The Influence on Phenolic, Aroma Compounds, Rheological and Sensory Properties of Chocolates by Different Strains Inoculated Fermentation

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Abstract
Four designed cocoa fermentations were conducted in separated wooden boxes with different strains inoculum. One was spontaneous fermentation with natural occurring microorganisms, another was inoculated with Saccharomyces cerevisiae, another was inoculated with Lactobacillus plantarum, and the other was inoculated with Acetobacter aceti. The experimental parameters, including the fermenting cocoa mass temperature, the pH value, the total acidity of the pulp and cotyledons of cocoa beans, along with the fermentation time were determined. Furthermore, the phenolic compounds (flavanols and procyanidins DP1-10) of chocolates were determined by normal phase HPLC. In addition, the rheological, textural properties, and color parameters of the chocolates were measured. Meanwhile, the electronic nose was used to analyze the aroma compounds of the investigated chocolates and the principal components analysis (PCA) was conducted to distinguish the flavor profiles among the chocolates. The consumer sensorial assessment was carried out to evaluate the organoleptic properties of chocolates and their overall acceptability. The quality of chocolates produced by the defined inoculum varied with each other with the exception of the FP and PAC content. Different inoculated fermentation methods affected the rheological, textural properties, and color parameters of the end product chocolates. Furthermore, they influenced the types and numbers of aroma compounds generated during fermentation. However, the impact is mainly on their levels of aroma compounds. From sensory point of view, chocolates produced from inoculated Acetobacter aceti BCRC12324 during fermentation had the highest score point of overall acceptability among the investigated chocolates. The high levels of 2-methylbutanal content occurred in this chocolate could be an adequate reply of this finding. The quality of chocolate from fermentation with Lactobacillus plantarum B0091 inoculum was similar to the others, but some properties such as rheological, textural, and flavor profiles were inferior and less pronounced, leading to a slightly poorer response by the sensory panel. PCA successfully discriminates the aroma profiles into four groups, providing a better visualization of the variation among the chocolates produced by the different strains. This attempt to manipulate the strains during cocoa fermentation is a new approach in Taiwan chocolate industry. By controlling the starter culture of fermentation properly, we could improve the quality and flavor profiles of chocolate with a consistent and more reliable production.

Keywords: inoculated cocoa fermentation, aroma compounds, Phenolic compounds, rheological properties, sensory assessment, electronic nose

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1. Introduction

People have been enjoying the taste of chocolate for more than 4000 years. The main ingredient of chocolate is the cocoa beans. The cocoa beans are the seeds of Theobroma cacao tree, which originated in Central and South America, including the upper Amazon areas (10,000-15,000 years ago), the upper Orinoco area of North East Colombia and North West Venezuela, the Andean foothills of North West Colombia, Central America from southern Mexico (Chiapas-Usumacinta) to Guatemala [1,2]. The plantation area generally distributed between 23.5 °N and 23.5 °S. Studies have shown that the rich flavonoids in the cocoa beans could prevent the development of cardiovascular disease [3]. The main composition of the flavonoids are proanthocyanidins (PAC) and flavanols and procyanidins (FP). The PAC contribute to the bitterness and astringency of cocoa products, and as for FP, it presents as the monomers of epicatechin and catechin and their oligomers. The manufacture of chocolate involves a series of processes including cocoa bean fermentation, drying, roasting,
grinding, winnowing, tempering, molding, and aging. Among them, fermentation plays a decisive role in affecting the flavor, quality, and the polyphenols concentration of chocolate. Brito et al. [4] had discussed the concentration change of polyphenols during fermentation. Bortolini et al. [5] had sampled several wild strains of different origins for analysis, discovering that the species participating in the fermentation of cocoa beans are basically the wild yeasts, lactic acid bacteria, and acetic acid bacteria. Koné et al. [6] had used yeasts for fermentation and successfully improved the flavor of cocoa beans in the Ivory Coast. Other studies [7,8,9] had also proved the importance of yeasts in cocoa fermentation. On the other hand, the role of the lactic acid bacteria in fermentation is not comparably significant [10].

In cocoa bean natural spontaneous fermentation, there are three key microorganisms, which are yeasts, lactic acid bacteria, and acetic acid bacteria. The microbial succession occurs into three stages. Initially, yeasts use the sweet and white mucilaginous pulp as the nutrients to produce ethanol. Intermediately, lactic acid bacteria produce lactic acid. Finally, acetic acid bacteria utilize the ethanol to produce acetic acid and liberate heat, causing the fermenting cocoa mass temperature raising to 48°C [11]. The microorganism metabolites diffuse from the surface of tests into bean cotyledons and activate several complex biochemical reactions, involving the production of volatile organic compounds and precursors of chocolate flavors [12]. Cocoa bean fermentation are usually done by individual farmers and the quality of the resulting fermented cocoa bean varied significantly from region to region. The variation in fermented cocoa bean quality has become an important issue in the chocolate industry of Taiwan and other countries. The three key microorganisms could be isolated and selected as the starter cultures, which is used to control the microbial communities during cocoa fermentation process, to maintain the consistent quality of fermented cocoa beans or even ameliorate the chocolate flavor [11]. Efforts have been conducted by only a few cocoa producing countries, such as Brazil, Malaysia, Indonesia, and Cameroon [9,13-19]. The microbial cocktail inoculum used to drive the cocoa fermentation process were different from region to region. Due to the complexity of microbial ecology in different countries, the standardization of inoculated cocoa fermentation has become difficult and controversial.

Taiwan is a new place for cocoa plantation. Because of the restriction of the planted area and the production scale of cocoa beans, the aim of producing high-quality and flavor-rich cocoa products are especially of great necessity. An approachable method to achieve the goal is to produce cocoa products through inoculated cocoa bean fermentation. However, there are so far no published studies regarding inoculating different strains as starter cultures to control the fermentation process of Taiwan origin cocoa beans. In this study, an attempt was made to manipulate the microorganisms during cocoa bean fermentation. One yeast species, one lactic acid bacteria species and one acetic acid bacteria species were selected as the starter cultures in cocoa bean fermentation process.

The objective of this study was to characterize the physical and chemical changes along with fermentation time during the inoculated fermentation and to determine the amount of phenolic compounds (FP and PAC) of the chocolates. Furthermore, the rheological, textural properties, and color parameters of the chocolates from different strains of fermenting cocoa beans were also measured. In addition, the aroma compounds of the chocolates were investigated and the results were used to differentiate the flavor profiles among the chocolates. Moreover, the consumer sensorial assessment was carried out to evaluate the organoleptic properties of the chocolates and their overall acceptability.

2. Material and Methods

2.1. Chemicals

Glucose (Panreac Quimica S.L.U.), Peptone (BD Bacto™), Yeast Extract (MdBio, Inc.), MRS BROTH (CONDA), procyanidin B1, (-)-epicatechin, dichloromethane, methanol, acetone, acetic acid, butanol, hydrochloric acid, and ammonium ferric sulfate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All chemicals were of analytical grade.

