MONASH University

Effect of selected yeast starter in cocoa fermentation: a study on antioxidant content, volatile organic compounds and sensory profile of Malaysian cocoa beans and chocolates produced

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Abstract

Antioxidant activity of cocoa beans is often influenced by drying and roasting stages. The first part of the study aimed to evaluate the effect of 13 naturally-existing yeast strains (indigenous yeast species that naturally present during cocoa fermentation) as starter culture in cocoa simulation media on total polyphenols content (TPC), total flavonoid content (TFC), fermentation index (FI), total soluble solids (TSS) and DPPH free radical scavenging activity (DPPH). The 13 naturally-existing yeast strains were namely *Pichia kudriavzevii* (GenBank accession number: MH979676, MH979680, MH979681, and MH979677), *Hanseniaspora thailandica* (GenBank accession number: MH979675), *Hanseniaspora* species (GenBank accession number: MH979678), *Wickerhamomyces* species (GenBank accession number: MH979679), *Saccharomyces cerevisiae* (GenBank accession number: MH979683), *Hanseniaspora oputiae* (GenBank accession number: MH979684) and *Candida quercitrusa* (GenBank accession number: MH979685, MH979687, MH979686 and MH979682). Yeasts were selected based on the phylogenetic analysis, where each species of the different genus (except the genus *Candida*) was used as a starter culture. Dried cocoa beans inoculated with isolates (*H. thailandica*, *P. kudriavzevii*, *H. opuntiae*, *Hanseniaspora* species, *Wickerhamomyces* species and *S. cerevisiae)* contained TPC, TFC and DPPH ranging from 21.82 to 69.81 mg/g Gallic acid, 1.68 to 6.33 mg/g Catechin and 113.85 to 328 μmoles/g Trolox, respectively. Based on the screening, *H. thailandica* and *P. kudriavzevii* are the potential starter cultures that result in cocoa beans with higher antioxidant content ($p \leq 0.05$) compared to natural fermentation.

Selected yeast species (*H. thailandica* (HT), *P. kudriavzevii* (PK) and a mixture of the two yeasts (Mix)) were used as starter cultures, respectively, in cocoa field fermentation (consisted of 20kg beans). The second part of the study aimed to determine the influence of these selected

yeast starters on the antioxidant content, volatile organic compounds (VOC) and sensory qualities of cocoa beans fermented. The correlation analyses suggested that phenolics and flavonoids compounds may not be the main constituents that led to the antioxidant activities of the fermented samples. Principal Component Analysis showed distinct differences in VOC profiles between spontaneous fermentation and fermentation using yeast. Sweet and spicy notes were the unique flavours that were only found in cocoa liquor produced using beans fermented with yeast starter cultures.

The third part of the study aimed to determine the rheological, melting properties, antioxidant content, VOC and sensory profile of chocolates produced using cocoa beans fermented with yeast starter cultures (PK, HT or Mix). The high enthalpy of melt of chocolate produced using cocoa beans fermented with HT was possibly associated with the higher fat content presented in the beans. The chocolate produced using cocoa beans fermented with HT resulted in the highest methylxanthines (caffeine and theobromine). VOC profiles of chocolates produced using beans fermented with HT, PK or Mix were noticeably different from Ghana and control chocolates (spontaneously fermented beans). Bitterness and astringency were the more intense flavour attributes in chocolates produced using beans added with yeast starters. The chocolate produced using beans fermented with PK was the most acidic; whereas chocolate produced using beans fermented with Mix had the sweetest taste. The usage of HT in cocoa fermentation is recommended as a potential yeast starter to improve the content of methylxanthines in cocoa.

Declaration

I hereby declare that this thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Print Name: Ooi Teng Sin

Date: 29-12-2020

Publications during enrolment

International peer-reviewed journals

i. Ooi, T. S., Ting, A. S. Y. & Siow, L. F. (2020). Influence of yeast starter cultures on antioxidant capacity of control-fermented cocoa beans: a simulation study, *LWT- Food Science and Technology, 122*. https://doi.org/10.1016/j.lwt.2019.108977
ii. Ooi, T. S., Ting, A. S. Y. & Siow, L. F. (2020). From field fermentation to cocoa liquor: a study on physicochemical properties, antioxidant content, volatile organic compounds and sensory profile of Malaysian cocoa beans added with yeast starter cultures. Submitted to *Journal of Food Preservation and Processing.*
iii. Ooi, T. S., Ting, A. S. Y. & Siow, L. F. (2020). Physicochemical properties, volatile organic compounds and sensory profile of dark chocolates made with Malaysian cocoa beans fermented with selected yeast starters. Submitted to *Journal of the Science of Food and Agriculture.*

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Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in peer-reviewed journals and two submitted publications. The core theme of the thesis is to study the influence of yeast starters (*P. kudriavzevii* and *H. thailandica)* towards the antioxidant, flavour and sensory of cocoa beans and chocolates produced. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Science under the supervision of Assoc. Prof. Siow Lee Fong.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researches and acknowledges input into team-based research.

Chapter 1 of the thesis is an introduction chapter. Chapter 5 of the thesis consists of general conclusions and future work. As for Chapter 2-4, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Publication Status	Nature and % of student contribution	Co-authors' name(s), nature and % of Co-author's contribution	Is the co-author a Monash student (Y/N)?
2	Influence of selected native yeast starter cultures on the antioxidant activities, fermentation index and total soluble solids of Malaysia cocoa beans: A simulation study	Published	70%, experimental design, conceptualization, methodology, experiment conduct, data collection, result interpretation, software, statistical analysis, validation, writing original draft, manuscript preparation and submission	A/P Siow Lee Fong 20% supervision, reviewing, resources and funding acquisition A/P Adeline Ting Su Yien 10% co-supervision, reviewing and resources	No No
3	Physicochemical properties, antioxidant content, volatile organic compounds and sensory profile of cocoa beans fermented with yeast starter cultures	Submitted	70%, experimental design, conceptualization, methodology, experiment conduct, data collection, result interpretation, software, statistical analysis, validation, writing original draft, manuscript preparation and submission	A/P Siow Lee Fong 20% supervision, reviewing, resources and funding acquisition A/P Adeline Ting Su Yien 10% co-supervision, reviewing and resources	No No
4	Physicochemical properties, volatile organic	Submitted	70%, experimental design,	A/P Siow Lee Fong	No

	compounds and sensory profile of dark chocolates made with Malaysian cocoa beans fermented with selected yeast starters		conceptualization, methodology, experiment conduct, data collection, result interpretation, software, statistical analysis, validation, writing original draft, manuscript preparation and submission	20% supervision, reviewing, resources and funding acquisition A/P Adeline Ting Su Yien 10% co-supervision, reviewing and resources	No

I have renumbered sections of submitted or published papers to generate a consistent presentation within the thesis.

Student name: Ooi Teng Sin

Student signature: **Date: 29-12-2020**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name: Assoc. Prof. Siow Lee Fong

Main Supervisor signature: **Date: 27-12-2020**

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List of abbreviations and acronyms

α	Alfa
β	Beta
BLAST	Basic Local Alignment Search Tool
CFU	Colony forming unit
℃	Degree Celsius
DNA	Deoxyribonucleic Acid
DSC	Differential scanning calorimetry
FI	Fermentation index
GAE	Gallic acid equivalents
GCMS	Gas chromatography mass spectrophotometer
h	Hour
ΔH	Melting enthalpy
HCL	Hydrochloric acid
HPLC	High performance liquid chromatography
HS-SPME	Headspace solid phase micro extraction
ITS	Internal Transcribed Spacer
MCB	Malaysian Cocoa Board
MeOH	Methanol
mg	Milligram
µl	Microliter
mL	Milliliter
Na_2Co_3	Sodium bicarbonate
OD	Optical density
PCR	Polymerase Chain Reaction
SD	Standard deviation
TAE buffer	Tris-acetate-EDTA buffer
T_c	Crystallisation temperature
TE buffer	Tris-EDTA buffer
T_m	Melting temperature

v/v	volume per volume
YEPD media	Yeast extract peptone dextrose media

Chapter 1

1.0 Introduction and Literature Review

1.1 Introduction

Cocoa fermentation is essential for the development of chocolate precursors in cocoa beans (Lopez & Dimick, 1995). There are two stages of cocoa fermentation which are the external and internal fermentations (Lopez & Dimick, 1995). External fermentation is where the microbes metabolizing on the mucilaginous pulp of cocoa beans; whilst internal fermentation is where biochemical reactions occur inside the cotyledon of cocoa bean for the formation of chocolate precursors (Lopez & Dimick, 1995). The microbial succession in the fermentation process is dominated by yeast, lactic acid bacteria, acetic acid bacteria, and spore-forming filamentous fungi (Schwan & Wheals, 2004).

Spontaneously fermented cocoa beans in Malaysia are often high in acidity, low in chocolate flavour and presence of undesirable flavours. Several starter cultures have been applied in cocoa fermentation and different flavours of chocolates were reported (Lefeber et al., 2012). The importance of yeast in cocoa fermentation and the development of chocolate flavour in cocoa beans have been extensively studied (Ho et al., 2014; Batista et al., 2016). However, there is no specific study on the influence of yeast as a starter culture on the antioxidant properties of the cocoa bean. Therefore, the current study determined the influence of selected yeast starters (*Hanseniaspora thailandica*, *Pichia kudriavzevii* and mixture of the two yeasts) on antioxidant content, volatile organic compounds (VOC) and sensory profile of cocoa beans produced during field fermentation (20 kg of cocoa beans). Two batches of cocoa fermentation were carried out to determine the reproducibility of the antioxidant content and organoleptic properties of cocoa beans produced by using the selected yeasts. The current study also provided insights on the rheological and melting properties, antioxidant content, VOC and sensory of chocolates produced using cocoa beans fermented with yeast starter cultures.

1.2 Literature review

1.2.1 *Theobroma cacao*

Cocoa (*Theobroma cacao* L.) is a member of the Sterculiaceae family. It is the main ingredient for chocolate and beverage (Kongor et al., 2016). Cacao can be referring to the botanical name of the cacao tree, while cocoa is usually referring to the fermented seed or fruit of the cocoa tree (Katz et al., 2011). Cocoa clones can be categorized into Forastero, Criollo, Trinitario and Nacional (Aprotosoaie et al., 2016). Forastero requires 3 to 7 days of fermentation while Criollo clone is fermented for a shorter duration of 2 to 3 days (Wood & Lass, 1985). The cocoa seed consists of the testa and white mucilaginous pulp. Cocoa pulp consists of 82-87% water, 10% sugar, 2-3% pentosans, 1-3% citric acid, and 1-1.5% pectin (Schwan & Wheals, 2004). The mucilaginous pulp is important for fermentation of cocoa beans (Lopez & Dimick, 1995).

Cocoa bean comprises of two types of storage cells: polyphenolic cells and lipid-protein cells. The polyphenolic cells contribute to 14-20% of dry bean weight. It is a single vacuole filled with polyphenols and alkaloids (caffeine, theobromine and theophylline) (Kongor et al., 2016). The lipid-protein cells contain cytoplasm which tightly packed with multiple small proteins, lipid vacuoles and starch granules which are necessities for the development of cocoa flavour and aroma (Kongor et al., 2016).

Cocoa fat consists of 95% triacylglycerols, 2% diacylglycerols, <1% monoacylglycerols, 1% polar lipids and 1% free fatty acids (Kongor et al., 2016). Cocoa butter comprises of saturated fat (stearic; 18:0, 35% and palmitic; 16:0, 25%), monosaturated fat (oleic; 18:1, 35%) and polyunsaturated linoleic (3%) (Kongor et al., 2016). The quality of

cocoa beans is determined by its flavour volatiles, nutritional composition, polyphenolic content and fermentative quality (Kongor et al., 2016).

1.2.2 Cocoa fermentation

Cocoa fermentation is the most crucial process in determining the flavour and quality of cocoa beans produced (Crafack et al., 2014). There is no chocolate flavour development without a proper fermentation (Ho, Zhao, & Fleet, 2014). The seeds within the ripe pod are freed from microorganisms (Schwan & Wheals, 2004). Once pods are opened, the pulp becomes contaminated with microorganisms which would kickstart the fermentation process. These microorganisms could come from the hands of workers, knives, unwashed equipment, baskets and boxes used during the pods handling process (Schwan & Wheals, 2004).

1.2.3 External fermentation

Cocoa fermentation consists of two stages, which are external and internal fermentation. External fermentation begins with the mucilaginous pulps, where the microorganism metabolizes the pectin and sugar in pulps around the beans (Lopez & Dimick, 1995).

Anaerobic and the aerobic phases are the two phases involved in the external fermentation (Lopez & Dimick, 1995). During the anaerobic phase, yeast metabolizes sugar and citric acid of pulps to produce ethanol, acetic acid and carbon dioxide. The initial pH of mucilaginous pulp at this stage is around 3.3 to 4.0. During microbe colonization, pulps collapse and lead to sweating. Sweating would then increase the pH and the condition becomes aerobic (Lopez & Dimick, 1995).

During the aerobic condition, lactic acid bacteria convert sugar into either lactic acid or acetic acid. Yeast activity is suppressed when the pH, alcohol level and aeration in the substrate

increase (Schwan & Wheals, 2004). However, acetic acid bacteria start to grow when the aeration is good. These bacteria involve in the exothermic process where ethanol is being oxidized into acetic acid and acetic acid is further oxidized into water and carbon dioxide (Schwan & Wheals, 2004). The exothermic process raises the temperature of fermenting mass to around 50 ˚C (Schwan & Wheals, 2004). Further increment of temperature above 50 ˚C is not conducive for the growth of acetic acid bacteria (Schwan & Wheals, 2004). When the condition becomes more aerobic, aerophilic spore-forming bacteria dominates the environment until the end of fermentation (Lopez & Dimick, 1995). A combination of ethanol, water and acetic acid diffuse into the cotyledon and induce the biochemical reaction inside the cocoa bean which is important to form chocolaty flavour (Schwan & Wheals, 2004).

1.2.4 Internal fermentation

Internal fermentation is where the biochemical reaction occurs inside the cotyledon of cocoa bean (Lopez & Dimick, 1995). Internal fermentation is vital for the development of chocolate flavour (Lopez & Dimick, 1995). Diffusion of alcohol and acetic acid into the cotyledon leads to nibs' acidification which then kickstarts the biochemical reaction in the cotyledon (Lopez & Dimick, 1995). There are two stages of internal fermentation which are anaerobic hydrolytic phase followed by an oxidative condensation phase (Lopez & Dimick, 1995).

