sample to FD 64 in the UHT-treated. Contrarily, the FD factors of the other substances decreased strongly with ultra-high temperature treatment.

Our descriptive testing of the orthonasal characteristics (3.2.1) showed a significant difference for the attribute *tropical fruit-like* between the fresh and UHT-treated pulp. The perception of highly volatile compounds, methyl 2-methylbutanoate (*fruity, banana-like,* FD < 1 in fresh and FD 64 in UHT-treated pulp), 2,3-pentanedione (*butter-like*, FD 64 and FD 256), hexanal (*green,* FD 32 and FD 16) and 3-methylbutyl acetate (*fruity*, FD 4 and FD 128) may have been contributed for the difference in this attribute. As aforementioned, their quantification and OAV calculation in cocoa pulp may help comprehend better the changes taking place during thermal treatment.

Pasteurised and UHT-treated pulp. The thermally treated pulps exhibited two compounds not perceived in the fresh material, δ–decalactone (*coconut-like*, RI 2188) and 5-(hydroxymethyl)furfural (*butter, caramel-like, fatty,* RI 2511). The substance 5-(hydroxymethyl) furfural showed FD factors of 256 in pasteurised and 16 in UHT-treated pulp. The heating of monosaccharides under acidic conditions, e.g. in juice preservation, leads to the formation of a large number of furanoid and pyranoid compounds. These arise from enolizations and dehydrating reactions of carbohydrates (Belitz et al., 2004). Murkovic and Bornik (2007) described the formation of 5-(hydroxymethyl)furfural (HMF) during coffee roasting, which peaked at 240 °C in the first 3 min of roasting. In cocoa pulp, the higher FD factor in pasteurised pulp may be attributed to the pulp's longer exposure to heat. While UHT-treatment was short, with only 30 s exposure to 135 °C and immediate cooling in an ice bath, pasteurised pulp was kept hot for 20 min, possibly giving rise to increased HMF formation.

3.3. Storage stability of thermally treated cocoa pulp

3.3.1. Microbiological analysis

In order to assess the shelf-life of the pulps, microbiological analyses (yeasts, moulds and aerobic mesophilic bacteria) of the fresh pulp and the thermally-treated pulps were carried out directly after processing. In addition, the thermally-treated pulps were also investigated after 6, 12 and 24 weeks of storage (Table 3). The colony forming units per gram of pulp [CfU/g] of yeasts and moulds were below 100 prior and after thermal treatment. Independent from the storage temperature and the sampling time point, the total mould and yeast count remained unchanged (<100 CfU/g). Furthermore, we investigated the presence of viable bacterial cells. The aerobic mesophilic bacteria in the fresh pulp accounted for 4.6×10^3 CfU/g. After pasteurisation, 6.3×10^2 CfU/g were found, indicating a reduction of approximately one logarithmic unit compared to the fresh material. The inactivation of the aerobic mesophilic bacteria was more effective with the ultra-high temperature treatment. The bacterial count after UHT-treatment was below 100 CfU/g, representing an inactivation of over two logarithmic units or 99% of the initial bacterial count. In addition, the total aerobic mesophilic bacterial count of the pasteurised and the UHT samples remained constant throughout storage, indicating the remaining viable bacterial cells were not able to grow and proliferate. Accordingly, differences between the two storing temperatures in either the pasteurised and the UHT-treated pulps could not be observed. The constancy in the total yeast and mould count as well as in the aerobic mesophilic bacterial count might be explained by the low pH-value of cocoa pulp, which conceivably acted as a hindering factor for the growth of microorganisms (Doyle and Buchanan 2013). Some approaches for the microbial inactivation of cocoa pulp for various products have been previously described (Escalante et al., 2015; Puerari et al., 2012). However, the microbial load of the stabilised cocoa pulp over a longer storage was first described in this study. Additionally, in a recent study, Firdaus et al. (2022) produced cocoa pulp syrups using different sugar types from West Sumatra. The syrups were not subject to any thermal treatment for preservation. The microbiological tests for fungi and salmonella showed that the syrup kept at 5 °C could only be consumed safely during the first five days, highlighting the importance of preserving the cocoa pulp to reduce possible health hazards to the consumers.

3.3.2. Colour properties

Despite inactivation of the peroxidase activity and probably also of the more thermolabile enzymes (3.1), a strong browning was observed during storage. The L*a*b*-values of pasteurised and UHT-treated pulps stored at 4 °C and 23 °C for 24 weeks are shown in Supplementary Table 3. Over time, the L* value of the thermally treated samples decreased, indicating the samples became darker. However, the decrease in the L* values was more distinct at 23 °C. The a* values increased over time, while the b* values decreased when the samples were kept at 23 °C, indicating a yellowing. At 4 °C, the a* values

Table 3
Microbiological analysis of fresh, pasteurised and UHT-treated cocoa pulp at different time points.

Sample	Total yeast and mould count [CfU/g]						
	t_0	4 °C /6 W	23 °C/6 W	4 °C/12 W	23 °C/12 W	4 °C/24 W	23 °C/24 W
Fresh	<100						
Pasteurised	<100	<100	<100	<100	<100	<100	<100
UHT-treated	<100	<100	<100	<100	<100	<100	<100
Sample	Aerobic mesophilic bacterial count [CfU/g]						
	t_0	4 °C /6 W	4 °C/12 W	4 °C/24 W	23 °C/4 W	23 °C/12 W	23 °C/24 W
Fresh	$4.6 * 10^3$						
Pasteurised	$6.3 * 10^2$	$8.0 * 10^2$	$1.1 * 10^3$	$5.2 * 10^2$	$5.9 * 10^2$	$5.6 * 10^2$	$4.9 * 10^2$
UHT-treated	<100	<100	<100	<100	<100	<100	<100

W=weeks in storage, t_0 = time point zero, sample taken directly after stabilisation.

increased from week 2 to week 12, then dropped after 24 weeks; the b* values decreased slightly from week 12 to week 24. The BI of the stored pulps was calculated and plotted over time (Fig. 2). The browning effect was more pronounced in samples stored at 23 °C than in the samples stored at 4 °C, being very similar to the fresh ones (Table 1). Various authors attribute the browning of thermally processed fruit juices and fruit juice concentrates to non-enzymatic reactions, such as Maillard reactions or the oxidative degradation of ascorbic acid (Ibarz 1990; Selen Burdurlu and Karadeniz 2003). In our case, both reactions could be responsible for the browning, as free amino acids and reducing sugars as well as vitamin C can be present in the pulp (Pettipher 1986). However, the reason for the browning needs to be investigated in further studies.

4. Conclusions

The valorisation of the complete cocoa fruit pulp could help to increase the sustainability along the cocoa bean supply chain, improve the livelihoods of many cocoa farmers and promote the development of tasty new food products. Due to the fresh pulp's high water activity and sugar content, a stabilisation step is recommendable to slow down spoiling reactions and increase its shelf-life for transport and food applications. Pasteurisation and UHT-treatment were shown to be effective technologies for the preservation of cocoa pulp, as microorganisms and enzymes were successfully inactivated. Sensory evaluations suggested substantial changes of the pulps' aroma-active substances, which were confirmed by identification experiments by means of cAEDA, GC-MS/O, SBSE-GC-MS/O and HS-SPME-GC-MS/O. For a more thorough characterisation of the pulps' aroma profiles, quantitative analyses with subsequent calculation of odor activity values (OAV) as well as recombination and omission experiments should be carried out in future studies. Considering the higher degree of microbiological inactivation, the retention of a larger number of aroma-active regions as well as the higher score in the intensity of attribute *tropical fruit-like*, a UHT-treatment followed by a cold storage is the recommended technological approach for the preservation of cocoa pulp.

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Thomas Bickel Haase: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Project administration. **Susanne Naumann-Gola:** Conceptualization, Writing – review & editing, Supervision, Project administration. **Eva Ortner:** Methodology, Data curation, Writing – review & editing, Supervision. **Holger Zorn:** Writing – review & editing, Supervision, All authors have read and agreed to the published version of the manuscript. **Ute Schweiggert-Weisz:** Conceptualization, Funding acquisition, Writing – review & editing, Supervision.

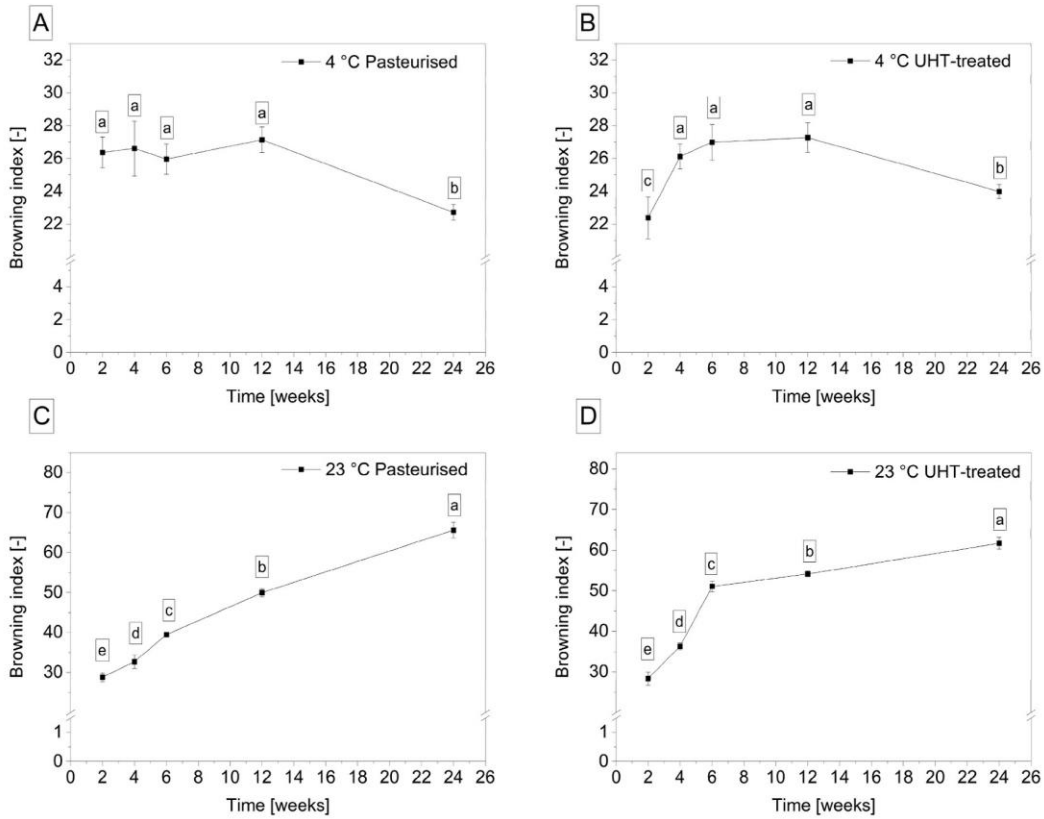

Fig. 2. Browning index of pasteurised and UHT-treated cocoa pulp stored at 4 °C and 23 °C for 24 weeks. Different letters indicate significant differences ($p < 0.05$) between the time points.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2023.100549.

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Chapter 3: Enzyme-assisted hydrolysis of *Theobroma cacao* L. pulp

Summary: Effective technologies are vital to remove the tightly adhering cocoa pulp without affecting the beans' quality, optimise the pulp yield and facilitate further processing. This chapter describes the effects of temperature, enzyme activity and enzyme combinations on the viscosity, particle diameter ($d_{v,0.9}$) and particle size distribution, browning index (BI) and total soluble solids of cocoa pulp by using the cell-wall degrading enzymes endo-polygalacturonase (P), endo-cellulase (C) and hemicellulase (H). A reduced quadratic model fitted the relative reduction in viscosity, the $d_{v,0.9}$ and the BI. P showed the strongest influence on the viscosity, while C proved the least effective. Moreover, synergistic effects were observed when combining P with C and/or H. Maximal reductions in viscosity by 70% with 300 U P and 300 U H at 40° C could be predicted. When combined, P, C and H reduced the $d_{v,0.9}$ to 418 µm (40 °C, 580 U) compared to the $d_{v,0.9}$ of fresh pulp, which was 613 µm. The BI was only affected significantly by the temperature, and the terms corresponding to enzyme activity and combinations were eliminated by backward regression analysis. Validation trials confirmed the models obtained for the relative reduction in viscosity and the $d_{v,0.9}$. This chapter offers important knowledge for the enzyme-assisted processing of cocoa pulp, increasing the sustainability of the cocoa supply chain through its valorisation and offering food producers innovative ingredients.

Keywords: Cocoa pulp, D-optimal design, enzyme-assisted de-pulping, by-products, valorisation, pulp processing

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Enzyme-assisted hydrolysis of *Theobroma cacao* L. pulp

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ABSTRACT

Cocoa pulp, a by-product of the cocoa supply chain, has gained the food sector's attention for its pleasant flavour. Enzyme-assisted technologies to facilitate its separation from the cocoa seeds remain largely unexplored. We studied the effects of temperature, enzyme activity and enzyme combinations on the physicochemical properties of cocoa pulp by Response Surface Methodology and D-optimal design. Endo-polygalacturonase, endo-cellulase and hemicellulase activities were investigated. A reduced quadratic model described the relative reduction in viscosity, of the particle diameter $d_{v,0.9}$ and of the browning index. Polygalacturonase had the strongest influence on viscosity, while cellulase had the least effect. Synergistic effects of polygalacturonase with cellulase and/or hemicellulase were identified. The model predicted maximal reductions in viscosity by 70.3 % with 275 U of polygalacturonase and 275 U of hemicellulase at 40 °C. Fresh pulp's $d_{v,0.9}$ was 613 μm. Combining all enzyme activities reduced the $d_{v,0.9}$–418 μm (40 °C, total activity: 580 U). Only the temperature proved to significantly influence the browning index, with higher temperatures causing a higher colouring effect. Lastly, the model was successfully validated for the relative reduction in viscosity and the $d_{v,0.9}$. Our study provides key insights into the enzyme-assisted processing of cocoa pulp, facilitating its separation from the seeds. By utilising the pulp, innovative ingredients can be developed for the food sector, and at the same time, the added value in the cocoa supply chain can be improved.

1. Introduction

Cocoa beans are one of the major agricultural export commodities worldwide as well as vital constituents in the economies of many countries in West Africa, South America and South-East Asia [1]. The International Cocoa Organization reported a worldwide cocoa bean production of around 4.9 million tonnes in the 2021/2022 cocoa season, providing a livelihood to approximately 50 million people worldwide [2,3]. Nonetheless, the cocoa industry faces several challenges, with one of the most critical being the generation of substantial quantities of side-streams, posing significant environmental and economic concerns [4]. Traditionally, the beans are considered the commercially important fraction of the cocoa fruit, which make up only a small portion of the total weight. The cocoa pod husks and the cocoa pulp —lost during the cocoa bean fermentation— contribute to ~90 % of the fruit's mass but are still considered as side streams [5]. The cocoa pulp has been ascribed an important role for the final aroma quality of fermented cocoa beans [6]. Notwithstanding, past studies suggest that partial de-pulping prior to fermentation is possible [7,8]. Previously, this has been mainly done using fruit de-pulpers. However, it has been reported that fruit de-pulpers often cannot remove the tightly adhering mucilaginous layer on cocoa beans without compromising their structural integrity [9]. Following de-pulping, the obtained pulp may be used as a food ingredient, thereby increasing the fruit's value, and creating opportunities for cocoa and food producers. Previously described uses of the pulp include applications in jams, vinegar and soft drinks [10,11].

The composition of cocoa pulp has been described to vary between cocoa cultivars, harvest season and country of origin [12]. These differences, especially with regard to the cocoa pulps' flavour profiles, highlight their great potential for various applications [13]. Cocoa pulp generally contains over 80 % water, around 13 % sugars and 1.5 % organic acids, such as citric acid and malic acid [14,15]. Cocoa pulp's major polysaccharides have been reported to be pectin (3.7–6.6 %), hemicellulose (1.5–2.8 %) and cellulose (4.7–5.1 %) with low

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Table 1
Parameters used to assess the responses during enzymatic hydrolysis of cocoa pulp.