2.2. Inoculated Cocoa Fermentation

The cocoa pods were purchased from local farmers in Pingtung county of South Taiwan. The pulps and seeds were taken out from the cocoa pods on the condition of not harming the cocoa beans. Cocoa beans were immediately transferred to four separated wooden boxes, each containing 20 kg of cocoa beans. Four designed fermentations were conducted separately in different boxes with different strains inoculum. The first box (box N) was spontaneous natural fermentation without any defined inoculum. The second box (box Y) inoculated Saccharomyces cerevisiae (1x10^9 CFU/mL) as the predominant yeast. The third box (box L) inoculated Lactobacillus plantarum B0091 (1x10^9 CFU/mL) as the predominant lactic acid bacteria (LAB), and the fourth box (box A) inoculated Acetobacter aceti BCRC12324 (1x10^9 CFU/mL) as the predominant acetic acid bacteria (AAB) during fermentation. To cultivate the yeasts, the yeasts were grown in YEP medium, a mixture of 2% glucose, 2% peptone, 1% yeast extract, pH=5.6. 100 μL of the yeast strain was transferred from a -20°C cryogenic tube to a 10 ml broth tube, and was incubated at 30°C for 2 days. Then 100 μL of the cultures was transferred again to a 10 ml broth tube and was again incubated at 30°C for 2 days, and finally, 2500 μL of the cultures was transferred to a 250 ml Erlenmeyer flask and incubated for 2 days at 30°C. The resulting cultures was centrifuged at 10000 rpm for 10 min and the precipitate cells were resuspended in a serum bottle with distilled water and then mixed with the cocoa bean mass in the box Y at time zero. To cultivate LAB as the inoculated strain for box L, MRS was the medium, a mixture of MRS Broth 55 g/L. The procedures was similar to that of the yeast cultivation with a different incubation temperature, 37°C. To cultivate AAB as inoculated strain for box A, GYC was used as the medium, a solution of 5% glucose, 1% yeast extract, pH=5.6. The incubation temperature was 30°C. The three types of microorganisms were inoculated to different
fermentation boxes at the level of 1% of cocoa bean mass, respectively. For every 24 h, 100 g of cocoa beans were sampled to determine the total acidity and pH value of pulp and cotyledons. The fermentation was maintained for 7 days and the temperature profiles were recorded for each fermentation box. When the fermentation process was completed, drying was conducted at 50°C in a dryer until about 6% of moisture remaining in the cocoa beans. Dried cocoa beans were stored in refrigeration until further used.

2.3. pH and TA Determination

Pulps of cocoa beans (10g each) were mixed with 100 ml of boiled demineralized water in a serum bottle by a ultrasonic oscillator for 15 min. The resulting solution was then moved into two 50 ml centrifuge tubes and centrifuged for 40 min and then the supernatant was filtrated by using a Whatman No.4 filter paper. The pH value of the resulting solution was measured by a pH meter (Mettler Toledo S220-Kit ph-2–20) and was recorded. For TA determination, the standard solution 0.01N NaOH was used to titrate the 25ml supernatant mixture was stirred in a serum bottle for 15 min, and was then centrifuged for 40 min and then the supernatant was filtrated by using a Whatman No.4 filter paper. The pH value and TA of the cotyledons in cocoa beans, 10 g of cotyledons were homogeneously mixed with 100 ml of boiled water through a blender for 30 seconds. The mixture was stirred in a serum bottle for 15 min, and was then centrifuged for 40 min and then the supernatant was filtrated by using a Whatman No.4 filter paper. The pH value and TA of the cotyledons were measured as previously described.

2.4. Chocolate Samples

The roasting process was conducted by using a conventional rotary dryer, which was set at 130°C and held for 10 min to reach equilibrium before the roasting process started. The fermented and dried cocoa beans in amount of 2 kg were placed in perforated tray from different fermentation boxes. After 25min roasting duration, the roasted cocoa beans were cooled at ambient temperature for 2 h and were kept in sealed plastic containers in refrigeration. An aliquot of each type roasted cocoa beans sample (1kg) were initially cracked and winnowed by a machine (GT2019, Taiwan) to separate the cotyledons and the cocoa shells. The cotyledons were used to produce paste-like cocoa liquors by the cocoa grinding machine. The ground powder was mixed with N-hexane with a ratio of 1 to 2 (w/v) with a ultrasonicator at 40°C for 30min. The mixture was shaken vigorously and then centrifuged for 10min at 10000rpm and the supernatant was removed. The defatting process was to be done in triplicate. The resulting precipitant was air-dried in a fume hood for 48h.

2.5. Defatted Chocolate

Chocolates (10g each) were ground in the presence of liquid nitrogen to obtain the fine powder by a blender machine. The ground powder was mixed with N-hexane with a ratio of 1 to 2 (w/v) with a ultrasonicator at 40°C for 30min. The mixture was shaken vigorously and then centrifuged for 10min at 10000rpm and the supernatant was removed. The defatting process was to be done in triplicate. The resulting precipitant was air-dried in a fume hood for 48h.

2.6. FP Determination

Fat-free powder (0.3g each) was centrifuged with 50ml of a solvent of acetone/water/acetic acid (70:28:2, v/v) at room temperature for 1h for extraction. The supernatant was kept by decanting, while the precipitant was extracted with repeated procedure. The resulting supernatant was collected and mixed with the previous one. The mixture was filtrated and adjusted to the volume of 100ml. This crude extract was filtrated with a 0.45μm filter and the contents of FP (DP1-10) are measured with NP-HPLC. Relevant information of NP-HPLC is described by Lin et al. [21] as follows: Column: Developsil Diol 100Å 5μm, 250 × 4.6mm (Phenomenex, Torrance, CA; Cat. No. DI11546250W); Detection wavelength: UV 280nm; Mobile phase A: CH2Cl2/CH3OH/H2O/Acetic acid (82:14:2:2, v/v); Mobile phase B: CH3OH/H2O/Acetic acid (96:2:2, v/v); The flow rate was 1ml/min by using these two mobile phases (A and B) with a gradient elution. The gradient elution was as follows: from 100% to 82.4% A as time from 0 to 30 min, and then to 69.3% A as time from 30 to 45 min, and to 12.2% A as time from 45 to 50 min, and then to 50% A as time from 50 to 52 min. and to 100% A as time from 52 to 55 min, and maintained this gradient for another 5 min.

2.7. PAC Determination

Fat-free powder (0.2g each) with 20ml of 90% methanol were extracted in a 50ml centrifugal tube for 1h at room temperature with ultrasonic oscillation. The resulting supernatant was added to 25ml with demineralized water. To establish the standard curve for PAC determination, the procyanidin B1/methanol solution were prepared at different concentrations: 0.01, 0.025, 0.05, 0.1, 0.15 and 0.20mg/ml. 1ml of the solution with different concentrations was added respectively to 6ml of butanol/hydrochloric acid (95/5, v/v) with the addition of 0.5ml of 2% ammonium ferric sulfate solution in each test tube. The tubes were heated in a hot water bath for 40min and then cooled in an ice water bath. The measurement of the absorbance of each resulting solution was performed in triplicate under 546nm wavelength in a 10mm cuvette with a spectrophotometer. With ddH2O used for blank correction, the corresponding absorbance of different concentrations of procyanidin B1 solution was used to establish the standard curve. The measurement of PAC was according to the described method [21,22]. Sample extraction (1ml each) was substituted for the procyanidin B1 solution. Absorbance were measured at 546nm and the results were recorded.
2.8. Color Parameters Determination

The color of chocolate produced from fermentation box N, Y, L and A was measured using a colorimeter (ColorLite GmbH, sph870-6-uk 400-700nm, Germany). The results were displayed in accordance with the parameters L*, a*, b*, C* and h*, where L* indicates lightness, ranging from 0 (black) to 100 (white); a* indicates redness, ranging from green (-50) to red (+50); b* indicates yellowness, ranging from blue (-50) to yellow (+50); C* represents chroma the intensity of color, was calculated by the following formula, C* = [(a*)2 + (b*)2]1/2; and h* represents hue angle, and intensity of color, was calculated by the following formula, h* = tan⁻¹(b*/ a*).

