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Immobilized Pichia kudriavzevii UNJCC Y-137 and Lactobacillus plantarum NP-57 on Gluten Pellets as Starter Cultures for Controlled Cocoa Fermentation: Effects on Physicochemical Properties and Volatile Compounds

Prof Dr.Dalia Sukmawati M.Si

Dalia-Sukmawati@unj.ac.id

State University of Jakarta

Salma Cut Desi Ayu

State University of Jakarta

Rizal Koen Asharo

State University of Jakarta

Tri Handayani Kurniati

State University of Jakarta

Laith Khalil Tawfeeq Al-Ani

School of Biology Science,Universiti Sains Malaysia

Catur Sriherwanto

Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Cibinong, West Java 16911, Indonesia

Sulistiani Sulistiani

Research Collaboration Centre for Traditional Fermentation, Surakarta 57126, Central Java, Indonesia

Fera R. Dewi

Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Cibinong, West Java 16911, Indonesia

Mohd Hafiz Arzmi

Cluster of Cancer Research Initiative IIUM (COCRII), International Islamic University Malaysia, Kuantan Campus, Kuantan, Pahang 25200, Malaysia

Patricia Akua Sitsofe Nyahe

Department of Crop Science, University of Ghana, P. O. Box LG 44, Legon, Accra, Ghana; and Ghana Cocoa Pest and Disease Control Unit, Ghana Cocoa Board, Accra, Ghana

Seidu Abu

Department of Crop Science, University of Ghana, P. O. Box LG 44, Legon, Accra, Ghana; and Ghana Cocoa Pest and Disease Control Unit, Ghana Cocoa Board, Accra, Ghana

Lobna Hajj Hedfi

Research Laboratory of Agricultural Production Systems and Sustainable Development (LR03AGR02), Carthage University, Tunisia

Muhammad Irfan

Department of Biotechnology, University of Sargodha, Sargodha, Pakistan

Mavis Agyeiwaa Acheampong

Department of Crop Science, University of Ghana, P. O. Box LG 44, Legon, Accra, Ghana; and Ghana Cocoa Pest and Disease Control Unit, Ghana Cocoa Board, Accra, Ghana

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Immobilized *Pichia kudriavzevii* UNJCC Y-137 and *Lactobacillus plantarum* NP-57 on Gluten Pellets as Starter Cultures for Controlled Cocoa Fermentation: Effects on Physicochemical Properties and Volatile Compounds

Dalia Sukmawati[1,5*]; Salma Cut Desi Ayu[1]; Rizal Koen Asharo[1]; Tri Handayani[1]; Laith Khalil Tawfeeq Al-Ani[2]; Catur Sriherwanto[3]; Sulistiani[4,5]; Fera R. Dewi[4,5]; Mohd Hafiz Arzmi[6]; Patricia Akua Sitsofe Nyahe[7]; Seidu Abu[7]; Lobna Hajj-Hedfi[8,9]; Muhammad Irfan[10]; Mavis Agyeiwaa Acheampong[7]*

[1] Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Negeri Jakarta, Rawamangun, Jakarta 13220, Indonesia

[2] School of Biology Science, Universiti Sains Malaysia, 11800 Minden, Pulau Pinang, Malaysia

[3] Biotech Center BPPT, Building 630 PUSPIPTEK Area, Setu, Tangerang Selatan, Banten 15314, Indonesia

[4] Research Center for Applied Microbiology, National Research and Innovation Agency (BRIN), Cibinong, West Java 16911, Indonesia

[5] Research Collaboration Centre for Traditional Fermentation, Surakarta 57126, Central Java, Indonesia

[6] Cluster of Cancer Research Initiative IIUM (COCRII), International Islamic University Malaysia, Kuantan Campus, Kuantan, Pahang 25200, Malaysia

[7] Department of Crop Science, University of Ghana, P. O. Box LG 44, Legon, Accra, Ghana; and Ghana Cocoa Pest and Disease Control Unit, Ghana Cocoa Board, Accra, Ghana

[8] Regional Centre of Agricultural Research of Sidi Bouzid, Gafsa Road Km 6, B.P. 357, Sidi Bouzid 9100, Tunisia

[9] Research Laboratory of Agricultural Production Systems and Sustainable Development (LR03AGR02), Carthage University, Tunisia

[10] Department of Biotechnology, University of Sargodha, Sargodha, Pakistan

*Corresponding authors: Dalia Sukmawati (Dalia-Sukmawati@unj.ac.id); Mavis Agyeiwaa Acheampong (maacheampong@ug.edu.gh)

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Abstract

The quality of Indonesian cocoa beans is deemed inferior due to inadequate fermentation. Incorporation of microbial starters during fermentation, utilising immobilised cells on gluten pellets, may enhance fermentation efficiency. This study assessed the efficacy of immobilised Pichia kudriavzevii UNJCC Y-137, Lactobacillus plantarum NP-57, and a combination of both on gluten pellets at three immersion durations (3, 6, and 9 h) on the physicochemical properties and volatile compounds of fermented cacao beans. The greatest viability of P. kudriavzevii and L. plantarum on gluten pellets was $6.67 \times 10^8 \pm 4.7 \times 10^7$ CFU/mL and $2.52 \times 10^9 \pm 1.3 \times 10^8$ CFU/mL, respectively after 9 h immersion. Scanning electron microscope analysis confirmed successful immobilisation after 9 h of soaking. The combination of P. kudriavzevii and L. plantarum yielded superior fermentation quality, achieving a fermentation percentage of 85.67 ± 2.73% and antioxidant activity of 86.04 ± 0.16%. The volatile compounds on cocoa beans comprised organic acids,

alcohols, aldehydes, amines, alkaloids, esters, and ketones. In conclusion, the use of immobilised yeast P. kudriavzevii UNJCC Y-137 and bacterium L. plantarum NP-57 on gluten pellets, particularly their combination with extended immersion duration, significantly enhanced fermentation quality, antioxidant activity, and volatile compound profile of cocoa beans.

Keywords

Cocoa fermentation; Immobilized starter culture; Gluten pellet; *Pichia kudriavzevii*; *Lactobacillus plantarum*; Volatile compounds

1 Introduction

Cocoa (*Theobroma cacao* L.) is a major plantation crop and an important agricultural commodity in Indonesia, contributing significantly to the national economy (De Boer et al., 2019; Neilson et al., 2020). However, the quality of Indonesian cocoa beans remains low in terms of both physical attributes and flavor (Schwab et al., 2008). Unlike other cocoa-producing countries, fermentation is not commonly practiced in Indonesia, despite its significant role in improving cocoa bean quality (Effendy et al., 2019; Ambarawati & Esterina, 2024). Effendy et al. (2019) examined 284 cocoa farmers in Central Sulawesi and discovered that more than 50% did not ferment their cocoa beans, mainly due to a lack of interest in improving quality. While Ambarawati and Esterina (2024) found that the probability of Indonesian farmers fermenting their cocoa beans increases with higher production volumes. However, many farmers remain reluctant to adopt fermentation due to various socio-economic factors. The absence of proper fermentation results in lower physical and chemical quality, reducing the market value of Indonesian cocoa beans.

