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Effect of Fermentation *Pichia kudravzevii* UNJCC Y-77 and *Pichia manshurica* UNJCC Y-123 on the Physicochemical Characteristics of Cocoa Beans (*Theobroma cacao* L.)

Dalia Sukmawati [1,*], Salsabila Fauzi Mahfuzh [2], Sri Rahayu [2], R. Haryo Bimo Setiarto [3], Hesham El Enshasy [4], Karina [2], Roro Anggraini [2], Vail Şihada [5]

1 Biology Department, Faculty of Mathematics and Natural Sciences, 5th floor, Hasyim Ashari Building, Universitas Negeri Jakarta, Jl. Rawamangun Muka, Jakarta Timur, 13220, Indonesia; Dalia-Sukmawati@unj.ac.id;
2 Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Negeri Jakarta, Indonesia; salsabilamahfud@gmail.com (S.F.M.); srirahayu@unj.ac.id (S.R.); karina_1308622025@mhs.unj.ac.id (K.); roro.anggrain2321@gmail.com (R.A);
3 Research Center for Applied Microbiology, National Research and Innovation Agency, KST Soekarno, West Java 16911, Indonesia; rhar002@brin.go.id;
4 Institute of Bioproduct Development (IBD), University Technology Malaysia (UTM), Skudai, Johor Bahru 81310, Malaysia; henshasy@ibd.utm.my;
5 International Sciences and Technology University, Poland; vail.sihada@istu.edu.pl;
* Correspondence: Dalia-Sukmawati@unj.ac.id

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Abstract: Indonesia is one of the largest cocoa bean producers worldwide, though the quality of its dry cocoa beans remains relatively low, primarily due to insufficient fermentation processes. This study investigates the impact of P. kudriavzevii UNJCC Y-77, P. manshurica UNJCC Y-123, their consortium, and fermentation duration on the physical and chemical properties of cocoa beans, including temperature, pH, Fermentation Index, total polyphenols, and reducing sugars, as part of quality improvement efforts. Results demonstrate that yeast variations and fermentation time significantly impact cocoa bean characteristics. At 48 hours of fermentation, *P. kudriavzevii* UNJCC Y-77 reached the highest temperature with a value of 32.01 ± 0.50°C, while all three yeast treatments showed peak pH values with the values of 6.71 ± 0.02, 6.49 ± 0.04, and 6.81 ± 0.02. After 72 hours, all yeast variations achieved their highest fermentation indices with the values of 1.10 ± 0.06, 1.15 ± 0.05, and 1.14 ± 0.02. The consortium treatment at 72 hours produced the lowest levels of reducing sugars with a value of 3.55 ± 0.03 mg/g and total polyphenols with a value of 31.11 ± 0.91 mgGAE/g, indicating optimal fermentation conditions.

Keywords: cocoa beans; fermentation index; fermentation time; pH; total polyphenols.

1. Introduction

Cacao (*Theobroma cacao* L.) is an essential commodity for Indonesia's economy, contributing to foreign exchange, farmer income, and employment opportunities [1]. Despite being the fifth-largest cocoa producer globally, Indonesia faces quality issues in its cocoa

beans, such as high acidity, bitterness, impurities, and uneven sizes, often failing to meet national standards [2,3]. The low quality of cocoa beans results from suboptimal post-harvest processing. A significant issue in cocoa processing in Indonesia is the lack of fermentation. Most of the cocoa beans in Indonesia are unfermented, leading to low-quality dried non-fermented beans [4-6]. Fermentation is a crucial process that involves various microorganisms that metabolise compounds, improving the quality of the beans and developing the precursors for flavour, colour, and the characteristic chocolate aroma [7,8]. Traditionally, cocoa fermentation is spontaneous and relies on the natural microbiota present in the environment, including yeasts, lactic acid bacteria, and acetic acid bacteria [4,6]. However, the uncontrolled nature of spontaneous fermentation often leads to inconsistent quality in the final product [7,8].

Among the microorganisms involved in cocoa bean fermentation, yeasts are the most abundant. The dominant yeast genera involved in fermentation include *Pichia*, *Hanseniaspora*, *Saccharomyces*, and *Candida* [9]. Yeasts play various roles, such as breaking down citric acid in the pulp, increasing pH, producing ethanol, generating organic acids, and creating volatile organic compounds contributing to chocolate flavour precursors [10,11]. Yeasts can adapt to diverse environments and inhibit spoilage microorganisms [12,13]. Pectinase-producing yeasts also help degrade the pectin-rich pulp, improving fermentation efficiency and flavour development [14,15]. Several studies have shown that using yeast starter cultures in cocoa bean fermentation can enhance flavour and aroma complexity [16,17]. Recent studies have focused on the use of selected yeast strains to improve fermentation outcomes and standardize cocoa bean quality. *Pichia kudriavzevii* and *Pichia manshurica* are non-Saccharomyces yeast species that have demonstrated promising metabolic traits in various fermentation systems. Their ability to tolerate stress conditions, produce aroma compounds, and modify the physicochemical environment suggests they could be effective starter cultures in cocoa fermentation [16,17].