A hydrolytic phase is started by bleaching of anthocyanin pigment from the storage cells, then following the diffusion of acids and water into the bean cotyledon (Lopez & Dimick, 1995). Majority of the flavour precursors are developed during the hydrolytic phase (Lopez & Dimick, 1995). A series of enzymatic oxidative reactions are initiated and resulted in browning of the cotyledons (Lopez & Dimick, 1995). During the anaerobic hydrolytic phase, cocoa glycosidases are triggered immediately after seed death and it hydrolyzes anthocyanin

pigments which are β-D-galactosidyl cyanidin and 3-α- L-aribinosidly cyanidin into sugar and cyanidin during the fermentation process (Lopez & Dimick, 1995). Both pigments are responsible for the purple colour of cocoa beans (Lopez & Dimick, 1995). This process decolorizes the purple colour in the cotyledon of cocoa beans (Lopez & Dimick, 1995).

The oxidative condensation phase occurs when oxygen starts diffusing into the cotyledon (Lopez & Dimick, 1995). The diffusion of oxygen into cotyledon activates the oxidase enzyme, leading to the oxidation of polyphenols (Lopez & Dimick, 1995). Production of o-quinones which happens during this stage is important and capable of reacting with compounds formed at the next stage of fermentation. O-quinones may polymerize to form diphenols and diphenol-quinones (Lopez & Dimick, 1995). Quinones take part in oxidation-reduction reactions with compounds that contain active hydrogen. They combine with amines, amino acids and thiols. Other aromatic compounds can be produced during this stage (Lopez & Dimick, 1995).

Oxidase activity continues during the drying stage under the sufficient moisture condition (Lopez & Dimick, 1995). The Maillard reaction occurs when amino acid and reducing sugars react with polyphenols oxidase enzymes for the browning process of cocoa beans (Barišić et al., 2019). Subsequently, the chocolate flavour is developed through these reactions (Barišić et al., 2019). Changes that occur in the pulp and the cotyledon of the cacao bean during fermentation are shown in Figure 1.1.

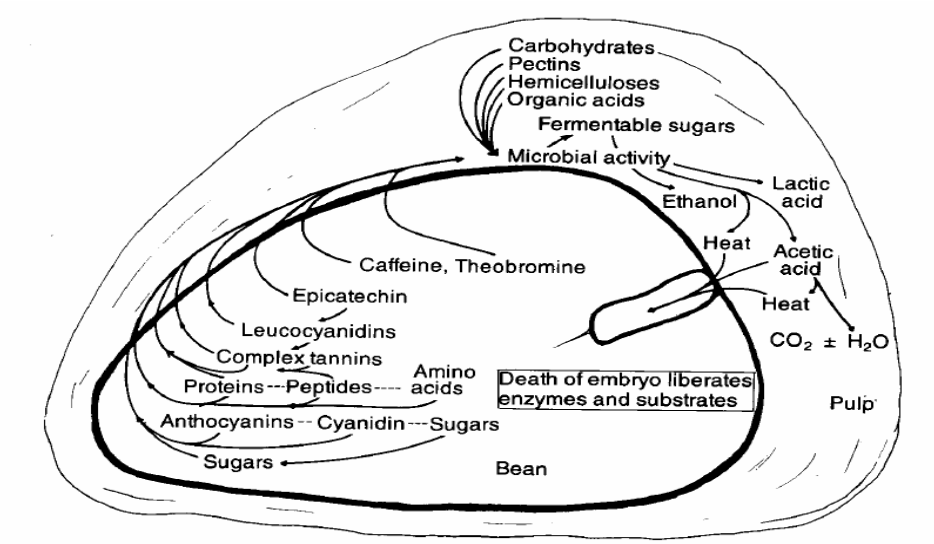


Figure 1.1 Biochemical changes in the cocoa bean pulp and cotyledon during fermentation (Lopez and Dimick, 1995)

1.2.5 Polyphenol compounds in cocoa beans

Polyphenols are closely associated with antioxidant and it has preventive effects towards cancer, cardiovascular, diabetes and age-related disorders (Wollgast & Anklam, 2000). Cocoa bean contains of 12-18% of polyphenols in the dry bean (Kim & Keeney, 1984). The colour and flavour of the cocoa beans are closely related to the polyphenols (Kim & Keeney, 1984). Polyphenols are derived from the secondary metabolism of plants (Wollgast & Anklam, 2000). Polyphenols of cocoa are stored in the pigment cells of cotyledons and depending on the number of anthocyanins. These pigments cells determine the colour of the bean cotyledons from white to deep purple (Wollgast & Anklam, 2000).

Polyphenols can differentiate into three main groups such as catechins or flavan-3-ols (37%), anthocyanins (4%) and proanthocyanidins (58%) (Wollgast & Anklam, 2000). It is reported that 35% of the polyphenol content of unfermented Forastero cocoa beans is mainly related with (-)-epicatechin (Kim & Keeney, 1984). (+)-catechin such as (+)-gallocatechin and (-)-epigallocatechin can also be found in smaller quantity (Wollgast & Anklam, 2000).

Cyanidin-3-α-Larabinosid and cyanidin-3-β-D-galactosid are main constituents of anthocyanin in cocoa beans (Wollgast & Anklam, 2000). Procyanidins consist mostly of flavan-3,4-diols (Wollgast & Anklam, 2000).

Camu et al. (2008) reported that alkaloids such as methylxanthines (caffeine and theobromine) and polyphenolic compounds such as proanthocyanidins and flavan-3-ols (epicatechin and catechin) give bitterness and astringency flavour in cocoa. During fermentation, 30% of alkaloids and 20% of polyphenols diffuse out of the beans, leading to a significant reduction in bitterness and astringency in cocoa bean (Camu et al., 2008). Cocoa beans polyphenol pigments are white to deep purple colour depending on the number of anthocyanins. The diffused polyphenols during fermentation would undergo oxidation and complexation to form insoluble tannins. Epicatechin and free anthocyanidins are converted by polyphenol oxidases into quinones. Quinones and polyphenols would interact with other polyphenols, proteins and peptides to form complexes, which contribute to the decrement of diffusion and astringency, therefore, giving rise to the brown colouration in cocoa beans (Camu et al., 2008). The summary of different polyphenols was shown in Table 1.1.

Table 1.1 Classification of polyphenols in cocoa beans or cocoa products (Wollgast & Anklam, 2000)

Types of polyphenols	
Catechins	• (-)-epicatechin • (+)-catechin • (+)-gallocatechin • (-)-epigallocatechin
Procyanidins	• Procyanidin B1= epicatechin-(4β→8)-catechin • Procyanidin B2= epicatechin-(4β→8)-epicatechin • Procyanidin B3= epicatechin –(4α→8)-catechin • Procyanidin B4 = catechin –(4α→8)-epicatechin • Procyanidin B5 = epicatechin –(4β→6)-epicatechin • Procyanidin C1= epicatechin (4β→8)-epicatechin- (4β→8)-epicatechin • Procyanidin C1= epicatechin –(4β→8)-epicatechin- (4β→8)- epicatechin • Procyanidin D = epicatechin –(4β→8)- epicatechin- (4β→8)- epicatechin-(4β→8)- epicatechin • Higher oligo- and polymers, mostly homologues of epicatechin with 2 to 18 monomeric units
Anthocyanins	• Cyanidin-3-α-L-arabinosid • Cyanidin- 3-β-D-galactosid
Flavanol glycosides	• Quercetin • Quercetin-3-*O*-glucoside (isoquercitin) • Quercetin-3- *O*-galactoside (hyperoside) • Quercetin-3-*O*-α-D-arabinoside • Quercetin-3-*O*-β-D-glucopuranosid
Flavone	• Apigenin • Apigenin-8-C-glucoside (vitexin) • Apigenin-6-C-glucoside (isovitexin) • Luteolin • Luteolin-7-O-glucoside • Dihydroquercetin • Dihydroxykaempferol • Kaempferol-rutinoside • Naringenin • Naringenin-glucoside • Myricetin-glucoside
Others	• Clovamide • Dideoxyclovamide • Caffeic acid • Chlorogenic acid • Coumaric acid • Ferulic acid • Phenylacetic acid • Phloretic acid • Protocatechuic acid • Syringic acid • Vanillic acid

1.2.6 Cocoa fermentation methods

1.2.6.1 Standard practice

The practice of cocoa fermentation methods is different according to a different country (Lopez & Dimick, 1995). Several basic fermentation methods such as shallow box fermentation and heap fermentation methods are common practice in countries such as Ghana and Malaysia (Wood & Lass, 1985). These methods involve heaping fresh beans to allow the proliferation of microorganism (Wood & Lass, 1985).

Malaysian Cocoa Board is practising shallow box fermentation in which the shallow box is covered with gunny sack to reduce heat loss. Shallow box fermentation is usually conducted in a building and covered by gunny sacks or banana leaves to retain the heat (Wood & Lass, 1985). Heap fermentation is placed on banana leaves which are spread out in a circle on the ground and covered with more leaves (Wood & Lass, 1985). Basket and tray fermentations are the other methods available to ferment the cocoa bean mass (De Vuyst & Weckx, 2016). Basket fermentation is commonly performed in Nigeria where the type of basket used can be of no definite size and are layered with leaves. The cocoa beans are also covered with leaves (Wood & Lass, 1985). Tray fermentation involves beans that are loaded into the trays (0.9m x 0.6m x 13 cm depth) and trays are stacked up to twelve or fourteen trays high (Wood & Lass, 1985).

1.2.6.2 Methods of quality enhancement by using starter cultures

Spontaneous fermentation often leads to cocoa beans with inconsistent quality (De Vuyst & Weckx, 2016). Several types of research have studied the influence of starter cultures in cocoa bean fermentation processes to enhance the sensory of chocolate produced. Two starter cultures composed of *Saccharomyces cerevisiae* H5S5K23, *Lactobacillus fermentum*

222 and *Acetobacter pasteurianus* 386B and another starter cultures composed of *Lactobacillus fermentum* 222 and *Acetobacter pasteurianus* 386B had been implemented (Lefeber et al., 2012). The application of both starter culture added fermentations produced chocolates with consistent chocolate flavour which is independent of cocoa producing region and fermentation method (Lefeber et al., 2012).

A recent study also applied microbial cocktail consists of *Saccharomyces cerevisiae* UFLA CCMA 0200, *Lactobacillus plantarum* CCMA 0238 and *Acetobacter pasteurianus* CCMA 0241 in cocoa fermentation and resulted in chocolate with attributes such as bitter, sweet and cocoa tastes (Moreira et al., 2018). Overall, the use of starter culture in cocoa bean fermentation is proven to overcome the variability in fermentation and flavour as compared to spontaneous fermentation (Lefeber et al., 2012; Moreira et al., 2018).

According to Batista et al., (2015), *Saccharomyces, Hanseniaspora* (anamorph *Kloeckera*) and *Pichia* are the leading genera during cocoa fermentation. It is found that fermentation with inoculated yeasts such as *Saccharomyces cerevisiae* and *Pichia kluyveri* prone to produce beans with coffee, sour and bitter flavour chocolate (Batista et al., 2015). Crafack et al., (2013) also mentioned that cocoa fermentation inoculated with *Kluyveromyces marxianus* KM16-6/ *Lactobacillus fermentum* L18/ *Acetobacter pasteurianus* A149 has been shown to produce the most bitter sour and astringent un-conched chocolate with the lowest sweetness and the lowest score for general liking. Thus, the use of yeast as starters, whether is used as a single or as a mixed starter culture, influenced the sensory outcome of the cocoa beans and its chocolate.

1.2.7 Yeast in cocoa fermentation

Yeasts are essential in cocoa fermentation and they are responsible for metabolizing sugar of cocoa pulp, production of ethanol and secondary metabolites (Ho, Zhao & Fleet, 2015). The ethanol enables the growth of acetic acid bacteria which causes nib's acidification and initiates the internal fermentation to produce chocolate precursors (Graham & Hugh, 2007). The by-products like secondary metabolites produced by yeast are mostly flavour-active and likely to be absorbed by cotyledons, therefore posing impact on chocolate flavour (Graham & Hugh, 2007). Studies have found that a mixed culture of yeasts as starter culture in cocoa fermentation produced beans of acceptable quality and flavour (Dircks, 2009). Besides, it has been reported that different starter cultures can influence the level of procyanidins in cocoa beans (Dircks, 2009).

Selected yeast species was also applied in green coffee bean fermentation where increment of antioxidant activity, TPC and TFC were observed (Haile & Kang, 2019b). During fermentation, fungal species such as *Rhizopus oligosporus* was reported to increase secretion of extracellular enzymes such as proteases and polysaccharide-degrading enzymes (cellulase, arabinose, xylanase and so on). The green coffee beans produced were also noted with improved aroma precursors (pyrazines, guaiacols, furanones and thiols) (Haile & Kang, 2019b).

A previous report had shown interesting correlations between yeast strain used for winemaking and phenolic composition of wine, revealing that yeast strain behaviour could somewhat modify chromatic properties, phenolic profile and antioxidant power of wine (Caridi et al., 2004). According to Caridi et al (2017), wine yeast starters were able to capture the greatest amount of anthocyanins and phenolic compounds from grape skins and the lowest amount of tannins from grape seeds.

The primary role of wine yeast in synthesizing flavour-active compounds is shown in Fig. 1.2 (Swiegers et al., 2005). Acetaldehyde is a major intermediate synthesized from yeast metabolism during fermentation (Fig. 1.2). It is a volatile compound with apple-like and nutty aroma (Swiegers et al., 2005). The amount of acetaldehyde increases over time due to oxidation of ethanol, the activity of film yeast, aeration and increase of fermentation temperature in wine (Swiegers et al., 2005).