Fermentation is a crucial stage in cocoa processing to enhance its quality. It is proven that fermentation induces biochemical changes that enhance the flavor, color, and aroma of chocolate (Fang et al., 2020; Ruiz-Santiago et al., 2024). During fermentation, the cocoa pulp undergoes microbial activity that breaks down glucose and polysaccharides, producing metabolites that serve as aroma and flavor precursors for chocolate (Ho et al., 2014; Sukmawati et al., 2021; Sukmawati et al., 2023). The microbial community involved in cocoa fermentation primarily consists of yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). Among these, yeasts play a dominant role, particularly those from the genera *Pichia*, *Hanseniaspora, Saccharomyces*, and *Candida* (Díaz-Muñoz et al., 2021; Sukmawati et al., 2021; Díaz-Muñoz & De Vuyst, 2022). Yeasts contribute to pulp degradation, ethanol production, organic acid formation, and the generation of volatile organic compounds, which influence cocoa quality (Gutiérrez-Ríos et al., 2022; Díaz-Muñoz et al., 2023).

LAB also play a vital role in fermentation. Among the LAB species, *Lactobacillus plantarum* is commonly used in food fermentation (Sukmawati et al., 2021; Ardilla et al., 2022). LAB metabolize glucose and citric acid to produce lactic acid, which enhances the flavor and aroma of cocoa (Viesser et al., 2021; Korcari et al., 2023). Korcari et al. (2023) concentrated on the fermentation of premium chocolate using specific lactic acid bacteria, notably *Lactobacillus fabifermentans* SAF13. The research indicated that inoculation with these bacteria affected the free amino acid profile and volatile chemical composition of the cocoa, resulting in improved sensory attributes in the chocolate produced. The presence of lactic acid bacteria correlated with the generation of flavor precursors and volatile compounds that enhance the distinctive taste and aroma of the premium chocolate. Those studies provide evidence that LAB plays a crucial role in cocoa fermentation by metabolizing sugars and acids to produce lactic acid and other compounds, thereby enhancing the flavor and aroma profiles of cocoa and its derived products.

Numerous investigations have indicated that although indigenous bacteria inherently aid in cocoa fermentation, their numbers frequently lack the adequacy to guarantee uniform and superior fermentation results. This has resulted in the investigation and utilization of starting cultures to improve the fermentation process. Falconí et al. (2023) proved that inoculating cocoa fermentations with a mixed local microbial starter culture led to a 19.7% enhancement in fermented beans relative to spontaneous fermentation. Furthermore, Campos et al. (2025) concluded that the microbial dynamics in cocoa fermentation emphasise the intentional application of starting cultures that not only expedites fermentation

but also enriches the flavour richness of cocoa beans. This indicates that dependence exclusively on indigenous bacteria may not produce the necessary level of quality and consistency in cocoa products.

Immobilized microbial starters have been extensively studied due to their ability to improve fermentation efficiency, enhance product quality, and protect live microbial cells (Burgin et al., 2011; Mitropoulou et al., 2013; Carbonero-Pacheco et al., 2024). Yeast immobilization is a method in which yeast cells are encapsulated within a support matrix, allowing them to maintain metabolic activity and be utilized in several fermentation cycles. Surface attachment via natural adsorption is a commonly employed technique for yeast immobilization (Altieri et al., 2019; Estela-Escalante et al., 2019). This methodology has been extensively utilized in diverse fermentation sectors, such as wine and beer manufacturing, to improve process efficiency and product uniformity (Moreno-García et al., 2018; Araujo et al., 2021). Nonetheless, its use in cocoa fermentation is presently constrained.

Gluten pellets were chosen as the immobilization support in this study because of their advantageous characteristics, such as biocompatibility, porosity, and cost-effectiveness. In contrast to conventional carriers like alginate or cellulose, gluten-based matrices provide a protein-dense framework that promotes microbial adhesion and metabolic activity. It is also biodegradable and food-grade, rendering them especially appropriate for food fermentation applications (Altieri et al., 2019; Carbonero-Pacheco et al., 2024). Furthermore, the fibrous and porous structure of gluten increases the surface area for microbial adhesion, hence enhancing cell survival and fermentation efficacy (Mitropoulou et al., 2013; Estela-Escalante et al., 2019). These benefits render gluten pellets, a viable substitute for traditional immobilization materials, particularly in scalable and sustainable fermentation systems.

This study investigated the application of immobilized microbial starters (*P. kudriavzevii* UNJCC Y-137 and *L. plantarum* NP-57) in cocoa fermentation, with a focus on improving physicochemical properties and volatile compound profile.

2 Materials and Methods

2.1 Preparation of Microbes

2.1.1 Biomass Suspension

Pichia kudriavzevii strain UNJCC Y-137 and *Lactobacillus plantarum* NP-57 were obtained from Universitas Negeri Jakarta Culture Collection (UNJCC). The preparation of the yeast (*P. kudriavzevii* UNJCC Y-137) and bacterial (*Lactobacillus plantarum* NP-57) (LAB) suspensions followed the method described by Sukmawati et al. (2023) and Sulistiani (Sulistiani & Handayani, 2018). The yeast and LAB biomass suspensions were adjusted to a concentration of 36 g/mL for subsequent cell immobilization. A yeast suspension was prepared by inoculating 600 mL of the yeast suspension in Yeast Malt Broth (YMB) medium. *P. kudriavzevii* UNJCC Y-137 isolate from 12 test tubes was suspended in 10 mL of sterile distilled water per tube and homogenized using a vortex. A 20% (v/v) yeast suspension was transferred into three 250 mL Erlenmeyer flasks, each containing 160 mL of YMB medium, and incubated in a shaker at 35 rpm for 48 h.

LAB biomass suspension was prepared by inoculating an 800 mL LAB suspension in de Man, Rogosa, and Sharpe Broth (MRSB) medium (Himedia, M369). L. plantarum NP-57 isolated from eight test tubes was suspended in 10 mL of sterile distilled water per tube and homogenized using a vortex. A 10% (v/v) of LAB suspension was transferred into four 250 mL Erlenmeyer flasks, each containing 180 mL of MRSB medium, and incubated in a shaker at 35 rpm for 24 h.

2.1.2 Preparation of Gluten Pellet Stock

Gluten pellet stock was prepared following the method of Bardi et al. (1996). Wheat gluten flour and distilled water were mixed at a 1:1 ratio (1000 g:1000 mL) and kneaded until smooth. The dough was manually shaped into pellets (2–3 cm diameter) and dried in an oven at 105°C for 5 h. Dried gluten pellets were stored in a jar at room temperature overnight before use in cell immobilization.

2.1.3 Cell Immobilization Preparation with Gluten Pellets

Cell immobilization was conducted following the method of Bardi et al. (1996). Yeast and LAB suspensions (600 mL and 800 mL, respectively) were centrifuged at 4000 rpm at 4°C for 20 min using a high-speed centrifuge (Hitachi CF16RXII) to form pellets. The resulting pellets were adjusted to 1 g/mL in YMB and MRSB media, corresponding to cell densities of 10^7 CFU/mL for yeast and 10^8 CFU/mL for LAB.

Yeast and LAB biomass suspensions (36 g/mL) were suspended at 2% (w/v) into six 500 mL Erlenmeyer flasks containing 300 mL glucose medium (36 g glucose, 0.3 g (NH4)2SO4, 0.3 g KH2PO4, 1.5 g MgSO4, and 1.2 g yeast extract in distilled water). Dried gluten pellets (100 g) were added to each flask and homogenized using a shaker at 35 rpm for 3, 6, and 9 h. Following incubation, the liquid medium was discarded via sterile filtration, and the immobilized gluten pellets were stored in sterile jars at 4°C. A viability test was conducted to determine optimal soaking time and cell density for fermentation.