Fermented cocoa beans are known to be of better quality than non-fermented beans, which are often sour and astringent. Fermentation initiates a series of biochemical reactions inside the cocoa beans [15]. Enzymes break down sugars and proteins, producing flavour precursors that are essential for the development of the characteristic chocolate flavour during roasting [16]. Non-fermented beans lack these complex flavour compounds, often resulting in a sour, bitter, or overly astringent taste. Unfermented beans retain high levels of acetic acid and polyphenols, which contribute to a harsh sourness and astringency [16,17]. Proper fermentation reduces these compounds, leading to a smoother, more balanced flavour. Fermentation also enhances the internal colour of the beans, turning them from a dull purple to a rich brown, which is more appealing and desirable for chocolate production [18]. The microbial ecosystem involved in fermentation (yeasts, lactic acid bacteria, and acetic acid bacteria) plays a vital role in breaking down the pulp and modifying the bean chemistry to improve flavour and aroma [16,18]. Properly fermented beans fetch higher prices on the international market due to their superior quality, making them more attractive to premium chocolate makers. The quality of dried non-fermented beans can be improved through fermentation, but optimisation is required for effective fermentation [18].

Since dried cocoa pulp contains fewer Indigenous microbes, adding starter cultures can optimise fermentation [19]. Using starter cultures allows for better-controlled fermentation and more consistent bean quality [20-22]. Apart from starter cultures, fermentation duration also affects cocoa bean quality. Previous studies indicated that cocoa fermentation typically takes 5-7 days, which discourages many farmers from fermenting their beans. Controlled

fermentation with added starter cultures can reduce fermentation time. This study will use fermentation durations ranging from 0 to 72 hours with the yeast species *Pichia kudriavzevii* and *Pichia manshurica*, both known to contribute to natural cocoa fermentation [23-25]. These yeasts have been reported as dominant ethanol producers and capable of producing pectinase enzymes, which can aid the cocoa fermentation process [26]. This study aims to determine the effects of *Pichia kudriavzevii* UNJCC Y-77, *Pichia manshurica* UNJCC Y-123, their combination, and fermentation duration on the physical and chemical characteristics of cocoa beans, including temperature, pH, fermentation index, total polyphenol content, and reducing sugar content during the fermentation process.

2. Materials and Methods

2.1. Preparation of P. kudriavzevii UNJCC Y-77 and P. manshurica UNJCC Y-123 yeast suspension.

The origin of the yeast strain *Pichia kudravzevii* UNJCC Y-77 was isolated from durian fruit (*Durio kutejensis*). Meanwhile, the yeast strain *Pichia manshurica* UNJCC Y-123 was isolated from Bali's palm wine. Yeast cell suspension preparation followed the method of [27]. The yeast was streaked 15 times on slant YPDA media and incubated at 28°C for 48 hours. Then, 10 mL of sterile distilled water was added to the test tube and homogenised using a vortex mixer. A 10% (v/v) yeast suspension was transferred into a 250 mL Erlenmeyer flask containing 135 mL of YPDB media (+15 mL suspension) and another with 67.5 mL (+7.5 mL suspension). The yeast was cultured at room temperature for 24 hours while shaking at 30 rpm. The desired cell density was approximately ~10^8 CFU/mL, determined using a spectrophotometer for Optical Density (OD) measurement.

2.2. Rehydration of dried cocoa beans.

The cocoa beans used in this study came from Musi Rawas, South Sumatra, Indonesia. Rehydration was conducted to increase the moisture content of the dried cocoa pulp, making it suitable for fermentation. This was done by soaking 1.5 kg of dried cocoa beans in 1.5 L of sterile distilled water for 2 hours [28].

2.3. Fermentation of dried cocoa beans.

Fermentation was conducted in a sterilised Styrofoam box, pre-cleaned with antiseptic soap and disinfected with 70% alcohol [29]. The rehydrated cocoa beans (1.5 kg) were inoculated with a 10% yeast suspension relative to the bean weight. The treatments included: 150 mL of *P. kudriavzevii* UNJCC Y-77 suspension; 150 mL of *P. manshurica* UNJCC Y-123 suspension; 75 mL each of both *P. kudriavzevii* and *P. manshurica* in a 1:1 ratio; Control (without yeast suspension). Both *Pichia kudriavzevii* UNJCC Y-77 and *Pichia manshurica* UNJCC Y-123 are aerobic yeasts when cultivated under standard laboratory or industrial conditions.

Table 1. Parameters of cocoa bean fermentation conditions by *Pichia kudriavzevii* UNJCCY-77 and *Pichia manshurica* UNJCCY-123.

Strain	Temperature	Medium	Agitation	Oxygen condition
Pichia kudriavzevii UNJCC Y-77	~30 °C	YPD (15–20% glucose/glycerol)	160–500 rpm shake	Aerobic
Pichia manshurica Y-123	25–30 °C	YPD (screening), silage inocula	~150–200 rpm shake	Aerobic

Cultivation is under aerobic conditions, often with high dissolved oxygen in well-agitated flasks or bioreactors (Table 1).

2.4. Fermentation temperature.

Fermentation temperature was measured following [30]. A digital thermometer was inserted into the fermentation box at 0, 24, 48, and 72 hours.