Factors	A: Temperature	B: Enzyme activity[a]	C: Enzyme ratio – (P:C:H)
Type of response	Numeric	Numeric	Categorical
Units	°C	Units	–
No. Levels	4	3	7
Type	Discrete	Discrete	Nominal
1	30	10	1:1:1
2	40	100	0:0:1
3	50	1000	1:0:0
4	60	–	0:1:0
5	–	–	1:1:0
6	–	–	0:1:1
7	–	–	1:0:1

[a] Total enzyme activity comprising endo-polygalacturonase (P), cellulase (C) and hemicellulase (H).

concentrations of lignin [14]. Moreover, the total dietary fibre content of cocoa pulp makes up to approximately 16.9 g per 100 g dry matter and is mainly composed of a soluble fraction (approximately 95 %) [16]. Soluble fibre can retain water, acting as a strong thickening, gelling, foam and emulsion stabilizing agent [17]. The high viscosity of cocoa pulp is believed to be mainly caused by pectins. Their degradation during the cocoa bean fermentation has been ascribed to the activity of pectinases produced by yeasts [18]. Nunes et al. [19] investigated the use of cocoa pulp from Brazil in the production of beer, for which they determined the pulp's rheological behaviour. The pulp exhibited an apparent viscosity of 95 mPas at 30 °C and was attributed a pseudo-plastic behaviour. Due to the pulp's high viscosity, the authors recognized the importance of appropriately designing the machinery to prevent potential losses, fouling, and contaminations during the valorisation of cocoa pulp. Additionally, this suggests the crucial need for developing processing protocols specifically tailored for cocoa pulp. To facilitate the processing of viscous pulps, the fruit processing industry has often made use of cell-wall degrading enzymes to increase the product yields, reduce the turbidity and the viscosity as well as to modify the sensory properties of the fruit-derived products [20]. Moreover, custom-made protocols consisting of tailored enzyme preparations have been reported for the extraction of valuable compounds from vegetal sources, such as flavours and colourants from tomatoes, red beets, and others [21–23]. Regarding the utilization of cocoa pulp, effective de-pulping and processing technologies remain largely underexplored but are imperative for its separation and valorisation, aiming to achieve optimal yields, minimize waste generation, and ensure the unimpeded utilization of cocoa beans.

To facilitate its valorisation, this study seeks to elucidate the influence of various activities and combinations of cell-wall degrading enzymes (endo-polygalacturonase, endo-cellulase, and hemicellulase), alongside the incubation temperature, on the viscosity, particle diameter ($d_{v,0.9}$) and particle size distribution, colour, and total soluble solid content of cocoa pulp by means of a D-optimal design.

2. Materials and methods

2.1. Recovery of the fresh cocoa pulp

Fresh cocoa pods from Cameroon were imported to Germany in a cool shipment directly after harvest. Immediately after arrival, the fruits were washed, cut open and the cocoa pulp was separated by means of a mechanical de-pulper with a 2.8 mm sieve mesh (Fructmas P006, Karl Bockmeyer Kellereitechnik GmbH, Germany). After de-pulping, the fresh pulp was portioned and vacuum-sealed in odourless plastic bags (PA/PE 90/130 × 280 mm, Dagema eG, Germany) and immediately frozen and kept at −20 °C until analysis.

2.2. Enzymes

Endo-polygalacturonase (E-PGALUSP, EC 3.2.1.15, 1100 U/mL) from *Aspergillus aculeatus* and endo-cellulase (E-CELAN, EC 3.2.1.4, 1200 U/mL) from *Aspergillus niger* were purchased from Megazyme (NEOGEN, Michigan, USA) as suspensions. Hemicellulase (H2125, CAS No.: 9025-56-3, EC 232-799-9, <3 U/mg powder preparation) from *Aspergillus niger* was purchased as a powder from Sigma Aldrich (Merck KGaA, Darmstadt, Germany) and was suspended in 100 mM sodium acetate buffer (Merck KGaA, Darmstadt, Germany) at pH 5.5 (600 U/mL). More details on the enzymes, such as the batch numbers and suppliers' recommendations, can be found in Supplementary Table 1.

2.3. Experimental design

The effects of temperature (factor A), enzyme activity (factor B) and enzyme combinations (factor C) (Table 1) on the relative reduction in viscosity, the particle diameter $d_{v,0.9}$, the content of total soluble solids (° Bx) and the browning index (BI) of cocoa pulp were studied. The experimental design was created and evaluated using the software Design Expert 8 (StatEase, Minneapolis, MN, USA). A D-optimal design was chosen following an optimal set of candidate runs proposed by the software's algorithm, where the set of treatment runs consisted of all possible combinations of the various factor levels (Supplementary Table 2). The enzymes endo-polygalacturonase (P), cellulase (C) and hemicellulase (H) were used and coded following this order: the number 1 was assigned to the run when the enzyme was present, whereas a 0 was assigned in the absence of the enzyme. The enzyme combinations were chosen based on previous literature on the cell-wall composition of cocoa pulp, focusing on the relative proportions of pectin, hemicellulose, and cellulose [12,14,24]. Moreover, the enzyme activities described the sum of the total final activities. For example, 600 U of a 1:1:1 ratio would correspond to the combined volumes of all three enzyme preparations (P, C, and H), each with an activity of 200 U (c.f. 2.2) (e.g., 333.3 μL of H preparation). Enzyme activities were varied with a 10-fold increase (10 U, 100 U and 1000 U) as described in literature [24]. Hydrolyses were conducted using 20 g of fresh pulp, resulting in dosages of 0.5 U/g for 10 U, 5 U/g for 100 U, and 50 U/g for 1000 U. In addition, the temperature was varied in 10 °C steps ranging from 30 °C to 60 °C.

A modified cubic model was chosen to study the individual as well as the interactive effects of the factors (A, B, C) on the responses Y_i (equation (1), Eq. (1)). The terms involved in the model were A, B, C, AB, AC, BC, ABC, A^2, B^2, A^2B, B^2A, A^2C, B^2C.

$$Y_i = \beta_0 + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{2}\beta_i x_i^2 + \sum_{i=1}^{3}\beta_{ij} x_i x_j + \sum_{i=1}^{3}\beta_{ijk} x_i x_j x_k + \sum_{i=1}^{3}\beta_{ij} x_i^2 x_j + \sum_{i=1}^{3}\beta_{ij} x_i x_j^2 \quad \text{(Eq. 1)}$$

Where $\beta_0, \beta_i, \beta_{ij}, \beta_{ijk}$ are the constant regression coefficients of the model and x_i, x_j and x_k are independent variables for the response Y_i. To assess the adequacy of the second order polynomial function, the variance was separated into linear, quadratic, cubic, and interaction components for each element in the experiment. An appropriate model was selected based on a significant p-value ($p < 0.05$), an insignificant lack of fit value ($p > 0.05$), and an adjusted as well as a predicted R^2 close to 1.0. A backward regression was performed on the original model with an alpha value of 0.100 to eliminate non-significant terms ($p < 0.10$). After regression, the newly obtained reduced model was checked for a better fit (maintaining $p < 0.05$ and a lack of fit >0.05) compared to the originally proposed model. Additionally, the adequate precision value (>4) and the difference in the adjusted and the predicted R^2, being optimally below ≤0.2, were considered when evaluating the reduced

model, aiming to have an adjusted R^2 higher than the original model. The terms were further assessed for the normal distribution of residuals. Internally studentized residuals against predicted response values and run values were examined to identify any abnormalities or trends in the data points. The Box-Cox plot was used to determine whether a power transformation (λ) of the data was necessary to fit the suggested model. Additionally, to reduce the risks of overseeing false-positive results (type I errors), the data was revised to determine if a Bonferroni correction was needed. A backward regression and analysis of variance (ANOVA) were used to determine the statistical significance of the D-optimal design study.

2.4. Response 1: Relative reduction in viscosity

The viscosity was measured with a Rapid-Visco-Analyser (RVA) (PerkinElmer LAS Ltd., Llantrisant, UK). Aliquots of 20 g of cocoa pulp were used per run. Prior to enzyme addition, the pulp was tempered to the desired temperature using a water bath (K20 mgw, LAUDA Dr. r. Wobser GmbH & Co. KG, Lauda-Königshofen, Germany). The temperature of the pulp was monitored with a thermometer (Checktemp 1, Hanna Instruments Deutschland GmbH, Vöhringen, Germany). Next, the pulp was transferred into the RVA sample cup with a paddle stirrer, and the enzyme/enzymes were added according to the run sheet (Supplementary Table 2). The method set in the TCW3 software (PerkinElmer LAS Ltd., Llantrisant, UK) was modified according to the temperature requirement of each run, which was held constant throughout the measurement. The rotation speed of the paddle was set to 160 rpm to minimize the effect of stirring on the viscosity of the pulp without compromising the efficient mixing. The viscosity [mPas] was measured against time [s] in intervals of 16 s, with the last measurement at 3600 s (1 h). The first data point was considered as the initial viscosity of the cocoa pulp (prior to hydrolysis), while the viscosity at 3600 s corresponded to the final viscosity. The reduction in viscosity [%] was calculated for all the runs, and the values were entered as the first response in the design (Design-Expert 8, StatEase®, Minneapolis, USA). As replicates were included in the experimental design, measurements were conducted once for each run.

2.5. Response 2: Particle diameter $d_{v,0.9}$

The particle size distribution was determined with a static laser diffraction instrument (Mastersizer 3000, Software version 2.15, Malvern Instruments Ltd., Worcestershire, United Kingdom), a sample dispersion unit MS 1 and a 300 mm RF lens (Malvern Instruments Ltd., Worcestershire, UK). Distilled water was chosen as the dispersant. The refractive index of the particles was determined to be 1.46, and the refractive index of the dispersant was 1.33. Particles were assumed to be irregularly shaped, so the Mie theory was applied as the optical model. Immediately after hydrolysis, samples were equilibrated to room temperature (~21 °C) using running water to cool samples down when necessary. Samples were then added to the dispersing unit, the obscuration was set between 10 % and 15 %, and the stirring speed was kept constant at 2000 min^{-1}. The measurement was conducted 2 min after sample introduction to allow for uniform dispersion, and it was repeated 1 min later to ensure the stirring did not affect the particle size distributions. Triplicates were carried out per sample, and a mean of all six values determined was calculated. Particle diameters were reported as $d_{v,0.9}$, representing a 90 vol% on a relative cumulative particle size curve.

2.6. Response 3: Total soluble solids (° Bx)

The total soluble solids (° Bx) were determined using a digital handheld refractometer DR301-95 (A. Krüss Optronic GmbH, Hamburg, Germany) at room temperature. Measurements were performed in triplicate, and the means were entered as the response.

2.7. Response 4: Browning index (BI)

The DigiEye colour imaging system (DigiEye V2.62, VeriVide, Leicester, UK) was used for colour measurements. This comprised an illumination box with diffuse illuminant D65 and a Nikon D90 digital camera. Digitizer calibration charts were used to calibrate the system. For the colour determinations, the pulp was evenly distributed in a white sample cup (Aqualab, Meter Group Europe, Munich, Germany) and the average surface colour was expressed as CIE L*a*b*-values with L*, a* and b* ranging from black [−] to white [+], from green [−] to red [+], and from blue [−] to yellow [+], respectively. The browning index was calculated using the L*a*b* values and following the formula described by Bal et al. [25]. Analyses were performed immediately after enzyme-assisted hydrolysis. Four colour measurements were performed from each run, and the results were averaged before being included in the model.

2.8. Validation trials

To confirm the results predicted by the model and validate these for the reduction in viscosity and the particle diameter $d_{v,0.9}$, confirmation runs (n = 3) were conducted for an enzyme combination suggested by the final model. This was selected following the software's numerical optimization options, where the enzyme combination was set to have a medium importance, and the goal was set to minimize the activity, representing the more cost-effective alternative for food producers. Regarding the responses, the goal was set to maximize a reduction in viscosity and obtain a minimal particle diameter. The outcome was compared with the predicted responses and checked if it was within the 95 % prediction interval (PI) and 95 % confidence interval (CI). Additionally, the accuracy of the model was investigated for the reduction in viscosity for the enzymes individually at 50 °C and 500 U and 800 U, as these activities were predicted to cause strong responses (n = 3).

3. Results and discussion

3.1. Response 1: Effect of the enzyme-assisted hydrolysis on the relative viscosity of cocoa pulp

3.1.1. Model output on response 1

The empirical data (n = 60) proved a reduction in viscosity between 11.2 % and 64.9 %, where the average reduction equalled 41.7 ± 12.8 %. The software could not corroborate the reduced cubic model pre-set in the design and was marked as aliased due to insufficient design points to fit this type of polynomial. Therefore, the data was fitted into a quadratic model with a significant p-value of 0.0003 and a lack of fit of 0.1372. The interactive effects of factors A and B (term AB), as well as of factors A and C (term AC), were eliminated by backward regression ($p > 0.1$), corresponding to the interaction between the temperature and the enzyme activity and the interaction between the temperature and the enzyme combination. The reduced quadratic model showed a better fit with an overall p-value of $p < 0.0001$, a lack of fit of 0.1252, an adjusted R^2 of 0.8462, a predicted R^2 of 0.774, and a precision value of 16.68. The latter indicates that the model was suited to navigate the design space. Furthermore, the proposed model accounted for the variance present in the data, as indicated by a normal distribution of the residuals. No Box-Cox transformation was needed, hinting at a close-to-normal distribution of the data points [26]. No outliers were detected, and the data points were within Bonferroni corrector limits. The following quadratic model described response 1 (% reduction in viscosity) (equation (2), Eq. (2)):

$$Y_1 = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{2} \beta_i x_i^2 + \sum_{i=1}^{3} \beta_{ij} x_i x_j \quad \text{(Eq. 2)}$$

The individual terms represent the temperature (factor A or x_1), the

Table 2
Final equations for the reduction in viscosity (response 1) of cocoa pulp for the different levels of factor C (enzyme combination).

Factor C: Enzyme combination[b]	Equations as expressed by the software[a]:
1:0:0	$-+5.00221+2.18941*A+0.061368*B-0.026323*A^2-5.94*10^{\ 5}*B^2$
0:1:0	$=-24.0895+2.18941*A+0.073811*B-0.026323*A^2-5.94*10^{-5}*B^2$
0:0:1	$=-17.6288+2.18941*A+0.081426*B-0.026323*A^2-5.94*10^{-5}*B^2$
1:1:0	$=+8.85547+2.18941*A+0.058698*B-0.026323*A^2-5.94*10^{-5}*B^2$
1:0:1	$=+3.17511+2.18941*A+0.071611*B-0.026323*A^2-5.94*10^{-5}*B^2$
0:1:1	$=-18.37158+2.18941*A+0.073645*B-0.026323*A^2-5.94*10^{-5}*B^2$
1:1:1	$=+1.92339+2.18941*A+0.068320*B-0.026323*A^2-5.94*10^{-5}*B^2$

[a] Where A is the temperature and B represents the enzyme activity.
[b] An assigned number 1 indicates the presence and a 0 indicates the absence of the enzyme. The first position corresponds to endo-polygalacturonase, the second to cellulase, and the third to hemicellulase.

enzyme activities (factor B or x_2) and the combination of enzymes (factor C or x_3). The term BC was statistically significant, indicating an interaction between the enzyme activities and the enzyme combination. In addition, the model terms A^2 and B^2 were also shown to contribute significantly to the response. The final equations in terms of the actual factors can be found in Table 2.

3.1.2. *Interpretation of the reduced model for the relative viscosity of cocoa pulp*

Considering the final equations outlined in Tables 2 and it becomes evident that the temperature (factor A) exerted a positive influence on the response. This assumption is underscored by the coefficient +2.18941 associated with the term, hinting at a reduction in viscosity with rising treatment temperatures. This observation aligns with findings from a prior study, wherein higher temperatures, combined with the enzymatic activity of pectinolytic enzymes from *Aspergillus niger* and *Aspergillus aculeatus*, led to a heightened reduction in the apparent viscosity of cocoa pulp [19].