2.1.4 Viability Test of Yeast and LAB Cells Immobilized on Gluten Pellets

Viability testing followed the TPC method of Marham et al. (2016). Yeast (*P. kudriavzevii* UNJCC Y-137) and LAB (*L. plantarum* NP-57) immobilized on gluten pellets were tested after 3, 6, and 9 h of soaking in glucose medium. For yeast, 10 g of gluten pellets from each treatment were inoculated into 90 mL of Yeast Mannitol Broth (Himedia, M716) and incubated at 35 rpm for 48 h. A 1 mL suspension was transferred to a Corning 15 mL tube containing 9 mL of sterile PBS, vortexed for 30 s, and serially diluted to 10^{-6}. A 0.1 mL aliquot from the final dilution was spread onto yeast mannitol agar (Himedia, M715) and incubated at 30°C for 48 h.

For LAB, 10 g of gluten pellets from each treatment were inoculated into 90 mL of MRSB and incubated at 35 rpm for 24 h. A 1 mL suspension was transferred to a Corning 15 mL tube containing 9 mL of sterile PBS, vortexed for 30 s, and serially diluted to 10^{-7}. A 0.1 mL aliquot from the final dilution was spread onto de Man, Rogosa, and Sharpe Agar (MRSA) (Himedia, M641) and incubated at 37°C for 24 h.

The Colony Forming Unit (CFU) count was determined using the formula below:

$$\text{CFU/mL} = \text{Average number of colonies formed} / (\text{Inoculum volume} \times \text{Dilution factor}).$$

2.2 Fermentation of Cocoa Beans (Clone MCC 02)

Fermentation was conducted following the method described by Scollo et al. (2020) using a 1000 mL thin-walled container. Before use, the fermentation container was cleaned with antiseptic soap, rinsed with 70% ethanol, and air-dried. Fermentation was carried out by placing 500 g of rehydrated dried cocoa beans (clone MCC 02 obtained from Lampung plantation) into the container and treating them with gluten pellets containing *P. kudriavzevii* (cell density of 10^8 CFU/mL) and *L. plantarum* (cell density of 10^9 CFU/mL) at 10% (w/w) of the bean weight. The treatments included: 50 g of *P. kudriavzevii* UNJCC Y-137; 50 g of *L. plantarum* NP-57; a combination of *P. kudriavzevii* UNJCC Y-137 and *L. plantarum* NP-57 in a 1:1 ratio (each at 25 g); and a control (cocoa beans without microbial addition). The fermentation process was carried out for 0, 72, 96, and 120 h in a closed container at 30–34°C.

2.2.1 Physical and Chemical Characteristics of Fermented Cocoa Beans

Physical quality assessment was performed using a cut test, chemical quality analysis was conducted following Melo et al. (2021), and antioxidant activity was assessed according to Shekhar and Anju (2014).

Cut test

The cut test was performed to determine the fermentation level of cocoa beans by splitting 50 dried fermented cocoa beans longitudinally and observing the color (Guzmán-Armenteros et al., 2023). The classification criteria were as follows: slaty beans (unfermented), purple-dominant with some brown (underfermented), and predominantly brown (fully fermented) beans (Tomlins et al., 1993). The fermentation success rate was calculated as follows:

$$\text{Cut test (\%)} = \sum \frac{Number\ of\ fully\ fermented\ beans}{Total\ number\ of\ cocoa\ beans\ split} \times 100\%$$

pH measurement

Briefly, 10 g of cocoa bean powder was dissolved in 90 mL of distilled water in a 250 mL beaker. The mixture was stirred until homogeneous and filtered using Whatman No. 41 filter paper. The filtrate was collected in a 50 mL beaker, and the pH value was recorded using a pH meter after stabilization.

Antioxidant activity (2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay)

A 0.1 mM DPPH solution was prepared by dissolving 3.95 mg of DPPH in 100 mL of methanol. Cocoa extract (100 mg) was dissolved in 100 mL of methanol and diluted to obtain final concentrations of 50, 100, 150, 200, and 250 ppm. Each sample (2 mL) was mixed with 2 mL of DPPH solution, vortexed for 30 s, and incubated at room temperature for 30 min in the dark. Absorbance was measured at 517 nm using a UV-Vis spectrophotometer. The percentage inhibition was calculated as follows:

$$(\%)\ \text{Inhibition} = \frac{Absorbance\ of\ blank - Absorbance\ of\ sample}{Absorbance\ of\ blank} \times 100\%$$

2.3 Sample Preparation and Volatile Compound Analysis by Gas Chromatography-Mass Spectrometry (GC-MS)

Sample preparation and volatile compound analysis were conducted according to Yang et al. (2022). Roasted cocoa beans (50) were ground and sieved through a 20-mesh sieve. A total of 12 g of cocoa powder was mixed with 100 mL of methanol p.a., macerated in a rotary shaker at 150 rpm for 24 h at 25°C in the dark, and filtered through Whatman No. 41 paper. The supernatant was concentrated using a rotary evaporator at 40°C for 20 min and further dehydrated using a speed vacuum. Extracts were stored at 4°C in the dark for subsequent GC-MS analysis.

Volatile compound analysis was performed using an Agilent Technologies GC-MS system (6890 GC-5973 MS). The cocoa extract (10 mg/mL in methanol (Supelco, 1.06012)) was directly injected into the GC-MS vial. The chromatographic conditions were as follows: injector temperature, 250°C (Splitless mode); pressure, 68.0 kPa; column flow rate, 0.8 mL/min; column type, SH-Rxi-5Sil MS Cap. column (30 m × 0.25 mm × 0.25 μm). The column temperature was programmed as follows: initial temperature, 100°C; ramped to 180°C (hold time, 2 min); ramped to 250°C (hold time, 5 min); and finally increased to 300°C (hold time, 10 min). Chromatogram data were analyzed using the National Institute of Standards and Technology (NIST) Library.

2.4 Scanning Electron Microscopy (SEM)

SEM analysis was conducted following Bekatorou et al. (2001) to confirm yeast and LAB adherence to gluten pellet surfaces. Gluten pellet samples containing *P. kudriavzevii* UNJCC Y-137, *L. plantarum* NP-57, and a control sample (without microbial addition) were dried in an electric oven at 50°C for 24 h. The dried samples were gold-coated (10 nm thickness) using a LuxorAu machine for 3 min to enhance electron conductivity. SEM imaging was performed using a Thermo Scientific Prisma E microscope at 2500× magnification for *P. kudriavzevii* samples and a Phenom ProX Desktop SEM at 5000× magnification for *L. plantarum* samples. SEM imaging was conducted by directing an electron beam onto the sample surface and detecting the reflected electrons to generate high-resolution images.

2.5 Statistical Analyses

This research encompasses a combination of both qualitative and quantitative data. The qualitative aspect involves analyzing volatile compounds through Gas Chromatography Mass Spectrometry (GCMS) and conducting morphological assessments of gluten pellet samples via Scanning Electron Microscopy (SEM). For the quantitative data, which includes viability tests of yeast cells and lactic acid bacteria (LAB) immobilized with gluten pellets, one-way ANOVA followed by Tukey's HSD test ($\alpha = 0.05$) was performed using IBM SPSS Statistics 25.0. Additionally, the physical and chemical characterization of cocoa beans, including the cut test, pH assessment, and antioxidant evaluation, was carried out using one-way ANOVA at the 5% level of significance.