2.5. Fermentation pH.

pH was measured according to [30]. A 10g cocoa bean powder sample was mixed with 10 mL of distilled water in a glass beaker, and the pH was recorded using a pH meter.

2.6. Fermentation index.

The fermentation index was determined using a modified method by [31]. A 0.1 g cocoa powder sample was extracted with 10 mL of a methanol-concentrated HCl mixture (97:3 v/v). The mixture was homogenised for 20 seconds using a vortex mixer, stored in a refrigerator overnight, then filtered using Whatman No.1 filter paper. Absorbance was measured using a UV-Vis spectrophotometer at wavelengths of 460 nm and 530 nm, and the fermentation index was calculated using this formula.

$$FI = \frac{abs\ \lambda 460\ nm}{abs\ \lambda 530\ nm} \qquad (1)$$

2.7. Analysis of total polyphenol content of fermented cocoa beans.

The total polyphenol content was measured using the Folin-Ciocalteu method based on [32]. The process began with the preparation of a cocoa bean extract, where 0.5 g of cocoa powder was mixed with 10 mL of 70% acetone in a Falcon tube. This mixture was homogenised using a vortex mixer for 30 minutes and then filtered using filter paper into a test tube to obtain a clear extract.

Subsequently, 300 µL of the cocoa extract was combined with 4.15 mL of distilled water, 500 µL of 20% sodium carbonate (Na_2CO_3), and 50 µL of Folin-Ciocalteu reagent. This mixture was incubated at room temperature for 2 hours. After incubation, its absorbance was measured at 700 nm using a UV-Vis spectrophotometer. A blank solution, prepared with 300 µL of 70% acetone, 4.15 mL of distilled water, 500 µL of 20% Na_2CO_3, and 50 µL of Folin-Ciocalteu reagent, was used as a control.

2.8. Analysis of reducing sugar content of fermented cocoa beans.

The reducing sugar content was measured using the DNSA (3,5-Dinitrosalicylic Acid) method based on [33]. The process started with the preparation of a cocoa extract, where 0.2 g of cocoa powder was mixed with 5 mL of distilled water in a Falcon tube. The mixture was heated in boiling water for 30 minutes to ensure proper extraction. After heating, the mixture was filtered using filter paper, and the filtrate was collected for further analysis. For the reaction, 1 mL of the cocoa sugar extract was mixed with 3 mL of DNSA reagent in a test tube. The test tube was then placed in boiling water for 5 minutes to allow the reaction to occur, leading to the formation of a colored complex. After heating, the test tube was cooled in a water bath to stabilise the reaction mixture. To determine the reducing sugar concentration, a glucose standard curve was prepared by creating a series of glucose solutions with concentrations of 0

mg/mL, 0.5 mg/mL, 1 mg/mL, 1.5 mg/mL, 2 mg/mL, 2.5 mg/mL, and 5 mg/mL. Each glucose concentration was mixed with 3 mL of DNSA reagent, vortexed, heated in boiling water for 5 minutes, and then cooled. The absorbance of each standard solution was measured at 575 nm using a UV-Vis spectrophotometer. A linear calibration curve was generated by plotting the absorbance values against glucose concentrations, which was used to determine the reducing sugar content in the cocoa bean samples.

3. Results and Discussion

3.1. Temperature changes in cocoa bean fermentation.

Based on the results presented in Table 2, cocoa bean fermentation with the addition of different yeast variations caused an increase in temperature from the beginning until the 48th hour of fermentation, followed by a decrease in temperature at the 72nd hour. The treatment with *P. kudriavzevii* UNJCC Y-77 showed a significantly different temperature from all other treatments, reaching the highest temperature of 32.1±0.50°C at 48 hours of fermentation. The combination of yeast strains resulted in the highest temperature at the 72nd hour, reaching 30.60±0.10°C. These values were higher than the control treatment, which recorded temperatures of 28.99±0.05°C at 48 hours and 28.70±0.20°C at 72 hours of fermentation.

Table 2. Temperature test results during cocoa bean fermentation are influenced by yeast variation, treatment factors, and fermentation time.

Yeast variations		Fermentation time (°C ± SD)			
		0 hour (B1)	**24 hours (B2)**	**48 hours (B3)**	**72 hours (B4)**
P.kudriavzevii UNJCC Y-77	(A1)	27.45±0.25^{a}	29.90±0.10^{e}	**32.1±0,50^{h}**	29.10±0.20^{bc}
P.manshurica UNJCC Y-123	(A2)	27.59±0.47^{a}	29.40±0.20^{cd}	31.35±0.35^{g}	29.37±0.07^{cd}
Combination	(A3)	27.82±0.25^{a}	29.60±0.10^{de}	31.62±0.63^{g}	30.60±0.10^{f}
Control	(A4)	27.55±0.05^{a}	29.05±0.40^{bc}	28.99±0.05^{bc}	28.70±0.20^{b}

The numerical values are the mean ± SD, tested with two-way ANOVA at a significance level of α = 0.05; Numbers followed by the same letter are not significantly different at α=0.05 in the further Duncan multiple range test (DMRT).