The contour plots displayed in Fig. 1 show the interaction of factors A and B for the seven enzyme combinations tested (factor C) on response 1. Strong reductions in viscosity are shown by the colour gradient changing towards orange and red, whereas blue indicates a low effect (Fig. 1). For the polygalacturonase alone (1:0:0), the model predicted a strong reduction in viscosity by at least 65 % in a range between 35 and 49 °C at approximately 370–670 U. For this enzyme, a maximal possible reduction by 66.4 % at around 42 °C and 509 U was predicted. The viscosity reduction of cocoa pulp has been mainly attributed to the role of pectin-degrading enzymes produced by yeasts during the fermentation of cocoa beans [18]. Accordingly, Nunes et al. [19] reported a decrease in the viscosity of cocoa pulp by 64.8 % and 65.7 % at 42.5 °C with technical pectinolytic enzymes (Novozymes Pectinex Ultra Clear). Meersman et al. [24] demonstrated a viscosity reduction of only 18.8 % using 1000 U endo-polygalacturonase to hydrolyse cocoa pulp. A likely explanation could be the higher amount of cocoa pulp used for hydrolysis, which was 50 g instead of 20 g as in this study. Another possible explanation for this disparity is their enzymatic inactivation procedure, involving a 10-min incubation at 100 °C prior to the viscosity measurement, which differs from the approach taken in this study. Their method prevents the further cleavage of cell-wall polysaccharides but overlooks factors such as pectin gelling upon heat exposure and its potential impact on the sample's viscosity [27]. In addition, the composition of cocoa pulp may vary strongly between cocoa varieties, pod ripeness, harvesting season and origin, accounting for extra variability between studies [5,28]. The second highest reduction in viscosity by single enzyme use was described for hemicellulase alone, corresponding to 55.8 %, predicted at

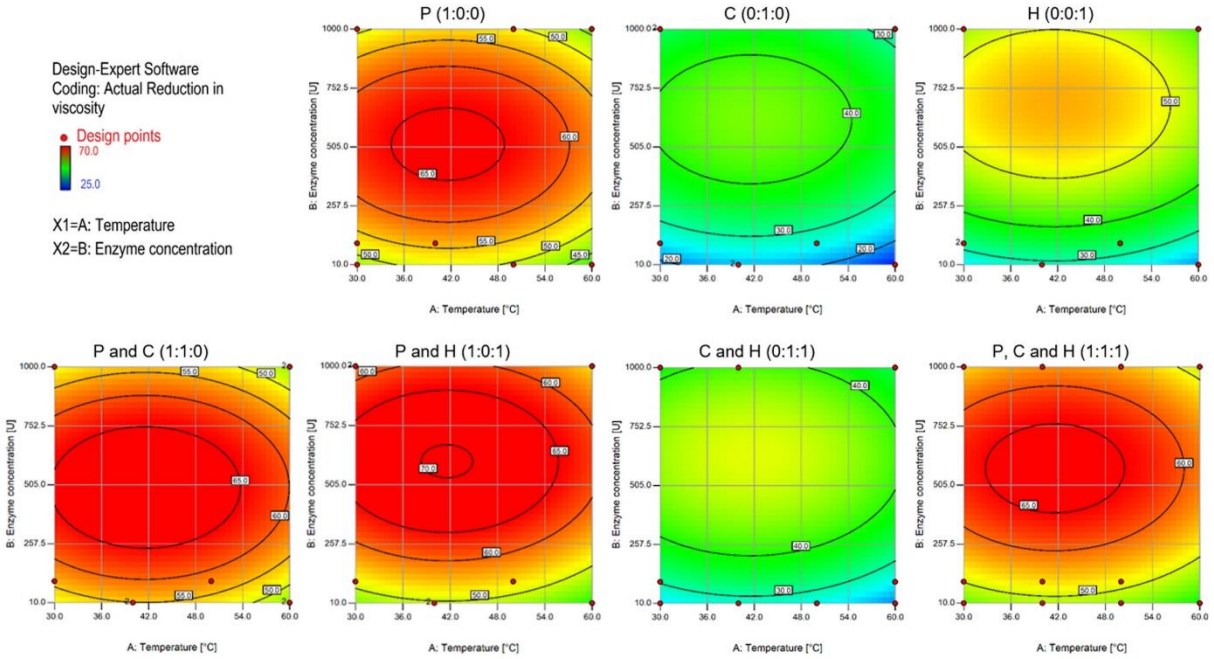

Fig. 1. Contour plots showing the interactions between temperature (A) and enzyme concentration (B) of the different enzyme combinations on the viscosity of cocoa pulp (response 1). The first position corresponds to endo-polygalacturonase, the second to cellulase and the third to hemicellulase, respectively (P:C:H). Whereas an assigned number 1 represents the presence and a 0 the absence of the enzyme in the treatment.

41.6 °C and approx. 685 U. Thanks to their abundance of hydrophilic groups, highly branched polymers like hemicelluloses have greater water retention capacities when compared to linear molecular structures like cellulose. Thus, this results in high viscosities [29]. The hemicellulase used in this study has been described to possess xylanase and mannase activities (Supplementary Table 1), which presumably contributed to the release of the neutral sugars bound to the cocoa pulp's pectin chains and helped reduce the viscosity [24,30]. In comparison, the endo-cellulase showed the lowest reduction in viscosity (0:1:0) with a maximum effect of 44.0 % at approximately 42 °C and 600 U. Although hemicellulase and cellulase could reduce the viscosity of cocoa pulp, their effects were lower than those of the polygalacturonase. These findings seem to be in line with the cell-wall composition described for cocoa pulp, with reported pectin concentrations between 3.7 % and 6.6 %, hemicellulose contents of up to 3 % and cellulose contents ranging from 0.7 % to 5.1 % [14,15,24]. Therefore, formulating the enzyme mix based on the exact cell-wall composition of cocoa pulp, as described in prior studies on the extraction of valuable compounds from vegetal sources, could be a promising approach for future studies [21]. This tailored-made protocol could potentially enhance the efficacy of the enzymatic treatment and further improve the utilization of cocoa pulp. However, this approach would be most effective when the composition of the cocoa pulp is well-characterized, and the natural variability in the polysaccharide composition across different cocoa pulp sources is taken into account [5,28].

The prominent red-orange region in the contour graph signifies a wide range of temperature–enzyme activity combinations resulting in a substantial viscosity reduction. When polygalacturonase was combined with other enzymes, the red section in the contour graphs tended to be equal to or even broader than the contour observed with polygalacturonase alone (Fig. 1). To facilitate comparison between treatments, the combinations are first compared at 45 °C, which is within the optimal temperature range of the enzymes studied [31–33]. At 45 °C, our model predicted the strongest effects for the combinations of polygalacturonase and cellulase (1:1:0) and polygalacturonase and hemicellulase (1:0:1), showing a possible reduction of up to 70.0 % and 69.3 % at 500 U, respectively. When combining cellulase and hemicellulase under the same conditions (0:1:1, 45 °C and 500 U), the viscosity could be reduced by only 48.9 %. The combination of all enzymes (1:1:1, 45 °C and 500 U) enabled a viscosity reduction by 67.1 %. Pectin is prevalent in the middle lamella of the cell-wall of higher plants, serving as a hydrating agent and cementing material within the cellulosic network [34]. Through the action of cellulolytic and hemicellulolytic enzymes, the pectin fraction can become more accessible for breakdown. Therefore, it is reasonable to infer a synergistic effect of pectinases with cellulases and hemicellulases on the degradation of the plant cell-wall [35,36]. Accordingly, when the entire temperature range was considered, the overall strongest response (70.3 %) was observed with the combination of polygalacturonase and hemicellulase at 41 °C and around 550 U. This enzyme pair demonstrated the broadest range of effectiveness, with predicted viscosity reductions exceeding 65 % across temperatures from 30 to 54 °C and enzyme activities of 320–900 U. Interestingly, the combination of polygalacturonase and cellulase also showed a similar range of efficacy, achieving over 65 % viscosity reduction within the temperature range of 30–54 °C and enzyme activities of 250–750 U. Notably, this combination required slightly lower enzyme activities compared to polygalacturonase and hemicellulase while still achieving a 65 % or higher response. When all three enzymes were combined in equal proportions (1:1:1), the 65 % contour became narrower, spanning temperatures of 32–50 °C and enzyme activities of 390–760 U. This was possibly due to the relative lower polygalacturonase activity in the combination. The expansion of the operational range, as shown by larger red areas in the contour graphs, could allow for more adaptability in industrial cocoa pulp processing. This would offer flexibility to vary the enzyme concentration and the temperature while still obtaining a strong reduction in viscosity, potentially leading to improved efficiency and productivity.

Table 3
Final equations for the particle diameter $d_{v,0.9}$ (response 2) of cocoa pulp for the different levels of factor C (enzyme combination).

Factor C: Enzyme combination†	Equations as expressed by the software[a]:
1:0:0	$= 553.75230 - 2.20586*A - 0.38184*B + 0.041928*A^2 + 3.69241*10^{-4}*B^2$
0:1:0	$= 702.00643 - 4.16732*A - 0.39382*B + 0.041928*A^2 + 3.69241*10^{-4}*B^2$
0:0:1	$= 647.77027 - 3.24938*A - 0.46840*B + 0.041928*A^2 + 3.69241*10^{-4}*B^2$
1:1:0	$= 643.93027 - 3.68362*A - 0.43496*B + 0.041928*A^2 + 3.69241*10^{-4}*B^2$
1:0:1	$= 573.62436 - 2.49811*A - 0.41367*B + 0.041928*A^2 + 3.69241*10^{-4}*B^2$
0:1:1	$= 660.56820 - 3.33931*A - 0.48559*B + 0.041928*A^2 + 3.69241*10^{-4}*B^2$
1:1:1	$= 599.81986 - 3.13061*A - 0.42698*B + 0.041928*A^2 + 3.69241*10^{-4}*B^2$

[a] Where A is the temperature and B represents the enzyme activity. †An assigned number 1 indicates the presence, and a 0 indicates the absence of the enzyme. The first position corresponds to endo-polygalacturonase, the second to cellulase, and the third to hemicellulase.

3.2. Response 2: Effect of the enzyme-assisted hydrolysis on the particle diameter $d_{v,0.9}$ of cocoa pulp

3.2.1. Model output on response 2

The proposed modified cubic model was analysed for its fit to the data, yet it was found that the model was aliased due to insufficient design points to fit the higher order polynomial model. Nonetheless, a quadratic model could be chosen based on a highly significant $p < 0.0001$. By backward elimination regression, the term AB was excluded. The obtained model presented an adjusted R^2 of 0.8702 and a predicted R^2 of 0.7761. The adequate precision value 14.367 concluded the fit of the model. The residuals displayed a normal distribution, suggesting the model accounted for the variance present in the data. Internally studentized residuals showed a random scatter, demonstrating a constant variance. The software did not recommend any transformation for the given data using the Box-Cox plot. Lastly, using Bonferroni corrector limits one outlier was found (run 3, (40 °C, 10 U, 1:1:0)) and excluded from the analysis.

3.2.2. Interpretation of the reduced model for the particle diameter $d_{v,0.9}$ and particle size distribution

The model analysis revealed that the linear terms A, B, and C had a significant influence on response 2, suggesting that the temperature, the combination of enzymes, and the variation of their activities independently affected the particle size of cocoa pulp. The response equations (Table 3) hinted at a negative effect of factor A on the particle diameter $d_{v,0.9}$, expressed by negative coefficients. This indicates that the $d_{v,0.9}$ decreased with increasing treatment temperature. In concentrated suspensions like fruit pulps, a weak network can develop between polysaccharides, resulting in gel-like structures [37]. With this in mind and after the observations made by Nunes et al. [19], it is likely that the increase in temperature helped weaken the interactions between cocoa pulp components, breaking up possible aggregates and, thus causing lower $d_{v,0.9}$ values. Additionally, the software showed the influence of the diverse enzyme ratios in combination with the temperature (AC) and the different enzyme activities (BC) to be significant. This was also the case for the quadratic products of temperature and enzyme activity (A^2 and B^2).

To investigate the effect between the enzyme activity and the enzyme combination (term BC) on the response, the interaction was

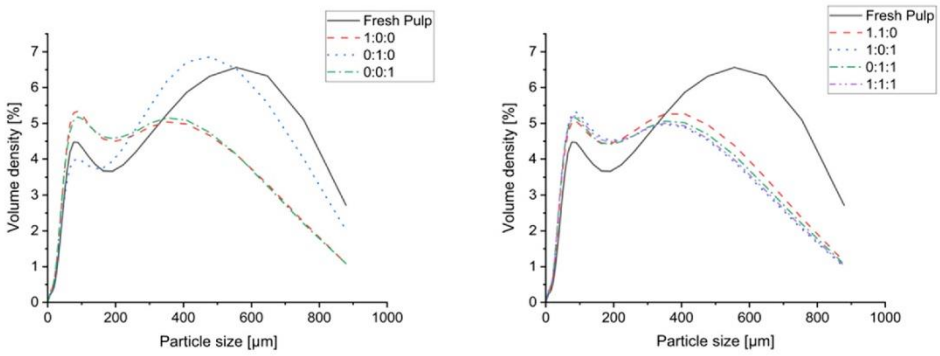

Fig. 2. Particle size distributions of fresh and enzymatically treated cocoa pulp by using one enzyme preparation (left) or combinations thereof (right). Samples as displayed in these graphs, were the results of enzyme-assisted hydrolyses at 30 °C and 1000 U of endo-polygalacturonase, endo-cellulase and/or hemicellulase. The first position corresponds to endo-polygalacturonase, the second to cellulase and the third to hemicellulase, respectively (P:C:H). An assigned number 1 indicates the presence and a 0 indicates the absence of the enzyme.

evaluated at 45 °C (c.f. 3.1.2). The $d_{v,0.9}$ of non-hydrolysed pulp was found to be 613 µm. The model predicted a maximal reduction in particle diameter resulting in a minimal particle $d_{v,0.9}$ for all enzyme combinations in a broad range between 500 U and 700 U. At 45 °C, the lowest $d_{v,0.9}$ (420 µm) was expected when combining all three enzymes (1:1:1) at 580 U. This trend was followed by the combination of polygalacturonase with hemicellulase (1:0:1) at 560 U with 430 µm. When using polygalacturonase and cellulase with a total activity of 590 U, the $d_{v,0.9}$ could be reduced to 435 µm. Interestingly, similar results could be observed for the combination cellulase and hemicellulase (0:1:1) at 660 U as well as for hemicellulase alone with an activity of 630 U. Polygalacturonase individually decreased the $d_{v,0.9}$ considerably with a minimal predicted $d_{v,0.9}$ of 440 µm and 520 U. On the other hand, cellulase led to a slightly weaker response with a $d_{v,0.9}$ of 490 µm at 540 U. It has been described that cellulases can disrupt the plant cell-walls with the help of other enzymes such as pectinases and hemicellulases [38]. Consequently, cellulase alone may not be able to degrade the cocoa pulp cell-walls fully, explaining the slightly larger $d_{v,0.9}$ in pulp treated only with this enzyme.

As the foreseen $d_{v,0.9}$ for the three enzymes were in a similar range, we opted to also evaluate the particle size distributions (Fig. 2). Overall, all treatments showed bimodal distributions. Moreover, all treatments lead to a reduction in the particle sizes compared to the fresh pulp. Cellulase had the lowest effect on the distribution compared to the fresh material, showing only a minimal shift of the second peak, and showing the closest resemblance to the fresh cocoa pulp. Polygalacturonase and hemicellulase individually shared similar trends. When combining the various enzymes, the particle size distributions were comparable (also to polygalacturonase and hemicellulase individually). This indicates that the enzyme-assisted hydrolysis of the pulps caused the particles to be reduced effectively in size, if polygalacturonase or hemicellulase were added. This was also the case for the combinations of polygalacturonase and cellulase, as well as cellulase and hemicellulase, as both exhibited a noticeable shift in the curve. This further emphasizes the effect previously noted with cellulase alone on the $d_{v,0.9}$, suggesting that this alone couldn't degrade the pulp independently. It's worth emphasizing: the Mastersizer's underlying principle assumes particles to be spherical in shape. Given that cocoa pulp contains fibres, the outcomes may exhibit variability based on the orientation of these fibres and the angle at which the laser interacts with the particle [39–41].

3.3. Response 3: Effect of the enzyme-assisted hydrolysis on the total soluble content (°Bx) of cocoa pulp

It was found that the cubic model was aliased and could not describe the response. Hence, the software suggested a linear model. Nonetheless, prior to elimination regression, the software showed a possible p-value of 0.2975, a lack of fit of 0.8095 as well as a low R^2 of 0.0317 and a predicted R^2 of −0.1534 for the linear equation. By backward elimination regression, all terms were excluded, resulting in an equation composed only of the mean value of all sample runs. The new R^2 and the new predicted R^2 were 0.1679 and −0.1440, respectively. A negative predicted R^2 suggests that the overall mean is a better predictor of the response than the current model. The mean value for the total soluble sugars was 16.5°Bx with a standard deviation of 0.9, whereas the minimal value was 15.1 °Bx and the maximum accounted for 18.6 °Bx. A possible reason for these results may be inhomogeneities in the raw material that conceal the effects of the enzymatic treatments. Despite the pulp being retrieved from a single batch of cocoa fruits and being mixed before its use, slight differences between samples regarding the ratio of fibrous material and a naturally occurring cocoa pulp exudate due to phase separation cannot be entirely excluded. Moreover, differences in the ripeness of the fruits may also have played a role. Moretti et al. [42] reported that pulp from ripe cocoa pods exhibited elevated levels of fructose and glucose, whereas that from unripe fruits predominantly displayed sucrose. These differences, in turn, may likely influence the total soluble content. A contrary reason for the lack of a model fit is that the enzyme-assisted hydrolysis, as investigated here, does not have a foreseeable effect on the total soluble solid contents of the pulp. This seems unlikely as previous studies indicate changes in the total soluble solids of juices when treated with cell-wall degrading enzymes [43,44]. Such is the case for four varieties of guava juices treated with pectinase, for which the total soluble solids increased compared to the treatment group without pectinase. This was attributed to the enzyme's influence on the pectins within the juices, leading to the breakdown of their chains and the release of soluble compounds [45]. In a different investigation by Leo et al. [46], the combination of cellulase, pectinase, and hemicellulase not only improved the juice yield but also resulted in an increased level of soluble sugar content.