3 Results and Discussion

3.1 Cultivation of Yeast *P. kudriavzevii* UNJCC Y-137.

The yeast isolate *P. kudriavzevii* UNJCC Y-137 was cultured using YMA to reactivate the isolate previously stored in a dormant state at 4°C. Macroscopic and microscopic morphological observations of the yeast are presented in Figure 1. After incubation at 35°C for 48 h, *P. kudriavzevii* UNJCC Y-137 formed round colonies with a butyrous texture, convex elevation, smooth edges, and a cream-white color. Microscopic examination revealed oval-shaped cells exhibiting monopolar budding. These observations are consistent with the findings of Wulan et al. (2021), who reported that *P. kudriavzevii* isolates exhibit ovoid cell morphology (5–8 µm in size) and reproduce asexually via monopolar budding. Furthermore, the colonies were characterized as round, cream-white, smooth-edged, and convex.

YMA is a widely used medium for yeast cultivation and isolation, including *P. kudriavzevii*. The medium comprises yeast extract, malt extract, peptone, glucose, and bacteriological agar. Glucose and malt extract serve as carbon sources, whereas yeast extract and peptone supply nitrogen essential for yeast growth (Hahn-Hägerdal et al., 2005; Wulan et al., 2021). The pH of YMA medium typically ranges from 4.5 to 6.5, with optimal yeast growth occurring at pH 3.0–6.0 (Membré et al., 1999; Narendranath & Power, 2005). These findings align with the study by Chu et al. (2023), which demonstrated that *P. kudriavzevii* exhibits acid tolerance and efficiently ferments ethanol at low pH, particularly at pH 3.0.

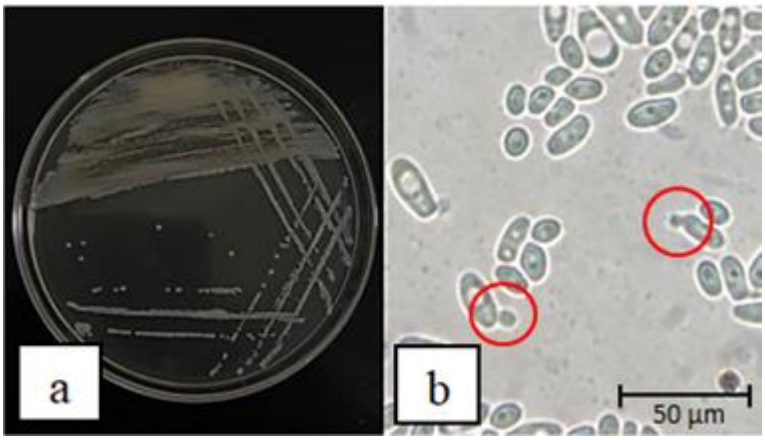

Fig. 1 Morphological characteristics of *P. kudriavzevii* UNJCC Y-137: (a) macroscopic and (b) microscopic views. The red circle indicates budding yeast cells

3.2 Cultivation of LAB *L. plantarum* NP-57

The lactic acid bacterium (LAB) *L. plantarum* NP-57 was cultivated using MRSA medium to reactivate the isolate previously stored at 4°C. Macroscopic and microscopic morphological observations are shown in Figure 2. After incubation at 37°C for 24 h, *L. plantarum* NP-57 formed round colonies with convex elevation, a milky-white color, and smooth edges. Microscopic analysis indicated that the cells were rod-shaped and arranged in short chains. These findings correspond with those of Qian et al. (2018) and University of California Davis (2025), who described *L. plantarum* colonies as small (2–3 mm in diameter), milky-white, convex, and smooth-edged. Microscopically, *L. plantarum* cells are Gram-positive, rod-shaped (1.5–10 µm in length), and arranged in short chains.

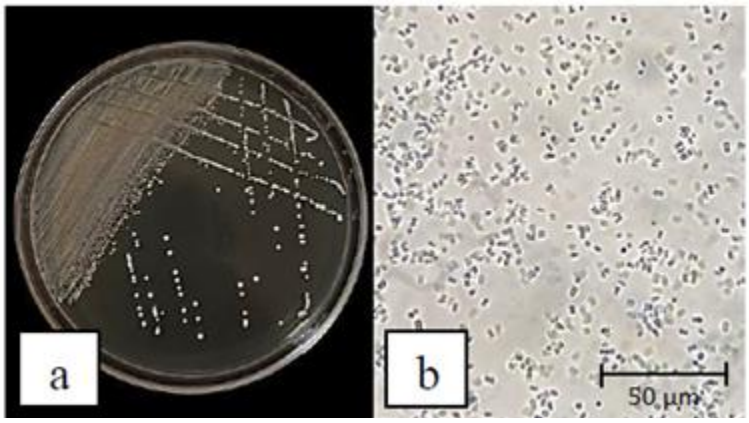

Fig. 2 Morphological characteristics of *L. plantarum* NP-57: (a) macroscopic and (b) microscopic views

3.3 Viability of Yeast and LAB Cells Immobilized on Gluten Pellets

A viability test was conducted to assess the survival of yeast and LAB cells immobilized on gluten pellets following treatments and to determine the cell density required as a fermentation starter. The viability results for yeast and LAB cells immobilized on gluten pellets are summarized in Table 1.

Table 1. Viability of *P. kudriavzevii* UNJCC Y-137 cells immobilized on gluten pellets.

Immersion Time (h)	*P. kudriavzevii* UNJCC Y-137 **(CFU/mL ± SE)**	*L. plantarum* NP-57 **(CFU/mL ± SE)**
3	$3.70 \times 10^8 \pm 0.26$a	$1.28 \times 10^9 \pm 0.05$a
6	$4.53 \times 10^8 \pm 0.34$a	$1.86 \times 10^9 \pm 0.04$b
9	$6.67 \times 10^8 \pm 0.47$b	$2.52 \times 10^9 \pm 0.13$c

The immersion time in glucose medium influenced yeast viability (Table 1). The highest viability (6.67×10^8 CFU/mL) was observed after 9 h of immersion, whereas shorter immersion times yielded lower viability. This suggests that prolonged immersion enhances nutrient absorption, thereby increasing viability.

Similarly, LAB viability increased with immersion time, reaching 2.52×10^9 CFU/mL after 9 h (Table 1). This indicates that per 10 g of gluten pellets, *P. kudriavzevii* and *L. plantarum* attain cell densities of 10^8 and 10^9 CFU/mL, respectively, which are suitable for cocoa fermentation. Dussap and Poughon (Dussap & Poughon, 2017) reported that yeast starters are typically inoculated at 10^6–10^8 CFU/mL to facilitate efficient alcohol fermentation, whereas LAB starters range from 10^6 to 10^{10} CFU/mL, significantly impacting pH and lactic acid production (Pereira et al., 2020).

The viability results indicate that prolonged immersion in glucose medium enhances microbial survival by providing essential nutrients. Arbianti et al. (2013) suggested that microbial colony expansion is influenced by environmental conditions, particularly nutrient availability. The glucose medium comprises glucose, $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, and yeast extract. Glucose acts as a carbon source, whereas yeast extract provides proteins, amino acids, and vitamins (Kligler, 1918; Zarei et al., 2016). KH_2PO_4 supplies phosphate for microbial growth, and $MgSO_4$ serves as a mineral source. Additionally, ammonium sulfate ($(NH_4)_2SO_4$) provides nitrogen, further supporting microbial proliferation.

3.4 Characterization of Yeast and LAB Cells on Gluten Pellets via SEM Analysis

The presence of yeast and lactic acid bacteria (LAB) cells on gluten pellets was confirmed through SEM analysis (Wilcox, 1993; Woo et al., 2023).

SEM imaging confirmed the successful attachment of yeast cells (Figure 3) and LAB cells (Figure 4) to the gluten pellet matrix (Nowosad et al., 2021).