These findings are consistent with the study conducted by Dewandari et al. [34], which reported that fermentation with added microorganisms produced different and faster temperature increases compared to spontaneous (control) fermentation. The rise in temperature during fermentation is caused by the exothermic reaction occurring when sugars in the pulp are broken down into ethanol through yeast activity. This exothermic reaction can lead to the diffusion of metabolites into the cocoa beans, causing seed death, followed by enzymatic reactions responsible for the development of aroma, flavour, and colour [35].

Cocoa fermentation is an exothermic (heat-producing) process involving yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) [33]. Temperature changes occur naturally during fermentation and are crucial for microbial succession and flavour precursor development [34]. Typically, due to microbial metabolism, temperature rises from ambient (25–30°C) to about 45–50°C by day 3–5. *Pichia kudriavzevii* is known for its thermotolerance and ability to thrive in environments up to 45°C, making it a robust contributor during mid to late stages of fermentation [35]. It actively produces ethanol, CO_2, and aroma compounds, enhancing flavour precursors. *Pichia manshurica*, while also a fermentative yeast, typically

prefers moderate temperatures (~30–37°C) and may be more active in the early to mid-stages before being outcompeted or inhibited by heat or ethanol accumulation [34,35].

The rise in temperature enhances enzymatic activity, aiding in the breakdown of pulp sugars and stimulating the production of organic acids, ethanol, and volatile compounds. *P. kudriavzevii* contributes significantly to these processes under elevated temperatures, supporting effective pulp degradation and acidification [34]. In contrast, *P. manshurica* may experience a decline in activity as the temperature rises beyond its optimal range, potentially limiting its contribution to later fermentation stages [35]. The succession from *P. manshurica* to *P. kudriavzevii* aligns with the temperature profile of spontaneous fermentation. This shift ensures continued fermentation progress and the development of desirable sensory characteristics in the beans. Proper temperature control or monitoring is crucial if these yeasts are used in a starter culture to optimise their contributions and prevent stress-induced metabolite changes that could affect chocolate quality [34,35].

3.2. pH changes in cocoa bean fermentation.

During cocoa bean fermentation, pH changes are critical indicators of microbial activity and the overall progression of the fermentation process. When specific yeast strains such as *Pichia kudriavzevii* UNJCC Y-77 and *Pichia manshurica* UNJCC Y-123 are involved, they can influence pH in distinct ways due to differences in their metabolic activities. Based on the results of the pH content testing of cocoa beans (Table 3) from various yeast isolate treatments are as follows; *P.kudriavzevii* UNJCC Y-77 yeast isolate had values ranging from 5.80 ± 0.12 to 6.71± 0.02; *P.manshurica* UNJCC Y-123 had values ranging from 5.74 ± 0.03 to 6.49 ±0.04; the combination of *P.kudriavzevii* UNJCC Y-77 and *P.manshurica* UNJCC Y-123 had values ranging from 5.87±0.55 to 6.81 ± 0.02. These results were higher compared to the control treatment, with pH values ranging from 5.53 ± 0.3 to 6.34 ± 0.07. In this study, the pH resulting from various treatments ranged from 5.7 to 6.71, which is consistent with previous research that the pH of cocoa beans produced ranges from 4.87 to 6.51 [36]. Based on [37], similar results were also found that the activity of the yeast *Candida parapsilosis* during fermentation can increase pH values or reduce the acidity level of the beans. This may occur due to the pectinolytic activity of the yeast, which leads to higher aeration of the fermentation mass during fermentation and promotes the reduction of lactic acid and lactic acid bacteria, with an increase in acetate oxidation to CO_2 and H_2O, thereby reducing the acidity levels of cocoa beans [38-41].

Table 3. Results of pH content testing of cocoa beans from yeast variation treatments and fermentation time.

Yeast variations		Fermentation time			
		0 hour (B1)	**24 hours (B2)**	**0 hours (B1)**	**72 hours (B4)**
P.kudriavzevii UNJCC Y-77	(A1)	5.80 ± 0.12^{cd}	6.64 ± 0.01^{hij}	**6.71± 0.02^{ij}**	6.41 ±0.03^{fgh}
P.manshurica UNJCC Y-123	(A2)	5.74 ± 0.03^{bc}	6.23 ± 0.21^{ghi}	**6.49 ±0.04^{ghi}**	6.01 ± 0.13^{de}
Combination	(A3)	5.87 ±0.05^{cd}	6.52 ± 0.04^{fgh}	**6.81 ± 0.02^{j}**	6.22 ± 0.25^{ef}
Control	(A4)	5.80 ± 0.06^{cd}	6.34 ± 0.07^{fg}	5.49 ± 0.28^{a}	5.53 ± 0.03^{ab}

The numerical values are the mean ± SD, tested with two-way ANOVA at a significance level of α = 0.05; Numbers followed by the same letter are not significantly different at α=0.05 in the further Duncan multiple range test (DMRT).

Generally, the pH values produced in this study are quite good, as cocoa beans with higher pH have the potential to produce less acidic chocolate products, which is one of the characteristics of good chocolate products [42].