3.4. Response 4: Effect of the enzyme-assisted hydrolysis on the colour of cocoa pulp

We assessed the suitability of the pre-set model by examining how well it fitted the data of the colour measurements. It was noticed that the model became distorted, as there weren't data points to support its higher order polynomial structure. Again, a quadratic model was chosen following the software's suggestion. Several terms were removed (B, C, AC, AB, BC and B^2). The chosen reduced model showed strong statistical significance with a p-value lower than 0.0001. Even though, the adjusted R^2 value (0.3860) and the predicted R^2 (0.3409) were low. With a precision value of 9.576, the model could be used to navigate the

Table 4
Confirmation runs (n = 3) for the reduction in viscosity (response 1) and the particle diameter (response 2). Carried out at 41.6 ± 0.2 °C with 10 U endo-polygalacturonase and cellulase (1:1:0).

	Reduction in viscosity [%]	Particle diameter $d_{v,0.9}$ [μm]
Predicted response	55.0	558.9
95 % Prediction interval -Low	43.8	525.6
95 % Prediction interval -High	66.1	592.2
95 % Confidence Interval -Low	50.2	542.8
95 % Confidence interval-High	59.7	575.1
Mean response	54.9 ± 2.8	548.2 ± 27.3

design space. When analysing the normal plot of residuals, it was found that they followed a normal distribution, suggesting that the model effectively accounted for variations in the data. Moreover, the studentized residuals showed random scatter hinting at consistent variance. Based on Box-Cox plot analysis no data transformation was deemed necessary by the software. The final equation (Eq. (3)) in terms of coded factor was the following:

$$BI = 25.33 + 0.86*A + 2.41*A^2 \quad \text{(Eq. 3)}$$

In our previous study on the thermal stabilisation of cocoa pulp, the browning index of pasteurised pulp differed significantly from pulp treated by ultra-high temperature processing. Latter's higher browning index was attributed to the higher processing temperature, causing the rate of non-enzymatic browning reactions to increase [47]. Similar could be inferred from this study. The browning index of the treated samples proved higher in samples treated at higher temperatures (50 °C and 60 °C). Consequently, this led to a model equation with only the temperature (factor A) as a significant variable. Notably, previous studies described a significant increase in the L* value (white colour component) of juices clarified with cell-wall degrading enzymes like pectinases and hemicellulases, causing them to become lighter [45,48]. This trend could not be corroborated by our data set.

3.5. Validation of the model

Confirmation runs (n = 3) were carried out to corroborate the outcomes predicted by the model. The recommended parameters (41.6 °C, 1:1:0, 10 U) (Table 4) were meticulously recreated using the same methodology (2.4 and 2.5), and the relative reduction in viscosity and the particle diameter $d_{v,0.9}$ were subsequently determined. The software proposed a 55.0 % reduction in viscosity and the prediction intervals ranged from 44.8 % (lower bound) to 66.1 % (upper bound). The empirical decrease in viscosity was determined to be 54.9 ± 2.8 %. This value falls within the range established by the prediction intervals and closely aligns with the software's initial prediction. For the particle diameter $d_{v,0.9}$, a predicted response of 558.9 μm was obtained. The measured mean equalled 548.2 ± 27.3 μm, falling also into the confidence and predicted intervals (Table 4). Regarding the reduction in viscosity for the single enzymes, the model accuracy could again be confirmed, as the means of the measurements were commensurate with the predictions made (Table 5). The results further substantiate the suitability of the quadratic models obtained for the enzyme-assisted processing of cocoa pulp.

4. Conclusions

The degradation of the cell-wall and its constituents in cocoa fruit pulp could be carried out successfully using enzymes in different activities and combinations, as well as in an extended temperature range. Enzyme-assisted hydrolyses offer promising solutions for cocoa pulp processing, facilitating efficient separation. By these, challenges such as incomplete pulp separation and damage to the fresh seeds by mechanical de-pulpers may be prevented, ensuring higher product qualities, and easing cocoa pulp valorisation. Our results indicate that endo-polygalacturonase plays the primordial role in the hydrolysis of cocoa pulp, while the roles of endo-cellulase and hemicellulase appear more auxiliary. The observed synergistic effects among the enzymes may offer substantial benefits for the industrial processing of cocoa pulp, such as enhancing the adaptability of the overall process, potentially leading to improved efficiency and productivity. Moreover, the design of experiments (DoE) proved a suitable tool to advance cocoa pulp processing. Expanding the experimental space and data set could provide deeper insights into the effects of temperature, enzyme concentration, and enzyme combinations on the investigated responses, particularly on the total soluble solids and BI, which were not fully ascertained in this study. To understand the effects on the particle structures, we recommend morphological particle determinations in fresh and enzymatically treated cocoa pulps. Lastly, a sensory evaluation of the enzyme-hydrolysed pulp is imperative for future applications of cocoa pulp in beverages and other food products.

CRediT authorship contribution statement

Thomas Bickel Haase: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Rukaiya Huseini Babat:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Holger Zorn:** Writing – review & editing, Supervision, Funding acquisition. **Susanne Gola:** Writing – review & editing, Supervision, Project administration. **Ute Schweiggert-Weisz:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

Table 5
Confirmation runs for the reduction in viscosity (response 1) using the enzymes endo-polygalacturonase, endo-cellulase and hemicellulase individually at 50 °C using 500 U and 800 U enzyme (n = 3).

Enzyme conc. [U]	Endo-polygalacturonase		Endo-cellulase		Hemicellulase	
	Predicted [%].	Determined [%]	Predicted [%].	Determined [%]	Predicted [%].	Determined [%]
500	64.5	66.5 ± 1.3	41.6	43.6 ± 1.9	51.9	49.6 ± 3.6
800	59.8	59.5 ± 4 0.2	40.6	40.3 ± 3.9	53.2	52.0 ± 2.1

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Data availability

Data will be made available on request.

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Chapter 4: Fermentation of cocoa pod husks with *Pleurotus salmoneo-stramineus* for food applications

Summary: The primary by-product of the cocoa industry, the cocoa pod husks (CPH), were investigated for their suitability as a growth medium to produce food ingredients. A fungus from the Basidiomycetes class was selected, as these were reported to be able to metabolise lignin and grow on woody structures. The strain of choice was *Pleurotus salmoneo-stramineus* (PSS), which after 8 days submerged cultivation resulted in a novel ingredient (fermented cocoa pod husks or CPHF) characterised in this chapter. The biomass of PSS was evaluated by means of ergosterol, an indicator of fungal growth. After 8 days of submerged cultivation, the mycelium comprised 54% of the total biomass. Moreover, fermentation proved successful in increasing the protein content in CPH. CPHF had a protein content of 18.9 g/100 g DM compared to 7.3 g/100 g DM in CPH. CPHF also exhibited a high biological value of 86 for the protein, as well as water and oil binding capacities of 3.5 mL/g and 2.1 mL/g, respectively. CPHF's particle diameter $d_{v,0,90}$ was 373 µm, while this was 526 µm in CPH. In terms of total dietary fibre, CPHF contained 73.4 g/100 g DM, whereas CPH had 63.6 g/100 g DM. The soluble fibre content proved notably lower in CPHF at 2.3 g/100 g DM compared to 10.1 g/100 g DM in CPH, with the insoluble fraction accounting for 71.1 g/100 g DM and 53.6 g/100 g DM, respectively. Furthermore, the integration of CPH and CPHF into food applications was investigated. Bread doughs incorporating different concentrations of either CPH or CPHF were assessed for texture, colour, and farinographic properties. With increasing CPH concentration, dough hardness, consistency, and browning index rose. Conversely, for CPHF, springiness and peak viscosities decreased. This study demonstrates the successful conversion of CPH into nutritious and functional ingredients using PSS fermentation, showcasing potential for upcycling this cocoa by-product.

Keywords: cocoa fruit, ergosterol, fermented by-products, fungal mycelium, techno-functional properties

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ORIGINAL ARTICLE

Fermentation of cocoa pod husks with *Pleurotus salmoneo-stramineus* for food applications

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Abstract

Cocoa pod husks (CPHs), the major side-stream from cocoa production, were valorized through fermentation with *Pleurotus salmoneo-stramineus* (PSS). Considering ergosterol as a biomarker for the fungal content, the mycelium accounted for 54% of the total biomass after 8 days in submerged cultures. The crude protein content of fermented CPH (CPHF) increased from 7.3 g/100 g DM in CPH to 18.9 g/100 g DM. CPH fermentation resulted in a high biological value of 86 for the protein. The water and oil binding capacities of CPHF were 3.5 mL/g and 2.1 mL/g, respectively. The particle diameter $d_{v,0,90}$ of CPHF was 373 μm as compared to 526 μm for CPH. The total dietary fiber was 73.4 g/100 g DM in CPHF and 63.6 g/100 g DM in CPH. The amount of soluble fiber was 2.3 g/100 g DM in CPHF and 10.1 g/100 g DM in CPH; the insoluble fraction accounted for 71.1 g/100 g DM and 53.6 g/100 g DM, respectively. Bread doughs with CPH or CPHF were characterized for texture, color, and farinographic properties. The dough hardness, consistency, and browning index increased with the concentration of CPH, whereas for CPHF, springiness and peak viscosities declined. We demonstrate the upcycling of CPH into nutritious and functional ingredients through PSS fermentation.

KEYWORDS
cocoa fruit, ergosterol, fermented by-products, fungal mycelium, techno-functional properties

1 | INTRODUCTION

With the introduction of the 17 Sustainable Development Goals, the UN highlighted the importance of facing poverty and other deprivations with strategies that promote health, education, equality, and strengthen economic growth. Accordingly, there is a growing demand for strategies to create safe and nutritious foods (United Nations, 2022). Therefore, effort should be made on the transformation of agricultural side streams, such as those from the cocoa industry, into nutritious and functional foods. In the present study, we propose the fungal fermentation of cocoa pod husks as an innovative approach to creating fiber- and protein-rich food ingredients.

The production of cocoa beans is forecasted for 2022/2023 to amount to approximately 5.0 million tons (International Cocoa Organization, 2022). The cocoa beans contribute to approximately

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10% of the total mass of the fruit, while the cocoa pod husk (CPH) represents the major fraction with about 70%—80% (Prabhakaran Nair, 2010). The large amounts of husks pose severe disposal challenges, as the uncontrolled accumulation may lead to the transmission of cocoa pests and diseases (Guest, 2007). CPH comprises the epicarp, mesocarp, endocarp, and a sclerotic portion (Campos-Vega et al., 2018). CPH has been reported to contain 76.6 g/kg crude protein in the dry matter, 325 g/kg crude fiber, and 101 g/kg ash. The energy content is 4.72 MJ/kg. CPHs consist mainly of fibrous materials, including 19.7%—26.1% cellulose, 8.7%—12.8% hemicelluloses, 14%—28% lignin, and 6.0%—12.6% pectin (Donkoh et al., 1991). The high proportion of lignin in CPH results in a woody, coarse texture. This makes the husks hardly digestible for humans and impedes their direct use in food products. Nonetheless, CPH contains soluble and insoluble dietary fiber, which could be interesting for the food sector. Some other applications for the use of CPH have been described. For instance, CPHs have been used in animal feed (Donkoh et al., 1991; Sobamiwa & Longe, 1994), as a feedstock for soap making (Gyedu-Akoto et al., 2015), and as an alternative energy source (Agyeman & Oldham, 1986). Biotechnological uses of CPH include its incorporation into food systems (Vriesmann et al., 2011) and biofuel production (Vásquez et al., 2019). Nevertheless, suitable transformation technologies are necessary to make cocoa pods accessible for human nutrition and food applications.

In recent years, the fermentation of side streams by higher mushrooms of the Basidiomycota division has gained increasing attention. The Basidiomycota include about 30,000 described species, which comprise, among others, fruiting body-forming fungi (Watkinson et al., 2016). The fruiting bodies of several edible mushrooms have become popular foods mainly due to their pleasant taste and their beneficial nutritional properties. Some of the health-promoting characteristics ascribed to them are the high-quality protein, the high fiber content, the presence of vitamin D_2 and several B vitamins, as well as their low fat content (Ahlborn et al., 2018; Manzi et al., 1999). Their high protein content makes them especially interesting as vegan protein sources (Stephan et al., 2018). As an alternative to the traditional production of fruiting bodies, fungal mycelia can be cultivated in submerged fermentations in the form of mycelial pellets (Ahlborn et al., 2019; Trapp et al., 2018). The mushroom mycelium exhibits similar positive nutritional properties as the fruiting bodies and is considered a promising alternative to plant-based proteins (Stephan et al., 2018). High biological values have been reported for fungal mycelial proteins previously (Ahlborn et al., 2019). In addition, mycelium growth has been shown to depend, among other things, on the carbon to nitrogen ratio, the type of carbon or nitrogen source, and the temperature (Hoa et al., 2015; Hoa & Wang, 2015). The application of mycelium from side stream fermentations can be performed in different ways: the processing of the mycelium–substrate complex as a whole, the mycelium separated from the substrate, the protein extracted from the mycelium, and the protein secreted by the fungus (Scholtmeijer et al., 2023). Furthermore, the fermentation of higher mushrooms offers possibilities to improve the organoleptic properties of agricultural side-streams, such as by-products from the cocoa industry (Klis et al., 2023).

Fermentation of CPH by fungi might be an interesting approach to creating protein- and fiber-rich ingredients for the food industry, thereby increasing the sustainability of the cocoa supply chain and addressing the challenges caused by the constantly growing world population. By giving additional value to CPH, cocoa farmers may benefit from new sources of income and better livelihoods, fomenting a more sustainable cocoa production (Vásquez et al., 2019) and thereby complying with the Sustainable Development Goals. This study explores possibilities for the valorization of CPH by fermentation with *Pleurotus salmoneo-stramineus* (PSS). The fermentation conditions were optimized, and the techno-functional properties of the fermented cocoa pod husks (CPHF) were evaluated for their use in food products using enriched bread doughs as demonstrators.

2 | MATERIALS AND METHODS

2.1 | Fermentation of cocoa pod husks with *Pleurotus salmoneo-stramineus*

The fermentation was performed according to Bosse et al. (2013) and Trapp et al. (2018). CPH from the cocoa variety SUL1 were obtained in 2020 from the cocoa germplasm collection garden of the Indonesian Coffee and Cocoa Research Institute (ICCRI), located in Nogosari village, Jember Regency, East Java. Prior to processing, the cocoa pod husks were washed thoroughly with water and left to dry at room temperature. A fine-grained CPH powder was obtained by drying, crushing, and homogenizing CPH. Using a Retsch SM 2000 (Retsch GmbH), the sun-dried husks were milled and sieved to a particle diameter of less than 1—2 mm. The ground CPH was stored at room temperature. Prior to fermentation, CPH were autoclaved to inhibit the growth of undesirable microorganisms. PSS was obtained from the Institute for Molecular Wood Biotechnology and Technical Mycology, Göttingen, Germany, and was selected from a total of 43 screened basidiomycetes as the most promising fungus for further analysis. For strain maintenance, the fungus was kept on malt extract agar plates (20 g/L malt extract, 15 g/L agar agar) and transferred to a new plate every 6 days using a spatula and by cutting out a 1 cm^2 piece of overgrown agar. For the pre-cultures, 200 mL of malt extract medium (20 g/L malt extract in drinking water) was placed in a 500 mL narrow-neck Erlenmeyer flask and inoculated with 1 cm^2 of mycelium. Homogenization was performed by using an Ultraturrax (IKA Works Inc.) at 10,000 rpm for 30 s. Cultivation took place on a horizontal shaker at 150 rpm at 24°C in the dark for 6 days. To ensure the comparability of the pre-cultures, these conditions were replicated between the different approaches. The main cultures were based on CPH medium consisting of 20 g/L finely ground husks and 3.6 g/L mono-sodium aspartate as a nitrogen supplement. 2 L of the main culture medium was added to a 5 L Erlenmeyer

flask. The pre-culture was homogenized using an Ultraturrax, and 200 mL was added to the main culture flask. A fermentation time of 8 days was selected. Finally, the harvested material was passed through a cheese cloth to separate the mycelium–CPH composite (CPHF) from the supernatant. This was washed with drinking water, and the resulting biomass was dried by lyophilization.

2.2 | Estimation of the fungal content via quantitation of the biomarker ergosterol

Freeze-dried mycelia were ground for 3 min in a vibrating mill (25 Hz) (MM 400, Retsch GmbH), and approximately 50 mg was weighed into a pyrex™ tube. After addition of 25 mg sodium ascorbate as an antioxidant, 0.25 mL internal standard (IST) (0.5 mg/mL 7-dehydrocholesterol ((>95% Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) in 2-butanone), and 5 mL methanolic NaOH (5% NaOH in 95% methanol)), the samples were vortexed and saponified for 60 min at 80°C in a water bath. After cooling in the dark, the samples were filtered through a membrane filter (0.45 µm (LLG-Labware, Meckenheim, Germany)) and extracted three times with 5 mL of n-hexane. The organic phases were combined in a 15 mL volumetric flask, made up *ad* mark with n-hexane, and dried over Na_2SO_4. Six milliliter of the sample (dilution factor = 2.5) was transferred to a fresh pyrex™ tube and dried under an N_2-stream. The residue was dissolved in 0.5 mL of tetrahydrofuran (THF) and 0.5 mL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) (Fluorochem Ltd.) and vortexed. After incubation at 70°C for 2 min, silylation was performed at room temperature overnight. Quantitation was performed by external calibration via the IST in the range of 10–100 µg/mL. Therefore, an ergosterol stock solution of 200 µg/mL (>95% TCI Deutschland GmbH) was prepared in 2-butanone. The individual calibration standards were prepared in 10 mL volumetric flasks by adding 1 mL of IST and between 0.5 and 5.0 mL of ergosterol stock solution *ad* 10 mL. One mL of the samples was concentrated to dryness under N_2 and then treated in the same way as the samples. For quantitative estimation of the fungal content, the ergosterol value of CPHF was compared to the reference value for pure PSS mycelium grown in malt extract medium (434 ± 31 mg/100 g), which was set to 100%.