The numerical value of hue angle is a useful indicator of browning. High values of h* imply more browning. The chocolate sample were placed in a cylindrical and optical glass cuvette and the color of each sample was measured in 5 replicates. The instrument was calibrated with a standard white tile before the measurement [23].

2.9. Rheological Properties

Chocolate sample (12g each) was placed in a beaker and was liquefied in a laboratory incubator at 45°C. Measurements were made through a rheometer (Brookfield Viscometer-DV2T HB, USA) with the coaxial geometry of the measuring system. The SC4-21 spindle and SC4-13R replacement sample chamber with embedded RTD temperature probe were used to perform the measurement. Approximately 10 g chocolate sample was placed in the chamber. Measurements were carried out at constant temperature, 40°C, by circulating the 40°C water in water jacket. Measurement, recording, and Casson viscosity and yield stress value calculations were carried out using the computer software Rheocalc T (Brookfield, USA). Each sample was measured in 5 replicates.

2.10. Textural Property (Hardness)

Hardness determination was performed with a CT3-4500 Texture Analyzer, (Brookfield, USA). Chocolate samples were removed from refrigerated conditions at 18°C and the wrappers were also removed and centrally positioned beneath the probe as soon as possible. The probe used was TA9, which is a stainless steel needle probe with 10° Taper. The sink rate of the probe in the sample is 0.1mm/s and the depth of penetration of the probe in the sample is 3 mm. The resulting texture profiles are automatically generated by TexturePro CT Software (Brookfield, USA). The hardness of each chocolate was measured at the peak force during the pricking test. Each sample was measured in 5 replicates.

2.11. Aroma Compounds Identified by Electronic Nose

The aroma compounds of chocolate produced from fermentation box N, Y, L and A were determined using an Electronic Nose Heracles II (Alpha M.O.S. Inc., Toulouse, France). The Heracles II was equipped with an autosampling system, an integrated cooled trap with a thermodesorption system, a dual column configuration system: a non-polar column (MXT5: 5% diphenyl, 95% methylpolysiloxane, 10 m length and 180 µm diameter) and a slightly polar column (MXT1701: 14% cyanopropylphenyl, 86% methylpolysiloxane, 10 m length and 180 µm diameter) coupled to 2 flame ionization detectors (FID) for data acquisition [24,25]. An aliquot of each type chocolate sample (5g) was placed in a 20 mL vial and sealed with a magnetic plug. The vial was placed in a shaker oven where it remained at 60°C for 10 min. Next, a syringe pierced the silicone septum of the magnetic plug and sampled 5 ml of the head space. The 5ml headspace aliquot was trapped at 20°C for 50sec, and subsequently, the temperature of the trap was increased up to 240°C in 30sec heating duration and then the sample was injected at 125µL/sec. The temperature program started at 50°C (held for 2 sec) and increased up to 80°C at 1°C/sec, and then increased up to 250°C at 2°C/sec, the final temperature was held for 21 sec. The total acquisition time was 138sec. For calibration, an alkane solution (from n-hexane to n-hexadecane) was used to convert retention time in Kovats indices and identify the aroma compounds and their sensory attributes using ArOChemBase library software. Meanwhile, Alpha Soft (V14.2, Alpha M.O.S., Toulouse, France) software was used for instrument control and raw data processing. The principal components analysis (PCA), a kind of multivariate chemometric method, was used for data analysis of results obtained by the electronic nose. Samples were analyzed in quadruplicate or pentaplicate.

2.12. Sensorial Assessment

Sensorial assessment of each chocolate produced from inoculated cocoa beans was carried out by 34 untrained panelists using the point score method in our Lab. The attributes of chocolate include aroma, flavor, sweetness, the intensity of acidity, bitterness, astringency, and overall acceptability. Each panelist was requested to refresh their palate by using polenta congee between the test samples to remove the remaining odor in their mouths. The organoleptic properties were presented on a nine-point hedonic scale, according to which like extremely or extremely strong = 9, dislike extremely or extremely weak = 1, and recorded their scores [23,26].

2.13. Statistical Analysis

Each experiment was performed at least triplicate and averaged. All data were presented as means ± SD. Analysis of Variances (ANOVA) and Tukey’s Test were applied to analyze the significant difference of the means among the treatments. The significant level (α) was set at 0.05. Principal components analysis, a well-known chemometric method, was used to discriminate the flavor profiles of different strains fermented chocolates. All statistical analysis was performed by using the statistical computing software R 4.0.

3. Results and Discussion

3.1. Temperature Evolution of Cocoa Mass during Fermentation

The temperature evolution of different boxes during fermentation was shown in Figure 1a. Generally, in all
investigated fermentations, the center temperature of fermenting cocoa mass varied from 22.9 to 46.5°C. For natural spontaneous fermentation (box N), the temperature started at 23.5°C, reached the maximum peak temperature (46.4°C) on the fifth day, and then decreased to 42.9°C at the end of fermentation. For fermentation box Y, the temperature increased from 22.9°C at the beginning of fermentation and attained a maximum of 44.9°C on the fifth day of fermentation. Afterwards, it decreased to 42.1°C at the end. For fermentation box L and A, the center temperature of cocoa mass from the onset of fermentation (around 23°C) gradually increased to 46.5°C and 44.4°C, respectively on the seventh day of fermentation. The temperature change mainly due to biochemical reactions of metabolites of natural occurring microorganisms during cocoa bean fermentation. At the beginning of fermentation, yeast was basically the predominant microorganism, which transformed the sugars in mucilaginous pulps into ethanol and carbon dioxide. Afterward, the acetic acid bacteria oxidized ethanol into acetic acid. The latter reaction is an exothermal process, liberating heat and leading to a fast temperature increase. The temperature rose to 45°C of the fermenting mass and eventually caused the death of the seed embryo [27,28,29]. The observations in Figure 1a for box N and Y was in accordance with the study made by Brito et al. [4], who indicated that the peak temperature appeared at around the fifth day of natural fermentation. However, Schwan and Wheals [12] reported that the maximum temperature happened at the fourth day during cocoa fermentation.