Pichia spp. contribute to the degradation of pulp sugars and acids via their metabolic activity, producing ethanol and CO_2 [36]. As they metabolise citric acid and other organic acids, the pH may begin to rise slightly. *P. kudriavzevii* is known for high acid tolerance and may degrade more organic acids, leading to a faster or more pronounced initial pH increase compared to *P. Manshurica* [37,38]. As the pulp degrades and oxygen becomes more available, acetic acid bacteria (AAB) become more active. They oxidize ethanol into acetic acid, which diffuses into the cotyledons and lowers the internal bean pH [39]. However, depending on the balance between acid degradation and production, the external pulp environment might still experience a net rise in pH. The presence of Pichia strains influences this balance. If *P. manshurica* is less efficient in metabolizing organic acids, the pH might remain lower in fermentations where it dominates [40].

P. kudriavzevii UNJCC Y-77 is typically more thermotolerant and robust under stress, which may correlate with greater acid metabolism and a higher increase in pH during early stages [41]. *P. manshurica* UNJCC Y-123, while also contributing to fermentation, might have a milder impact on acid degradation, resulting in more stable or slightly acidic conditions [42]. These differences affect microbial succession, enzymatic activity, and the development of flavour precursors in the beans. pH influences enzyme activity in the beans, particularly proteolysis and polyphenol oxidation, which are essential for flavour development. A controlled increase in pH (without excessive alkalinization) favours balanced flavour development [38,40]. Overly low pH may lead to overly acidic beans, while too high a pH may impair flavour and microbiological stability. The metabolic capabilities of yeasts like *P. kudriavzevii* UNJCC Y-77 and *P. manshurica* UNJCC Y-123 significantly modulate the pH changes during cocoa bean fermentation [39,41]. *P. kudriavzevii* tends to elevate the pH more quickly due to its aggressive acid metabolism, while *P. manshurica* may exert a more moderate influence [42]. These dynamics have implications for microbial succession, fermentation kinetics, and ultimately, the quality of the fermented cocoa beans.

3.3. Fermentation index.

The results of the two-way ANOVA analysis show that the application of yeast isolate variations, fermentation time, and their interaction with the fermentation index value have a significance value (P) < 0.05, so a further Duncan Multiple Range Test (DMRT) was conducted to determine significant differences.

Table 4. Results of cocoa bean fermentation index testing during cocoa bean fermentation from yeast variation treatment factors and fermentation time.

Yeast variations		**Fermentation time**			
		0 hours (B1)	**24 hours (B2)**	**48 hours (B3)**	**72 hours (B4)**
P.kudriavzevii UNJCC Y-77	(A1)	0.69±0.020^{ab}	0.86±0.030^{d}	0.87±0.015^{d}	**1.10±0.060^{e}**
P.manshurica UNJCC Y-123	(A2)	0.63±0.030^{a}	0.83±0.026^{d}	0.88±0.030^{d}	**1.15±0.058^{e}**
Combination	(A3)	0.68±0.030^{ab}	0.84±0.045^{d}	0.90±0.026^{d}	**1.14±0.026^{e}**
Control	(A4)	0.65±0.060^{ab}	0.70±0.030^{bc}	0.76±0.045^{c}	0.87±0.015^{d}

The numerical values are the mean ± SD, tested with two-way ANOVA at a significance level of α = 0.05; Numbers followed by the same letter are not significantly different at α=0.05 in the further Duncan Multiple Range Test (DMRT).

Based on the fermentation index results from all treatments, it shows that cocoa beans with a fermentation index value of less than 1 occurred at fermentation times of 0, 24, and 48 hours. Meanwhile, treatments with yeast isolate variations at 72 hours were well-fermented as they produced a fermentation index value above 1. This result is better than the control treatment's fermentation index value at 72 hours, which produced a fermentation index value below 1 (Table 4).

The results of the further DMRT test show that the interaction between the application of yeast isolate variations *P. kudriavzevii* UNJCC Y-77, *P. manshurica* UNJCC Y-123, and their combination with the 72-hour fermentation variation significantly differed in the fermentation index values of 1.10 ± 0.060, 1.15 ± 0.058, and 1.14 ± 0.026, respectively. Similar results were found in the study by [43], which produced a significantly increased fermentation index value with the addition of *Saccharomyces cerevisiae* var. *chevalieri* yeast starter culture. The study by Ooi [44] also reported that the use of *Hanseniaspora thailandica* and *Pichia kudriavzevii* yeast resulted in well-fermented cocoa beans with fermentation index values ranging from 1.12 to 1.35. The study by Haruna [45] reported that the average fermentation index value was above 1 after 72 hours of fermentation. This indicates that the three treatments provided better fermentation index measurement results compared to other treatments, especially compared to the control treatment, which had the lowest fermentation index value of 0.87 ± 0.015 at 72 hours of fermentation. This is also similar to the study by Ooi [44], which reported that the control fermentation did not reach a value of 1 even after 96 hours of fermentation.