Gas chromatographic (GC-FID) analysis was performed using a gas chromatograph (GC) (Agilent 7890, Agilent Technologies Inc) equipped with an auto sampler (Agilent 7683B) and a flame ionization detector (FID). One microliter of the sample was injected at 250°C via a split/splitless injector in splitless mode. A 30 m × 0.32 mm × 0.25 µm DB5ms column (Agilent Technologies, 123–5532) was used. The oven temperature program started at 100°C for 3 min, was heated to 280°C with 30°C/min, held for 12 min, heated to 320°C with 30°C and held for another 5 min. H_2 was used as carrier gas at a constant flow rate of 2.2 mL/min. The flow rates of gases in the FID were set as follows: H_2: 40 mL/min; air: 400 mL/min; and makeup gas (N_2): 25 mL/min. The method was validated regarding linearity, recovery, method precision, limit of detection (LOD), and limit of quantification (LOQ) following DIN 32645.

2.3 | Characterization of CPH and CPHF

Prior to the chemical characterization, the wet CPHF mass was freeze-dried and milled to obtain a homogeneous sample. Milling was carried out with a Grindomix GM 200 (Retsch GmbH) for 15 s at 7500 rpm. The non-fermented, dried, and milled CPH was used for comparison. The composition of wheat flour type 550 from Mühlen König (Frießinger Mühle GmbH) was also analyzed as it was used in the development of bread doughs.

2.3.1 | Determination of the dry matter content

Samples were dried in a LECO TGA 601 (LECO Instrumente GmbH) oven in a ceramic crucible at 105°C (Lebensmittel- und Futtermittelgesetzbuch, 2021). The dry matter (DM) was determined gravimetrically by differential weighing.

The residual moisture of fermentates during optimization of the growth time of PSS on CPH was determined with a moisture analyzer (MA 35, Sartorius AG).

2.3.2 | Determination of the crude protein and ash contents

The crude protein content of the samples was derived from the nitrogen content determined according to the Dumas method. This method can be applied to all foodstuffs and animal feed in a range of 1.9—23.9 mg N absolute in solid samples and is described in § 64 LFBG (2013). Analyses were carried out with a TruMac N Nitrogen Determinator (LECO Instrumente GmbH). The crude protein contents of the PSS fermentates during optimization trials were determined according to Kjeldahl as described in AOAC 2001.11 (AOAC, 2005). In both cases, to calculate the protein content of the samples, the nitrogen content was multiplied by the protein factor 6.39 for CPH and 6.00 for CPHF obtained from the amino acid distribution (2.3.3).

The ash was determined by means of LECO TGA 601 (LECO Instrumente GmbH). The samples were incinerated in a ceramic pan at 550°C (Lebensmittel- und Futtermittelgesetzbuch, 2021). The ash content was determined gravimetrically by differential weighing.

2.3.3 | Determination of amino acids and calculation of the biological value

Amino acid analyses were carried out, according to Ahlborn et al. (2019). Approx. 30 mg of CPH or CPHF was mixed with 2.5 mL

of 6 M HCl (containing 1 g/L phenol) and incubated at 110°C for 24 h for total hydrolysis. After cooling, 1.5 mL of sodium hydroxide solution (7.5 M) was added, and the pH was carefully adjusted to 2.2 with 7.5 and 1 M sodium hydroxide solutions. The volume was adjusted to 20 mL with citrate buffer (11 g trisodium citrate dihydrate, 6 g citric acid, 14 mL thiodiglycol, 12 mL of a 32% (*w*/*w*) HCl, and 2 g phenol made up to 1 L with *dd*H_2O and adjusted to pH 2.2) in a volumetric flask, and the solution was filtered through a syringe filter (0.45 µm) into a vial. For determination of cysteine and methionine, oxidation was performed prior to the hydrolysis with 0.5 mL oxidation mix (0.05 mL hydrogen peroxide (w = 30%), mixed with a 0.45 mL phenolic formic acid (889 g formic acid mixed with 111 g *dd*H_2O, containing 4.73 g phenol)) for 16 h at 4°C. The oxidation was stopped by the addition of 0.084 g of sodium disulfite. For quantitation of tryptophane, an alkaline hydrolysis was carried out with 2.5 mL of phenolic sodium hydroxide solution (5 M sodium hydroxide solution containing 0.1% phenol). After the addition of 1.5 mL of 0.5 M phosphoric acid, the pH was adjusted to 2.2 with 3.75 M and 1 M HCl.

Amino acid analysis was carried out with an amino acid analyzer S433 (Sykam GmbH) with an LCA K13/Na and a gradient program of two sodium citrate buffer solutions (A: 0.12 N, pH 3.45; B: 0.20 N, pH 10.85) and a regeneration solution (20 g sodium hydroxide and 0.2 g EDTA per liter *dd*H_2O) with a flow rate of 0.45 mL/min. Post-column derivatization was carried out with 0.2 M ninhydrin (pH 10.85) with a flow of 0.25 mL/min. The injection volume was 150 µL. Identification and quantification were carried out by external calibration in a range of 10–200 nmol/mL with an amino acid calibration mix (Sykam GmbH, Fürstenfeldbruck, Germany) ($R^2_{\text{all amino acids}} > 0.999$). The calibration mix (standard solution H-Ox, Sykam) contained all amino acids, including Cys-Ox and Met-Ox, except for tryptophane. A tryptophane stock solution (L-tryptophane ≥99% Carl Roth GmbH + Co. KG) was prepared separately.

To calculate the pure protein content, the AA_{res} value was calculated for each amino acid, taking into account the loss of one water molecule per peptide bond. The sum of all AA_{res} gives the pure protein content. To evaluate the protein quality, the biological value (BV) was calculated via the essential amino acid index (EAAI) (Ahlborn et al., 2019), related to the reference protein defined by FAO/WHO (1973) with a BV of 100.

Based on the determined amino acid profiles, corrected nitrogen to protein conversion factors were calculated for CPH ($F_{CPH} = 6.39$) and CPHF ($F_{CPHF} = 6.00$).

2.3.4 | Determination of total dietary fibers

The determination of the total dietary fiber content as well as the contents of soluble and insoluble dietary fiber was carried out according to the AOAC 991.43 (Association of Analytical Chemists [AOAC], 2000) method using an enzyme assay kit (K-TDFR-200A, Megazyme Ltd).

2.3.5 | Scanning electron microscopy

Scanning electron microscopy was performed under high-vacuum using an EVO LS 10 SEM (Zeiss, Jena, Germany), equipped with a secondary electron detector (Everhart–Thornley detector). The excitation voltage was 15.00 kV. All samples were sputtered with gold.

2.3.6 | Determination of the water-binding capacity

The water-binding capacity was determined by combining 2.0 g of sample with an approximately 20-fold excess of water. After mixing well and 24 h of soaking, the samples were centrifuged at 2500 *g*, at 20°C for 5 min. After centrifugation, the supernatant (unbound water) was discarded, and the samples were turned upside down and drained for 25 min. The weight of the water-saturated sample was determined, and the water-binding capacity [g/g] was calculated (American Association of Cereal Chemists [AACC], 1978).

2.3.7 | Determination of the oil binding capacity

The oil binding capacity (OBC) of CPH, CPHF, and flour was analyzed using the method described by Ludwig et al. (1989) at room temperature (~21°C). In scaled centrifuge tubes, a 1.5 g sample was dispersed in an excess of corn oil (10 mL). After vigorously mixing, the sample was centrifuged at 700 *g* and 20°C for 15 min. The volume of free oil was read on the side of the centrifuge tube and calculated as described by Ludwig et al. (1989).

2.3.8 | Particle size distribution

The particle size distributions of flour, CPH, and CPHF were measured using a static laser diffraction instrument (Malvern Mastersizer 3000, Software version 2.15, Malvern Instruments Ltd). The refractive index of the particles was determined to be 1.53, the absorbance was 0.005 [L/mol cm], and the refractive index of the dispersant was 1.40. The particles were irregularly shaped, and the Mie theory was applied as an optical model. The samples were mixed with butanol in a 1:1 ratio, equilibrated to room temperature, and then added to the dispersing unit. Butanol (99.4%, Sigma Aldrich, Merck KGaA) was used as the dispersant. To standardize the sample concentration, the obscuration was adjusted between 10% and 15%. The stirring speed of the dispersion unit was set to 3000 min^{-1}. To ensure a homogenous dispersion of the sample, measurements were started after 2 min and repeated 1 min later. Samples were measured in triplicate. As the stirring did not affect the particle sizes (second measurement), all six values were taken into consideration when calculating the means. The particle size measurements are reported as $d_{v,0.1}$, $d_{v,0.5,}$ and $d_{v,0.9}$. The diameters $d_{v,0.1}$, $d_{v,0.5,}$ and $d_{v,0.9}$ correspond to 10, 50, and 90 vol% on a relative cumulative particle size curve, respectively.

2.3.9 | Determination of protein solubility

1.50 g of sample was added to 35 mL of a 0.1 mol/L sodium chloride solution while stirring until it was suspended. The initial pH value was noted and then adjusted to 7.0. The samples were held at this pH value for 1 h and the pH value was measured after 30 min and 1 h. The suspension was then transferred to a 50 mL volumetric flask and filled up to the mark. After vigorous shaking, 20 mL of the solution was centrifuged for 15 min at 20,000 *g* and 15°C. The supernatant was filtered through a Whatman No.1 filter (Whatman GmbH), and the nitrogen content was determined in the filtrate after Dumas (1831) (2.2.3). The protein content was calculated with the protein factor 6.39 for CPH, 6.00 for CPHF, and 5.81 for wheat flour. The protein solubility was determined using Equation 1:

$$\text{Protein solubility } [\%] = \frac{\text{Volume NaCl solution } [mL] * \text{Protein in the supernatant } \left[\frac{mg}{mL}\right]}{\text{Sample mass } [mg] * \text{Protein in the DM } [\%] \text{ x DM of the sample } [\%]} * 100 \quad (1)$$

2.3.10 | Determination of color and browning index

The DigiEye color imaging system (DigiEye V2.62, VeriVide), comprising an illumination box with diffuse illuminant D65 and a Nikon D90 digital camera, was used for color determinations. Digitizer calibration charts were used to calibrate the system. For the color measurements, the sample was evenly distributed in a white sample cup (Aqualab, Meter Group), and the average surface color was expressed as CIE L*a*b*-values with L*, a*, and b* ranging from black (0) to white (100), from green (−128) to red (+127), and from blue (−128) to yellow (+127), respectively (Kumah et al., 2019). The browning index was calculated using the L*a*b* values and following the formula described in Bal et al. (2011).

2.3.11 | Determination of pasting properties

The AACC 76-21 500 test was conducted to evaluate the viscosity of the samples in excess of water during temperature-controlled cycles (pasting properties) (AACC, 2000). For this analysis, the RVA 4500 (PerkinElmer Inc) with paddle stirrers (PerkinElmer) was used.

All measurements were carried out in triplicate. Three different CPH and CPHF concentrations (2.5% (*w/w*), 5% (*w/w*) and 10% (*w/w*)) were added to the wheat flour. The pure flour as well as the CPH and CPHF were also investigated. A total of 3.5 g of flour-blend was weighted into the sample cups and mixed with 25 mL of water until the sample was homogenously suspended. Next, the sample cup was assembled into the RVA, and the test was immediately started.

2.4 | Analysis of white bread dough with added CPH and CPHF

Different dough samples were prepared by substituting proportional weight amounts (0% (*w/w*) (standard wheat bread dough), 2.5% (*w/w*), 5% (*w/w*), and 10% (*w/w*)) of flour Type 550 with CPH and CPHF. Doughs were prepared by adding 53.5% (*w/w*), water. No further ingredients, such as salt or yeast, were added to the blends to keep the dough as simple as possible and to enable the comparison between samples.

2.4.1 | Farinographic measurements

As wheat flours and their capacities for water uptake may differ between producers and production batches, the water absorption of the flour and the resistance of the various doughs to mixing were determined following the International Association for Cereal Science and Technology (ICC) Standard No. 115/1 (International Association for Cereal Science and Technology, 1992). With a farinograph (Successor Farinograph TS, Brabender GmbH & Co. KG), the kneading curve was plotted in a force–time diagram from which parameters such as the optimal water absorption, dough development time, dough stability, and dough softening were derived. To determine the optimal water absorption of the wheat flour type 550, a dough with a consistency of 500 Farinograph Units (FE) was prepared in a 50 g measuring cell using sigma blades. The value 500 FE was chosen, as it is a popular and desired value for the texture and dough performance of bread doughs (Miś et al., 2012). Measurements were conducted at 30°C for 20 min. The optimal water absorption of the wheat flour accounted for 53.5% (*w/w*). The amount of water added to the flour blends was kept constant to determine how the addition of CPH or CPHF affected the doughs' properties. The water content of the flour was determined to be 13.7%, while the water contents of the flour blends were calculated, taking the DM of CPH and CPHF as well as their concentrations in the blends into account. These values were entered into the equipment's software prior to measurements. The obtained doughs were evaluated based on their textural profiles (2.4.2).

2.4.2 | Texture profile analysis (TPA)

A texture analyzer (TA. XT plus Stable Micro Systems) was used to assess the hardness and springiness of the dough samples made with different weight percentages of CPHF and CPH. 20 g of dough was carefully rolled into a ball and left 10 min to rest in closed vessels to prevent drying out. The TPA analyses consisted of two cyclic compression steps of 40% deformation. A Plexiglas cylinder probe with a diameter of 25 mm (TA. XT plus Stable Micro Systems) was chosen. The hardness was defined as the peak force in Newton [N] during the first compression cycle, whereas the springiness described the height that the dough recovered during the time elapsed between compressions (Bourne, 1978).

2.5 | Statistics

The results were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using Tukey's multiple comparison

test ($p < .05$) to determine significant differences between two groups using Origin 2022b (OriginLab Corporation). With the same software, Spearman's list-wise correlation analysis was performed to establish associations between the results.

3 | RESULTS AND DISCUSSION

3.1 | Fermentation of CPH with *Pleurotus salmoneo-stramineus*

3.1.1 | Validation of the GC-FID method for quantitation of ergosterol

The method was validated regarding linearity in a range of 10—100 µg/mL, corresponding to 50—500 mg/100 g ($n = 3$; $R^2 = .9999$). The linearity test, according to Mandel, proved the linearity in the given range. The average recovery in the CPH matrix at ten points in the same range was between 97.016% and 101.83% ($n = 3$). The method precision showed a relative standard deviation (RSD) of 4.0%. The LOD determined by means of the calibration method following DIN 32645 was 0.42 ± 0.11 µg/mL respectively 2.1 mg/100 g ($n = 3$) and the LOQ was 1.44 ± 0.38 µg/mL respectively 7.2 mg/100 g ($n = 3$). Considering the ergosterol contents of various basidiomycetes (e.g. 210 mg/100 g DM for *Phanerochaete chrysosporium* or 375 mg/100 g DM for *Pleurotus sapidus*, depending on culture media and fermentation time) described in the literature (Ahlborn et al., 2018; Niemenmaa et al., 2008), this method is applicable to many different fungi and serves the purpose intended here. Data on the response-, residue- and error plots, as well as calibration curves and detailed results, are reported in the supplementary materials (Figures S1–S2, Tables S1–S4).