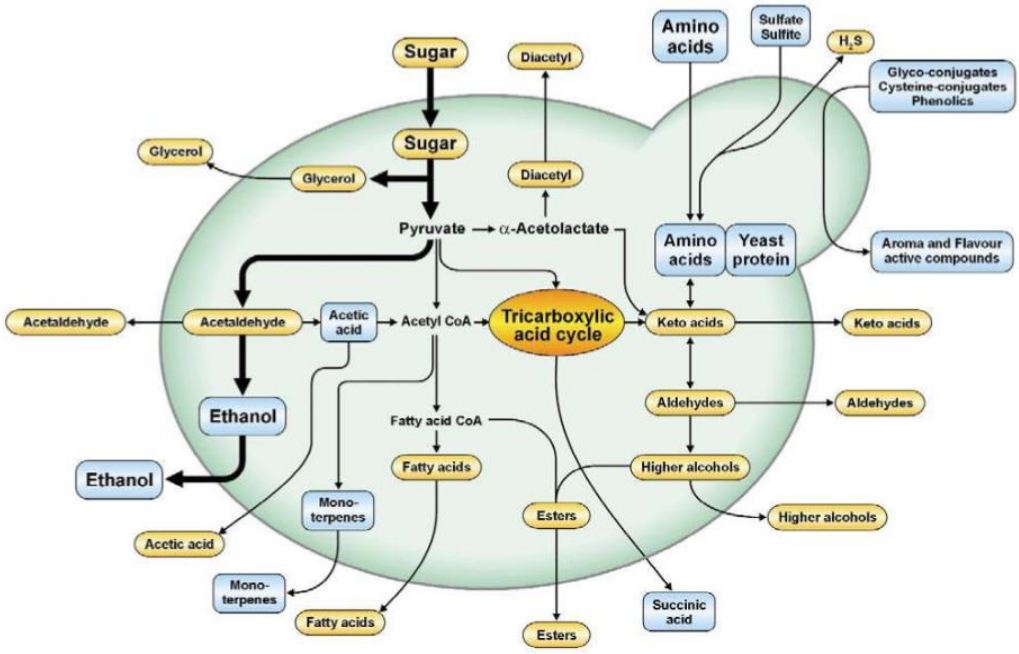


Figure 1.2 Synthesis of flavour-active compounds from sugar, amino acids and sulfur metabolism by wine yeast (Swiegers et al., 2005)

1.2.8 Drying of cocoa beans

Cocoa beans are dried to reduce its moisture content (6 to 8%) to prevent mould infestation during storage (Kongor et al., 2016). Methods such as sun-drying, artificial or forced air drying are used depending on the climatic conditions (Koya & Faborode, 2009). Sun drying is a simple, low cost and effective method as it does not need expensive devices as used in the artificial dryers. It is commonly carried out for 3 to 7 days under the sun by spreading beans

on the ground with one bean thickness to ensure even drying (Koya & Faborode, 2009). The artificial drying method is performed by the air-ventilated oven. It takes a shorter time for bean to be dried and thus is an advantage during the rainy season. Cocoa beans that are dried under forced convective drying are more acidic (Koya & Faborode, 2009).

Rapid drying process retains a higher amount of acetic acid in beans, leading to off-flavour (Kongor et al., 2016). Slow drying process leads to beans with low acidity, poorer colour and mould formation (Kongor et al., 2016). During drying, the initiation of polyphenol oxidase leads to major polyphenol oxidizing reactions in beans and results in brown colour beans (Kongor et al., 2016). Acidity, astringency and bitterness of cocoa beans can also be reduced through the drying process (Koya & Faborode, 2009).

1.2.9 Roasting of cocoa beans

Roasting is one of the crucial steps that govern the quality of cocoa beans produced. During roasting, volatile acids evaporate and lead to a decrement in acidity, sourness and bitterness of cocoa beans (Kongor et al., 2016). Flavour precursors such as short-chain peptides, free amino acids and reducing sugars also undergo the Maillard reaction and Strecker degradation to develop volatile organic compounds such as aldehydes, alcohols, esters, ketones, pyrazines and furans (Kongor et al., 2016). During Strecker degradation, the reaction of carbonyl derivative with free amino lead to the formation of aldehydes which eventually aids in flavour development (Kongor et al., 2016).

It is well known that temperature and roasting time affect the chemical and physical changes in cocoa beans (Kongor et al., 2016). The roasting conditions are often performed at 130˚C to 150 ˚C for 15 to 45 min (Kongor et al., 2016). However, these parameters vary depending on the type of cocoa clones, final cocoa product and the type of cocoa material

(beans or nibs) (Wood & Lass, 1985; Kongor et al., 2016). The manufacturer decides the degree of roasting according to preference (Reed, 2010). A low roast is generally preferred in Europe as it gives fruitiness for some of the cocoa beans varieties, leading to acidic and low bitter chocolate (Reed, 2010). Medium roast (115 to 135˚C) is preferred in the United States as cocoa flavours can be optimized using these processing conditions (Reed, 2010). High roasts (>140 ˚C) are applied for the blending of cocoa beans due to imbalance of cocoa flavours and high carbon burnt flavours (Reed, 2010). The Malaysian cocoa beans are reported to have the optimum cocoa flavour at a roasting temperature of 150 ˚C, for 30 to 40 mins (Ramli et al., 2006). Generally, the raw cocoa beans are roasted according to the properties of cocoa beans, typically at roasting temperature of 130 to 150 ˚C, for 15 to 45 mins (Ramli et al., 2006).

1.3.0 Cocoa bean flavour

The determining factor for which the cocoa bean is commercially accepted is the flavour (Kongor et al., 2016). Often, the cocoa beans flavour quality is influenced by several factors such as genotype and origin of the cocoa tree, pod storage, fermentation, drying and roasting of cocoa beans (Kongor et al., 2016). The origin of cocoa beans is one of the major causes of variation that results in different cocoa bean flavours (Reed, 2010). Forastero beans are considered as bulk chocolate while Criollo and Trinitario (a hybrid between Forastero and Criollo) are considered as bulk or fine cocoa (Santander et al., 2019). It is known that Criollo beans give rise to nutty flavour while Trinitario beans give rise to chocolate flavour with some fruitiness and other supplementary flavours (Wood & Lass, 1985).

Several major off-flavours that are commonly detected in cocoa beans are of excessive acidity, mouldy, smoky flavours and under-fermented beans (Wood & Lass, 1985). Excessive acidity is mainly associated with the acetic acid produced by bacteria which penetrate into cocoa bean cotyledons. Mould formation is not only resulting in off flavour beans but also

affect the appearance of cocoa beans. These mouldy beans would turn into black colour externally (Wood & Lass, 1985). Filamentous fungi particularly *Aspergillus, Penicillium* and *Fusarium* species may also lead to the development of mycotoxin in cocoa beans (Copetti et al., 2011). The smoky flavour is associated with contamination by smoke during drying or storage. These off-flavours are difficult to remove during cocoa beans processing (Wood & Lass, 1985). Under-fermented beans are bitter and astringent due to the high levels of polyphenols (Schwan & Wheals, 2004).

1.3.1 Volatile organic compounds in cocoa beans

The volatile organic compounds are derived from aroma precursors, which are formed during fermentation. Most of the volatile organic compounds are developed during the roasting stage (Castro-Alayo et al., 2019). There are more than 600 flavour compounds detected in cocoa beans and cocoa products (Crafack et al., 2014). Several main groups of volatile organic compounds are known as aldehydes, esters, ketones, alcohols, pyrazines and acids. Other compounds such as furans, pyrones, quinoxalines, lactones, pyrroles and diketopiperazines are also reported (Aprotosoaie et al., 2016).

Alcohol compounds are developed during the fermentation process as a result of microbial activity and heat degradation of amino acids (Aprotosoaie et al., 2016). Alcohols such as 3-methyl-1-butanol, 3-methyl-2-butanol, 2 methyl-1-propanol, 1,3-butanediol, 2,3-butanediol and phenylethyl alcohol are produced during the fermentation (Rodriguez-Campos et al., 2012). While alcohol compounds such as 3-methyl-1-butanol and phenyl-lacetaldehyde are produced as a result of amino acids catabolism during the fermentation (Rodriguez-Campos et al., 2012). Alcohols mainly deliberate fruity, green and floral aromas (Aprotosoaie et al., 2016). High intensity of alcohol content is preferred as it leads to chocolate with flowery and candy notes (Rodriguez-Campos et al., 2012).

Aldehydes and ketones are also vital for a good chocolate flavour (Aprotosoaie et al., 2016). These compounds are products of Strecker degradation particularly during roasting (Aprotosoaie et al., 2016). Aldehyde compounds such as 2-methylbutanal and 3-methylbutanal confer malty and chocolate notes, while 5-methyl-2-phenyl-2-hexenal confers a bitter cocoa note in cocoa (Aprotosoaie et al., 2016). Acetophenone and acetoin are two key compounds for ketones (Aprotosoaie et al., 2016). Acetophenone confers sweet and floral notes in cocoa beans, whereas acetoin is the precursor of tetramethylpyrazine which confers chocolate and cocoa flavours (Rodriguez-Campos et al., 2012).

Esters as the second most important volatile compound group confer a fruity flavour in cocoa (Rodriguez-Campos et al., 2012). During fermentation, esterification of amyl alcohols to amyl acetates should be avoided as it would lead to the bad flavour of cocoa beans (Rodriguez-Campos et al., 2012). The 2-phenylethyl acetate is produced as a result of yeast metabolism during the fermentation (Rodriguez-Campos et al., 2012). This compound is favourable in the cocoa aroma as it confers flowery and honey flavour notes (Rodriguez-Campos et al., 2012).

Acids are often associated with acetic acids which confer sour and vinegar-like aroma in the cocoa beans (Aprotosoaie et al., 2016). Several acids that often produce off-flavour in cocoa beans are known as isobutyric, isovaleric and propionic acids. These acids are produced by *Bacillus* spp. during the end of fermentation (Rodriguez-Campos et al., 2012). Isobutyric, isovaleric and propionic acids result in rancid, buttery and hammy flavour notes which are undesirable for cocoa products (Rodriguez-Campos et al., 2012).

Pyrazines consist of 40% of the aromas in roasted cocoa (Rodriguez-Campos et al., 2012). Tetramethylpyrazine is one of the key pyrazine compounds that deliberate roasted, nutty and chocolate flavour aromas (Rodriguez-Campos et al., 2012). It is known that clone

variations have different concentration of pyrazines (Aprotosoaie et al., 2016). The criollo clone has the highest pyrazine, whereas Nacional clone has the lowest pyrazine concentrations (Aprotosoaie et al., 2016). The study also reported that the absence of yeast in fermentation results in a product with fewer pyrazines and chocolate aromas (Aprotosoaie et al., 2016).

1.3.2 Processing of cocoa beans into chocolate

The processes of chocolate making were shown in Fig. 1.3. After the roasting process, cocoa beans were deshelled into cocoa nibs during the winnowing process (Wood & Lass, 1985). Then, alkalization is performed to enhance the colour and flavour of cocoa (Aprotosoaie et al., 2016). Alkalization can also be done prior to roasting. This process would reduce the beans' astringency and bitterness by complex polymerization of polyphenols (Aprotosoaie et al., 2016). Blending and milling are carried out subsequently for cocoa liquor production. Cocoa mass is separated into cocoa powder and cocoa butter during the hydraulic pressing process (Wood & Lass, 1985).

To produce plain chocolate, a mixture of cocoa nibs, cocoa butter and sugar are used (Wood & Lass, 1985). The mixture is ground and refined to smooth chocolate during the conching process. Conching improves the flavour of chocolate as off-flavours would diminish during the conching process. Overall, dark chocolate is conched at 70 to 82˚C (Aprotosoaie et al., 2016). The tempering process comprises of the pre-crystallization procedure to obtain stable βV form by exposing the chocolate to a specific temperature (Svanberg et al., 2013). Moulding and enrobing are applied right after the tempering process (Wood & Lass, 1985).

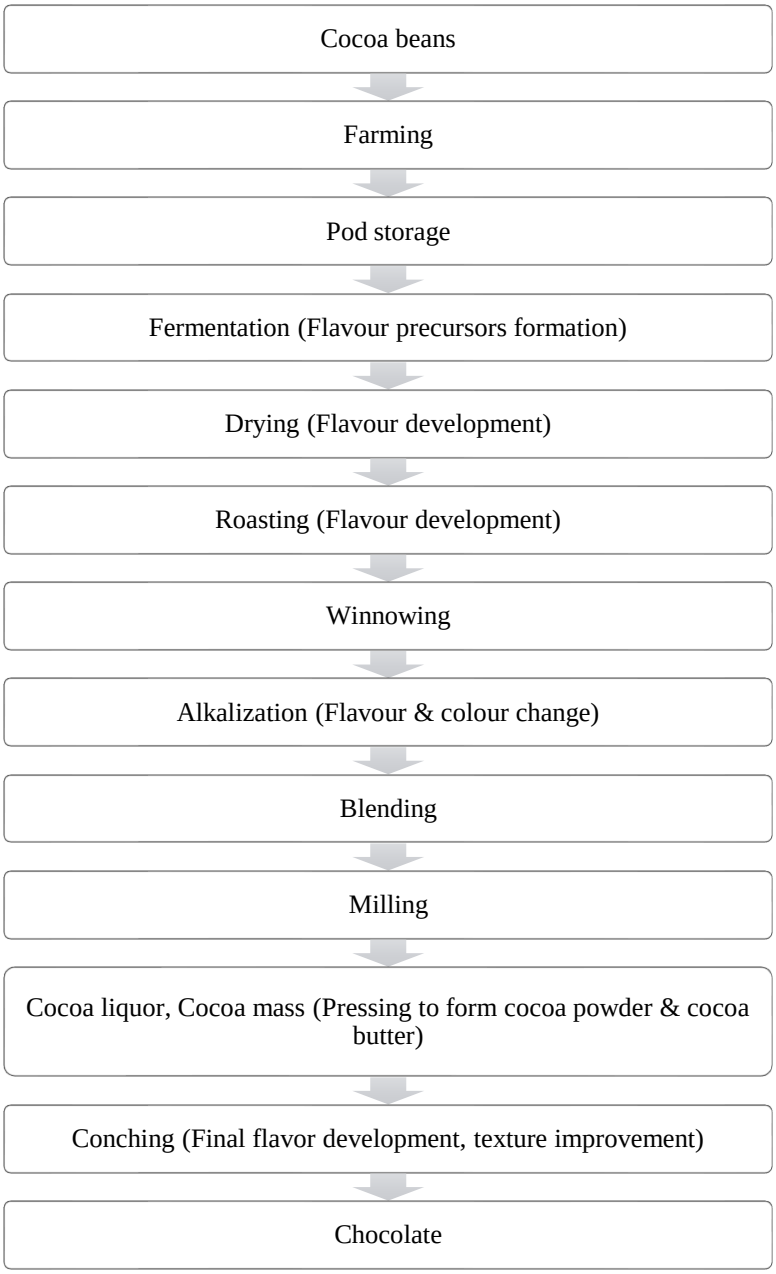


Figure 1.3 Chocolate making processes (Aprotosoaie et al., 2016)

1.4 Problem Statement

Spontaneous cocoa fermentation often leads to inconsistency of beans quality. Several studies from preliminary work, lab scale to pilot scale fermentations have been done to control cocoa fermentation and also to examine the microbial influence on the final quality of cocoa beans (Meersman et al., 2013; Racine et al., 2019). Research has reported that the usage of several starter cultures in fermentation can produce unusual flavours of cocoa beans. Despite the existing literature has demonstrated the potential of yeast starter culture in controlled fermentation to improve the flavour of cocoa beans; there is a lack of literature showing how such fermentation would impact the antioxidant activities of the cocoa beans. Hence, it is crucial to have a better understanding of the antioxidant activities of cocoa beans inoculated with starter cultures consisting of isolated yeast species from Malaysia. The 1st part of the study involves a simulation study that provides insight on the antioxidant activities of dried cocoa beans inoculated with yeast starter cultures.