The binding of microbial cells to gluten pellets is substantially influenced by the physicochemical attributes of gluten and the biological traits of the bacterium. Electrostatic forces, hydrophilic contacts, and hydrophobic connections are crucial in this adhesion process. The composition of gluten, especially its proteins and polysaccharides, is essential for supporting these interactions. Nawrocka et al. (2017) investigated the influence of dietary fibre polysaccharides on the structural and thermal characteristics of gluten proteins. Their studies demonstrated that the presence of polysaccharides modifies the secondary structure of gluten, thus impacting its interaction with microbial cells. In addition, Wieser (2007) published an extensive investigation of gluten proteins, stressing their significance in defining the specific baking quality of wheat through the provision of water absorption capacity, cohesiveness, viscosity, and elasticity to dough. The interaction between proteins and water molecules is marked considerably by the presence of functional groups, including amine (-NH_2) and carboxyl(-COOH), on the protein surface. These groups enhance electrostatic interactions and hydrogen bonding with water molecules and charged cellular surfaces, greatly influencing protein solubility and interactions (Wohlfahrt, 2005). Polysaccharides such as arabinoxylan and β-glucan contain hydroxyl (-OH) groups that interact with microbial cells, contributing to viscosity and stability. The hydrophilic segment of gliadin interacts with water molecules or hydrophilic cell surfaces, whereas the hydrophobic domain of glutenin interacts with hydrophobic cell membrane components, such as lipids, further enhancing microbial adhesion (Xu et al., 2024). These findings collaboratively highlight the crucial role of gluten's physicochemical characteristics and composition in microbial adhesion mechanisms.

Yeast and LAB cells generally possess negatively charged surfaces due to the presence of phosphate or carboxyl groups on their outer membranes (Ahimou et al., 2002; Bergey & Boone, 2009). This creates electrostatic interactions between the negatively charged microbial surfaces (-) and the positively charged gluten matrix (+), which contains amino groups in its protein structure. Such electrostatic attraction facilitates microbial adhesion and adsorption onto gluten pellets. This adhesion mechanism enhances cell retention on gluten surfaces, improving the efficiency of biotechnological processes involving immobilized cells (Moreno-García et al., 2018).

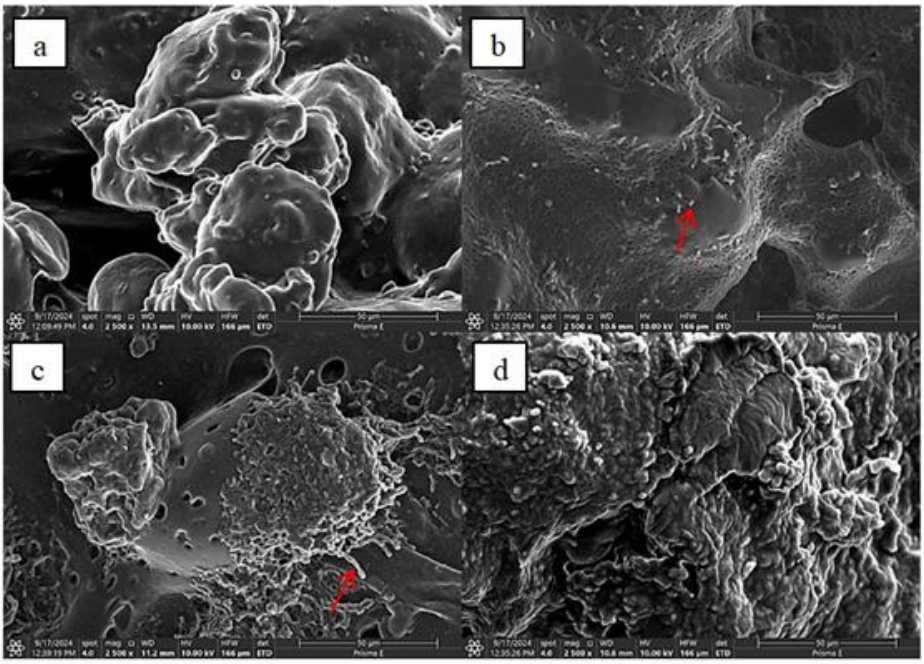

Fig. 3 SEM images of *P. kudriavzevii* UNJCC Y-137 on gluten pellets (magnification: 2,500×). (a) Control, (b) 3-h immersion, (c) 6-h immersion, and (d) 9-h immersion

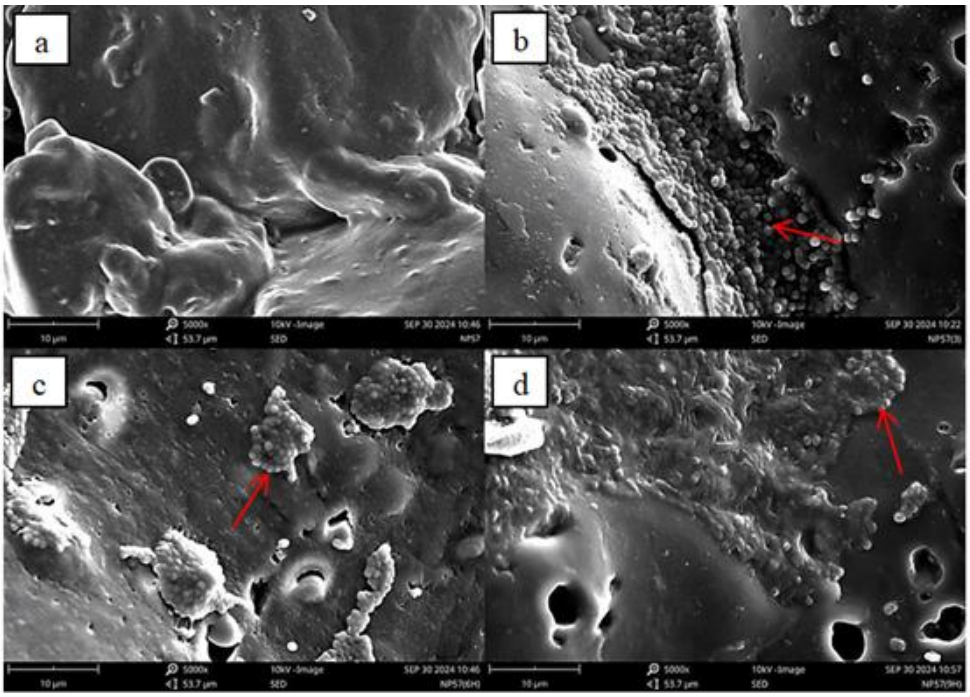

Fig. 4 SEM images of L. plantarum NP-57 on gluten pellets (magnification: 5,000×). (a) Control, (b) 3-h immersion, (c) 6-h immersion, and (d) 9-h immersion

3.5 Physical and Chemical Characteristics of Fermented Cocoa Beans

Cut test

The cut test is a physical assessment conducted after fermentation to evaluate the success of the fermentation process based on the color of cocoa bean nibs. Variations in microbial addition and fermentation duration influence the color of cocoa beans (Table 2). The highest percentage of fully fermented cocoa beans was observed in the *L. plantarum* LAB treatment, reaching 86.33%, compared to the control treatment, which had a lower percentage of fully fermented beans at 71.00%. This result is attributed to the formation of melanoidins or dark-colored compounds in the cocoa beans due to *L. plantarum* activity during fermentation. A good cut test result is approximately 3.00, where beans typically exhibit a purple-brown appearance with a dominant brown color. Cocoa beans are considered acceptable if more than 50% are fully fermented with a dominant brown color (Guehi et al., 2010; Oliveira et al., 2021).