3.1.2 | Submerged cultivation of PSS in CPH-medium

The fermentation experiments were started with 20 g of CPH DM/L. The CPH had a crude protein content of 7.3 ± 0.1 g/100 g DM, and the initial fungal content was 0% since no ergosterol was detected in the CPH. Since ergosterol is found almost exclusively in the cell membranes of fungi, it may serve as a biomarker for fungal growth. Therefore, ergosterol can also be used to exclude unintended fungal infestation of the substrate prior to use as a fermentation substrate (Ibrahim et al., 2022; Osswald et al., 1986). The crude protein content increased until main culture day 8 (up to 22.2 ± 0.1 g/100 g DM; no significant difference to main culture day 10, $p < .05$) as well as the fungal content (up to 54%; 233 mg/100 g DM ergosterol; no significant difference to main culture day 10, $p < .05$) (Figure 1). For economic reasons, a longer cultivation time with consistent results is not beneficial. The dry biomass of the fermentates was approximately halved during fermentation and harvesting. This loss of biomass can be attributed to the dissolution of components during fermentation,

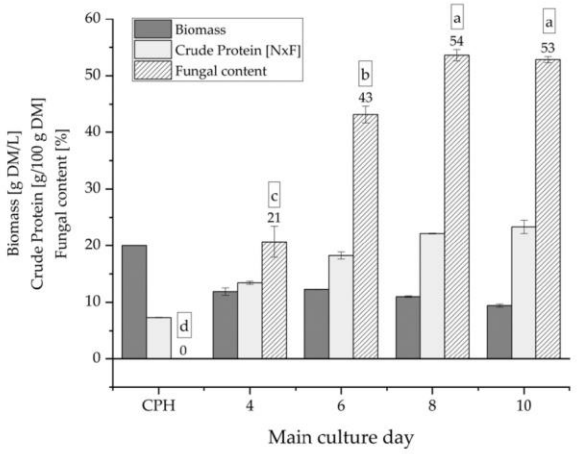

FIGURE 1 Growth curve of *Pleurotus salmoneo-stramineus* (PSS) in cocoa pod husk (CPH)-medium over 10 days ($n = 2$). DM: dry matter; NxF: nitrogen content multiplied by protein factor F. Means with no letter in common indicate significant differences ($p < .05$).

which are separated by the harvesting step. The harvest of the non-inoculated CPH-medium as a blank yielded a dry biomass of 11.9 ± 0.1 g DM/L. Vriesmann et al. (2011) reported a yield of water-soluble pectins after aqueous extraction at 100°C of 12.6%. During media preparation, the CPHs were autoclaved for 20 min at 120°C. This represents an aqueous extraction under the influence of high temperatures. Furthermore, CPHs contain between 8.4% and 10.4% reducing sugars and between 9.6% and 11.4% soluble dietary fiber (Vriesmann et al., 2011; Yapo et al., 2013). In addition to the leaching of soluble components, losses of biomass may also be attributed to the fungal metabolism (Kirk et al., 1975). This explains the decrease in recovery of solid biomass with increasing fungal content.

Due to the low nitrogen content of the CPH, it was necessary to supplement the CPH-based medium with a nitrogen source to improve fungal growth. Fraatz et al. (2014) showed that supplementation with asparagine and aspartic acid may boost the growth of *Pleurotus* species in submerged cultures. Therefore, the optimum amount was determined in preliminary tests by quantifying residual amounts of aspartate in the supernatant at the end of the culture period (data not shown).

The upcycling of different side streams and agricultural wastes with mushrooms has been described in several studies. Ahlborn et al. (2019) upcycled apple pomace with different basidiomycetes and obtained mycelial biomasses with crude protein contents between 9.6% with *Wolfiporia cocos* and 25.4% DM with *Pleurotus sapidus*. The corresponding yields of dry biomass ranged from 13.2–14.5 g DM/L. In a similar study, *Ganoderma lucidum*, *Lentinula edodes*, and *Pleurotus ostreatus* were cultivated on winery waste. Crude protein contents between 17.6 and 19.7 g/100 g DM were achieved (Petre et al., 2016). The results obtained in this study, especially for crude protein content and yield of biomass, are comparable with those from those two studies. In terms of fiber content, CPH lies within common ranges for fruit pomaces. Vriesmann et al.

[10] showed a lignin content of 21.4 g/100 g DM in CPH, while apple pomace has a lignin content of 15.4 g/100 g and grape pomace of 56.7 g/100 g DM (Okoro et al., 2021). A study by Manu-Tawiah and Martin (1987) showed better growth of *Pleurotus ostreatus* on a complex medium (peat-extract medium) than on a synthetic medium. Recently, basidiomycetes were used to upcycle side streams of the palm oil industry to serve as a rearing substrate for insects. The fermentation positively influenced the development of the insect larvae (Klüber et al., 2022). All mentioned studies, including the present one, demonstrate that basidiomycetes are able to grow on different side stream-based media. In addition, the protein content of CPHF resembled that of PSS grown on a standard nutrition medium (malt extract) with 26.1 ± 0.1 g/100 g DM. The use of agricultural side streams, such as CPH, is advantageous regarding valorization and cost savings (Bosse et al., 2013). To make this approach even more efficient, replacing the supplementation with aspartic acid with another side stream rich in aspartic acid might be addressed in future studies.

The surface structures of CPHs were compared to those of CPHF and pure PSS mycelium by means of scanning electron microscopy. The surface structure of CPHs (Figure 2a) differed significantly from the structure after fermentation (Figure 2b), on which fungal mycelium could be observed evenly distributed on the particles' surface. The pure PSS mycelium (Figure 2c) showed composites formed by smaller particles, while the CPH particles were larger and displayed a more uniform surface. Zhu et al. (2016) applied scanning electron microscopy to investigate the morphological changes in Jerusalem artichoke stalks after inoculation with different fungal strains. The different fungal species caused varying levels of degradation to the stalks' cell walls, with *P. chrysosporium* causing the most extensive decay and *G. trabeum* leading to disrupted and weakened cell wall structures. The physical invasion of cell walls by the mycelia through the fissures between cells was assumed to be dependent on degradative mechanisms for polysaccharide depolymerization by the different species. In that regard, the surface coverage of CPH by PSS may suggest a fungal capacity to degrade the husk's woody cell wall matrix, enabling the growth of mycelium in the CPH's porous structures and in intercellular spaces. Moreover, the similarity of CPHF compared to pure PSS mycelium may allow for the detection of fungal growth at the microscopic level.

3.1.3 | Evaluation of CPH and CPHF for the development of food products

The DM content of CPH and CPHF accounted for 89.2% and 94.3% (Table 1), respectively, while the DM content of the flour was 86.3%, well in accordance with values reported in the literature (86.7%) (Chandra et al., 2015). The chemical composition of the flour is described in Table S5 in the supplementary materials.

Proteins have an extensive influence on the functional, nutritional, and textural properties of raw materials, processed foods, and formulated foods (Zayas, 1997). In general, basidiomycetes exhibit a high protein content and a good protein quality, whether as a fruiting body or mycelium (Berger et al., 2022; Yu et al., 2017). The crude protein content of wheat flour was 9.8 g/100 g DM (Table S5), as reported previously (Belitz et al., 2004). The protein content of CPH was 7.3 g/100 g DM, and it increased by fermentation to 18.9 g/100 g DM in CPHF. The growth of fungal mycelium thus leads to an increased protein content compared to the initial substrate (Ahlborn et al., 2019; Petre et al., 2016), giving the fermentate a significant advantage in terms of nutritional value. The BV of CPH and CPHF was comparably high at 85 and 86, respectively (Table 2). These values are significantly higher than those of soy protein (BV 74) or French beans (BV 58) (Zajul, 2017). The BV of CPHF is comparable with that of pork meat (86) and beef (83) (Rimbach et al., 2015), making it a good alternative for these animal-based proteins. Zajul (2017) showed BV for basidiomycetes proteins of *Pleurotus sapidus*, grown on isomaltulose-molassis, of 82; *Pleurotus sajor-caju*, grown on isomaltulose-molasses, of 65; and *Lentinula edodes*, grown on carrot pomace, of 66. In this study, we could additionally demonstrate the dependence of protein formation and amino acid composition on the culture medium. Regarding the pure protein content, the value for pure PSS mycelium was slightly higher than for PSS mycelium grown in CPH medium, yet the BV of the pure PSS mycelium accounted only for 75. The share of glutamic acid/glutamine was high in pure PSS mycelium with 31.2%, in contrast to CPH with 14.1% and CPHF with 18.5%. At the same time, the share of essential amino acids was lower. While tryptophane was the first limiting amino acid in CPH and CPHF, the sum of cysteine and methionine was limiting in the pure PSS mycelium, followed by isoleucine and leucine.

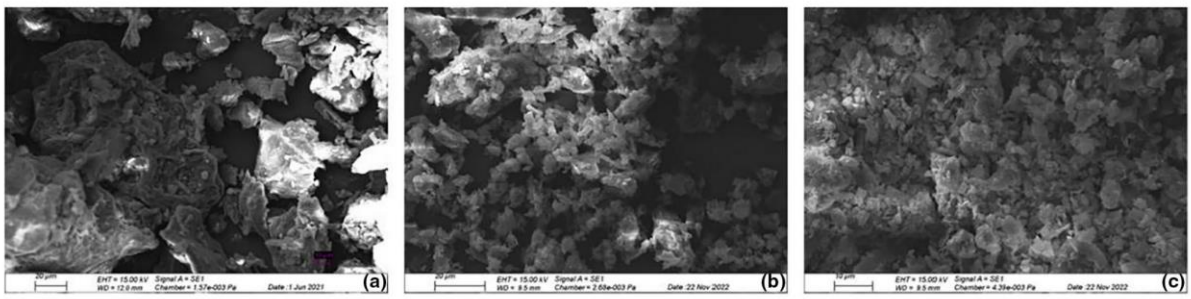

FIGURE 2 Environmental scanning electron microscopy recordings from cocoa pod husks (CPH) (a), pure *Pleurotus salmoneo-straimenus* (PSS) mycelium (b), and fermented cocoa pod husks (CPHF) (c).

TABLE 1 Chemical composition and techno-functional properties of cocoa pod husks (CPH) and cocoa pod husks fermented with *Pleurotus salmoneo-stramineus* (CPHF) ($n=3$).

	CPH	CPHF
Dry matter (DM) [%]	89.2 ± 0.8^a	94.3 ± 3.8^a
Crude protein [g/100 g DM]	07.3 ± 0.1^b	18.9 ± 0.1^a
Ash [g/100 g DM]	09.1 ± 0.2^a	04.8 ± 0.1^b
Protein solubility [%]	14.1 ± 1.8^a	13.0 ± 0.7^a
Water-binding capacity (WBC) [mL/g]	06.2 ± 1.6^a	03.5 ± 0.1^b
Oil-binding capacity (OBC) [mL/g]	1.1 ± 0.1^b	02.1 ± 0.2^a
Total dietary fiber [g/100 g DM]	63.6 ± 6.6^a	73.4 ± 5.4^a
Insoluble dietary fiber [g/100 g DM]	53.6 ± 4.2^b	71.1 ± 5.3^a
Soluble dietary fiber [g/100 g DM]	10.1 ± 0.1^a	02.3 ± 1.4^b

Note: Means in the same row with no letter in common indicate significant differences ($p<.05$).

Fraatz et al. (2014) showed a similar effect for *Pleurotus sapidus*, for which the medium composition significantly influenced the amino acid composition. By calculation of AA_{res}, the pure protein content of CPH was quantified to 4.9 ± 0.1 g/100 g DM and to 17.7 ± 0.8 g/100 g DM for CPHF. This underlines that the quantitation of protein, according to Kjeldahl or Dumas, typically overestimates the true protein contents. For fungal mycelia, the chitin content of the cell wall contributes to the total nitrogen content (Di Mario et al., 2008; Tshinyangu & Hennebert, 1996). Regarding the protein solubility, this did not differ significantly between the flour, CPH, and CPHF and ranged between 13.0% and 16.8% (Table S5 and Table 1) and will, hence, not be further discussed. Altogether, despite the lower tryptophane concentration and lower ratio of glutamic acid/ glutamine in CPHF compared to PSS mycelium, the results prove PSS suitable for the fermentation of by-products of the food industry, resulting in an increase in the

TABLE 2 Amino acid composition and biological value of cocoa pod husks (CPH), fermented cocoa pod husks (CPHF), and pure *Pleurotus salmoneo-stramineus* (PSS) mycelium ($n=3$).

	Amino acids [g/100 g DM] (% amino acid of total amino acids)			% Amino acid of total amino acids
	CPH	CPHF	PSS mycelium	Reference protein FAO/WHO (1973)
Alanine	0.34 ± 0.01^b (5.9%)	1.28 ± 0.01^a (6.2%)	1.02 ± 0.01^a (4.4%)	-
Arginine	0.20 ± 0.03^c (3.6%)	1.01 ± 0.05^b (4.9%)	1.53 ± 0.03^a (6.6%)	-
Aspartic acid/asparagine	0.98 ± 0.02^c (17.2%)	2.91 ± 0.15^a (14.1%)	1.72 ± 0.05^b (7.4%)	-
Cysteine	0.08 ± 0.00^a (1.4%)	0.30 ± 0.01^a (1.4%)	0.31 ± 0.00^a (1.3%)	-
Glutamic acid/glutamine	0.80 ± 0.02^c (14.1%)	3.82 ± 0.07^b (18.5%)	7.21 ± 0.49^a (31.2%)	-
Glycine	0.27 ± 0.00^b (4.8%)	0.93 ± 0.04^a (4.5%)	0.92 ± 0.02^a (4.0%)	-
Histidine	0.24 ± 0.00^c (4.2%)	1.42 ± 0.07^b (6.9%)	2.38 ± 0.07^a (10.3%)	-
Isoleucine	0.23 ± 0.00^b (4.0%)	0.88 ± 0.05^a (4.3%)	0.64 ± 0.03^a (2.8%)	4.0%
Leucine	0.39 ± 0.01^c (6.8%)	1.51 ± 0.07^a (7.3%)	1.12 ± 0.05^b (4.8%)	7.0%
Lysine	0.44 ± 0.01^b (7.7%)	0.93 ± 0.05^a (4.5%)	1.07 ± 0.04^a (4.6%)	5.5%
Methionine	0.10 ± 0.00^a (1.7%)	0.34 ± 0.01^a (1.7%)	0.24 ± 0.01^a (1.0%)	-
Phenylalanine	0.28 ± 0.01^b (4.8%)	0.92 ± 0.05^a (4.5%)	0.70 ± 0.02^a (3.0%)	-
Proline	0.28 ± 0.01^b (4.9%)	0.78 ± 0.04^a (3.8%)	0.72 ± 0.02^a (3.1%)	-
Serine	0.34 ± 0.00^b (5.9%)	1.07 ± 0.05^a (5.2%)	1.02 ± 0.00^a (4.4%)	-
Threonine	0.24 ± 0.00^b (4.1%)	0.91 ± 0.05^a (4.4%)	1.04 ± 0.01^a (4.5%)	4.0%
Tryptophane	0.03 ± 0.01^a (0.5%)	0.12 ± 0.01^a (0.6%)	0.20 ± 0.00^a (0.9%)	1.0%
Tyrsoine	0.17 ± 0.01^a (3.0%)	0.34 ± 0.03^a (1.7%)	0.39 ± 0.01^a (1.7%)	-
Valine	0.31 ± 0.01^b (5.4%)	1.13 ± 0.05^a (5.5%)	0.91 ± 0.02^a (3.9%)	5.0%
Cysteine+Methionine	0.17 ± 0.00 (3.1%)	0.64 ± 0.01 (3.2%)	0.55 ± 0.02 (2.5%)	3.5%
Phenylalanine+Tyrosine	0.47 ± 0.03 (8.2%)	1.26 ± 0.09 (5.9%)	1.09 ± 0.02 (4.9%)	6%
pure protein (AAres)	4.89 ± 0.10	17.68 ± 0.75	19.99 ± 0.24	-
BV	85 ± 2	86 ± 0	74 ± 3	100
1st limiting AA	Trp	Trp	Cys + Met	-
2nd limiting AA	Cys + Met	Lys	Ile	-
3rd limiting AA	-	Cys + Met	Leu	-

Note: Means in the same row with different letters indicate significant differences ($p<.05$).

total protein concentration and proteins with high BV. PSS fermentations of agricultural side-streams rich in tryptophane and aspartic acid—also in combination with materials with low concentrations thereof—may offer solutions to reduce supplementation costs and optimize protein yields and protein qualities.

The WBC of a material describes its capacity to retain water under centrifugal pressure or compression. It represents the sum of bound, hydrodynamic, and physically trapped water (López et al., 1996). The water-binding capacities of flour (Table S5), CPH, and CPHF were determined (Table 1). The WBC of CPH accounted for 6.2 mL/g and was, thus, higher than the WBC of CPHF (3.5 mL/g) and flour (0.62 mL/g). In the literature, values of 0.84 mL/g for flour (Soral-Śmietana et al., 2003) and of 5.8 mL/g for CPH have been reported (Figueroa et al., 2020). The minor deviations from the values previously reported might be explained by natural variations in the materials. The chemical composition of cocoa, especially the fiber fraction, can be affected by the harvest season, the maturation degree of the fruits, and the cocoa variety (Balladares et al., 2016). Furthermore, the WBC is closely associated with the hydration levels of protein and fiber. Excessive WBC of a particular ingredient can lead to dehydration of other components in formulated foods, negatively affecting the texture, color, and overall sensory properties of the final product. This is the case in baked goods, where the proper hydration of all flour components, especially protein and starch, is essential for the appropriate dough formation (Zayas, 1997). Overall, the WBC of CPH and CPHF were higher than values reported for flours conventionally used in bakery products (Mesías & Morales, 2017), suggesting the use of CPH and CPHF in formulated foods may exert a strong influence on the organoleptic quality and shelf-life of the final product, such as dry mouthfeel or slower water release rates (syneresis).