The usage of certain yeast species in fermentation to improve the phenolic content of food is well known in wine, wheat bran, coffee bean and tea industries (Caridi et al., 2017; Kwak et al., 2018; Moore et al., 2007). However, this is yet to be further understood for cocoa fermentation. In a similar study, the usage of *P. kudriavzevii* in cereal-mix fermentation leads to a more organoleptically accepted product as well as increases the overall antioxidant capacity of cereal-based fermented product (Ogunremi et al., 2015). Though it is well known that starter culture affected the flavour profile of cocoa beans, few literatures on the antioxidant content of the cocoa beans produced by using selected yeast starter (*H. thailandica, P. kudriavzevii* and a mixture of these two yeasts) in field fermentation were reported. Since yeast has been shown to enhance the antioxidant properties of fermented food products, it is our research interest to determine if the selected yeast starter influences the antioxidant content of

cocoa beans. In view of this, the influence of yeast starter on antioxidant content, volatile organic compounds (VOC) and sensory profile of cocoa beans produced during the field fermentation were determined. Two batches of cocoa fermentation were carried out to determine the reproducibility of the antioxidant content and organoleptic properties of cocoa beans produced by using the selected yeasts.

Cocoa is one of the richest sources of antioxidant and it has a protective effect on the cardiovascular system (Urbańska & Kowalska, 2019). Often, these antioxidant compounds are degraded and destructed by high temperature applied during the cocoa beans processing (Urbańska & Kowalska, 2019). It is known that the source of beans, and chocolate making processes influence the polyphenols content of chocolate products (Maleyki & Ismail, 2008). Furthermore, the chocolate making processes and sensorial characteristics of chocolate are often determined by parameters based on the rheological properties (Cahyani et al., 2019). It is known that certain yeast strain produces wine with higher values of total polyphenols and monomeric anthocyanins (Ooi et al., 2020). However, there is a limited report available on the rheological and melting properties, antioxidant content, VOC and sensory of chocolates produced using cocoa beans fermented with the selected yeast starter culture. Therefore, it is vital to have a better understanding of the rheological and melting properties, antioxidant content, VOC and sensory profile of chocolates produced using beans fermented with the selected yeast starter culture.

1.5 Research questions

The specific research questions were:

1. How would the naturally-existing yeasts isolated from spontaneous fermentation (indigenous yeast species that naturally present during cocoa fermentation) influence the fermentation parameters and antioxidant properties of cocoa beans during a lab simulation? (Chapter 2)
2. What is the effect of selected yeast towards the fermentation parameters (pH of pulps, pH of nibs, total soluble solids, fermentation index, cut test and pH of dried nibs) and antioxidant properties (TPC, TFC, DPPH, ABTS scavenging activity) of cocoa beans in the field fermentation (20kg cocoa beans)? Is it reproducible when the same selected yeast is applied in a different batch of fermentation? (Chapter 3)
3. Does the addition of selected yeast starters in the field fermentation (20kg cocoa beans) influence the volatile organic compounds and sensory of cocoa beans produced? (Chapter 3)
4. Does the addition of yeast starters in fermentation influence the physicochemical properties, antioxidant properties of phytochemicals (TPC, TFC and DPPH free radical scavenging activity), polyphenols (epicatechin and catechin), methylxanthines (theobromine and caffeine), flavour (VOCs) and sensory of the produced chocolates as compared to chocolates made from spontaneously fermented beans or standard Ghana chocolate? (Chapter 4)

1.6 Objectives:

i. To evaluate the effect of isolated yeast as starter culture in cocoa simulation media on total polyphenols content (TPC), total flavonoid content (TFC), fermentation index (FI), total soluble solids (TSS) and DPPH free radical scavenging activity (DPPH). (Chapter 2)

ii. To determine the effect of selected identified yeast species on the fermentation parameters (~~pH of pulps,~~ pH of nibs, total soluble solids, fermentation index, cut test and pH of dried nibs) and antioxidant properties (TPC, TFC, DPPH, ABTS scavenging activity) of fermented beans produced from 2 batches of field fermentation (20 kg of cocoa beans). (Chapter 3)

iii. To determine the volatile organic compounds (VOC) of cocoa beans using Head-space Solid Phase Micro Extraction (HS-SPME) Gas Chromatography-Mass Spectrophotometry (GCMS) and to determine the sensory acceptance of cocoa liquor produced. (Chapter 3)

iv. To characterize the physicochemical properties, antioxidant properties of phytochemicals (TPC, TFC and DPPH free radical scavenging activity), polyphenols ((-)-epicatechin and (+)-catechin), methylxanthines (theobromine and caffeine), flavour (VOCs) and sensory acceptance of chocolates produced from controlled fermented beans, spontaneously fermented beans and standard Ghana beans. (Chapter 4)

Chapter 2

Work presented in this chapter was **published** in *LWT-Food Science and Technology,* Volume 122, March 2020, 108977, pp. 1-8, as an article entitled “Influence of selected native yeast starter cultures on the antioxidant activities, fermentation index and total soluble solids of Malaysia cocoa beans: A simulation study”. DOI: 10.1016/j.lwt.2019.108977

2 Influence of selected native yeast starter cultures on the antioxidant activities, fermentation index and total soluble solids of Malaysia cocoa beans: A simulation study

2.1 Introduction

The rising popularity towards dark chocolate is stimulated by a growing interest in healthy living. Cocoa contains flavonoids (antioxidants) which are beneficial to improve cardiovascular health, blood flow and cognitive functions (Katz et al., 2011). Cocoa beans undergo fermentation to give rise to the chocolaty flavour of the cocoa beans. Cocoa fermentation involved microbes such as yeast which dominates the process, followed by lactic acid bacteria, and acetic acid bacteria that grow on the mucilaginous pulp of the cocoa beans. During fermentation, alcohols and acetic acid produced by microbes diffuse into the cotyledon of cocoa beans, leading to biochemical reactions within the bean, thus producing the chocolate precursors (Misnawi, 2008).

Yeasts have been used as starter culture in fermentation. Fermentations inoculated with yeasts such as *Saccharomyces cerevisiae* and *Pichia kluyveri* prone to produce beans with coffee, sour and bitter flavour chocolate (Batista et al., 2015). Crafack et al., (2013) also reported that cocoa fermentation inoculated with mixed inoculum (*Kluyveromyces marxianus* KM16-6/ *Lactobacillus fermentum* L18/ *Acetobacter pasteurianus* A149) has been shown to produce a high level of bitter-sourness and astringent chocolate with the lowest score of sweetness and general liking. There is no specific study on the influence of yeast as a starter culture on the antioxidant properties of the cocoa bean. However, wine yeast (*Saccharomyces cerevisiae)* is well known to cause a decrease in the phenolic content of wines (Caridi et al., 2004). In a study using different strains of *Saccharomyces cerevisiae* strain Sc2659 was reported to not only metabolized sugar (Brix) faster than strain Sc1483, but it also produced a

wine with significantly higher values of colour intensity, total polyphenols and monomeric anthocyanins compared to strain Sc1483 (Caridi et al., 2004). These preliminary results had shown interesting correlations between yeast strains used for winemaking and phenolic composition of wine, indicating that yeast strain behaviour may modify chromatic property, phenolic profile and antioxidant power of wine (Caridi et al., 2004).

Despite existing literature has demonstrated the potential of yeast starter culture in controlled fermentation to improve the flavour of cocoa beans; there is a lack of literature showing how such fermentation would impact the antioxidant activities of the cocoa beans. To the best of our knowledge, this is the first study on determining the antioxidant activities of cocoa beans after inoculated with a single native yeast species as starter culture using a simulation medium. The present research aims to determine the antioxidant activities of cocoa beans inoculated with starter cultures consisting of isolated yeast species from Malaysia. This simulation study provides insight on the antioxidant activities of dried cocoa beans inoculated with yeast starter cultures.

2.2 Materials and methods

2.2.1 Isolation of naturally-existing yeasts isolates

The spontaneous cocoa bean fermentation was performed to obtain naturally-existing yeast isolates (indigenous yeast species that naturally present during cocoa fermentation). Yeast isolates after identification were then applied as a starter culture using a cocoa simulation medium. Cocoa shallow box fermentation (31 x 31 x 31 cm^3) with 20 kg of wet cocoa beans were used for yeast isolation according to a method of Sulaiman et al. (2014). The cocoa fermentation process was carried out for five days and the turning process of cocoa beans was completed at 72 h of cocoa fermentation. The shallow cocoa box was firstly sterilized with 99.9% ethanol prior to loading of cocoa beans. The cocoa beans were filled up to approximately

29 cm compared to the height of the box (31cm). The box was covered with gunny sack once the beans were loaded. Mixed cocoa clones such as PBM 123, BR 25, MCBC 1 and MCBC 8 were bought from Malaysian Cocoa Board Jengka, Pahang for the fermentation process. The reason of using mixed cocoa clones was because it was to simulate a standard fermentation practice by small holder where mixed cocoa clones were used. Usually, the estate or farm plants a mixed variety of cocoa clones and harvests these pods at the same season.

Sample collection was conducted according to a modified method by (Papalexandratou et al., 2011). Samplings were conducted by randomly collecting (at 0, 6, 24, 48, 72, 96 and 120 h fermentation) cocoa beans from different points of the box. Samples were mixed and kept in a sterile polybag followed by storage in a freezer at -20°C for laboratory and microbial analysis.

2.2.2 Isolation of yeast

Yeast isolates were obtained from the collected samples by bean swab method as suggested by Daniel et al. (2009). The bean swab method was carried out where the cocoa bean was swabbed on the yeast extract medium and incubated at 25°C for yeast growth. Serial dilution was conducted according to Pereira et al. (2017) where 25 g of cocoa beans with pulp was added to 225 mL saline-peptone water (v/v 0.1% peptone (Merck, Germany), v/v 0.8% NaCl (Merck, Denmark), followed by homogenization using a stomacher at a normal speed for 5 min.

Yeast extract medium was used for yeast isolation. The media consisted of glucose 50 g (Fisher Scientific, UK), yeast extract 3.0 g (Becton, Dickinson and Company, France), KH_2PO_4 0.1 g (Hamburg Chemicals, UK), NaCl 0.1 g (Merck, Denmark), $CaCl_2$ 0.013 g (Friendemann Schmidt, Australia), distilled water 1 L and agar 15 g (Becton, Dickinson and Company, France). Medium was adjusted to pH 5.5 before autoclaved (Hirayama Hiclave HVE-50, Japan) at 121°C for 15 min. Yeast culture agar plates were incubated at 30°C for 48-

72 h. Yeast species were selected for pure culture preparation and identification by molecular technique. Individual colonies of each morphotype were subcultured and purified. Purified yeast isolates were preserved in 25% glycerol at -20°C (Hamburg Chemicals, UK) as a stock culture.

2.2.3 Identification of yeast isolates via DNA sequencing

2.2.3.1 gDNA extraction from pure cultures

Genomic DNA (gDNA) extraction protocol was according to (Daniel et al., 2009). Colonies of yeast from the pure yeast agar plate after three days of growth at 30°C were picked by using sterilized tooth picks and placed into the Eppendorf tube filled with 200 µL of lysis buffer solution (Triton X-100 10 mL, SDS 5 g, NaCl 2.922 g and Tris HCl 0.7882 g at pH 8.0, EDTA 0.1461 g at pH 8.0). The tube was heated in 70°C water bath for 3 min and kept frozen for 3 min in -80°C freezer. The previous step was repeated once followed by addition of v/v Chloroform (J.T. Baker, UK): isoamyalcohol (analytical UNIVAR reagent, Australia) (24:1). Tubes were then centrifuged (13000 rpm, 10 min) and an aqueous phase was then transferred to a tube. Two volumes of absolute cold ethanol (R&M chemicals, UK) was added to the aqueous phase and then the mixture was incubated overnight at -20°C. The tube was then spun at 13000 rpm for 10 min. The supernatant was discarded, and the pellet was air-dried under the airflow laminar for 10 min. 50 µL of Tris-EDTA (TE) buffer was added and the tube was incubated at -20°C.

2.2.3.2 PCR amplification

Primers ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') were used for PCR amplification. The PCR amplification was performed in a final volume of 50 µL containing 17.45 µL of PCR master mix direct load

(Biotech rabbit), 4 μL of DNA template and 28.55 μL of deionized water. For the PCR reaction, initial denaturation was conducted at 94°C for 4 min, followed by 29 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 2 min, extension at 72°C for 2 min and lastly final extension at 72°C for 10 min followed by cooling at 10°C for infinity. PCR was performed using a thermocycler (MJMini BIO-RAD, US). PCR product was then loaded and separated by electrophoresis in 1% (w/v) agarose gels in 1x Tris/Borate/EDTA (TBE) buffer at 80 V, 120 A for 40 mins before viewing by gel imaging viewer. The PCR products were stored at -20°C before purification and sequencing.