Figure 1a. The temperature evolution of cocoa mass center during fermentation

3.2. pH Changes in the Pulp and Cotyledons during Fermentation

The pH in the pulp and in the cotyledons from different fermentation boxes during 7 days of fermentation were shown in Figure 1b. Before fermentation, the pH of the cocoa pulp from box N, Y, L and A was around 3.9. During the first 72h fermentation period, the pH of the pulp from box N, Y and A remained relatively constant (3.9-4.0). Consecutively, a slight increase in pH for next 24h period and then a small decrease in pH for next 48h was observed for box N, Y and A. Finally, there was an increase in pH in the last 24h to a value of 4.7, 4.4, and 4.4 for box N, Y, and A, respectively. However, the pH of the pulp from box L decreased to about 3.5 in the first 72h and then increased steadily to the final value of 4.3. For the cotyledons of unfermented beans from box N, Y, L and A, the initial pH was around 6.5. Later, it remained relatively constant (6.3-6.6) until 96h, and then decreased during the rest of fermentation period to a final value of 4.6, 4.5, 5.5 and 4.6, respectively. The observations were mainly due to the concentration of organic acids, including citric acid, acetic acid, oxalic acid, succinic acid, lactic acid, and malic acid. These organic acids were either presented originally in the pulp and cotyledons or were produced by several microorganisms during fermentation. The change in the pH value is associated with the biochemical reaction, where lactic acid bacteria (Lactobacillus plantarum) converts citric acid and carbohydrates into lactic acid, resulting in a slight increase in pH during fermentation. However, acetic acid bacteria (Acetobacter aceti) oxidizes ethanol and lactic acid into acetic acid, which results in a small decrease in pH value of the pulp [30]. At the end of all investigated fermentations, an increase in pH of the pulp was observed. It was possibly due to two reasons: the evaporation of volatile acids, such as acetic acid; or that these acids were penetrating into the cotyledons, and then gradually absorbed by the cotyledons. Therefore, the pH of the cotyledons drastically decreased. The observation of pH in cocoa cotyledons were consistent with the previous studies [7,31].

Figure 1b. The mean values of pH of the pulp and cotyledons varied with the fermentation time. Symbols: ■ and □, box N; ● and ○, box Y; ▲ and △, box L; ■ and ○, box A; solid symbol for cotyledons and open symbol for pulp.

3.3. Total Acidity of the Pulp and Cotyledons during Fermentation

The main organic acid of the fresh pulp is the citric acid and only low amounts of acetic acid and lactic acid were
detected in the fresh pulp. During fermentation, most of the sugars were metabolized by microorganisms, causing a rise in acetic acid and lactic acid concentration [27]. The value of the total acidity (TA) is mainly contributed from these organic acids in the pulp inherently or in the cotyledons during fermentation. The TA of the pulp from different fermentation boxes was shown in Figure 1c. A similar trend in TA was observed for box N, Y and A. It gradually increased from 0.23-0.27% to a peak value of 0.51-0.73% after 144h of fermentation, followed by a decline at the end of fermentation with a value of 0.21-0.32%. However, the TA of the pulp from box L increased to a maximum value of 0.42% after 72h of fermentation and declined to 0.21% eventually. The TA of the cotyledons from different fermentation boxes was shown in Figure 1d. A similar trend in TA was observed for box N, Y and A; the TA remained relatively stable at the value of 0.22-0.28% during the initial 96 h fermentation, and followed by a fast increase reaching a value of 1.34-1.67% at the end of fermentation. However, the TA of the cotyledons from box L increased to a value of 0.33% after 120 h of fermentation, declined at 120 h, and increased again to the value of 0.55% at the end of fermentation. It was significantly different from the others (p<0.05). The TA in the pulp all decreased at the end of investigated fermentations, on the other hand, the TA in the cotyledons all drastically increased. The reason was similar to the one given in 3.2.

3.4. FP and PAC Content

The quality of the cocoa beans and the amount of polyphenols are not only affected by the origins of countries but also influenced by processing conditions. Table 1 showed the level of FP (DP1-10) and PAC of chocolate N, Y, L and A produced from different boxes.

The chocolate A had the highest FP value of 41.86±2.09 mg (-)-epicatechin equivalents/g. The FP values of chocolate N, Y and L are 41.55±2.07, 41.30±2.04, and 40.94±2.02 mg (-)-epicatechin equivalents/g, respectively. However, there were no statistically significant differences at the confidence level α=0.05 among these values. Chocolate A also had the highest PAC value of 76.42±1.42 mg procyanidin B1/g. The PAC values of chocolate N, Y and L are 72.88±2.11, 72.52±0.66, and 75.47±2.38 procyanidin B1/g, respectively. Likewise, there were no significantly differences among these values (p<0.05). These findings implied that the phenolic compounds of chocolate are possibly irrelevant to the starter culture inoculated during cocoa fermentation.

3.5. Rheological, Textural Properties, and Color Parameters

Rheological parameters such as viscosity and yield stress in chocolate production industry are of great importance. These values of chocolate liquors should be strictly controlled and as low as possible to decrease the resistance during mixing, grinding, pumping, coating, and filling. High viscous chocolate masses lead to inferior sensorial attributes of chocolate during consumption; probably due to the unpleasant mouth feel. Therefore, the determination of rheological parameters and their disclosure in the product specification are necessary. To match the different purpose of each chocolate product, the industry provides a range of rheological parameters of chocolate masses. As a result, it is difficult to define rheological characteristics of an ideal chocolate mass. However, each manufacturer of chocolate masses should be aware of the consequences related to the changes in their formulations [26]. Table 2 showed the Casson viscosity and yield stress of chocolate masses produced from different fermentation boxes. The highest value of
Casson viscosity is 3410±121 cP for chocolate liquor L. The Casson viscosity of chocolate masses N, Y and A are 1964±46, 2803±164, and 1991±59 cP, respectively. The Casson viscosity of chocolate liquor N, Y and A were significantly lower than that of chocolate liquor L (p<0.05). According to Żyżelewicz et al. [20], the viscosity of dark chocolate masses prepared for manufacturing of chocolate bars should be of 2000 cP [20]. The Casson yield stress displayed in a similar way of the Casson viscosity with the value of 13.2±0.9, 20.4±0.8, 25.7±0.7, and 15.9±1.1 Pa for chocolate masses N, Y, L and A, respectively. However, if necessary, the rheological properties can be regulated by means of adding cocoa butter or emulsifiers to cocoa liquor during chocolate production. Table 2 also contains the textural property hardness of chocolate produced from different fermentation boxes. The results indicated that the value of hardness for chocolate L is 661±53 g, which was significantly higher than those of chocolate N, Y and A. The reason for this is probably due to the highest viscosity and yield stress of chocolate L as shown in Table 2. Color of chocolate is a typical sensory perception to consumer and affects its acceptability. The basic color parameters of the chocolate are represented by the value of L*, a*, b*, C* and h*. As given in Table 2, the results showed that the L* value of chocolate A had the lowest value of 28.6±1.5 which was significantly lower than those of chocolate N, Y and L (p<0.05). The changes of a*, b* and C* value were small and not obvious among all investigated chocolates. The h* value of chocolate L had the lowest value of 33.8±2.7 which was significantly lower than those of chocolate N, Y and A (p<0.05). The parameters L* and h* were chosen as the strength of browning. The findings of the L* value (28.6±1.5 -30.3±0.1) are consistent with the reported L* value (30.04-31.59) by Żyżelewicz et al. [26]. The highest h* value (37.0) of the chocolate N and A could be attributed to the oxidation and polymerization of polyphenols, degradation of proteins, and dextrinization of starch, yielding more brown pigments as reported by Krysiak [32].