The OBC is highly important for the texture and mouthfeel of baked goods, meat formulations, and soups (Belitz et al., 2004). The OBC of flour, CPH, and CPHF accounted for 1.0 mL/g, 1.1 mL/g, and 2.1 mL/g, respectively (Table 1 and Table S5). The OBC capacity of CPH was in accordance with values reported by Figueroa et al. (2020), corresponding to 1.2 mL/g. The OBCs of CPH and wheat flour did not differ statistically from each other. Conversely, CPHF exhibited the highest OBC and differed significantly from CPH. The reason for this might be the larger particle surface areas of CPHF, caused by overall smaller particle size classes compared to CPH (Table 3) (Benítez et al., 2017). The use of CPHF may offer interesting perspectives for the use in foods with large contents of oils, such as chocolate fillings, as they may help reduce unwanted migration of lipids, deaccelerate the formation of fat blooms, and increase the products' shelf-life (Eibl & Rothkopf, 2018).

Due to its low caloric content and its health-promoting benefits, dietary fiber is an important nutrient often lacking in the Western diet. Positive correlations between the amount of ingested fiber and a risk reduction for metabolic and heart diseases have been proposed (Ötles & Ozgoz, 2014). Overall, the amount of total dietary fiber (TDF) in the fermented husks did not differ significantly from CPH. The TDF of CPH accounted for 63.6 g/100 g DM and 73.4 g/100 g DM for CPHF (Table 1). The total carbohydrate and TDF contents of CPH vary widely. TDF values between 18% and 59% of the total cocoa pod weight have been previously reported. Additionally, insoluble dietary fiber (IDF) has been described as the predominant fraction, accounting for 48%, while soluble dietary fiber (SDF) accounts for approximately 11% (Figueroa et al., 2020). The insoluble fiber fraction was predominant in CPH and CPHF. The relative amount of IDF increased with fermentation from 53.6 g/100 g DM in CPH to 71.1 g/100 g DM in CPHF, whereas the relative amount of SDF was reduced from 10.1 g/100 g DM to 2.3 g/100 g DM. The relative increase in TDF, especially in the IDF, can be explained by the solubility of the SDF and, thus, the loss through harvesting. The higher proportion of SDF in the unfermented husks may explain their higher WBC, as water-soluble fibers often present more hydroxyl groups able to interact with water through hydrogen bonds (Chen et al., 2021). The high fiber content of CPHF, together with its increased protein content compared to the unfermented material, may enable its use as a bulking agent to increase the protein and fiber contents of formulated foods.

The particle size distribution indicates the percentile of particles in a certain size class or fraction. The diameters $d_{v,0.1}$, $d_{v,0.5}$, $d_{v,0.9}$ correspond to 10, 50, and 90 vol% on a relative cumulative particle size curve, respectively (Servais et al., 2002). All three samples presented bimodal particle size distribution curves (Figure 3), indicating a higher predominance of two particle size classes on each curve. The $d_{v,0.9}$ of flour was 144 µm, while the $d_{v,0.9}$ of CPH and CPHF were 526 µm and 373 µm, respectively (Table 3). CPH showed a larger $d_{v,0.9}$

TABLE 3 Particle size classes ($n = 6$) and L*a*b* ($n = 3$) color components of wheat flour, cocoa pod husks (CPH), and cocoa pod husks fermented with *Pleurotus salmoeo-stramineus* (CPHF).

	Wheat flour	CPH	CPHF
$d_{v,0.1}$ [µm]	15.27 ± 0.05^{b}	19.78 ± 0.80^{a}	12.60 ± 0.17^{c}
$d_{v,0.5}$ [µm]	67.23 ± 0.34^{c}	210.0 ± 16.83^{a}	123.33 ± 3.20^{b}
$d_{v,0.9}$ [µm]	143.67 ± 1.37^{c}	525.67 ± 29.74^{a}	373.33 ± 8.11^{b}
L* [−]	91.46 ± 0.0^{a}	48.82 ± 0.14^{b}	40.36 ± 0.19^{c}
a* [−]	0.64 ± 0.01^{c}	12.70 ± 0.27^{a}	12.03 ± 032^{b}
b* [−]	9.35 ± 0.0^{c}	22.17 ± 0.18^{a}	20.55 ± 0.27^{b}
Browning index [−]	11.04 ± 0.0^{c}	78.08 ± 0.11^{b}	90.90 ± 0.83^{a}
Color [−]			

Note: Means in the same row with different letters indicate significant differences ($p < .05$).

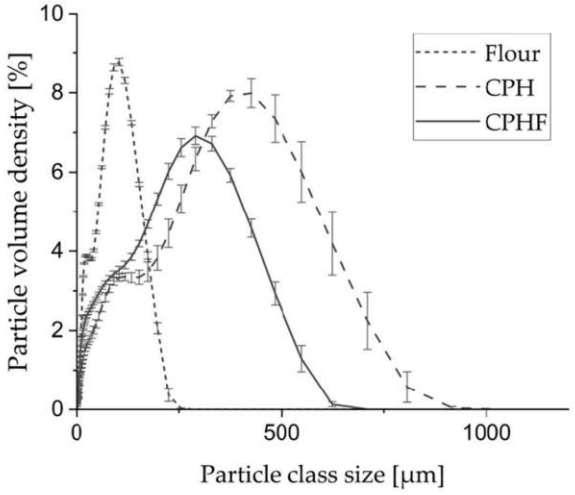

FIGURE 3 Particle size distribution of white flour, cocoa pod husks (CPH), and fermented cocoa pod husks (CPHF) ($n = 6$).

compared to CPHF, probably caused by the higher hardness of the material. The $d_{v,0.9}$ of CPHF was likely smaller due to the processing step the pod husks were subjected to. Previous studies have reported on different metabolic mechanisms of fungal species for the degradation of lignin and other cell wall polysaccharides (Zhu et al., 2016). The growth of PSS on the CPH and its putative cell wall degrading activities may have accounted for the disruption of the husks' structures, resulting in finer powders compared to the non-fermented husks when milled under the same conditions. The microscopic images (Figure 2) demonstrate a complete surface coverage of the fermented husks by mycelium, supporting this assumption.

In addition to the chemical composition, the color of the three samples was measured. The flour's L*a*b* values were 91.46, 0.64, and 9.35, respectively (Table 3). The L* of CPH and CPHF was 48.82 and 40.36, respectively, indicating a darker color of the fermented husks. The a* values of CPH (12.70) and CPHF (12.03) were in a closer range but still statistically different. The b* component of the CPH's color accounted for 22.17 and 20.55 for CPHF. The lower L* value of CPHF was also reflected in a higher browning index, 90.90 compared to 78.08 for CPH. The higher BI of CPHF may be attributed to the growth of the fungal species, an exposure to heat during autoclavation, and hence the formation of oxidation products as well as ongoing enzymatic activities. To understand the color differences better, further analyses of the composition of the CPH and the CPHF are needed and should be the focus of future studies.

3.2 | Textural properties of four blends and bread doughs with different concentrations of CPH and CPHF

Bread is a staple food that is commonly consumed in many countries. Traditional bread recipes primarily rely on wheat flour, which is produced by removing the bran and germ fractions of the wheat grain. By doing so, important nutrients are lost, and the bread made from white flour is limited in dietary fibers (Xu et al., 2019). Therefore, we investigated the addition of CPH and CPHF to bread doughs as a means to increase the protein and dietary fiber content of white bread.

The pasting properties of flour blends give important insights on the breads' final characteristics (Fu et al., 2008). Overall, the pasting temperature, which describes the temperature at which the viscosity begins to increase during the heating process, stayed constant for all flour-blends containing CPH as well as CPHF (Figure 4). A high peak viscosity (PV) hints at a high water-holding capacity (Balet et al., 2019). In spite of the higher WBC of the unfermented husks compared to CPHF, flour samples containing CPH did not exhibit a clear trend, besides showing lower PV compared to the flour. As wheat flour is mixed with water, a three-dimensional structure is created, where gluten particles are integrated into membranes that contain granules of starch and other flour components (Fu et al., 2008; Zayas, 1997). Therefore, the lower PV of the CPH samples may indicate an impediment in the formation of this network, resulting in a lower degree of starch-swelling and, thus, lower PV. The unfermented husks alone did not show a conventional pasting curve, hinting at a low starch concentration therein. The light increase in viscosity after thermal input may be explained by the presence of pectin in CPH. Cocoa husks have been reported to contain approximately 6% pectin (Sobamiwa & Longe, 1994). The gelling point of pectin has been determined to range between 51 and 86°C (Kastner et al., 2012).

The PV of samples with CPHF decreased with an increasing level of substitution. In the sample with pure CPHF, the viscosity remained constant throughout the pasting experiment. As the water was in abundance, it is possible that the fibers, predominantly water-insoluble, were completely hydrated and that their capacity for water uptake was already at its limit, resulting in a constant viscosity (Zhou et al., 2021). Furthermore, the final viscosity of 10% CPH was higher compared to the sample with equal CPHF concentration, possibly due to the higher WBC of the unfermented husks and a stronger competition for the water between the CPH and the flour. Two hypotheses have been proposed for the interaction of fibers with gluten networks. The first suggests that the dietary fiber competitively binds water, leading to the partial dehydration of gluten. Consequently, this induces a conformational change in the gluten matrix and causes the collapse of the polymeric network of gluten. According to this, the detrimental effects of fibers in dough can be mitigated by optimizing the water content. The second hypothesis hints at a gluten-diluting effect, whereby fibers physically disrupt the gluten network. This disruption occurs when fibers interfere with the cohesive structure of gluten, leading to its dispersion or the dispersion of gluten aggregates (Zhou et al., 2021). Therefore, as CPH and CPHF contained considerable amounts of dietary fiber, reduced gluten network formation due to the competition for water and physical hindrance by the fibers are possible.

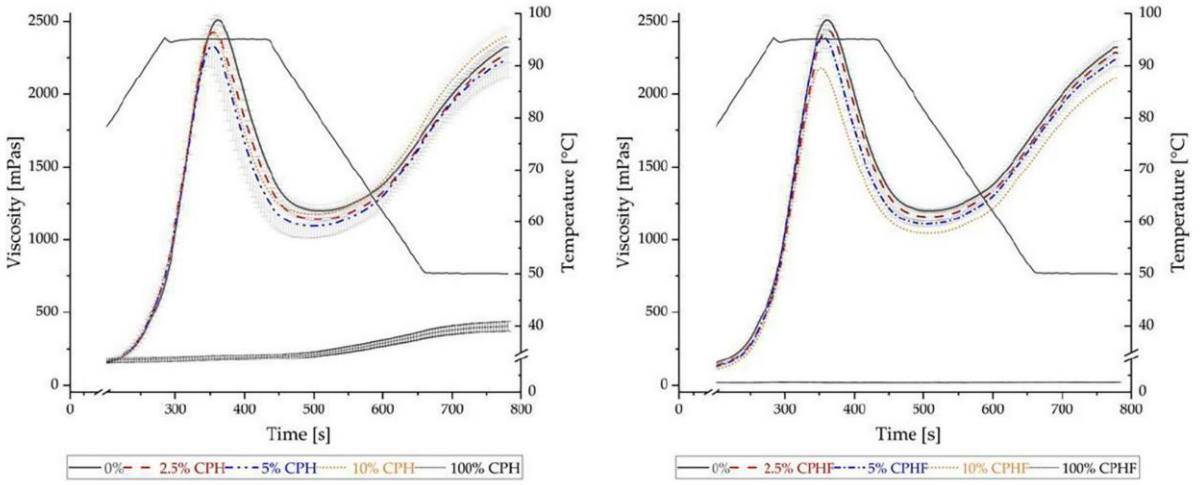

FIGURE 4 Pasting properties of flour blends with 0%—100% addition of cocoa pod husks (CPH) (left) and fermented cocoa pod husks (CPHF) (right) ($n = 3$).

Texture profile analyses of bread doughs containing 0% (*w/w*), 2.5% (*w/w*), 5% (*w/w*), and 10% (*w/w*) CPH and CPHF were performed. A replacement with 2.5% (2.24 N) and 5% CPH (2.25 N), as well as with 2.5% CPHF (1.54 N), led to a reduced dough hardness compared to the standard bread dough composed of only flour and water (4.25 N). The addition of 10% CPH (3.62 N) as well as 5% CPHF (4.64 N) led to a dough hardness similar to that of standard bread dough. The addition of 10% CPHF increased the hardness to 7.14 N. The samples' springiness was similarly high in all doughs with CPH and in the one with 2.5% CPHF, whereas the samples with 5% CPHF and 10% CPHF were similar to the standard dough. The hardest sample (10 % CPHF) presented the lowest springiness (25.66%). This was followed by 5% CPHF with 31.41% springiness and the white bread dough with 38.72% springiness. The three samples did not differ significantly from each other. However, they were distinct from the samples with 2.5% CPH (89.77%), 5% CPH (93.90%), 10% CPH (88.36%), and 2.5% CPHF (95.52%). The addition of different concentrations of CPH led to elasticities above those of the white bread doughs. As aforementioned, it is likely that the higher WBC of the CPH caused the water to be strongly bound to the CPH's fibers, limiting the unfolding of the gluten proteins and decreasing the degree of cross-linking between cysteine groups (Gras et al., 2001). Nawrocka et al. (2020) reported on the effect of insoluble and soluble fibers on disulfide-bond formation. The incorporation of more IDF into doughs led to a more intense abnormal folding of gluten protein. Fiber samples with high SDF content increased the number of stable cysteine bonds (SS bonds), whereas dough weakening, an indication of SS rupture, could be assigned to a high IDF content. As CPHF contained a higher IDF to SDF ratio compared to CPH, the increasing dough hardness and reduced springiness with increasing concentration may be attributed to a higher degree of destabilization of the gluten network by interference in the formation of stable cysteine bonds. Nonetheless, it is important to note that the kneading time and the amount of added water were kept constant, possibly causing the CPH doughs to remain partially undeveloped (Mirsaeedghazi et al., 2008). Experiments producing CPH and CPHF doughs with varying kneading times and water concentrations, as well as studies of the dough's rheological properties, may help elucidate the interactions within the doughs. These should be the focus of future studies.

In a preliminary trial, the optimal water concentration for the wheat flour type 550 was determined. The water necessary to develop the dough accounted for 53.5% (*w/w*) and was in accordance with values previously reported (54.1%) (Nikolic et al., 2013). All doughs developed consistencies above the 500 FE standard range (Table 4). Overall, the maximum resistance, expressed in FE, increased with increasing flour substitution. The addition of CPH led to higher torque values (higher consistency values) compared to CPHF. While 2.5% CPH showed a consistency of 818.5 FE, 2.5% CPHF addition led to a consistency of 808 FE. The differences between CPH- and CPHF-containing doughs became clearer with increasing concentration. Doughs made with 5% CPH showed 942.5 FE, and doughs made with 10% CPH showed 1080.5 FE, whereas doughs produced with 5% CPHF and with 10% CPHF showed values of 871 FE and 945.5 FE, respectively. The dough development time (DT) was also affected by the addition of CPH and CPHF. The DT was slightly shortened in the dough produced with 2.5% CPH, whereas it was prolonged for the other concentrations of either CPH or CPHF compared to white bread dough. This parameter describes the time it takes for the developing dough to reach maximum consistency. Therefore, the longer development times of the substituted doughs suggest it takes more kneading time to integrate the CPH and CPHF into the doughs. The larger particle size classes of CPH and CPHF compared with the flour may have played a role in prolonging the DT. In a study on bread doughs enriched with grape pomace fibers of different particle sizes, Mironeasa et al. (2019) described the effect of particle size on water absorption (WA). The authors observed a longer DT in doughs with larger particle size types. This was attributed

TABLE 4 Farinographic properties of bread doughs made with 0% (w/w), 2.5% (w/w), 5% (w/w), and 10% (w/w) cocoa pod husks (CPH) and fermented cocoa pod husks (CPHF) ($n = 2$).