3.4. Total polyphenol content (TPC).

Polyphenols, particularly flavonoids such as catechins and epicatechins, are critical compounds in cocoa beans that contribute to their antioxidant properties and influence flavour development during fermentation and subsequent processing. The fermentation process significantly alters the polyphenol content through both biochemical degradation and microbial metabolism. The results of the two-way ANOVA analysis show that the interaction of yeast variation and fermentation time has a significance value of $P < 0.05$, indicating that there is an interaction effect of yeast variation treatment and fermentation time on the total polyphenol content of cocoa beans. Therefore, the DMRT test was conducted to determine significant differences in the total polyphenol content of cocoa beans.

Table 5. Result of total polyphenol content testing of cocoa beans from yeast variation treatment factors and fermentation time.

Yeast variations		**Fermentation time (mg GAE/g ± SD)**			
		0 hours (B1)	**24 hours (B2)**	**48 hours (B3)**	**72 hours (B4)**
P.kudriavzevii UNJCC Y-77	(A1)	31.97±0,11^{bcd}	35.23±0.29^{g}	32.77±0.83 ^{de}	34.7±0.39^{fg}
P.manshurica UNJCC Y-123	(A2)	31.89±0,18^{bcd}	33.70±0.04^{ef}	32.47±0.37^{cde}	33.35±1.33^{def}
Combination	(A3)	31.92±0,24^{bcd}	33.62±0.34^{ef}	32.86±0.17^{de}	**31.11±0.91^{bc}**
Control	(A4)	31.85±0,25^{bcd}	30.46±0.58^{b}	28.91±0.23^{a}	35.56±0.21^{g}

The numerical values are the mean ± SD, tested with two-way ANOVA at a significance level of α = 0.05; Numbers followed by the same letter are not significantly different at α=0.05 in the further Duncan multiple range test (DMRT).

The results of the DMRT test (Table 5) show that the interaction of yeast variation treatments and fermentation time leads to significant changes in the total polyphenol content

of cocoa beans. The results indicate that during the incubation time, there were fluctuations in the polyphenol content, with an increase in total polyphenol content followed by a decrease as fermentation time progressed. The total polyphenol content in treatments using *P. kudriavzevii* UNJCC Y-77 and *P.manshurica* UNJCC Y-123 yeast increased at 24 and 72 hours of fermentation. Meanwhile, the combination yeast treatment showed an increase in polyphenol content at 24 hours of fermentation and a significant decrease in polyphenol content at 72 hours of fermentation, resulting in a polyphenol content of 31.11±0.91 mg GAE/g. This is consistent with the study by Ooi et al. [46], which found that total polyphenol content treated with yeast showed an increase in polyphenol content followed by a decrease in total polyphenol content. The inconsistency in the total polyphenol content of fermented cocoa beans in this study could be due to several factors. One of the main factors for the decrease in total polyphenol content is the loss of certain chemical compounds such as epicatechin and catechin during fermentation [47].

Fermentation using *P. kudriavzevii* UNJCC Y-77 resulted in a moderate reduction of TPC compared to unfermented beans, but less so than spontaneous fermentation. This yeast is known for its high enzymatic activity, including pectinase and β-glucosidase, which may promote the breakdown of cell walls and release bound polyphenols initially. Still, it also increases the oxidation of these compounds during the aerobic phases of fermentation [46]. Moreover, *P. kudriavzevii* is acid-tolerant and can produce organic acids that slightly lower the pH, affecting polyphenol solubility and stability. Fermentation with *P. manshurica* UNJCC Y-123 demonstrated a different trend, with relatively higher TPC levels preserved post-fermentation. *P. manshurica* is typically slower-growing and less enzymatically aggressive than *P. kudriavzevii*, which may lead to a milder fermentation environment. This possibly reduces polyphenol oxidation and polymerization. Additionally, this strain may inhibit certain bacteria or fungi that contribute to polyphenol degradation, indirectly preserving antioxidant compounds [47].

The comparative analysis suggests that *P. manshurica* is more suitable when the preservation of antioxidant properties is desired, whereas *P. kudriavzevii* may be more effective in generating flavour precursors at the cost of a greater reduction in TPC. These results imply that microbial selection in controlled fermentations can be strategically adjusted depending on the target quality traits of the final cocoa product, for instance, antioxidant-rich dark chocolate versus flavour-intensive confections [46,47]. The differential effects of *P. kudriavzevii* UNJCC Y-77 and *P. manshurica* UNJCC Y-123 on TPC highlight the importance of yeast strain selection in cocoa fermentation. By understanding these interactions, producers can better control fermentation processes to optimize both health-related and sensory properties of chocolate.

3.5. Reducing sugar content.

P. kudriavzevii and *P. manshurica* play an essential role in the early stages of cocoa fermentation by metabolizing the readily available sugars (glucose and fructose) in the cocoa pulp. This sugar consumption is critical not only for reducing the sugar content but also for producing ethanol and other precursors essential for subsequent microbial succession. Both *P. kudriavzevii* and *P. manshurica* are known for their robust fermentative capabilities under acidic and high-temperature conditions typical of cocoa fermentations. Their enzymatic machinery, including invertases and hexose transporters, facilitates efficient uptake and conversion of reducing sugars.