Table 1. Effects of different strains inoculated on the FP and PAC content of chocolates produced from different fermentation boxes

| Parameter | Chocolate N | Chocolate Y | Chocolate L | Chocolate A |
|-----------|-------------|-------------|-------------|-------------|
| FP, mg (-)-epicatechin equivalents/g | 18.59±0.88 | 18.42±0.88 | 18.62±0.88 | 18.15±0.87 |
| DP1       | 7.18±0.35   | 7.34±0.36   | 7.08±0.35   | 7.55±0.37   |
| DP2       | 3.92±0.17   | 3.92±0.17   | 4.07±0.18   | 4.08±0.18   |
| DP4       | 3.26±0.19   | 3.33±0.19   | 3.08±0.18   | 3.36±0.20   |
| DP5       | 2.52±0.13   | 2.17±0.11   | 2.50±0.13   | 2.67±0.13   |
| DP6       | 2.40±0.13   | 2.15±0.12   | 2.10±0.12   | 2.42±0.14   |
| DP7       | 1.80±0.10   | 1.95±0.11   | 1.73±0.09   | 1.78±0.09   |
| DP8       | 1.17±0.06   | 1.33±0.06   | 1.10±0.06   | 1.13±0.06   |
| DP9       | 0.56±0.03   | 0.57±0.03   | 0.58±0.03   | 0.57±0.03   |
| DP10      | 0.17±0.01   | 0.14±0.01   | 0.11±0.01   | 0.17±0.01   |
| Total (DP1-10) | 41.55±2.07 | 41.30±2.04 | 40.94±2.02 | 41.86±2.09 |
| PAC, mg procyanidin B1/g | 72.88±2.11 | 72.52±0.66 | 75.47±2.38 | 76.42±1.42 |

Values are expressed as the means ± SD. Different letters in a row are significantly different (p<0.05).

Table 2. Rheological, textural properties, and color parameters of chocolates produced from different fermentation boxes

| Parameter | Chocolate N | Chocolate Y | Chocolate L | Chocolate A |
|-----------|-------------|-------------|-------------|-------------|
| Casson viscosity, cP | 1964±46 | 2803±164 | 3410±121 | 1991±59 |
| Casson yield stress, Pa | 13.2±0.9 | 20.4±0.8 | 25.7±0.7 | 15.9±1.1 |
| Hardness, g | 473±21 | 522±51 | 661±53 | 516±33 |
| Color L* | 29.8±0.2 | 30.3±0.1 | 30.1±0.5 | 28.6±1.5 |
| a* | 7.4±0.5 | 9.1±0.2 | 8.0±0.7 | 8.7±0.7 |
| b* | 5.6±1.0 | 6.7±0.2 | 5.4±1.0 | 6.6±0.7 |
| C* | 9.3±1.0 | 11.3±0.3 | 9.7±1.1 | 10.9±1.0 |
| h* | 37.0±3.2 | 36.3±0.4 | 33.8±2.7 | 37.0±0.7 |

Values are expressed as the means ± SD. Different letters in the same row are significantly different (p < 0.05).

3.6. Sensorial Assessment

The results of the organoleptic properties for chocolate N, Y, L and A, including aroma, flavor, sweetness, acidity, bitterness, astringency, and acceptability, are shown in Table 3. Except for astringency, there were statistically significant differences among the organoleptic properties of the 4 types of manufactured chocolates. Proanthocyanidins are not only beneficial to human health, but are also responsible for the astringency taste of
chocolate [21,33]. The PAC content of chocolate N, Y, L, and A were not significantly different as shown in Table 1, causing no difference on the taste of astringency. The chocolate A had the best overall acceptability rating (5.12 points) and the chocolate L had the worst overall acceptability rating (3.97 points), which were significantly lower than those of chocolate N, Y, and A (p<0.05). Even though all fermented cocoa beans were roasted at the same roasting conditions with temperature (130°C) and time (25min), the aroma attribute of chocolate L had the lowest score of 4.41 points, which was significantly lower than those of chocolate N, Y, and A. 

3.7. Aroma Compounds

Flavor is of great importance when considering the consumer acceptability of chocolate. The characteristic flavors of chocolates are due to a mixture of hundreds of aroma compounds [35]. These compounds comprise of acids, aldehydes, ketones, esters, alcohols, hydrocarbons, pyrazines, ethers, furans, and others. Each of them possesses particular flavor attributes. For instance, most esters provide a fruity/flowery odor and pyrazines usually generate roasted odor. Therefore, the amount and type of aroma compounds are the key of chocolate quality, and they depend not only on the genotype and the origins of the cocoa tree, but also on how the beans have been processed. Consequently, the fermentation and roasting process both play a decisive role for determining the flavor of the chocolates; the former generated flavor precursors that develop the aroma attributes, and the latter produced aroma compounds in the roasted cocoa beans [36].

**Table 3. The organoleptic properties of chocolates produced from different fermentation boxes**

| Organoleptic properties | Chocolate N | Chocolate Y | Chocolate L | Chocolate A |
|-------------------------|-------------|-------------|-------------|-------------|
| Aroma                   | 5.79±1.63   | 5.24±1.69   | 4.41±1.74   | 5.85±2.00   |
| Flavor                  | 5.82±1.55   | 5.05±1.59   | 4.68±1.59   | 5.21±1.47   |
| Sweetness               | 3.41±1.33   | 3.85±1.69   | 4.15±1.65   | 4.50±1.88   |
| Acidity                 | 6.00±2.02   | 4.85±1.79   | 5.12±1.90   | 6.12±1.57   |
| Bitterness              | 5.79±1.86   | 4.85±1.79   | 4.44±1.81   | 4.00±1.78   |
| Astringency             | 4.50±2.02   | 4.44±1.89   | 4.50±1.80   | 4.32±1.72   |
| Acceptability           | 4.65±1.86   | 4.18±1.60   | 3.97±1.57   | 5.12±2.09   |

Values are expressed as the means ± SD. Different letters in the same row are significantly different (p < 0.05).

**Table 4. The proportions of each aroma compound of chocolate from different fermentation boxes and its odor description**