2.2.3.3 BLAST search for sequencing information

The sequencing information was edited and aligned by using Chromas Pro (version 1.7.4) and then analysed by Basic Local Alignment Search Tool (BLAST) through the National Centre for Biotechnology Information (NCBI) to identify the closest yeast species to the yeast isolates.

2.2.3.4 Fermentation of cocoa beans with the yeast starter culture

Cocoa medium was prepared in the laboratory according to a modified method of Pereira et al. (2012) in Erlenmeyer flasks. 500 mL of cocoa pulp media was placed in 500 mL of Erlenmeyer flasks to be used as a medium. The cocoa pulp media was added with 17 sg/L of fructose (Hamburg Chemicals, UK), 25 g/L of glucose (Fisher Scientific, UK), 10 g/L of citric acid (Fisher Scientific, UK), 5 g/L of yeast extract (Becton, Dickinson and Company, France), 5 g/L of peptone (Merck, Germany) and 20% (w/v) fresh cocoa seeds.

Yeast species was used as starter culture in fermentation after species identification. Yeasts were selected based on the phylogenetic outcome, where each species of the different

genus was used as a starter culture. The agar plate was prepared according to a method modified from (Pereira et al., 2012). Each yeast species was firstly cultured in 1.5 mL YEPD broth consisting of peptone (Merck, Germany) (1%), dextrose (Fisher Scientific, UK) (2%), and yeast extract (Becton, Dickinson and Company, France) (0.3%) (Shu & Johnson, 1947) (Pereira et al., 2012). The cultures were incubated overnight (12 h) at 30°C (Binder, Germany). The number of yeast cells in suspension was counted by hemocytometer in cells/ml (Hirschmann, Germany). Suspension of the culture with a concentration of 10^6 cells mL^{-1} was prepared by diluting the suspension with distilled water (Pereira et al., 2012). Yeast starter culture with 1.5 mL culture was mixed with 500 mL of cocoa pulps media in an Erlenmeyer flask and incubated at 30°C. Control was a spontaneous fermentation process with indigenous microorganisms present in the cocoa fruit. Samples were collected every 24 h until 120 h for analysis. Samples were then oven-dried at 38°C until 7.5% moisture content was obtained. Determination of moisture content was by gravimetric method with circulating air at 100˚C (AOAC 931.04).

2.2.4 Antioxidant activities

2.2.4.1 Sample extraction

Sample extraction was conducted according to a modified method of Ioannone et al. (2015). Ground sample (1.5g) was defatted 3 times by extracting with 10 mL of hexane and dried under a fume hood for 20 min. 1 g of dried and defatted material was extracted with 5 mL of 70:29.5:0.5 acetone/water/acetic acid (v/v/v) by mixing for 1 min in a vortex mixture and sonication for 10 min at room temperature. The mixture was spun at 2100 *g* for 10 min and then filtered with Whatman filter paper no 4. The filtered cocoa extract was used for all chemical analysis.

2.2.4.2 Total polyphenol content

The total polyphenol contents of the samples were determined according to a method of Ioannone et al. (2015). The sample extract was firstly diluted in a ratio of 1:999 µL methanol (J.T. Baker, UK), then 20 µL of diluted sample was taken to mix with 100 µL of Folin-Ciocalteau's reagent (diluted in 1:10 with water) (Merck, USA), followed by 75 µL of 10 % (w/v) sodium bicarbonate (Na_2CO_3, Friendemann Schmidt, Australia) (Hamburg Chemicals, UK) solution was added in the 96 wells plate. Colour was allowed to develop in the dark for at least 2 h. The absorbance of the sample was measured at 740 nm by using an ultraviolet-visible (UV-vis) spectrophotometer, (Perkin Elmer Lambda 25, USA). Standard curve to determine total polyphenol content was prepared using standard Gallic acid (Sigma Aldrich, USA) according to a modified method of Singleton and Rossi (1965). Standard Gallic acid (Sigma Aldrich, USA) samples of 10 known concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.00 mg/mL were prepared. The total polyphenol content of the sample was reported as milligram gallic acid equivalents (GAE) per gram defatted cocoa.

2.2.4.3 Determination of DPPH free radical scavenging activity

The DPPH free radical scavenging activity of well-dried cocoa samples was according to the modified method of Ee et al. (2019). 100 µL of the diluted sample was added to 200 µL of 2,2 diphenyl-1-pic-rylhydrazyl (DPPH) (Sigma Aldrich, USA). The mixture was then left to react for 30 min at room temperature in the dark. After incubation, absorbance was read at 515 nm using the microplate reader (Tecan, Switzerland). Ascorbic acid was used as a standard. Ethanol mixed with DPPH was used as a blank while water was used as a control. The scavenging effect was determined based on the percentage of DPPH free radical scavenging

activity using the equation below and expressed as micromole of Trolox per gram of defatted cocoa.

$$\%\ \text{DPPH radical scavenging activity} = \frac{\text{The absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \text{ x } 100\%$$

2.2.4.4 Total flavonoid content

Total flavonoid content was performed according to a modified method of Jia et al. (1999). The total flavonoid content was measured using spectrophotometric method. 0.25 mL of sample was mixed with 3 mL of deionized water and 0.3 mL of 5% $NaNO_2$ (R&M chemicals, UK). The mixture was then incubated at room temperature for 5 min. The mixture was then added with 1.5 mL of 2% aluminium chloride hexahydrate ($AlCl_3 \cdot 6H_2O$) (R&M chemicals, UK) and kept at room temperature for 5 min. Then, 2 mL of 1 mol/L sodium hydroxide (NaOH) (R&M chemicals, UK) was added. The flask was filled up to 10 mL with deionised water. The total flavonoid content was measured for its absorbance against a prepared reagent blank at 510 nm. Catechin (Sigma Aldrich, USA) was used as a standard and results are expressed as milligram catechin equivalents per gram of sample (mg CE/g).

2.2.4.5 Total soluble solids

The total soluble solids content of the fermented cocoa pulp for a sample was measured by using Digital Hand-held Pocket Refractometer PAL-1 (Atago PAL-1, Japan) where a small amount of the sample was placed on the refractometer to get reading. The cocoa pulp was firstly separated from its nib and placed into a Falcon tube. Distilled water was added volume per volume (v/v) into the tube and vortexed. The amount of total soluble solids content of pulp was recorded in Brix unit.

2.2.5 Fermentation index

Fermentation index of the fermented samples was determined according to a modified method of Gourieva and Tserevinov (1979). The cocoa nibs were ground with a blender (Panasonic MX-798S, Malaysia) into powder form. 0.5 g of cocoa powder was weighed by using a weight balance and put into a 100 mL of volumetric flask. Then, a mixture of methanol (J.T Bakers, UK) and hydrochloric acid (J.T Bakers, UK) (97:3) was added into the volumetric flask. A mixture of methanol and hydrochloric acid (97: 3) was used to penetrate the cocoa bean structure to degrade the pigment (polyphenol). The mixture was shaken and kept in refrigerated condition overnight. The samples were filtered using Whatman filter paper No.4. The absorbance of the filtrate was measured at wavelengths of 460 nm and 530 nm by using UV-visible spectrophotometer (Perkin Elmer Lambda 25, USA).

The ratio between absorbance 460 nm and 530 nm was used to measure the degree of fermentation of the cocoa beans. Fermentation index (FI) was calculated as follow:

Fermentation index (FI) = (Absorbance at 460 nm)/ (Absorbance at 530 nm)

According to Sulaiman (2014), fermented beans have FI value of >1, over-fermented beans have FI value of >1.6, whereby unfermented beans have FI value of <1.

2.2.6 Statistical analysis

Triplicates of analysis were performed. The data were analysed using one-way and two-way Analysis of Variance (ANOVA) followed by mean comparison using the Tukey test at $p \leq 0.05$ levels with Statistical Package for the Social Sciences (SPSS) version 23. The phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA X).

2.3 Results and Discussion

2.3.1 Isolation and identification of yeast species

In this study, 13 yeast isolates were obtained from the mucilaginous pulp. The DNA extraction followed by polymerase chain reactions (PCR) and subsequent sequencing of the DNA of 13 isolates led to the identification of 7 yeast species comprising of 5 genera namely: *P. kudriavzevii, H. thailandica, C. quercitrusa, S. cerevisiae, H. opuntiae, H.* species, and *W.* species (Table 2.1a, Table 2.1b and Figure 2.1a). The morphological characteristics of yeast identified were shown in Appendix A, Table A1.

The number of yeast colony during a spontaneous fermentation increased significantly from the beginning until after 120 h of fermentation (Table 2.1c, Figure 2.1b). There was no significant change in the number of yeast colony from after 0 h of fermentation until after 12 h of fermentation. The number of yeast colony increased insignificantly after 12 h of fermentation to after 24 h of fermentation stated as log 2.2 ± 0.38 CFU/ml. Then, there was a significant increment in the number of yeast colony from after 24 h of fermentation until after 36 h of fermentation which was recorded as log 2.9 ± 0.10 CFU/ml. After 36 h of fermentation, the number of yeast colony increased until after 48 h of fermentation, remained constant from 48 h to 60 h of fermentation then reduced after 72 h of fermentation. The number of yeast colony was found remained unchanged from after 72 h to 84 h of fermentation. The decrement or increment of number of yeast colony from after 36 h of fermentation until after 84 h of fermentation was not significant. In addition, the number of yeast colony decreased insignificantly from after 84 h of fermentation to after 96 h of fermentation recorded as log 2.8 ± 0.10 CFU/ml. There were no significant changes in the number of yeast after 96 h of fermentation until after 120 h of fermentation. The lowest number of yeast colony was found

after 0 h of fermentation and after 12 h of fermentation respectively recorded as log 2.1 ± 0.53 CFU/ml and log 2.1 ± 0.15 CFU/ml. Conversely, the highest number of yeast colony was after 48 h of fermentation and after 60 h of fermentation respectively stated as log 3.1 ± 0.12 CFU/ml and log 3.1 ± 0.10 CFU/ml.

The average of total soluble solids of freshly harvested cocoa beans from cocoa pod was 17.2 Brix. After 0 h of fermentation, the value of total soluble solids of cocoa beans was only 1.5± 0.06 Brix. Generally, the number of yeast colony was low when there were high total soluble solids presented on the cocoa beans at the beginning of fermentation until after 12 h of fermentation (Figure 1). After 12 h of fermentation, the number of yeast increased significantly until after 36 h of fermentation while the value of total soluble solids in cocoa beans reduced from after 12 h of fermentation to after 36 h of fermentation, stated as 0.6± 0.2 Brix. The number of yeast colony and total soluble solids of cocoa pulp remained neither significant increment nor decrement from after 36 h of fermentation until after 48 h of fermentation. After 60 h of fermentation, the number of yeast colony decreased insignificantly while the amount of total soluble solids in cocoa beans remained no significant changes until after 72 h of fermentation. The number of yeast colony does not have significant increment or decrement after 72 h of fermentation until after 120 h of fermentation in regardless of the fluctuation observed in total soluble solids of cocoa pulps from after 72 h of fermentation until the end of fermentation.

Based on the phylogenetic tree obtained (Fig. 2.1b), *H. thailandica, H. opuntiae, H.* species and *S. cerevisiae* were closely related to each other. While, *C. quercitrusa* and *W.* species were distant genera with the previous groups mentioned. *P. kudriavzevii* isolate 43, 7, 27 and 33 were from the same genus and were highly similar to each other based on the bootstrap value (Fig. 2.1). In the present study, *P. kudraivzevii* isolate 33 and isolate 43 were arbitrarily selected within isolates 43, 7, 27 and 33, *H. thailandica, S. cerevisiae, H. opuntiae,*

H. species and *W.* species were used as a starter culture in a cocoa pulp simulation medium in the subsequent study.

Table 2.1a Identification of yeast and their accession number deposited in NCBI gene bank

Yeast strain code	Accession number obtained from NCBI gene bank	Yeast identity
43	MH979676	*Pichia kudriavzevii*
6	MH979675	*Hanseniaspora thailandica*
27	MH979680	*Pichia kudriavzevii*
42	MH979685	*Candida quercitrusa*
47	MH979687	*Candida quercitrusa*
1	MH979686	*Candida quercitrusa*
33	MH979681	*Pichia kudriavzevii*
7	MH979677	*Pichia kudriavzevii*
4	MH979682	*Candida quercitrusa*
32	MH979683	*Saccharomyces cerevisiae*
46	MH979684	*Hanseniaspora opuntiae*
48	MH979678	*Hanseniaspora* species
49	MH979679	*Wickerhamomyces* species

Table 2.1b Sequencing information and similarity percentage (%) of yeast isolates

	Yeast isolate	Identities	Percentage, % matching with NCBI gene bank	Sequence ID obtained from NCBI gene bank	Accession number obtained from NCBI gene bank	Species
1.	43	384/437	88	FJ231424	MH979676	*Pichia kudriavzevii*
2.	6	376/376	100	AB501150	MH979675	*Hanseniaspora thailandica*
3.	27	426/426	100	KY457575	MH979680	*Pichia kudriavzevii*
4.	42	534/534	100	KF728785	MH979685	*Candida quercitrusa*
5.	47	477/477	100	MF574303	MH979687	*Candida quercitrusa*
6.	1	532/533	99	KF728785	MH979686	*Candida quercitrusa*
7.	33	427/427	100	KY457575	MH979681	*Pichia kudriavzevii*
8.	7	344/401	99	EU315755	MH979677	*Pichia kudriavzevii*
9.	4	537/537	100	KF728785	MH979682	*Candida quercitrusa*
10.	32	756/756	100	KF728774	MH979683	*Saccharomyces cerevisiae*
11.	46	661/662	99	KT226114	MH979684	*Hanseniaspora opuntiae*
12.	48	632/666	99	KM982972	MH979678	*Hanseniaspora* species
13.	49	466/506	92	JQ901906	MH979679	*Wickerhamomyces* species

Table 2.1c Measurements of temperature during fermentation, pH during fermentation, pH nibs, pH pulps, total soluble solids and yeast counts during a spontaneous fermentation from 0 to 120 fermentation periods (hrs)