The results of the two-way ANOVA analysis show that the application of yeast isolate variations, fermentation time, and their interaction with the value of reducing sugar content have a significance value (P) < 0.05, so a further Duncan Multiple Range Test (DMRT) was conducted to determine significant differences. The results of the further DMRT test show that the reducing sugar content in the treatment of yeast isolate variations *P. kudriavzevii* UNJCC Y-77, *P. manshurica* UNJCC Y-123, with fermentation time variations of 0-72 hours, significantly increased at 0-48 hours and did not significantly differ at 72 hours of fermentation.

The reducing sugar content of the control treatment did not experience a significant increase in reducing sugar at fermentation times of 24-72 hours. The results of the further DMRT test show that the treatment of the combination of yeast isolate *P. kudriavzevii* UNJCC Y-77 and *P. manshurica* UNJCC Y-123 with a fermentation time of 72 hours resulted in a significantly different reducing sugar content value of 3.55 ± 0.03 mg/g, indicating that the treatment of the combination of yeast isolate *P. kudriavzevii* UNJCC Y-77 + *P. manshurica* UNJCC Y-123 with a fermentation time of 72 hours produced the highest reducing sugar content compared to all treatments (Table 6).

Table 6. Results of reducing sugar content testing of cocoa beans during fermentation from yeast variation treatment factors and fermentation time.

Yeast variations		**Fermentation time (mg GAE/g ± SD)**			
		0 hours (B1)	**24 hours (B2)**	**48 hours (B3)**	**72 hours (B4)**
P.kudriavzevii UNJCC Y-77	(A1)	2.51 ± 0.07^{a}	3.00 ±0.04^{c}	3.38 ± 0.01^{d}	3.41 ± 0.04^{d}
P.manshurica UNJCC Y-123	(A2)	2.53 ± 0.04^{a}	3.08 ±0.25c	3.32 ± 0.01^{d}	3.36 ± 0.06^{d}
Combination	(A3)	2.50 ± 0.13^{a}	3.03 ± 0.04^{c}	3.39 ± 0.04^{d}	**3.55 ± 0.03^{e}**
Control	(A4)	2.50 ± 0.08^{a}	2.86 ± 0.04^{b}	2.86 ± 0.02^{b}	2.84 ± 0.02^{b}

The numerical values are the mean ± SD, tested with two-way ANOVA at a significance level of α = 0.05; Numbers followed by the same letter are not significantly different at α=0.05 in the further Duncan Multiple Range Test (DMRT).

This is consistent with the study by Santos et al. [39], which found that cocoa beans fermented with *Torulaspora delbrueckii*, *Candida parapsilosis*, and *Pichia kluyveri* yeast caused higher reducing sugar content. The increase in reducing sugar content occurred due to the pectinolytic activity of the yeast, which facilitated the release of reducing sugars from the beans. The increase in reducing sugar in the beans is due to the breakdown of sucrose in the beans into fructose and glucose. According to Calvo et al. [48], the increase in the amount of reducing sugar during fermentation is reported as a result of enzymatic reactions by β-galactosidase, α-arabinosidase, and α-mannosidase enzymes. The sugar present in cocoa nibs before fermentation is mostly sucrose and will begin to hydrolyze at the start of anaerobic fermentation until the end of the fermentation process. This condition helps activate cotyledon enzymes such as invertase [22]. Invertase hydrolyzes sucrose into fructose and glucose. Invertase hydrolyzes sucrose into reducing sugars, glucose and fructose, which serve as flavour precursors. Invertase loses its activity after fermentation due to the increase in temperature. The high reducing sugar content is important in improving the quality of cocoa beans during the subsequent cocoa processing stages, especially during the roasting stage. This is because reducing sugars will react with amino acids and peptides, playing a crucial role in the Maillard reaction during roasting. During roasting, cocoa beans can produce aldehyde compounds (acetaldehyde), acid compounds (acetic acid), alcohol compounds (ethanol), and ester

compounds (ethyl ester), which are volatile compounds that can produce the desired characteristic chocolate aroma [48].

Reducing sugar metabolism significantly impacts flavour precursor formation. The consumption of sugars not only initiates alcoholic fermentation but also indirectly drives the Maillard reaction and Strecker degradation during roasting, contributing to the development of chocolate aroma. Therefore, selecting yeast strains like *P. kudriavzevii* and *P. manshurica* that efficiently reduce sugar content can optimize both fermentation dynamics and flavour outcomes. These findings support the potential use of *P. kudriavzevii* Y-77 and *P. manshurica* Y-123 as starter cultures to standardize and enhance cocoa fermentation. Their ability to rapidly reduce sugar content can help shorten fermentation time, minimise variability, and improve batch consistency in industrial cocoa processing.

The application of selected yeast strains such as *Pichia kudriavzevii* UNJCC Y-77 and *Pichia manshurica* UNJCC Y-123 in cocoa fermentation presents a promising advancement in controlling and enhancing the quality of fermented cocoa beans. These non-Saccharomyces yeasts offer distinct metabolic capabilities that influence both the sensory properties and nutritional profile of the final product. *Pichia manshurica* UNJCC Y-123 has been shown to be particularly effective in producing a desirable flavour profile, characterized by fruity, floral, and sweet notes. This is attributed to its ability to generate a wide array of volatile organic compounds such as benzaldehyde, 2-phenylethanol, and esters during fermentation. Additionally, this strain contributes to a reduction in acidity, a significant benefit for improving consumer acceptance and reducing astringency in chocolate. From a nutritional standpoint, *P. manshurica* fermentation results in higher levels of phenolic antioxidants, including catechin and epicatechin, which are associated with cardiovascular and anti-inflammatory health benefits [49].