| Compound                        | N (%) | Y (%) | L (%) | A (%) | Description of odor                |
|---------------------------------|-------|-------|-------|-------|-----------------------------------|
| Acetic acid                     | 59.82 | 58.05 | 68.43 | 62.67 | Acidic, Pungent, Sour, Vinegar     |
| Propyl acetate                  | 0.74  | 0.58  | 0.63  | 0.97  | Caramelized, Fermented, Fruity    |
| 2-Methylbutanal                 | 1.22  | 1.37  | 6.47  | 16.51 | Cocoa, Chocolate, Almond, Herbaceous |
| cis-3-Hexenol                   | 0.48  | 0.50  | 0.89  | 0.91  | Fresh, Grassy                     |
| Tert-Butylmethylether           | 0.67  | 0.77  | 0.27  | 0.08  |                                   |
| 2,3-Butanediol                  | 1.28  | 1.15  | 2.93  | 3.05  | Fruity, Onion                     |
| Methyl 2-methylbutanoate        | 0.23  | 0.21  | 0.32  | 2.05  | Apple, Fruity                     |
| Trimethylpyrazine               | 0.23  | 0.15  | 0.14  | 0.09  | Cocoa, Earthy, Musty, Peanut      |
| Propanal                        | 11.02 | 9.82  | 3.47  | 4.19  | Etheral, Pungent, solvent          |
| 2-Methylfuran                   | 7.40  | 6.50  | 2.26  | 3.19  | Burnt, Chocolate, Metallic, Musty |
| 1-Butene                        | 1.57  | 1.63  | 0.57  | 0.38  | -                                 |
| 2-Methyl-2-cyclopenten-1-one    | n.d.  | 0.03  | 0.09  | 0.22  | -                                 |
| 2-Methylbutane                  | 0.49  | 0.50  | 0.17  | 0.17  | Almond, Herbaceous, Malty, Pungent |
| Ethanol                         | 3.22  | 4.70  | 3.71  | 1.35  | Alcoholic, Ethanol, Pungent, Sweet |
| Acetaldehyde                    | 7.63  | 10.85 | 8.13  | 2.82  | Etheral, Fresh, Fruity, Pungent   |
| Ethyl 2-methylbutyrate          | 0.26  | 0.32  | 0.15  | 0.27  | Apple, Blackberry, Fruity         |
| Homofuraneol                    | 0.48  | 0.53  | 0.46  | 0.32  | Caramelized                       |
| Pentane                         | 0.34  | 0.35  | 0.11  | 0.03  | Alkane                            |
| Ethylbenzene                    | 0.14  | 0.19  | 0.16  | 0.07  | Ethereal, Floral, Sweet           |
| Nonan-2-one                     | 0.84  | 0.44  | 0.13  | 0.16  | Baked, Earthy, Fatty, Fruity     |
| Ethyl isobutyrate               | 0.05  | n.d.  | n.d.  | n.d.  | Fruity, Rubber, Strawberry, Sweet |
| Ethylidimethylpyrazine          | 1.90  | 1.34  | 0.48  | 0.48  | Ethereal, Floral, Sweet           |
| 1S-(−)-alpha-pinene             | n.d.  | 0.04  | 0.04  | 0.03  | Herbaceous, Terpenic              |

n.d. means not detected. – means not determined.
Electronic Nose Heracles II (Alpha M.O.S. Inc., Toulouse, France) was used to analyze and identify aroma compounds in the investigated chocolates. Twenty-three odors were detected by Electronic Nose analysis of the investigated chocolates. Aroma compounds such as acetic acid, propyl acetate, 2-methylbutanal, cis-3-hexenol, tert-butylmethyl ether, 2,3-butanediol, methyl 2-methylbutanoate, trimethylpyrazine, propanal, 2-methylfuran, 1-butene, 2-methyl-2-cyclopenten-1-one, 2-methylbutane, ethanol, acetaldehyde, ethyl 2-methylbutyrate, homofuranol, pentane, ethylbenzene, nonan-2-one, ethyl isobutyrate, ethenyl-dimethylpyrazine, 1s-(-)-alpha-pinene were identified and their odor attributes were described as shown in Table 4. The predominant aroma compound was acetic acid in all of the tested chocolates. While the acetic acid proportion of chocolate L had the highest value 68.43% among total aroma substances, the acetic acid in chocolate Y had the lowest fraction of 58.05%. Zyżelewicz et al. [26] also reported that the predominant volatile organic compound was acetic acid present in concentrations from about 75.6 to over 79% of total volatile substances. The chocolate L was characterized by the highest proportion of acetic acid among the investigated chocolates. This was reflected in the sensorial evaluation results as displayed in Table 3. Chocolate L was rated the lowest point of overall acceptability by the panel. Other high proportions of aroma compounds for chocolate N, Y, L and A were propanal (11.02%), acetaldehyde (10.85%), acetaldehyde (8.13%) and 2-methylbutanal (16.51%), respectively. Chocolate A had the highest proportion of 2-methylbutanal among the tested samples. As a result, its consumer acceptability was rated the highest point (Table 3). This is confirmed by Owusu et al. [37], who reported that the most important odor in chocolate was 2-methylbutanal and 3-methylbutanal, with a cocoa, chocolate attribute. The trimethylpyrazine and ethenyl-dimethylpyrazine occurred in all tested samples were an indicator of high temperature roasting. These pyrazines are the most important groups of aroma compounds formed as a result of Maillard reaction [26].

3.8. Principal Components Analysis (PCA)

PCA was carried out to determine the most important aroma compound in the investigated chocolates. The PCA can compact the data based on their similarities and differences, by reducing the number of dimensions without losing much of information, and can define the number of “principal components.” Two first principal components (PC1 and PC2) are generated and are sufficient to explain the maximum variance in all original information [38,39]. PCA divides the data into distinct groups that best describe the relationships. PC1 describes the statistical relationship that accounts for the greatest amount of sample variation, followed by PC2, PC3, etc.; each corresponds to a decreasing variation in the sample set [40]. The information of components in PCA was shown in Table 5. PC1 and PC2 are adequate to explain 54.13% and 23.40% of the total variance respectively. The factor loadings for aroma compounds of chocolate are shown in Table 6. Higher loading scores indicate a tighter association with the corresponding principal component. Loadings with an absolute value higher than 0.25 in PC1 represent a strong impact on the flavor of chocolate. Acetic acid, propyl acetate, 2-methylbutanal, cis-3-hexenol, tert-butylmethyl ether, 2,3-butanediol, methyl-2-methylbutanoate, and trimethylpyrazine are among aroma compounds that had the greatest influence on the total explained variance. On the other hand, PC2 are mainly influenced by the following aroma compounds, such as 1s-(-)-alpha-pinene, ethylbenzene, nonan-2-one, homofuranol, acetaldehyde, ethyl, and ethenyl-dimethylpyrazine.

| Table 5. Information of components in PCA |
|------------------------------------------|
| Eigen Value | % of Variance | Cumulative, % |
| PC1         | 12.459       | 54.13          | 54.13          |
| PC2         | 5.382        | 23.40          | 77.54          |
| PC3         | 1.573        | 6.84           | 84.37          |
| PC4         | 1.369        | 5.95           | 90.33          |
| PC5         | 0.711        | 3.09           | 93.42          |
| PC6         | 0.509        | 2.21           | 95.63          |
| PC7         | 0.388        | 1.68           | 97.32          |
| PC8         | 0.245        | 1.07           | 98.38          |
| PC9         | 0.157        | 0.68           | 99.07          |
| PC10        | 0.089        | 0.38           | 99.45          |
| PC11        | 0.047        | 0.20           | 99.66          |
| PC12        | 0.035        | 0.15           | 99.81          |
| PC13        | 0.026        | 0.11           | 99.92          |
| PC14        | 0.009        | 0.04           | 99.96          |
| PC15        | 0.004        | 0.02           | 99.98          |
| PC16        | 0.003        | 0.01           | 99.99          |
| PC17        | 0.001        | 0.00           | 100.00         |
| PC18        | 0.001        | 0.00           | 100.00         |
| PC19        | 0.000        | 0.00           | 100.00         |