Parameters	**Fermentation periods (hrs)**										
	0	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**	**108**	**120**
Temperature during fermentation, ◦C	20.7 ±0.58 a	25.0 ±1.00 b	29.0 ±0.00 c	31.7 ±0.58 c	32.0 ±0.87 c	32.0 ± 1.00 c	32.0 ± 1.00 c	32.0 ± 1.00 c	44.3 ±2.31 de	42.7 ±0.58 d	46.3 ±1.16 e
pH during fermentation	3.77 ± 0.07 bc	3.67 ± 0.07 bc	3.99 ± 0.44 c	3.64 ± 0.04 bc	3.74 ± 0.46 bc	3.61 ±0.21 bc	3.57 ±0.25 bc	3.18 ± 0.13 ab	2.56 ± 0.11 a	2.80 ± 0.16 a	2.86 ± 0.13 a
pH nibs	6.77 ± 0.05 d	6.74 ± 0.19 d	6.91 ± 0.08 d	6.65 ± 0.10 cd	6.59 ± 0.04 cd	6.59 ± 0.06 cd	6.33 ± 0.12 c	5.53 ± 0.33 b	4.81 ± 0.05 a	4.71 ± 0.03 a	4.55 ± 0.10 a
pH pulps	3.70 ± 0.04 ab	3.74 ± 0.07 abc	3.68 ± 0.06 a	3.75 ± 0.05 abc	3.94 ± 0.08 bcd	4.13 ± 0.04 d	3.99 ± 0.06 cd	3.95 ± 0.04 cd	3.77 ± 0.10 abc	4.06 ± 0.09 d	4.46 ± 0.19 e
Total soluble solids (Brix)	1.5 ± 0.06 e	1.4 ± 0.12 de	1.2 ± 0.06 d	0.6 ± 0.2 abc	0.6 ± 0.06 ab	0.6 ± 0.06 bc	0.6 ± 0.06 bc	0.4 ± 0.06 a	0.6 ± 0.06 bc	0.7 ± 0.06 bc	0.8 ± 0.06 c
Yeast colony count, log CFU/ml	2.1 ± 0.53 a	2.1± 0.15 a	2.2 ± 0.38 ab	2.9 ± 0.10 c	3.1 ±0.12 c	3.1 ± 0.10 c	2.9 ± 0.10 c	2.9 ±0.06 c	2.8 ± 0.10 bc	2.6 ± 0.21 abc	2.8 ± 0.21 bc

The different letter indicates significant difference ($p \leq 0.05$) at different fermentation hours based on One-way ANOVA, Tukey test ($p \leq 0.05$). Bars indicated standard error of the mean (±SEM)

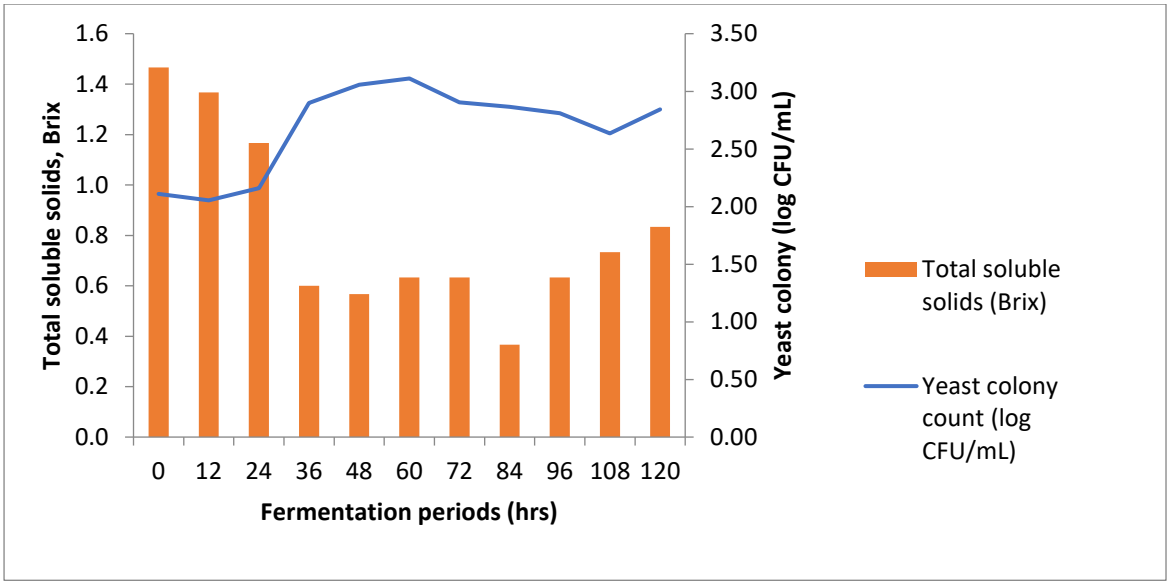


Figure 2.1a Total yeast population (log CFU/mL) with consumption of total soluble solids during a spontaneous fermentation.

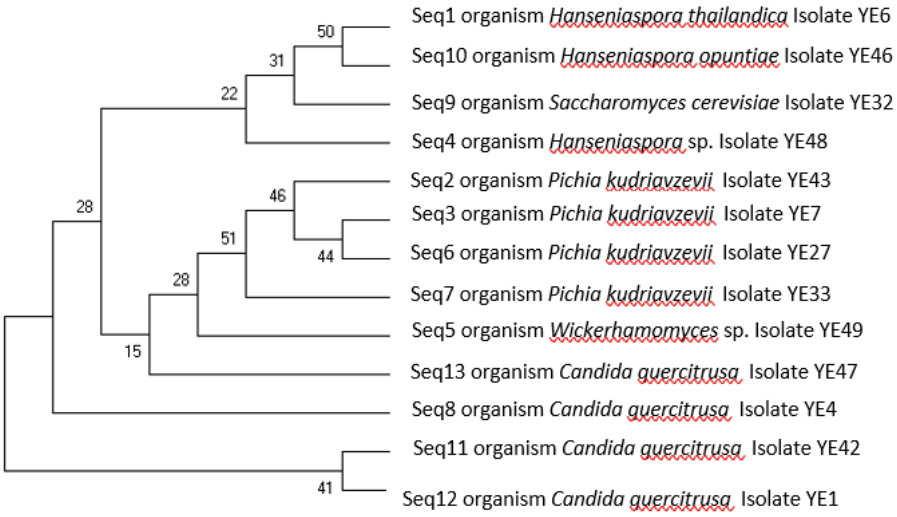


Figure 2.1b Phylogenetic tree generated consisted of 13 yeast species associated with cocoa fermentation by using a Maximum Parsimony analysis, conducted in MEGA X

In this study, the predominant yeast species identified during the cocoa fermentation was similar to previous literature. Previous literature reported that major yeast species found in a cocoa fermentation was ranging from 6 to 10 (Nielsen et al., 2007; Kone et al., 2016). The high diversity of yeast was previously reported by several studies (Nielson et al., 2007; Lefeber et al., 2012; Arana-Sánchez et al., 2015). Our finding was similar to findings reported by Kone

et al. (2016) where yeasts such as *S. cerevisiae, P. kudriavzevii, C. tropicalis* and *W. anomalus* were reported. Furthermore, Ho et al. (2014) also reported that *P. kudriavzevii, S. cerevisiae, Saccharomycopsis crataegensis,* and *Hanseniaspora guilliermondii* were the predominant yeasts during cocoa fermentation. The current study concluded that *P. kudriavzevii* and *C. quercitrusa* were the most prevalent species as both species were the most identified from the total yeast isolates. The diversity of yeast communities is influenced by factors such as the use of starter cultures, type of cocoa, ripeness of cocoa pod, and postharvest treatments (Schwan & Wheals, 2004; Kone et al., 2016). *C. quercitrusa* is excluded as a starter culture as they are well known for causing disease such as Candidemia (Westblade et al., 2015).

2.3.2 Determination of total polyphenols content

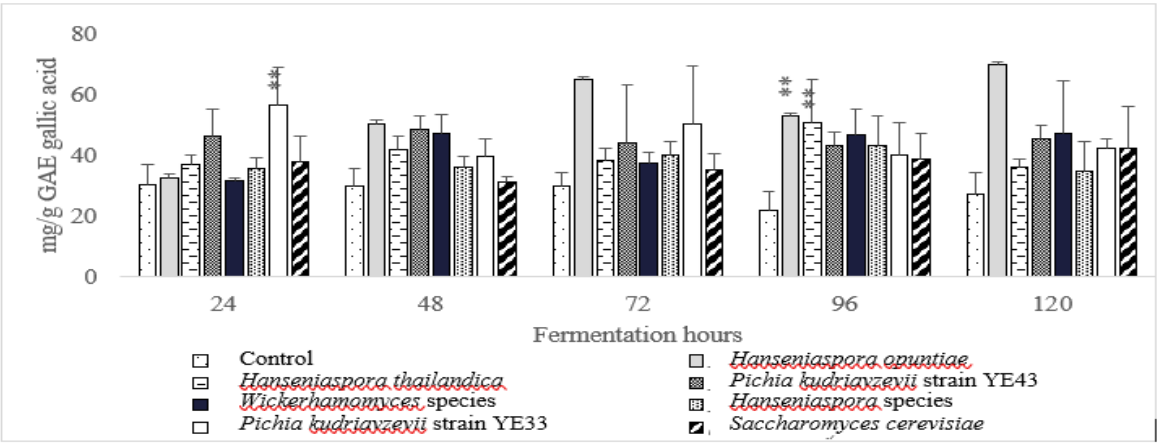
In this study, the phenolic compounds of dried cocoa beans ranging from 21.82±6.15 to 69.81±38.23 mg/g GAE after 120 h fermentation (Figure 2.2). There were no overall significant changes throughout 120 h fermentation, however, it was found that cocoa beans fermented with *P. kudriavzevii* isolate 33 had higher phenolic compounds than control ($p \leq 0.05$) at 24 h fermentation period while cocoa beans inoculated with *H. opuntiae* and *H. thailandica* had higher phenolic compounds than control ($p \leq 0.05$) at 96 h fermentation (Figure 2.2).

This study showed a lower phenolic content in dried cocoa beans compared to previous literature. The phenolic content of raw cocoa beans in Malaysia was previously reported to vary from 71.42 to 82.68 mg/g GAE (Hii et al., 2010). The lower range of TPC value found in the present study could be related to the usage of cocoa pods from a combination of various cocoa clones. It was reported that the difference in phenolic compounds was dependant on cocoa varieties and geographical regions. For instance, the raw cocoa beans seeds (Amazon hybrid variety Clone CCN51) from Ecuador was proven to contain 1-3 folds higher total phenolic content than the Trinitario cultivars from Venezuela (Oraz & Nesbeny, 2016). In this

study, despite all the mixed cocoa clones were obtained from a single region, clones contribute to various phenolic contents. Phenolic content was far more genotype-dependent than influenced by either organic or integrated grown (Veberic, 2016).

Phenolic compounds are secondary metabolites present in plants and can scavenge free radicals based on their electron donor ability (Haile & Kang, 2019a). The high phenolic compounds found in cocoa beans fermented with *H. opuntiae, H. thailandica* and *P. kudriavzevii* Ye 33 could be due to the release of the phenolic compounds, which were bound in the cellular structure of cocoa beans. These phenolic compounds are initially bound with sugar, reducing their availability to the organisms. During fermentation, organisms are capable in hydrolysing complexes of phenolics into simple, soluble-free phenols which are readily absorbed, leading to an increase in the phenolic content of cocoa seeds (Haile & Kang, 2019).

The cellular structure degradation of cocoa beans, which occurs during cocoa bean drying would also release the bound phenolic compounds, leading to the increment of phenolic content (Oraz & Nesbeny, 2016). In the current study, we suggest that both yeast species and cellular structure degradation during drying may aid in releasing the bound phenolic compounds.



(**) indicates significant difference compared to control at p≤ 0.05 at fixed interval based on two-way ANOVA, Tukey test (p≤0.05). Bars indicated standard error of the mean (±SEM)

Figure 2.2 Comparison of total polyphenols content (mg/g GAE Gallic acid) of cocoa beans at 24, 48, 72, 96 and 120 h fermentation

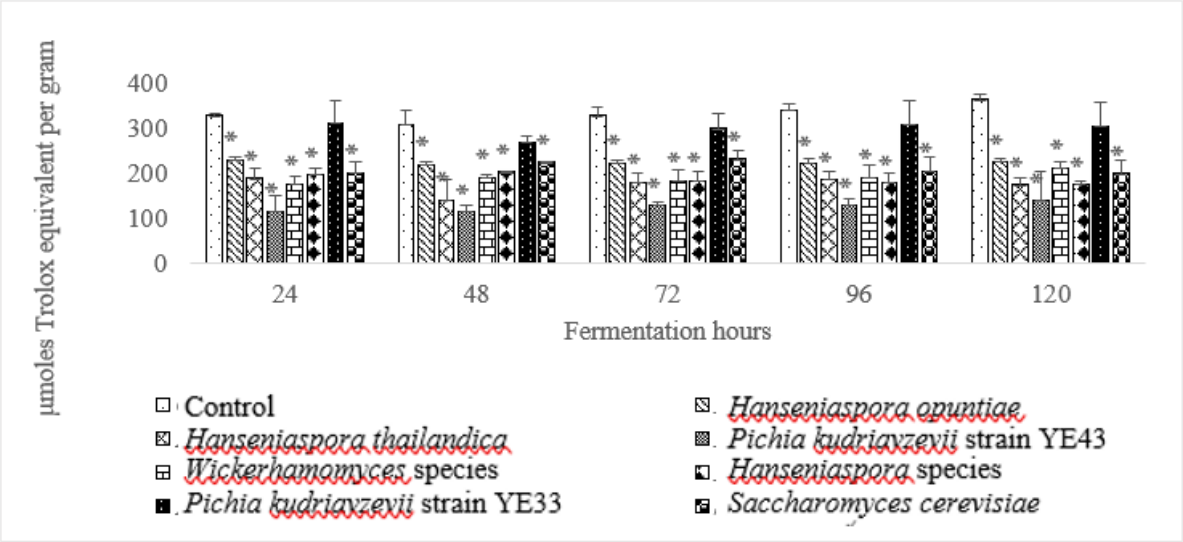
2.3.3 Determination of DPPH free radical scavenging activity

In this study, we also examined the effect of yeast starter culture towards the DPPH free radical scavenging activity of cocoa beans. The current study showed that the DPPH free radical scavenging activity ranging from 113.85±15.73 to 328±20.04 μmoles/g Trolox (TE) after 120 h fermentation. There was no change of DPPH free radical scavenging activity after 120 h fermentation. Nevertheless, the present research showed that all cocoa beans inoculated with yeast starter cultures except for *P. kudriavzevii* isolate 33 had lower DPPH free radical scavenging activity (at $p \leq 0.05$) than control at each fermentation hour (Figure 2.3).