Table 2. Cut test results for fully fermented cocoa beans (%) from different microbial treatments and fermentation times.

Microbes	**Fermentation time**			
	0 h	**72 h**	**96 h**	**120 h**
Control	23.33 ± 4.06^{ab}	48.67 ± 6.36^{c}	66.67 ± 1.76^{ef}	71.00 ± 1.15^{efg}
P. kudriavzevii UNJCC Y-137	24.67 ± 3.53^{ab}	57.33 ± 1.33^{d}	75.33 ± 2.85^{fgh}	84.67 ± 1.76^{ij}
L. plantarum NP-57	21.33 ± 2.40^{a}	62.67 ± 0.67^{de}	76.67 ± 1.33^{ghi}	86.33 ± 0.88^{j}
Combination	30.33 ± 2.33^{b}	72.67 ± 4.37^{fg}	85.67 ± 2.73^{j}	81.67 ± 1.76^{hij}

Values followed by the same lowercase letters are not significantly different at the 5% significance level ($p > 0.05$).

All treatments, including the control, *P. kudriavzevii* yeast, and *L. plantarum* LAB, exhibited an increase in color change over the 120-h fermentation period. However, the combination treatment showed a decrease after 120 h of fermentation. Initially slaty (purple-black), the cocoa beans progressively turned brown due to microbial activity, particularly from *P. kudriavzevii* UNJCC Y-137 and *L. plantarum* NP-57. P. kudriavzevii is known to produce organic

acids and ethanol under oxygen-limited conditions, which facilitates ethanol diffusion into the beans, leading to cellular death and activation of endogenous enzymes, such as polyphenol oxidase (Sukmawati et al., 2021; Meryandini et al., 2023). *Lactobacillus plantarum* is known to metabolize glucose and citric acid into lactic acid, contributing significantly to various fermentation processes. This metabolic activity not only enhances the flavor profile of fermented products but also improves their preservation qualities (Kennes et al., 1991a), which lowers the pH of cocoa beans and affects internal chemical reactions. This acidification, driven by lactic acid bacteria (LAB), facilitates the Maillard reaction, a chemical interaction between amino acids and reducing sugars that produces melanoidins or dark-colored compounds, contributing to the observed color change (Chang et al., 2024; Sahin et al., 2024).

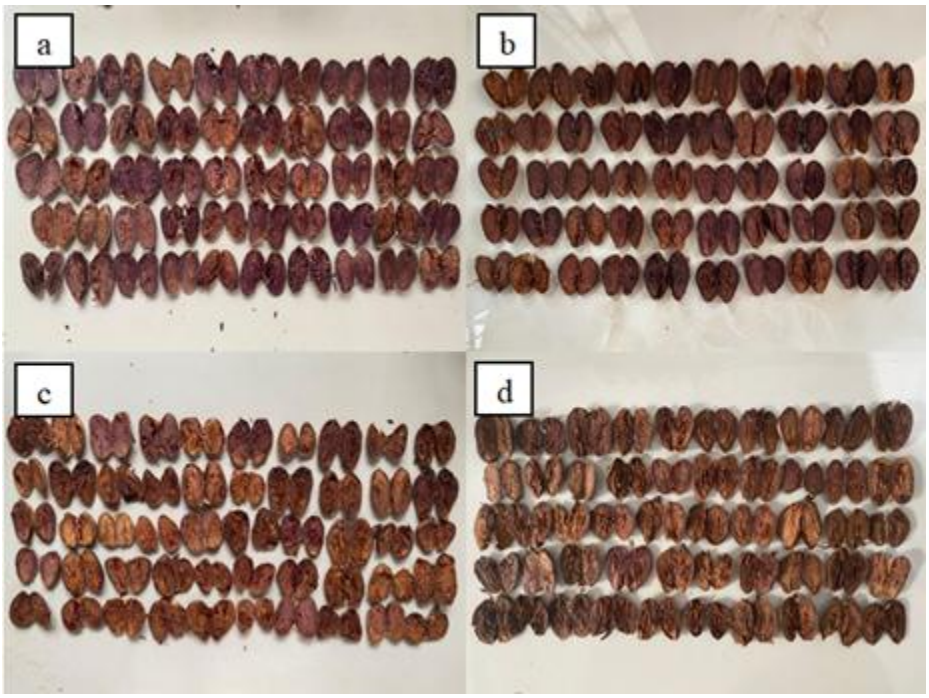

Fig. 5 Cut test results of cocoa beans after fermentation. (a) Control 0 h, (b) Yeast 120 h, (c) LAB 120 h, and (d) Combination 96 h

The decrease in fully fermented cocoa beans observed in the combination treatment after 120 h was likely due to mold growth, caused by an imbalance in lactic acid and ethanol production, which altered environmental acidity (Rahayu et al., 2021; Subroto et al., 2023). Evelyne et al. (2018) also reported that excessive lactic acid production can create overly acidic conditions, impeding optimal fermentation and promoting mold growth. Therefore, the best percentage of fully fermented cocoa beans in the combination treatment was achieved at 96 h (85.67%), demonstrating a significant improvement compared to the control.

Antioxidant Activity

Antioxidants inhibit oxidation-related deterioration, such as rancidity or off-flavors. Microbial variation and fermentation duration influenced the antioxidant activity of cocoa beans. As shown in Table 3, the combination treatment yielded the highest antioxidant activity (86.04 ± 0.16%) compared to the control (71.07 ± 0.00%). This enhancement is attributed to the synergistic interaction between *P. kudriavzevii* and *L. plantarum*, which produce lactic acid and maintain an acidic fermentation environment. Lower pH correlates with increased antioxidant activity. An antioxidant is considered effective if its inhibition percentage exceeds 50% (Molyneux, 2004; Mardani-Ghahfarokhi & Farhoosh, 2020).

Antioxidant activity in the control, *P. kudriavzevii*, and *L. plantarum* treatments increased up to 72 h of fermentation, then declined from 96 to 120 h, indicating a peak at 72 h. The addition of *P. kudriavzevii* enhanced antioxidant

properties, reaching 83.17 ± 0.00% at 72 h. *P. kudriavzevii* is known to produce organic acids, such as lactic acid, which enhance antioxidant activity (Savadogo et al., 2006; Zhao et al., 2021; Wang et al., 2023). Wang et al. (2023) identified an acid-tolerant strain of *P. kudriavzevii* (strain Y2) that can tolerate higher levels of acetic acid. The study underpinned the yeast's metabolic pathways associated with acid tolerance and production, indicating its potential for organic acid synthesis.

Table 3. Antioxidant activity of cocoa beans (%) under different microbial treatments and fermentation durations.

Microbes	**Fermentation time**			
	0 h	**72 h**	**96 h**	**120 h**
Control	74.25 ± 0.09^{f}	77.26 ± 0.09^{h}	73.22 ± 0.09^{e}	71.07 ± 0.00b
P. kudriavzevii UNJCC Y-137	71.95 ± 0.00^{c}	83.17 ± 0.00^{k}	79.50 ± 0.00^{j}	78.78 ± 0.10^{i}
L. plantarum NP-57	69.64 ± 0.09^{a}	78.74 ± 0.15^{i}	75.07 ± 0.00^{g}	72.35 ± 0.09^{d}
Combination	77.40 ± 0.07^{h}	79.73 ± 0.07^{j}	85.47 ± 0.00l	86.04 ± 0.16^{m}

Values followed by the same lowercase letters are not significantly different at the 5% significance level ($p > 0.05$).