Description	Flour	CPH 2.5%	CPH 5%	CPH 10%	CPHF 2.5%	CPHF 5%	CPHF 10%
Water absorption (WA) [%]	53.3 ± 0.3	53.4 ± 0.1	53.5 ± 0.1	53.35 ± 0.1	53.5 ± 0.1	53.8 ± 0.1	53.6 ± 0.2
Development time [mm:ss]	00:01:46 ± 0:00:06	00:01:40 ± 0:00:23	00:03:50 ± 0:01:26	00:05:39 ± 0:02:10	00:03:26 ± 0:02:41	00:06:01 ± 0:03:00	00:11:57 ± 0:02:41
Consistency [FE]	511 ± 6.4	818.5 ± 82.7	942.5 ± 132.2	1080.5 ± 181.7	808 ± 138.6	871 ± 151.32	945.5 ± 115.3
WA consistency [%]	54.1 ± 1.1	61.4 ± 1.9	64.6 ± 3.5	67.9 ± 4.5	62.8 ± 5.8	63.1 ± 3.9	64.7 ± 2.7
Dough stability [mm:ss]	0:01:17 ± 0:00:43	00:02:46 ± 0:01:02	00:04:54 ± 0:00:28	00:03:27 ± 0:00:57	00:04:41 ± 0:04:20	0:06:39 ± 0:02:24	00:07:13 ± 0:00:20

to slower water absorption by the larger particles, hampering the formation of the gluten network and increasing the time required for optimal dough development. Further studies using CPH and CPHF powders with finer particle sizes should be carried out in the future. In addition, the equipment's software proposed values for the water absorption to obtain doughs with 500 FE consistency. These were 61.4%, 64.6%, and 67.9% for 2.5%, 5%, and 10% CPH, respectively. The water absorption values for CPHF were 62.8%, 63.1%, and 64.7% for 2.5%, 5%, and 10% addition, respectively. A contributing factor for the higher water amounts recommended might be the higher protein content of the studied flour blends compared to the wheat flour. Thus, by replacing a part of the flour with CPH or CPHF, the relative amount of protein in the dough increased (Nikolic et al., 2013). Another factor to consider are changes in the fiber fraction of the doughs as well as their concentrations therein. Dietary fiber may increase water absorption, mainly due to the higher number of hydroxyl groups allowing for a stronger interaction with water through hydrogen bonding (Kurek et al., 2016). Consequently, there is stronger competition between the added proteins, fibers, and gluten in the available water. Future trials carried out with adapted water concentrations are necessary to determine if consistency more similar to an ideal standard for bread dough can be achieved.

The addition of CPH and CPHF caused the bread dough to change color (Table S6). With increasing concentrations of CPH and CPHF, the L* value of the dough decreased, indicating the dough became darker. CPHF had a stronger effect on this parameter compared to CPH. The same trend was observed for the a* component. On the other hand, the b* component was higher in dough made with 10% CPH. The b* values of doughs containing 10%, 5%, and 2.5% CPHF, and 5% CPH were commensurate. The changes in the L*a*b* values were also reflected in the BI of the doughs. Browning became higher with increasing concentrations of CPH and CPHF. CPHF caused the samples to darken more than CPH. The dark color of the dough may present a challenge in the use of CPHF as a food ingredient. There are, however, positive associations between brown food products, especially baked goods, and their acceptance by consumers, as these are sometimes associated with a higher fiber content and possible health-promoting benefits (Clydesdale, 1993; Downey, 2011). The belief that a darker color suggests a healthier product has encouraged the bakery industry to increase the use of food colorants (Downey, 2011). Therefore, CPHF could also be used as a natural food colorant (Grob et al., 2021).

In accordance with the results previously discussed, Spearman correlation analyses showed a positive association between the concentration of added CPH and the BI, the consistency, the water absorption, the dough development time, and the stability time (Figure 5a). Negative associations were determined between the concentration of CPH and the L* value and between the concentration and the PV. The darkening of wheat bread with the increasing addition of cocoa pod husk powders (CPHPs) was previously reported (Zamri et al., 2013). The PV and the water absorption, as well as the consistency and the PV, were also negatively associated, possibly relating to the reduction of starch and gluten—the main responsible

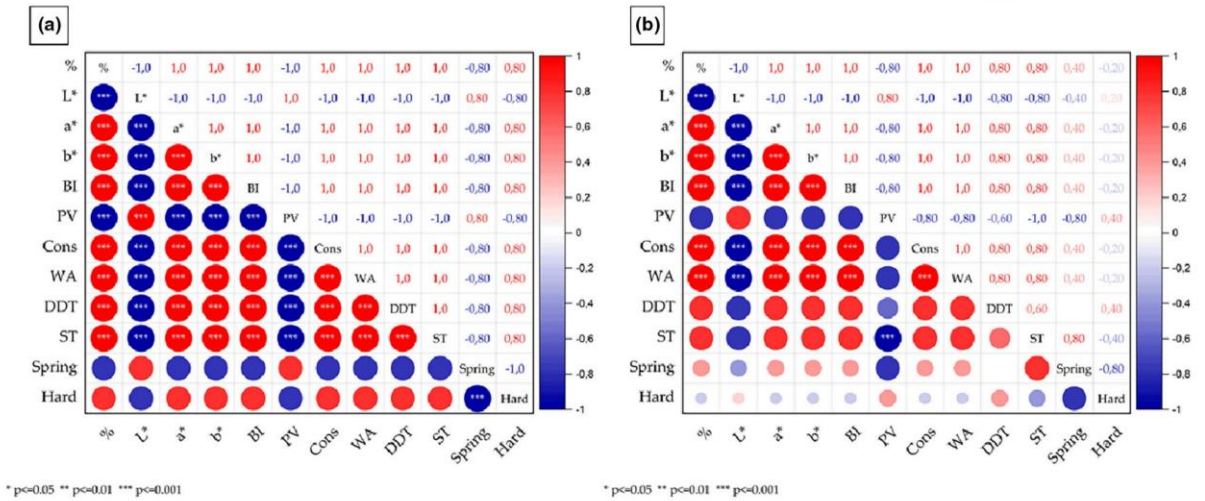

FIGURE 5 Spearman correlation matrices of dough properties produced with different concentrations of (a) cocoa pod husks (CPH) and (b) fermented cocoa pod husks (CPHF). % indicates the flour substitution in % (*w/w*), BI the browning index, PV the peak viscosity, Cons the consistency, WA the water absorption, DDT the dough determination time, ST the stability time, Spring the springiness, and Hard the hardness of the developed doughs. Red indicates a positive and blue indicates a negative association between the variables.

components for the formation of networks in wheat doughs—by dietary fibers. Moreover, the correlation analyses indicated a negative association between the hardness and the springiness. In addition, an increase in dough development time and water absorption can often be observed in dough formulations enriched with fibers. The strength and stickiness of such doughs are expected to be increased, while the tolerance for mixing and fermentation is diminished (Zhou et al., 2021). This was described for breads produced with CPHPs. The authors reported: "as the concentration of CPHP increased in the formulation, the specific volume, microscopic structure, and pore size of bread underwent a decrease, leading to an observable rise in the bread's final hardness" (Zamri et al., 2013). Furthermore, Spearman correlations suggested a significant positive relationship between the concentration of CPHF and the a*-value, the b*-value, the BI, the consistency, and the water absorption (Figure 5b). Similar to CPH, the concentration was negatively related to the L* value. The browning index was shown to be positively linked to the consistency and the water absorption. This was expected, as the three variables are dependent on the concentration of CPHF. Alike observations were made by Ulziijargal et al. (2013), where the replacement of 5% of wheat flour with mushroom mycelium powder led to bread loaves with lower lightness and white index values. Contrarily to CPH, the hardness and springiness were not significantly correlated in samples containing CPHF, possibly due to differences in the fiber compositions between the two ingredients.

4 | CONCLUSIONS

This paper explored the valorization of cocoa pod husks by fermentation with *Pleurotus salmoneo-stramineus* as an innovative approach to creating protein-rich and fiber-rich ingredients for the food industry. The fermentation conditions were successfully optimized, and the techno-functional properties of the fermented cocoa pod husks (CPHF) were evaluated for use in food products. We demonstrated that basidiomycetes are able to grow on cocoa pod husks and produce high-quality protein. We additionally proved the dependence of protein formation and amino acid composition on the culture medium, showing that for PSS, the CPH medium led to an improved protein quality compared to malt extract medium while maintaining the same protein content. The integration of different concentrations of CPH and CPHF in bread doughs was possible, yet their suitability for industrially produced bakery products still needs to be further explored. Experiments producing doughs containing CPH and CPHF by varying the kneading times and the water concentrations added may help elucidate the interactions within the developed doughs and improve the doughs' consistencies. By adding value to cocoa pod husks through fungal fermentation, cocoa farmers may profit from alternative sources of income and improve their livelihoods, fomenting additionally a more sustainable cocoa production.

AUTHOR CONTRIBUTIONS

Thomas Bickel Haase: Conceptualization (equal); formal analysis (equal); investigation (equal); methodology (equal); writing – original draft (equal); writing – review and editing (equal). **Victoria Klis:** Conceptualization (equal); formal analysis (equal); investigation (equal); methodology (equal); writing – original draft (equal). **Andreas Klaus Hammer:** Methodology (equal); validation (equal). **Claudia Pinto Lopez:** Formal analysis (equal); investigation (equal); validation (equal). **Christoph Verheyen:** Writing – review and editing (equal). **Susanne Naumann-Gola:** Funding acquisition (equal); project administration (equal); supervision (equal); writing – review and editing (equal). **Holger Zorn:** Funding acquisition (equal); project

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

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7 Concluding remarks and outlook

On the whole, the demand and production of cocoa beans have experienced a steady growth over the years. In 2023, the global cocoa market was valued to approximately US$ 15.16 billion. The total cocoa sales are predicted to grow at 4.7% CAGR from 2023 to 2033, increasing the market valuation to US$ 23.98 billion by the end of 2033 (Choudhury 2022). While cocoa beans are broadly utilized, the full potential of the total fruit remains untapped (Campos Vega et al. 2020). Considering that the beans amount to 21-23% of the cocoa fruit, the whole fruits could be valued at a much higher price if all fractions could be commercialised. However, data on these other fractions' market size is hitherto unavailable, so their market values still need to be defined (Campos Vega et al. 2020). Moreover, meeting the increasing demand for cocoa without compromising the environment remains difficult. Especially, as cocoa production is regarded as unsustainable and has been associated with the deforestation and the destruction of primary forests (Fountain and Huetz-Adams 2020; Potts et al. 2017). Accordingly, the European Parliament, Council of the European Union (6/9/2023), introduced on June 2023 Regulation (EU) 2023/1115 on deforestation-free products. The new regulation aims to avoid that listed products (e.g. cocoa and coffee) Europeans buy, use, and consume contribute to deforestation and forest degradation, intending to reduce carbon emissions and improve the sustainability of the various imported products. Consequently, this implies the need to optimise the cocoa supply chain. One potential approach to improve the sustainability of cocoa production involves the valorisation of side streams, putting into context the work carried out in this dissertation.

In a more focused scope, this dissertation laid important groundwork for the valorisation of the cocoa pulp and the cocoa pod husks, thereby assisting food producers in using these as new ingredients. Aroma analyses carried out in Chapter 1 revealed differences in the aroma compositions and qualities in cocoa pulps from four different origins. While some aroma-active substances were confirmed, such as linalool and 2-phenylethanol, others were detected in cocoa pulp for the first time, laying important foundations for future research. Moreover, the variations in aroma composition support the previous assumption that cocoa pulp aroma mirrors the fruits' quality, assisting in categorising them as either fine or flavour or as bulk cocoa (Eskes et al. 2007). Ergo, by evaluating the pulps' aroma composition, future correlations with the quality of the cocoa beans, the resulting chocolate, and the quality of the other cocoa fractions may be possible. With this in mind, the integration of online sensor technologies for the detection of characteristic aroma compounds might be applied directly on the farms as a mean to monitor the fruits' quality (Koehne et al. 2023). This could provide information on the suitability of the fruit's pulp for certain food applications or even offer a tool to monitor the microbial state of the material by the detection of microbially-formed VOCs, such as indole. Consequently, this would ease the selection of

cocoa fruits and complement human sensory on-site. Despite the results of this chapter being highly promising for the cocoa pulp valorisation, evaluations were limited to qualitative analyses and identification. Particularly as AEDA does not provide a definitive indication of which substances have the most significant impact on the overall aroma profile. Likewise, the FD factors ascribed in AEDA do not consider physiological impressions influencing the perception of odour character or the sensory thresholds of odour-active compounds in the food matrix (Marsili 2020). Based on the work carried out in this dissertation, there remains a knowledge gap that needs to be addressed by the quantification of aroma-active substances in cocoa pulps together with aroma reconstitution experiments, and the determination of odour activity values (OAV). Future endeavours should also include fruits from additional origins, different seasons, and diverse genetic backgrounds, helping establish the industrial processing of cocoa fruit pulp in different cocoa-producing regions.

To obtain high quality cocoa pulp with extended shelf-life, processing sites near farms may be beneficial, as the prolonged storage of cocoa fruits (e.g. during transportation to a processing centre) negatively affects the pulp yield (Afoakwa et al. 2013). That being the case, the economic challenges for small-scale cocoa pulp producers in Ghana were reported to include high haulage costs due to scattered cocoa farms, commonly with poor infrastructure (Adomako 2006). In view of this, it is of industrial and scientific interest to develop optimal processes for the preservation of cocoa pulp using easily accessible technologies. Chapter 2 of this dissertation demonstrated that pasteurization and ultra-high temperature treatment are suitable technologies for preserving cocoa pulp with minimal changes in organoleptic quality. The thermally treated pulps could be kept at 4 °C and 23 °C for 24 weeks without showing significant microbiological growth. This can be advantageous for the haulage of the processed cocoa pulp in comparison to cool transportation, as dispatching at a higher temperature might help improve energy efficiency and help maintain the costs low. Notwithstanding, thermally treated cocoa pulp still contains high concentrations of water, representing cost and logistic challenges for transport. Hence, drying technologies may offer alternatives for the pulp's preservation. Guirlanda et al. (2023) recently indicated that spray-drying may be suitable for the large-scale stabilisation of cocoa honey, with a lower heat exposure compared to other drying technologies. Additionally, the concentration of cocoa pulp through the evaporation of water may help increase the dry matter content of the pulp and reduce, thus, shipping costs. Nevertheless, the influences of the drying and concentration technologies on the organoleptic quality of cocoa pulp, as well as the costs associated with this processing, remain largely unclear. It is sensible to assume that the separation and preservation of cocoa pulp may be supported by an enzyme-assisted processing, as described in Chapter 3. By applying cell-wall degrading enzymes, such as endo-polygalacturonase, the physicochemical characteristics of the pulp might be also modified to meet requirements for certain processes and applications (e.g., the production of clear pulp concentrates). As to the pulp's recovery, enzymatic hydrolysis could support loosening the tightly adhering fibrous layer around the fresh cocoa seeds, improving the pulp yields, preventing seed damage during separation as well as reducing their fermentation time. While there are studies on the benefits of

the partial de-pulping of cocoa beans on their final quality, the amount of pulp around these may vary significantly between cultivars, ranging from 5% to 26% of the fruit weight (Adams et al. 1982; Dias et al. 2007). Because of this, there isn't a universal standard on how much pulp can be separated, and processing protocols will need to be adjusted accordingly, independently of the choice of separation technology. Furthermore, the primary concern for cocoa producers will continue to be ensuring the proper fermentation of the cocoa beans, as these are still the profit-making fraction of the fruits. Put differently, after an eventual de-pulping, it has to be ascertained that enough pulp remains available for fermentation. In the context of enzyme-assisted de-pulping, a complete removal of the pulp is the likely outcome due to its liquefaction. This approach could be particularly valuable in countries where cocoa beans are deemed of lower quality for chocolate production or where cocoa bean fermentation is not conducted, such as Indonesia (Ariningsig 2020). In such cases, utilizing the unfermented de-pulped cocoa beans for alternative purposes like cocoa butter, soaps, cosmetics, and more could be considered (Adomako 2006).

Fungal fermentation proved a fascinating approach to upcycle cocoa pod husks into protein- and fibre-rich ingredients. Nonetheless, this approach is accompanied by some limiting factors. First, several aspects related to food law and food safety also need to be considered (Steiß 2024). According to Regulation (EU) 2015/2283 (Regulation (EU) 2015/2283 of the European Parliament and of the Council 2015), fungal mycelium belonging to the Basidiomycota division is considered a novel food under Article 3(2)(ii), encompassing foods derived from microorganisms, fungi, or algae or isolated or produced from them. While certain products within this category have received Novel Food approval, such as mycelium-based shiitake fermented pea and rice proteins for use in various products, including baked goods and cocoa/chocolate items, the approval process for other fungi would require separate applications under Articles 10-12 of Regulation (EU) 2015/2283. It is also important to note that cocoa pod husks (CPH) themselves may be classified as Novel Food, as their consumption in significant quantities before May 15, 1997, is not well-documented (Regulation (EU) 2015/2283). Furthermore, potential residues and contaminants in CPH, such as Cadmium, need closer examination prior to their widespread use for human consumption (Vanderschueren et al. 2021).

Overall, this doctoral thesis provides valuable knowledge for optimising cocoa by-product utilization, reducing economic losses, and promoting sustainable practices in cocoa production. Recognising the challenges posed by the dynamics of the cocoa supply chain, this dissertation emphasizes the need for continued research to address gaps in quantification, scalability, infrastructure development and regulatory approval. Ultimately, a concerted effort is required to ensure that the knowledge generated in this work can be effectively translated into practical solutions within the cocoa industry.

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