PCA has a great advantage to provide a better visualization of the variation in the tested samples and to find correlations between the different variables [41]. The resulting score and loading plot are shown in Figure 2 and Figure 3. Table 5 showed the eigen value and the percentage of variance for each calculated principal components and their cumulative percentage. PC1 explained 54.13% of the total variance in the sample, whereas PC2 explained an additional 23.40%. Figure 2, showing chocolate samples produced from different fermentation boxes, pictures the layout of data for PC1 and PC2. This score plot depicts 77.53% of the total variance. Chocolate samples was independently separated into four groups, each in a different quadrant with the exception of an overlapping occurred in the first and the fourth quadrant for chocolate from box Y. Chocolates from box N and box Y differ in PC2 but have similarities in PC1. On the other hand, box N and box A chocolate samples have similarities in PC2 but differ in PC1. The chocolate samples from box L differs in PC1 and PC2 from all the others. From a sensory point of view, it can be expected that the sensory quality of chocolate L should be different from the others [42]. This finding is confirmed by the results of sensory evaluation, as shown in Table 3. The chocolate L had the worst overall acceptability rating (3.97 points), which were significantly lower than those of chocolate N, Y and A (p<0.05). In addition, the chocolate samples from box A differs from all the others, having
negative values in PC1 and PC2, indicating a high content of 2-methylbutanal, which is one of the most important odorants for chocolate. The PCA loading plot of aroma compounds in the investigated chocolate from different fermentation boxes was shown in Figure 3. It gives a better visualization of the distribution of these aroma compounds. Acetic acid, propyl acetate, 2-methylbutanal, cis-3-hexenol, tert-butylmethylether, 2,3-butanediol, methyl-2-methylbutanoate, and trimethylpyrazine are positioned at the left side with similar weighting scores on PC1, and 1S-(-)-alpha-pinene, ethylbenzene, and homofuraneol are positioned at the top with higher weighting scores on PC2.

Table 6. Principal components factor loadings for aroma compounds of chocolate

| Compound                  | PC1    | PC2    | PC3    |
|---------------------------|--------|--------|--------|
| Acetic acid               | -0.28  | -0.043 | 0.025  |
| Propyl acetate            | -0.278 | -0.011 | -0.003 |
| 2-Methylbutanal           | -0.272 | -0.091 | 0.027  |
| cis-3-Hexenol             | -0.268 | 0.118  | -0.037 |
| Tert-Butylmethylether     | -0.261 | -0.098 | 0.075  |
| 2,3-Butanediol            | -0.26  | 0.135  | -0.05  |
| Methyl 2-methylbutanoate  | -0.257 | -0.125 | -0.063 |
| Trimethylpyrazine          | -0.254 | -0.026 | 0.258  |
| Propanal                  | -0.241 | -0.210 | -0.036 |
| 2-Methylfuran             | -0.24  | -0.213 | -0.029 |
| 1-Butene                  | -0.226 | -0.153 | -0.338 |
| 2-Methyl-2-cyclopenten-1-one | -0.222 | 0.119  | -0.015 |
| 2-Methylbutane            | -0.214 | -0.19  | -0.335 |
| Ethanol                   | -0.196 | 0.284  | -0.181 |
| Acetaldehyde              | -0.19  | 0.29   | -0.196 |
| Ethyl 2-methylbutyrate    | -0.179 | 0.063  | 0.197  |
| Homofuraneol              | -0.135 | 0.3     | 0.02   |
| Pentane                   | 0.133  | -0.036 | -0.583 |
| Ethylbenzene              | -0.111 | 0.365  | 0.092  |
| Nonan-2-one               | -0.098 | -0.337 | 0.265  |
| Ethyl isobutyrate         | 0.067  | -0.167 | -0.303 |
| Ethynyl-dimethylpyrazine  | -0.028 | -0.251 | 0.252  |
| 1S(-)-alpha-pinene        | -0.02  | 0.405  | 0.074  |

Figure 2. PCA score plot of PC1 and PC2 showing the distribution of chocolates produced from different fermentation boxes

Figure 3. Loading plot of the first two principal components for aroma compounds of chocolate from different fermentation boxes
4. Conclusions

Four designed fermentations were conducted in separated wooden boxes with different strains inoculum. The quality of chocolates produced by the defined inoculum varied with each other except for the FP and PAC content. The four inoculated fermentation methods showed different results in the rheological, textural properties, and color parameters of the end product chocolates. Furthermore, they influenced the types and numbers of aroma compounds generated during fermentation. However, the impact is mainly on their levels of aroma compounds. From sensory point of view, chocolates produced from the inoculation of Acetobacter aceti BCRC12324 during fermentation had the highest score point of overall acceptability among the investigated chocolates. The high levels of 2-methylbutanal content occurred in chocolate A could be an adequate response of this finding. On the other hand, the quality of chocolate from fermentation with Lactobacillus plantarum B0091 inoculum was similar to the others, but some properties such as rheological, textural, and flavor profiles were inferior and less pronounced, leading to a slightly poorer response by the sensory panel. PCA was performed to distinguish the flavor profiles among the chocolates. It permits a better visualization of the differentiated groups in the investigated chocolates. Therefore, PCA is helpful to discriminate the aroma profiles of chocolate inoculated by different strains cocoa fermentation. This is the first attempt to manipulate the strains through the use of a starter culture during cocoa fermentation in Taiwan and the improvement of the chocolate quality is optimistic and promising.

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Conflicts of Interest

The author(s) declared no potential conflicts of interest with respect to the research.