The decline in antioxidant activity after 72 h is likely due to microbial metabolism shifts from anaerobic to aerobic conditions (Dinh et al., 2022). However, in the combination treatment, antioxidant activity continued increasing up to 120 h, reaching 86.04 ± 0.16%, significantly higher than the control. This improvement is attributed to the synergistic effects of *P. kudriavzevii* and *L. plantarum*, which stabilize antioxidant compounds. Adesulu-Dahunsi et al. (2020) also reported similar findings in fermented Nigerian foods and beverages, highlighting the effectiveness of this microbial combination in enhancing antioxidant stability.

pH

The pH measurement aimed to determine the acidity level resulting from the breakdown of organic acids produced during fermentation. Variations in microbial inoculation and fermentation duration influenced the pH value of cocoa beans (Table 4). The pH value of cocoa beans in the *L. plantarum* treatment was significantly ($p < 0.05$) lower (4.94 ± 0.03) compared to that in the *P. kudriavzevii* yeast treatment (5.93 ± 0.03). This reduction in pH is attributed to the production of lactic acid by *L. plantarum*, which diffuses into the cotyledons of cocoa beans. Torres-Moreno et al. (2021) reported that the final pH value of cocoa beans is crucial for optimal flavor development. Cocoa beans with a final pH between 4.75 and 5.19 are considered well-fermented, enhancing the formation of volatile compounds and aroma precursors.

Table 4. pH values of cocoa beans under different microbial treatments and fermentation durations.

Microbes	**Fermentation time**			
	0 h	**72 h**	**96 h**	**120 h**
Control	5.87 ± 0.05^{c}	5.72 ± 0.03^{c}	5.89 ± 0.08^{c}	5.92 ± 0.03^{c}
P. kudriavzevii UNJCC Y-137	5.91 ± 0.11^{c}	5.72 ± 0.15^{c}	5.84 ± 0.03^{c}	5.93 ± 0.03^{c}
L. plantarum NP-57	5.72 ± 0.03^{c}	4.94 ± 0.03^{a}	5.18 ± 0.08^{b}	5.21 ± 0.03^{b}
Combination	5.75 ± 0.03^{c}	5.21 ± 0.03^{b}	5.15 ± 0.05^{b}	5.09 ± 0.03^{ab}

Values followed by the same lowercase letters are not significantly different at the 5% significance level ($p > 0.05$).

The pH value decreased up to 72 h of fermentation in the control, *P. kudriavzevii*, and *L. plantarum* treatments before increasing between 96 and 120 h. This decrease is attributed to the production of organic acids, including lactic acid, through glucose metabolism by *P. kudriavzevii*, where ethanol is converted into lactic acid via the PDC1 (pyruvate decarboxylase 1) and d-LDH (d-lactate dehydrogenase) gene pathway. The diffusion of lactic acid generated by L. plantarum into the cocoa cotyledons resulted in a decrease in pH, as also demonstrated by Ho et al. (2015), who showed that the pH decline was due to lactic acid permeation into cocoa beans during fermentation (Ho et al., 2018; Lestari et al., 2020; Nurhayati & Apriyanto, 2021; Korcari et al., 2023). In the individual treatments (control, *P. kudriavzevii,* and *L. plantarum*), the pH increased between 96 and 120 h of fermentation. This increase is linked to a decline in *L. plantarum* activity and the role of acetic acid bacteria (AAB) in metabolizing sugars into acetic acid and CO_2, leading to a decrease in organic acid content and a subsequent rise in pH (Mamlouk & Gullo, 2013). In the combined treatment, the pH persisted in its decrease until 120 h, presumably attributable to the prolonged action of *L. plantarum*. The greater concentration of *L. plantarum* (10^9 CFU/mL) relative to *P. kudriavzevii* (10^8 CFU/mL) may have additionally affected this reduction. Melia et al. (2024) reported that higher *L. plantarum* concentrations could lower pH to below 5 under specific conditions (Qiao et al., 2018; Ntsamo et al., 2020). These findings emphasize the significance of microbial interactions and population equilibrium in influencing the pH profile and overall advancement of cocoa bean fermentation.

3.6 Analysis of Volatile Compounds in Fermented Cocoa Beans

Volatile compound analysis was conducted following fermentation and roasting. The aroma and taste of cocoa are influenced by microbial activity, which produces reducing sugars such as glucose and fructose through cotyledon invertase action and releases free amino acids via carboxypeptidase and aspartate protease activity. During roasting, these precursors participate in the Maillard reaction, forming various aroma compounds (Purbaningrum et al., 2023; Quelal et al., 2023).

GC-MS analysis (Table 5) identified 17 volatile compounds, including acids, alcohols, aldehydes, amines, alkaloids, ketones, and esters. Quelal et al. (2023) reported that esters, acids, alcohols, aldehydes, and ketones are commonly found in roasted cocoa beans and are influenced by roasting parameters and cocoa variety.

Table 5. Volatile compounds identified in fermented and roasted cocoa beans under different microbial treatments. A1 = Control (no inoculation); A2 = *P. kudriavzevii* UNJCC Y-137; A3 = *L. plantarum* NP-57; A4 = Combination of P. kudriavzevii and L. plantarum (1:1).

Compound	**CAS**	**Area (%)**				**Odor Description**
		A1	**A2**	**A3**	**A4**	
Acid:						
Acetic acid	64-19-7	-	-	28.54	14.96	Sour, vinegar (Ooi et al., 2021)
Total area percentage of acid compounds (%)	Total area percentage of acid compounds (%)	-	-	28.54	14.96	
Alcohols:						
2-Butanol, 3-methyl-	598-75-4	-	-	-	40.78	Malty, bitter, chocolat (Chóez-Guaranda et al., 2024)
2,3-Butanediol	513-85-9	-	2.4	-	11.16	Fruity, creamy, buttery

Compound	CAS	Area (%)				Odor Description
		A1	A2	A3	A4	
						(Nurhayati et al., 2019)
Erythritol	149-32-6	-	6.72	-	-	-
Glycerin	56-81-5	-	-	12.08	4.82	-
Total area percentage of alcohols (%)	Total area percentage of alcohols (%)	-	9.12	12.08	56.76	
Aldehydes:						
Butanal	123-72-8	-	-	-	0.82	Cocoa-like (Liu et al., 2022)
Butanal, 3-methyl-	590-86-3	-	-	-	8.54	Cocoa-like (Liu et al., 2022)
Pentanal	110-62-3	-	-	-	8.54	Almond, malt (Ooi et al., 2021)
Propanal, 3-methoxy-	2806-84-0	-	2.4	-	-	-
Total area percentage of aldehydes (%)	Total area percentage of aldehydes (%)	-	2.4	-	17.9	
Amines:						
2-Isopropoxy-ethylamine	81731-43-3	-	2.4	-	-	Mild, ethereal (NCBI, 2025)
Dimethylamine	124-40-3	-	2.54	-	-	Fishy (NCBI, 2025)
N.2-dimethylpropan-1-amine	625-43-4	-	-	8.65	-	Fishy (NCBI, 2025)
Total area percentage of amines (%)	Total area percentage of amines (%)	-	4.94	8.65	-	
Alkaloid:						
Caffeine	58-08-2	-	67.6	-	-	Bitter (Ooi et al., 2021)
Total area percentage of alkaloids (%)	Total area percentage of alkaloids (%)	-	67.6	-	-	
Ester:						
11-Aminolauric acid methyl ester	56817-92-6	-	5.22	-	-	-
Total area percentage of ester (%)	Total area percentage of ester (%)	-	5.22	-	-	
Ketones:						
1,2-Cyclopentanedione	3008-40-0	-	-	-	3.76	Maple, caramel-like (Wang et al., 2021)
2-hydroxy-2-cyclopenten-1-one	10493-98-8	-	-	-	3.76	Maple, caramel-like (Wang et al., 2021)