The current DPPH free radical scavenging activity of cocoa beans was lower compared to literature. Former literature showed that the DPPH free radical scavenging activity of cocoa beans was recorded at 323.8 to 1307.07 μmoles/g TE dry weight (Oraz & Nesbeny, 2016). A study carried out by Batista et al. (2016) had also found that there was an increment of DPPH free radical scavenging activity during fermentation which was contrary to the finding of the present study. The difference in findings could be related to the used of cocoa variety. The DPPH free radical scavenging activity of cocoa beans is also highly dependent on the composition of cocoa cultivars used. Various clones are known to possess a different level of polyphenols and antioxidants. Furthermore, the type of growing conditions in growing the cocoa fruit, harvesting time and drying condition applied to the cocoa beans would also contribute to the variation in antioxidant capacity (Batista et al., 2016; Oraz & Nesbeny, 2016).

Moreover, the species of yeast involved in fermentation is known to affect antioxidant concentration and activity in table olive, green coffee, cocoa bean and wine (Sharma et al., 2012; Batista et al., 2016; Haile & Kang, 2019). In a similar study where local, commercial and wild yeast strains were used as a starter culture in wine fermentation, the yeast strains were found to influence the parameters of the wine produced, including antioxidant activity (Sharma

et al., 2012). In the present study, it was found that apart from cocoa beans inoculated with *P. kudriavzevii*, a lower DPPH free radical scavenging activity was recorded for all other groups. In cocoa fermentation, enzymatic hydrolysis reaction causes polyphenols within the beans to be prone towards polymerization and the formation of complexes with proteins, which in turn decreases the solubility of the polyphenols and therefore their antioxidant activity (Misnawi, 2008). Enzymatic hydrolysis in fermentation is a microbial directed process, and it is thus suggested that the species of yeast involved could have influenced this process, and therefore produced the low DPPH free radical scavenging activity (Misnawi, 2008). However, the specific reasons why *P. kudriavzevii* did not show the same behaviour as other yeast starter cultures remain unclear and could be the subject for further study.



(*) Indicates significant difference compared to control at p≤ 0.05 at fixed interval based on two-way ANOVA, Tukey test (p≤0.05) between the treated fermentation. Bars indicated standard error of the mean (±SEM)

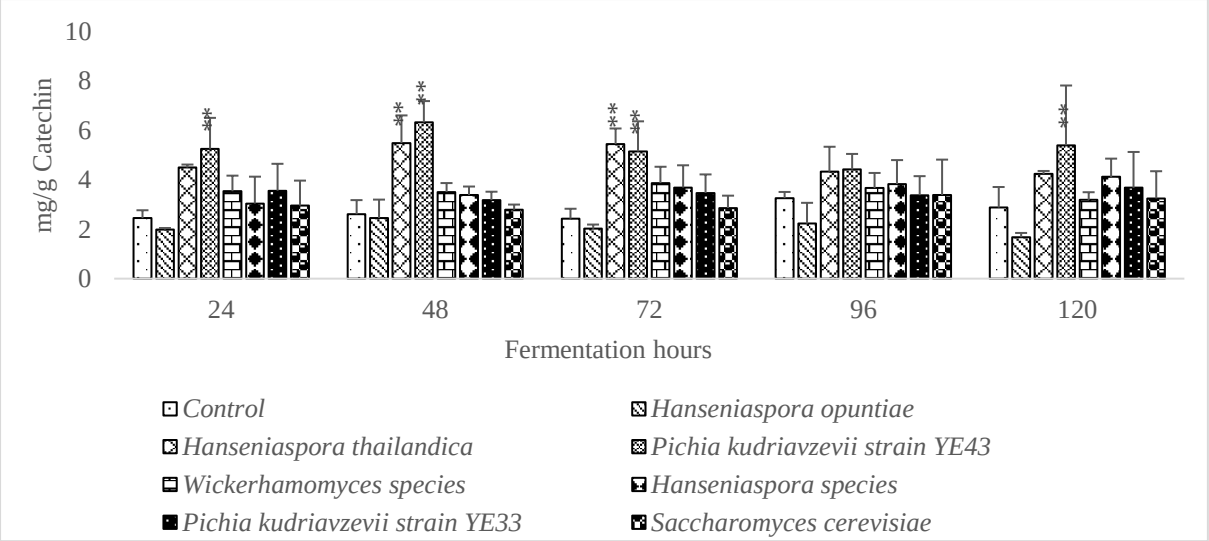
Figure 2.3 Comparison of DPPH content (µmoles/g TE) of cocoa beans at 24, 48, 96 and 120 h fermentation

2.3.4 Total Flavonoid Content (TFC)

The TFC of cocoa beans found in the present study ranging from 1.68 to 6.33 mg/g Catechin (Figure 2.4). There was no significant change (p≤0.05) in TFC of cocoa beans for all the fermentation after 120 h fermentation. Overall, the TFC of cocoa beans inoculated with *P.*

kudriavzevii isolate 43 was significantly higher ($p \leq 0.05$) than control except at 96 h fermentation (Figure 2.4). The TFC of cocoa beans inoculated with *H. thailandica* was also significantly higher ($p \leq 0.05$) than control at 48 and 72 h fermentation.

The range of TFC recorded in the present study was supported by previous literature. It was reported that the TFC was 8.33 mg/g epicatechin of raw cocoa powder and 3.50 mg/g to 12.62 mg/g epicatechin of dried cocoa bean (Zzaman et al., 2013; Fenglin et al., 2013). Based on a similar research carried out by Haile & Kang (2019), it was stated that there was no significant change ($p \leq 0.05$) in TFC of coffee bean produced from control and those coffee beans inoculated with yeast between different fermentation hours. Nonetheless, previous literature showed that the TFC was improved in fermented coffee added with yeast at 24 h fermentation (Haile & Kang, 2019). Yeast was claimed to be effective in increasing the number of flavonoids in coffee extracts during coffee fermentation (Kwak et al., 2018; Haile & Kang, 2019). This was in line with the present finding where cocoa beans inoculated with yeast as a starter culture produced high flavonoids content as compared to control. The increment of flavonoids content might be due to the conversion of insoluble phenolic compounds into soluble flavonoids during fermentation (Kwak et al., 2018; Haile & Kang, 2019). Therefore, we suggested that both *P. kudriavzevii* and *H. thailandica* improve the conversion of insoluble phenolic compounds into soluble flavonoids which eventually produced high flavonoids cocoa beans.



(**) indicates significant higher TFC than control at p≤ 0.05 at every fixed 24 h interval until 120 h based on two-way ANOVA, Tukey test (p≤0.05) between the treated fermentation. Bars indicated standard error of the mean (±SEM)

Figure 2.4 Comparison of total flavonoid content (mg/g Catechin) of cocoa beans at 24, 48, 96 and 120 h fermentation

2.3.5 Total soluble solids

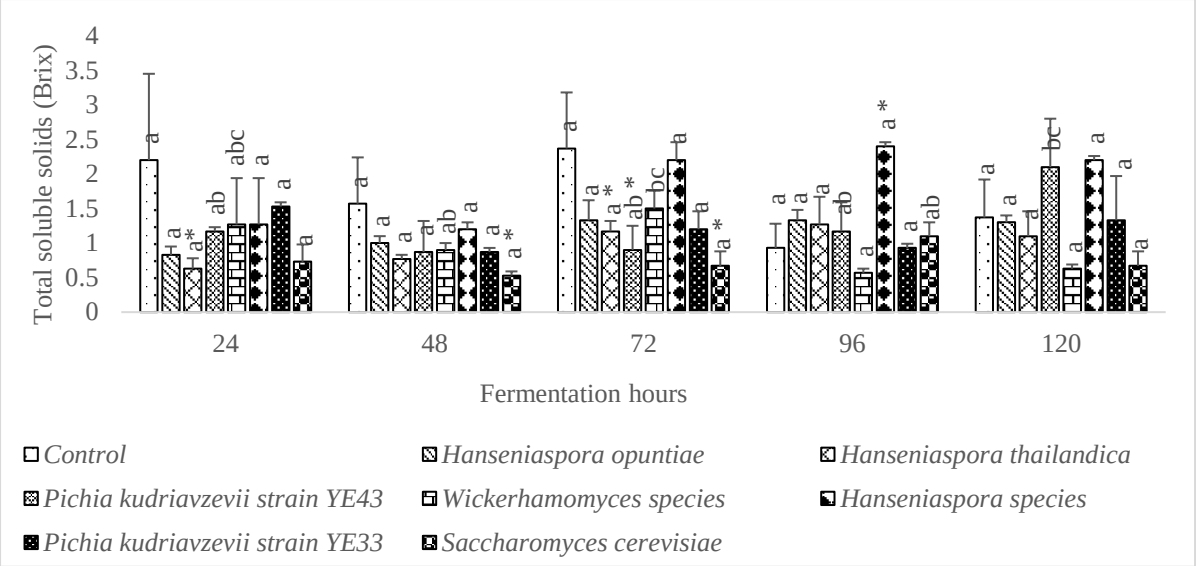
During the five days of fermentation, the total soluble solids varied from 0.53 to 2.4 Brix. There were no changes in total soluble solids of cocoa beans for all fermentations after 120 h (Figure 2.5). The low total soluble solids of cocoa beans compared to control (p≤0.05) were observed at several fermentations specifically: fermentation added with *H. thailandica* at 24 and 72 h; fermentation added with *S. cerevisiae* at 48 and 72 h; and fermentation added with *P. kudriavzevii* isolate 43 at 72 h. On the contrary, the total soluble solids of cocoa beans were higher than control (at p≤0.05) in fermentation added *Hanseniaspora* species at 96 h.

The range of total soluble solids of cocoa beans recorded in the current study was lower than literature. Previous literature reported that the total soluble solids of cocoa beans added with yeast starter cultures was 0.57 to 4.37 Brix (Ooi et al., 2016). The difference of total soluble solids of cocoa beans measured could be influenced by the losses of cocoa sweating during fermentation. During fermentation, microorganisms would secrete pectolytic enzymes to break down mucilage pulp forming by-product such as cocoa bean sweating. Cocoa sweating contains high concentrations of sugar, pectin and organic acids (Kong et al., 2018). The loss of

cocoa sweating could contribute to the lower value of total soluble solids measured in the present study.

Furthermore, Moreira et al. (2017) also stated that the microbial inoculation fastened sugar consumption in the first 24 h fermentation, which is in line with the current finding where cocoa beans fermented with selected yeasts showed lower total soluble solid contents than control. Moreover, total soluble solids of cocoa beans would also decrease as the fermentation prolonged due to loss of citrate acid and metabolism of pulp by yeast (Ho et al., 2014). Different microbial composition was reported to affect the cocoa bean fermentation, where the pulp was less metabolized during the fermentation with the addition of only acetic acid bacteria. It was also found that fermentation with *Saccharomyces cerevisiae* and *Lactobacillus plantarum* had significant ($p \leq 0.05$) lower sugar content while the addition of mixed microorganisms (yeast, lactic acid bacteria and acetic acid bacteria) in fermentation improved the pulp degradation (Kresnowati & Febriami, 2015). This simulation finding suggests that the addition of selected yeast species (*H. thailandica, P. kudriavzevii, H. opuntiae, W.* species and *S. cerevisiae)* aids in the breakdown of total soluble solids more efficiently.

On the other hand, the high total soluble solids of cocoa beans observed in fermentation added with *Hanseniaspora* species after 4 days fermentation could be due to the increment in releasing soluble solids such as glucose and fructose from the cocoa fruit pulp (Kong et al., 2018). Some yeast is capable of producing invertase enzyme which hydrolyses sucrose into glucose and fructose (Kong et al., 2018). It is postulated that *Hanseniaspora* species could be a potential species in producing such invertase enzyme during fermentation, leading to a higher total soluble solids content of cocoa beans.



(*) Indicates a significant difference of TSS as compared to control at p≤ 0.05 at every fixed 24 h interval until 120 h. Small letter (a) in the figure indicates comparison within the same species where the different small letter indicates a significant difference (p≤0.05) based on two-way ANOVA, Tukey test (p≤0.05). Bars indicated standard error of the mean (±SEM)

Figure 2.5 Comparison of total soluble solids content (Brix) of cocoa beans at 24, 48, 96 and 120 h fermentation

2.3.6 Fermentation index

Fermentation index measures the degree of fermentation based on the brownness formed in the cocoa beans. Over-fermented bean has fermentation index of 1.6 while under-fermented beans have fermentation index of less than 1.0. It indicates the completeness of the fermentation (Nsor-Atindana et al., 2012). Generally, there was no significant difference in fermentation index of cocoa beans fermented with and without yeast starter culture (Table 2.2). All cocoa beans were well fermented after 96 h fermentation. However, cocoa beans produced from control was over-fermented (>1.6) at 120 h (Table 3.2). Beans treated with *H. thailandica* were under-fermented at 0, 48, 72 and 120 h whereas beans treated with *W.* species were under-fermented at 24 and 72 h (Table 2.2).

All cocoa beans were well fermented after 96 h fermentation which were consistent with previous literature reported by Pereira et al., (2017). According to Ho et al. (2014), fermentation added with selected yeasts produced dried beans that were fully fermented. This is consistent with the present study where fully fermented beans were obtained from fermentation added with selected yeasts. The degree of fermentation in cocoa beans is due to

the diffusion of polyphenols during fermentation, followed by oxidation and reduction with other cellular compounds, which then turning into brown colour in cocoa beans (Kresnowati & Febriami, 2015; Hernandez et al., 2016). Polyphenol oxidase involved in oxidation reaction by catalysing o - diphenol to o - quinone leading to the brown colour formation in cocoa beans (Hernandez et al., 2016). The polyphenol oxidase works best at 42 to 45°C which usually achieves on day three of fermentation (Caligiani et al., 2007). This further corresponded to the formation of well-fermented beans which obtained after 72 h fermentation. Subsequently, over-fermented beans which detected in control after 120 h fermentation were undesirable as it would lead to off-flavours. If the fermentation continues for a long duration, growth of unwanted moulds and bacteria can generate off-flavours (Caligiani et al., 2007). Moreover, unfermented cocoa beans are also not desirable as they have dark grey colour and more astringent (Caligiani et al., 2007). Both over-fermented and under-fermented beans would give rise to a loss of flavour in the final product after subsequent cocoa processing. Based on our finding, it is suggested to perform yeast fermentation up to 96 h to avoid over-fermentation as well as the production of unfermented beans.