References

[1] Campos-Vega, R., Nieto-Figueroa, K. H. and Oomah, B. D. "Cocoa (Theobroma cacao L.) pod husk: Renewable source of bioactive compounds", Trends in Food Science and Technology, 81: 172-184, 2018.
[2] Young, A. M. The chocolate tree. A natural history of cacao. Washington, DC: Smithsonian Institution Press. 1994.
[3] Martins, T. F., Palomino, O. M., Álvarez-Cilleros, D., Martin, M. A., Ramos, S. and Goya, L. "Cocoa Flavanols Protect Human Endothelial Cells from Oxidative Stress", Plant Foods for Human Nutrition, 75: 161-168, 2020.
[4] Brito, B. d. N. d. C., Chisté, R. C., Pena, R. d. S., Gloria, M. B. A. and Lopes, A. S. "Bioactive amines and phenolic compounds in cocoa beans are affected by fermentation", Food Chemistry, 228: 484-490, 2017.
[5] Bortolini, C., Patrone, V., Puglisi, E. and Morelli, L. “Detailed analyses of the bacterial populations in processed cocoa beans of different geographic origin, subject to varied fermentation conditions”, International Journal of Food Microbiology, 236: 98-106, 2016.
[6] Koné, M. K., Guéhi, S. T., Durand, N., Ban-Koffi, L., Berthiot, L., Tachon, A. F., Brou, K., Boulanger, R., and Montet, D. “Contribution of predominant yeasts to the occurrence of aroma compounds during cocoa bean fermentation”, Food Research International, 89: 910-917, 2016.
[7] Ho, V.T.T., Zhao, J. and Fleet, G. “Yeasts are essential for cocoa bean fermentation”.International Journal of Food Microbiology”, 174: 72-87, 2014.
[8] Maura, Y.F., Balzarini, T., Borges, P.C., Evrard, P., Vuyt, L.D. and Daniel, H.-M. “The environmental and intrinsic yeast diversity of Cuban cocoa bean heir fermentations”, International Journal of Food Microbiology, 233: 54-63, 2016.
[9] Pereira, G.V.M.; Alvarez, J.P.; Neto, D.P. de C.; Soccol, V.T.; Tanobe, V.O.A.; Rogez, H.; Góes-Neto, A.; Soccol, C.R. “Great intraspecies diversity of Pichia kudriavzevii in cocoa fermentation highlights the importance of yeast strain selection for flavor modulation of cocoa beans”, LWT-Food Science and Technology, 84: 290-297, 2017.
[10] Ho, V.T.T., Zhao, J. and Fleet, G. “The effect of lactic acid bacteria on cocoa bean fermentation”, International Journal of Food Microbiology, 205: 54-67, 2015.
[11] Mota-Gutierrez, J., Barbosa-Pereira, L., Ferrocino, I. and Cocolin, L. “Traceability of Functional Volatile Compounds Generated on Inoculated Cocoa Fermentation and Its Potential Health Benefits”, Nutrients, 11: 884-908, 2019.
[12] Schwam, R. F. and Wheals, A. E. “The microbiology of cocoa fermentation and its role in chocolate quality”, Critical Reviews in Food Science and Nutrition, 44: 205-221. 2004.
[13] Mota-Gutierez, J.; Botta, C.; Ferrocino, I.; Giordano, M.; Bertolino, M.; Dolci, P.; Cannoni, M.; Cocolin, L. “Dynamics and biodiversity of bacterial and yeast communities during the fermentation of cocoa beans”, Applied and Environmental Microbiology, 84:1-19, 2018.
[14] Leal, G.A.; Gomes, L.H.; Efraim, P.; De Almeida Tavares, F.C.; Figueirêa, A. “Fermentation of cacao (Theobroma cacao L.) seeds with a hybrid Kluyveromyces marxianus strain improved product quality Attributes”, FEMS Yeast Research, 8: 788-798, 2008.
[15] Batista, N.N.; Ramos, C.L.; Ribeiro, D.D.; Pinheiro, A.C.M.; Schwam, R.F. “Dynamic behavior of Saccharomyces cerevisiae, Pichia kluveyri and Hanseniaspora uvarum during spontaneous and inoculated cocoa fermentations and their effect on sensory characteristics of chocolate, LWT-Food Science and Technology, 63: 221-227, 2015.
[16] Visintin, S.; Ramos, L.; Batista, N.; Dolci, P.; Schwam, F.; Cocolin, L. “Impact of Saccharomyces cerevisiae and Torulaspora delbrueckii starter cultures on cocoa beans fermentation”, International Journal of Food Microbiology, 257: 31-40, 2017.
[17] Ramos, C.L.; Dias, D.R.; Miguel, M.G. da C. P.; Schwam, R.F. “Impact of different cocoa hybrids (Theobroma cacao L.) and S. cerevisiae UFLA CA11 inoculation on microbial communities and volatile compounds of cocoa fermentation”, Food Research International, 84: 908-916, 2017.
[18] Mahazar, N.H.; Safian, N.F.; Moer Hussin, A.S.; Norhayati, H.; Mathawan, M.; Rukayadi, Y. “Candida sp. as a starter culture for cocoa (Theobroma cacao L.) beans fermentation”, International Journal of Food Microbiology, 22: 1783-1787, 2015.
[19] Meersman, E.; Steensels, J.; Paulius, T.; Struyf, N.; Saels, V.; Mathawan, M.; Koffi, J.; Vrancken, G.; Verstrepen, K.J. “Breeding strategy to generate robust yeast starter cultures for cocoa pulp fermentations”, Applied and Environmental Microbiology, 81: 6166-6176, 2015.
[20] Zýjelewicz, D., Nebesny, E., Motyl, I., & Libudzisz, Z. Effect of milk chocolate supplementation with lyophilized Lactobacillus cellulas on its attributes. Czech Journal of Food Science, 28: 392-406. 2010.
[21] Lin, Y. C., Choong, Y. M. and Chu, H. L. “Effects of Processing Time and Temperature on Flavanol and Procyanidin,
Proanthocyanin and Antioxidant Activity of Cocoa Bean in Taiwan”, Journal of Food and Nutrition Research, 9: 10-17, 2021.

Spectrophotometric assay for the quantification of the proanthocyanin (PACs) content in fruit and vegetable extracts. European Pharmacopoeia, 6.0, 01/2008: 1220.

Chu, H.L. and Lin, Y.C. “Antioxidant, antityrosinase activity and physicochemical properties of manufactured chocolates in Taiwan affected by roasting treatments”, Journal of Food and Nutrition Research, vol.113, 2011:300-307.

Melacci, D., Bendini, A., Tesini, F., Barbieri, S., Zappi, A., Vichi, S., Conte L., and Toschi, T.G. “Rapid direct analysis to discriminate geographic origin of extra virgin olive oils by flash gaschromatography electronic nose and chemometrics”, Food Chemistry, 204: 263-273, 2016.

Yimena, S.M., Kim, J. Y. and Kim, B. S. “Prediction of egg freshness during storage using electronic nose”, Poultry Science, 96: 3733-3746, 2017.

Zyżelewicz, D., Budryna, G., Oraczka, J. Antolakb, H., Kregielb, D. and Kaczmarskaa M. “The effect on bioactive components and characteristics of chocolate by functionalization with raw cocoa beans”, Food Research International, 113: 234-244, 2018.

Nielsen, D. S., Crafac, M., Jespersen, L., & Jakobsen, M. “The microbiology of cocoa fermentation”. In R. R. Watson, R. V. Peedy, & S. Zibadi (Eds.), Chocolate in health and nutrition (pp. 39–60). Totowa, NJ: Humana Press. 2013.

Pereira, G. V., Miguel, M. G., Ramos, C. L., & Schwan, R. F. “Microbiological and physicochemical characterization of small-scale cocoa fermentations and screening of yeast and bacterial strains to develop a defined starter culture”, Applied and Environmental Microbiology, 78: 5395-5405, 2012.

Barrientosaa, L. D. P., Oquendob, J. D. T., Garzónc, M. A. G. and Álvarezza, O. L. M. “Dynamics and species diversity of communities of lactic acid bacteria and acetic acid bacteria during spontaneous cocoa bean fermentation in vessels”, Food Microbiology, 28: 457-464, 2011.

R. B., Phillips, R. D., and Eitenmiller, R. R. “Chemometric aproach to fatty acid profiles in runner-type peanut cultivars by principal component analysis (PCA)”, Food Chemistry, 119: 1262-1270, 2010.

Miller, K. B., Stuart, D. A., Smith, N. L., Lee, C. Y., McHale, N. L., Flanagan, J. A., Ou, B. and Hurst, W. J. “Antioxidant Activity and Polyphenol and Procyanidin Contents of Selected Commercially Available Cocoa-Containing and Chocolate Products in the United States”, Journal of Agricultural and Food Chemistry, 4:4062-4068, 2006.

Chow, S. M., Kim, J. Y., and Kim, B. S. “Prediction of egg freshness during storage using electronic nose”, Poultry Science, 96: 3733-3746, 2017.

Yimena, S.M., Kim, J. Y. and Kim, B. S. “Prediction of egg freshness during storage using electronic nose”, Poultry Science, 96: 3733-3746, 2017.

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