Compound	CAS	Area (%)				Odor Description
		A1	A2	A3	A4	
4H-Pyran-4-one, 2,3-dihydro-3,5-	28564-83-2	-	-	-	0.89	-
Total area percentage of ketones (%)	Total area percentage of ketones (%)	-	-	-	8.41	

The formation of various volatile compounds, such as alcohols, aldehydes, acids, and esters, occurs via the amino acid degradation pathway. The deamination and transaminase processes convert amino acids produced by *P. kudriavzevii* and *L. plantarum* into precursor compounds, such as α-keto acids, which undergo various reactions, including oxidation to produce organic acids, reduction to produce alcohols, dehydration to produce aldehydes, and esterification to produce esters. Amine compounds are formed through the amino acid decarboxylation pathway, facilitated by decarboxylase enzymes that remove the -COOH group. Ketone compounds are generated via oxidative deamination, leading to the formation of α-keto acids, which then undergo decarboxylation to produce ketones. Meanwhile, alkaloid compounds are formed through amino acid decarboxylation, which serves as the initial step in alkaloid biosynthesis. These compounds can subsequently undergo methylation, cyclization, oxidation, or reduction to form various alkaloid derivatives (Schwab et al., 2008).

The results of the volatile compound analysis indicated that microbial treatment and fermentation duration influenced the volatile compound profile of cocoa beans. The greatest variation in volatile compounds was observed in treatment A4, which involved the combined inoculation of *P. kudriavzevii* and *L. plantarum*, producing 10 volatile compounds. Treatment A2, with *P. kudriavzevii* alone, yielded seven volatile compounds, while treatment A3, with *L. plantarum* alone, resulted in the formation of three volatile compounds. In contrast, no volatile compounds were detected above the GC-MS detection threshold in treatment A1 (control), where no microbial inoculation was applied.

The absence of volatile compounds in treatment A1, which involved unfermented cocoa beans, suggests that fermentation is a critical step in cocoa processing. Without fermentation, aroma precursors fail to develop, preventing the subsequent formation of volatile compounds (Castro-Alayo et al., 2019). This finding aligns with research by Britto de Andrade et al. (2021), which states that fermentation is essential for microbial metabolism, facilitating the biochemical reactions necessary for flavor development, including the Maillard reaction.

In treatment A2, caffeine was identified as the dominant compound, with a peak area of 67.60%. Caffeine and theobromine are alkaloid compounds commonly found in cocoa bean husks (Jokić et al., 2018; Djali et al., 2023). Cocoa bean shells contain significant amounts of methylxanthines, with theobromine concentrations ranging from 4.64 to 10.92 mg/g and caffeine levels between 1.59 and 4.21 mg/g, depending on the origin of the cocoa (Jokić et al., 2018). The researchers highlighted that during fermentation, methylxanthines migrate from the bean into the shell, contributing to these concentrations. While cocoa beans contain various saturated and unsaturated fatty acids, this particular compound is not commonly reported in cocoa.

In treatment A3, acetic acid was the dominant volatile compound (28.54%), contributing to a sour, vinegar-like aroma (Campos et al., 2025). The second most abundant compound, glycerin (12.08%), is a sugar alcohol frequently detected in fermented foods and beverages, such as beer, honey, vinegar, and wine (Zhao et al., 2015). The third most abundant compound, N,2-dimethylpropan-1-amine (8.65%), is not typically found in cocoa beans. N,2-dimethylpropan-1-amine is a volatile aliphatic amine distinguished by a powerful, fishy odour, characteristic of low-molecular-weight amines. Although specific investigations on the antibacterial action of this molecule are scarce, related research on structurally related aliphatic amines provide corroborative evidence (Serafim et al., 2019).

In treatment A4, the dominant volatile compound was 2-butanol, 3-methyl (40.78%). This compound is formed through amino acid metabolism during fermentation, involving decarboxylation and reduction, and contributes to a characteristic bitter chocolate aroma (Schwab et al., 2008; Chóez-Guaranda et al., 2024). The second most abundant compound, acetic acid (14.96%), enhances the fermentation quality and flavor profile of cocoa (Ooi et al., 2021). However, excessive acetic acid may impart an undesirable sour taste, reducing cocoa quality. Therefore, balancing

acetic acid levels is crucial for optimal fermentation outcomes (Rodriguez-Campos et al., 2011). The third most abundant compound, 2,3-butanediol (11.16%), contributes to chocolate aroma by imparting fruity, smooth, and buttery notes Nur (Nurhayati et al., 2019).

As presented in Table 5, microbial inoculation influenced the composition and quantity of volatile compounds in cocoa beans. *P. kudriavzevii* and *L. plantarum* exhibited distinct metabolic pathways, leading to varied volatile compound production. The combined inoculation of both microbes resulted in the highest number of volatile compounds, suggesting a synergistic effect on flavor development. Moreira et al. (2016) reported that microbial diversity significantly affects the sensory attributes of chocolate, highlighting the importance of fermentation in cocoa processing. The findings from this research have the potential to elevate the quality of cocoa fermentation in regions where traditional methods are inconsistent or underdeveloped. This work could contribute to producing higher-quality cocoa with improved flavor and aroma meeting the demands of the global chocolate industry and enhancing competitiveness in the international cocoa market.

4 Conclusions

The quality of cocoa beans after fermentation improved with the addition of immobilised *P. kudriavzevii* UNJCC Y-137 and *L. plantarum* NP-57 on gluten pellets, and fermentation time influenced both the physical (cut test) and chemical (pH and antioxidant activity) properties of the fermented cocoa beans. The combination treatment with 120-min immersion achieved the highest fermentation percentage (85.67 ± 2.73%) and antioxidant activity (86.04 ± 0.16%). Furthermore, the addition of immobilised starter cultures and extended fermentation time facilitated the formation of various volatile compound groups, including organic acids, alcohols, aldehydes, amines, alkaloids, esters, and ketones, whereas the control treatment did not yield detectable volatile compounds above the GC-MS detection threshold under the analytical conditions employed.

These findings suggest that gluten pellets represent a viable and practical immobilisation matrix for starter cultures in cocoa fermentation, offering potential advantages for standardising fermentation quality in smallholder settings. However, the present study was conducted at laboratory scale, and further investigation is needed to evaluate the scalability of this approach under field conditions. Future research should also assess the sensory quality of chocolate produced from cocoa beans fermented with immobilised starters, as well as the reusability and shelf life of the gluten pellet carriers.

Statements and Declarations

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Role of Funders

The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Competing Interests

The authors declare that they have no competing financial or non-financial interests related to this work.

Author Contributions

Conceptualization: D.S.; methodology: M.A.A. and R.K.A.; data curation: M.I. and R.M.; manuscript original draft preparation: D.S. and S.C.D.A.; investigation and formal analysis: R.K.A., L.H.H., and T.H.; supervision: C.S., S., and F.R.D.; writing, review, and editing: F.R.D., D.S., M.H.A., M.A.A., P.A.S.N., and S.A. All authors read and approved the manuscript.

Data Availability

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval

Not applicable.

Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

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