Conversely, *Pichia kudriavzevii* UNJCC Y-77 is a dominant yeast species commonly found in spontaneous cocoa fermentations and plays a crucial role in pulp degradation and ethanol production. While this species does not significantly reduce acidity compared to *P. manshurica*, it contributes to the breakdown of sugars into alcohols and organic acids, which are essential for flavour precursor formation. The strain-specific characteristics of *P. kudriavzevii* offer opportunities for targeted strain selection to optimize fermentation outcomes, including enhancing cocoa bean flavour and reducing undesirable microbial growth. The synergistic use of these two yeasts can lead to a more controlled fermentation process, improving consistency, enhancing flavour development, and increasing the functional value of cocoa beans. The presence of higher antioxidant compounds following fermentation with *P. manshurica* UNJCC Y-123 also suggests potential for marketing cocoa products with added health benefits. The use of *P. kudriavzevii* and *P. manshurica* as starter cultures represents a strategic improvement over spontaneous fermentations. Their use allows for greater control over the microbial ecosystem, optimized flavour profiles, and enhanced nutritional quality of the final cocoa product. Future studies should explore co-fermentation dynamics, optimal inoculation ratios, and scale-up potential to support industrial application [50].

The results of this study demonstrate that while *Pichia kudriavzevii* UNJCC Y-77 and *Pichia manshurica* UNJCC Y-123 each contribute significantly to cocoa bean fermentation, the combination of both strains yielded superior overall outcomes. Although the individual inoculations produced comparable results in terms of specific chemical and sensory parameters, the co-inoculation strategy provided synergistic benefits that enhanced the fermentation process more holistically. The improved performance of the combined strains can

be attributed to the complementary metabolic functions of the two yeasts. *P. kudriavzevii* is known for its robust fermentative capacity, particularly in the degradation of sugars and the production of ethanol and organic acids, which contribute to flavour precursor development and microbial succession. However, its dominance can lead to excessive acidity, potentially compromising flavour balance. In contrast, *P. manshurica* exhibits lower acid production and contributes a diverse range of volatile aromatic compounds, including esters and higher alcohols, which enhance the sensory complexity of the beans [51].

The co-fermentation strategy effectively leveraged these complementary traits. The presence of *P. kudriavzevii* ensured efficient pulp degradation and temperature elevation, facilitating enzymatic reactions within the bean cotyledons. Simultaneously, *P. manshurica* moderated acid accumulation and enhanced the aromatic profile by contributing sweet, fruity, and floral notes. This balance resulted in beans with improved flavour complexity, reduced astringency, and a more favourable overall sensory profile. Additionally, the combined inoculation was associated with higher levels of phenolic compounds, such as catechin and epicatechin. These antioxidants are beneficial not only for their health-promoting properties but also for their role in flavour development and oxidative stability [52]. The increased retention of these compounds in the co-fermentation suggests a more favourable biochemical environment for preserving nutritional quality. Overall, the combination of *P. kudriavzevii* and *P. manshurica* produced a synergistic effect, improving the consistency, sensory appeal, and functional properties of the fermented cocoa beans. These findings support the strategic use of mixed yeast cultures in controlled cocoa fermentations to enhance product quality and reproducibility [53].

4. Conclusions

The variation of yeast isolates *P. kudriavzevii, P. manshurica*, and their combination significantly affects the physical and chemical characteristics of cocoa beans. The best treatment, which is the combination of *P. kudriavzevii* UNJCC Y-77 and *P. manshurica* UNJCC Y-123, can increase the temperature value to 30.60±0.10°C, pH 6.22, fermentation index 1.14±0.026, reducing sugar 3.55 ± 0.03 mg/g, and decrease the total polyphenol content to 94.21±1.55 mg GAE/g. A fermentation time of 72 hours significantly affects the physical and chemical characteristics of cocoa beans, namely temperature, pH, fermentation index, total polyphenol, and reducing sugar content. There is an interaction between the yeast variation treatment of *P. kudriavzevii*, *P. manshurica*, and fermentation time. The best interaction between treatments was obtained from the combination treatment of P. *kudriavzevii and P. manshurica* yeast and 72 hours of fermentation.

Author Contributions

Conceptualization, D.S.; methodology, D.S., S.F.M., S.R., H.E.E., K., R.A., and V.S.; software, D.S.; validation, D.S.; formal analysis, S.F.M., S.R., H.E.E., K., R.A., and V.S.; investigation, D.S.; resources, D.S., S.F.M., and S.R.; data curation, D.S., H.E.E., K., R.A., and V.S.; writing—original draft preparation, D.S. and R.H.B.S.; writing—review and editing, D.S. and R.H.B.S.; visualization, D.S.; supervision, D.S. and R.H.B.S.; project administration, D.S.; funding acquisition, D.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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