Table 2.2 Comparison of fermentation index of cocoa beans at 24, 48, 96 and 120 h fermentation

h	Control	*H.opuntiae*	*H. thailandica*	*P. kudriavzevii* strain YE43	*Wickerha-momyces* species	*Hanseniaspora* species	*P. kudriav-zevii* strain YE33	*S. cerevisiae*
0	1.69± 0.35^{abA}	1.08±0.27^{a} ^{bA}	0.89±0.14^{a} ^{AB}	1.13±0.41^{a} ^{bA}	1.63± 0.33^{abB}	0.94±0.06^{abA}	1.35±0.39^{a} ^{bA}	1.16± 0.68^{abA}
24	1.14± 0.07^{aA}	1.8±0.84^{aA}	1.03±0.19^{a} ^{AB}	1.05±0.19^{a} ^{A}	0.89± 0.27^{aA}	1.55±0.38^{aA}	1.06±0.09^{a} ^{A}	1.12± 0.44^{aA}
48	1.3± 0.12^{abA}	1.35±0.13^{b} ^{A}	0.72±0.01^{a} ^{A}	1.11±0.36^{a} ^{bA}	1.05± 0.14^{abAB}	1.25±0.22^{abA}	1.47±0.23^{b} ^{A}	1.11± 0.26^{abA}
72	1.29± 0.18^{aA}	0.98±0.22^{a} ^{A}	0.98±0.12^{a} ^{AB}	1.28±0.21^{a} ^{A}	0.78± 0.08^{aA}	1.15±0.30^{aA}	1.51±0.54^{a} ^{A}	1.28± 0.48^{aA}
96	1.19± 0.31^{aA}	1.14±0.09^{a} ^{A}	1.07±0.10^{a} ^{B}	1.41±0.17^{a} ^{A}	1.31± 0.16^{aAB}	1.38±0.13^{aA}	1.23±0.35^{a} ^{A}	1.23± 0.69^{aA}
120	1.74± 0.47^{aA}	1.17±0.30^{a} ^{A}	0.98±0.14^{a} ^{AB}	1.57±0.36^{a} ^{A}	1.29± 0.33^{aAB}	1.13±0.06^{aA}	1.4±0.43^{aA}	1.11± 0.31^{aA}

The capital letter (A) in the table indicates comparison within the same species where the different capital letter indicates significant difference ($p \leq 0.05$) at different fermentation hours; small letter (a) indicating comparison for different yeast treated fermentations, different small letter indicating significant difference based on two-way ANOVA, Tukey test ($p \leq 0.05$) between the treated fermentation. Bars indicated standard error of the mean (±SEM)

2.4 Conclusion

Overall, the present study provides insights into the antioxidant activities of the cocoa beans produced after the addition of selected yeast starter culture. The current findings showed that the application of selected yeast starter culture in cocoa fermentation produces cocoa beans with higher total polyphenols and flavonoid content compared to control at particular fermentation periods. Certain yeast fermentation of cocoa beans causes bound phenolic compounds to be released which results in a higher phenolic content of cocoa seeds. We observed that with the addition of selected yeast starter culture (*H. thailandica, P. kudriavzevii, H. opuntiae, W.* species and *S. cerevisiae)*, the fermentation process proceeded efficiently in sugar metabolism. These findings suggested that *P. kudriavzevii* (MH979681) and *H. thailandica* (MH979675) are potential yeast species in modulating the antioxidant activities of the dried cocoa beans. Future study is needed to assess the effect of using these two yeast starter cultures in field condition, on the antioxidant activities of cocoa beans, sensory and antioxidant properties of resultant chocolate.

Chapter 3

Work presented in this chapter was submitted to *Journal of Food Preservation and Processing* as an article entitled “Physicochemical properties, antioxidant content, volatile organic compounds and sensory profile of cocoa beans fermented with yeast starter cultures”.

3 Physicochemical properties, antioxidant content, volatile organic compounds and sensory profile of cocoa beans fermented with yeast starter cultures

3.1 Introduction

Cocoa (*Theobroma cacao L.*) as one of the richest source of phenolic compounds is associated with various health benefits (Peláez-Soto et al., 2020). The precedence criteria for which the cocoa beans are commercially accepted is its aroma, which also determines its quality of final products. The formation of chocolate flavour precursors in cocoa beans is developed through the fermentation process. Cocoa fermentation is driven by microorganisms such as yeasts, which set as pioneer species in colonizing the fermentation, followed by lactic acid bacteria (LAB), acetic acid bacteria (AAB), aerobic spore-forming bacilli, and filamentous fungi (Batista et al., 2016). Often, Malaysian cocoa fermentation is conducted spontaneously without any starter culture using basic fermentation methods such as heap and shallow box methods (Sulaiman et al., 2014). Several common problems arise from spontaneous cocoa fermentation are high acidity or low in chocolaty flavour due to incomplete fermentation, off-flavours as a result of over-fermentation and beans spoilage that are leading to low crop value for smallholders and farmers (Schwan & Wheals, 2004).

As spontaneous cocoa fermentation often leads to inconsistency of beans quality, several studies from preliminary work, lab scale to pilot scale fermentations have been done to control cocoa fermentation and also to examine the microbial influence on the final quality of cocoa beans (Meersman et al., 2013; Racine et al., 2019). Research has reported that the usage of several starter cultures in fermentation can produce unusual flavours of cocoa beans. For instance, the application of *Pichia kluyveri* and *Cyberlindnera fabianii* in cocoa fermentation produce chocolate with high cocoa and roasting notes (Meersman et al., 2016). Usage of mixed

cultures of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* leads to beans with fruity flavour (Visintin et al., 2016).

The usage of certain yeast species in fermentation to improve the phenolic content of food is well known in wine, wheat bran, coffee bean and tea industries (Caridi et al., 2017; Kwak et al., 2018; Moore et al., 2007). However, this is yet to be further understood for cocoa fermentation. According to our lab-scale fermentation result, *H. thailandica* and *P. kudriavzevii* were the yeasts that exist in cocoa fermentation and the usage of these yeasts had led to cocoa beans with higher TPC as compared to the spontaneous fermentation (Ooi et al., 2020). In a similar study, the usage of *P. kudriavzevii* in cereal-mix fermentation leads to a more organoleptically accepted product as well as increases the overall antioxidant capacity of cereal-based fermented product (Ogunremi et al., 2015). Despite it is well known that starter culture affected the flavour profile of cocoa beans, the literature on the antioxidant content of the cocoa beans produced by using selected yeast starter (*H. thailandica, P. kudriavzevii* and mixture of these two yeasts) in field fermentation are still limited. Since yeast has been shown to enhance the antioxidant properties of fermented food products, it is our research interest to determine if the selected yeast starter influences the antioxidant content of cocoa beans. Hence, the present study aims to determine the influence of yeast starter on antioxidant content, volatile organic compounds (VOCs) and sensory profile of cocoa liquors produced after the field fermentation. The aim of this work was to compare the cocoa liquors produced from these controlled fermented beans. After well-drying the cocoa beans to 7.5% moisture content, the fermented beans were ground to a cocoa liquor (without addition of sugar). A chocolate mass was prepared from these cocoa liquors in Chapter 4, then were subjected to conching, tempering and moulding processes (Di Mattia et al., 2014). Two batches of cocoa fermentation were carried out to determine the reproducibility of the antioxidant content and organoleptic properties of cocoa beans produced by using the selected yeasts.

3.2 Materials and methods

3.2.1 Raw materials

Mixed clones of cocoa pods (PBM 123, BR 25, MCBC 1 and MCBC 8) were obtained from two farms in Pahang, Malaysia and were fermented with yeast starters (*Hanseniaspora thailandica* (MH979675) (HT), *Pichia kudriazevii* (MH979681) (PK) and a mixture of these two yeasts (Mix)). Ghana cocoa beans were purchased from Pastry Pro Pte Ltd and used as a reference for cocoa liquor. These beans were roasted and a commercial product which involved spontaneous fermentation.

3.2.1.1 Defined starter culture for cocoa fermentation

Yeast samples (*Hanseniaspora thailandica* (MH979675) and *Pichia kudriazevii* (MH979681)) used in this research were naturally existing yeast isolated from the spontaneous cocoa bean fermentation and identified in previous work by Ooi et al. (2020). Yeast culture was prepared according to Pereira et al. (2012).

3.2.1.2 Dual culture method to determine the relationship of *P. kudriavzevii* and *H. thailandica* as a mixed starter

The dual culture agar plating method was according to a modified method of Muniaraj et al. (2008). Yeasts were collected from a yeast extract peptone dextrose (YEPD) plate (less than 2 weeks old), diluted in water and adjusted to OD600 of 0.1. Then, 15µL was plated on YEPD plates (5.5cm in diameter) in quadruples. The plate was divided into two portions by making a line on the bottom of the petri dish. Then, each yeast was inoculated in one half of the plate by streaking with a minimum distance of about 2cm between each streak by using an inoculation loop. For control, yeast (*P. kudriavzevii* or *H. thailandica)* was respectively

streaked at one half of the plate and sterile YEPD agar on the other half of the plate. The streaks were streaked not closer than 2cm (a minimum of not more than 2 cm distance was maintained between two yeasts). All plates were incubated at 24±1˚C for 5 days. Each plate was observed for the presence of yeast colonies. The growth of *P. kudriavzevii* and *H. thailandica* in the same media was observed. The yeast isolates were observed if any of it grew faster than one another. If any yeast isolates secreted antagonistic substance, it would diffuse through the agar medium and may inhibit the growth of another species. If the yeast isolate did not release antagonistic substance, its growth will be unaffected and would be similar to control plates.

3.2.2 Cocoa fermentation

Two batches of fermentation were carried out using mixed clones of cocoa pods (PBM 123, BR 25, MCBC 1 and MCBC 8) obtained from two farms in Pahang, Malaysia. Cocoa shallow box fermentation method (31 x 31 x 31 cm ^{3}) with 20 kg of wet cocoa beans was carried out for 4 days at 30˚ C and turning process of cocoa beans was done at 72 h of fermentation. The box was firstly sterilized with 99.9% ethanol prior to loading of cocoa beans. Cocoa beans were filled up to approximately 29 cm compared to the height of the box (31 cm). Then, gunny sack was used to cover the cocoa beans. Control was a spontaneous fermentation process with indigenous microorganisms present in the cocoa fruit. For controlled fermentation, 250 mL of yeast suspensions with 10^6 cells/mL was sprayed on the cocoa beans mass and mixed respectively, and then left for 96 h of fermentation (Santos et al., 2020).

Cocoa beans that were randomly collected (at 0, 24, 48, 72 and 96 h) from different points of the box were mixed and kept in sterile polybag then stored in the refrigerator at -20˚ C for physicochemical analysis. Samples after 96 h of fermentation were oven-dried (Memmert, Germany) at 38°C until 7.5% moisture content was obtained.

3.2.3 Chemical analyses

3.2.3.1 Temperature of fermenting cocoa beans and pH of cocoa nibs

The temperature of fermenting cocoa beans was measured by inserting thermometers into ¾ height of the cocoa fermentation box. For the pH of cocoa nibs, five grams of cocoa nibs was weighed by using a weight balance (Mettler Toledo PL 4002, United States). Then, 45 mL of boiled distilled water was added into each sample and left cold. The pH was read by a pH meter (Eutech Cyber Scan pH 300, Singapore).

3.2.4 Quality evaluation of cocoa beans

Well-dried cocoa beans (moisture content at 7.5%) after deshelled were used for all the chemical analysis. Sample extraction for antioxidant activities of dried cocoa beans was conducted according to a modified method of Ioannone et al. (2015). These dried beans were oven roasted according to Sulaiman et al. (2014) at 145 ºC, 30 mins, deshelled and used for VOCs and sensory analysis.

3.2.4.1 Total polyphenol content (TPC)

The TPC of the samples was determined according to a method of Ioannone et al. (2015) and reported as milligram gallic acid equivalents per gram (mg/g GAE) of defatted cocoa.

3.2.4.2 Total flavonoid content (TFC)

The TFC was performed according to a modified method of Jia et al. (1999). The TFC was measured for its absorbance against a prepared reagent blank at 510 nm. Catechin (Sigma

Aldrich, USA) was used as a standard and results are expressed as milligram Catechin equivalents per gram of sample (mg/g Catechin).

3.2.4.3 Determination of DPPH free radical scavenging activity

The DPPH free radical scavenging activity of well-dried cocoa beans was determined according to a modified method of Ee et al. (2019). 100 μL of the diluted sample was added to 200 μL of 2,2 diphenyl-1-pic-rylhydrazyl (DPPH) (Sigma Aldrich, USA). The mixture was then left to react for 30 mins at room temperature in the dark. After incubation, absorbance was read at 515 nm using the microplate reader (Tecan, Switzerland). Ascorbic acid was used as a standard. Ethanol mixed with DPPH was used as a blank while water was used as a control. The scavenging effect was determined based on the percentage of DPPH free radical scavenging activity using the equation below and expressed as micromole of Trolox per gram of defatted cocoa.

$$\%\ \text{DPPH radical scavenging activity} = \frac{\text{The absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}}\ \text{x}\ 100\%$$

3.2.4.4 Determination of ABTS radical scavenging assay

The ABTS radical scavenging assay of cocoa samples was determined according to a method of Belščak et al. (2009). The ABTS solution was diluted with ethanol to obtain an absorbance of 0.70 ±0.02 at 734 nm. Ethanol was used as a blank and Trolox (100 – 1000 μM) was used as a standard to obtain the calibration curve. All results were expressed as Trolox equivalents.

3.2.4.5 Fermentation index

Fermentation index of dried cocoa beans was determined according to a method of Sulaiman et al. (2014). Fermented beans have FI value of >1, over-fermented beans have FI value of >1.6, whereby unfermented beans have FI value of <1. Fermentation index (FI) was calculated as following:

Fermentation index (FI) = (Absorbance at 460 nm)/ (Absorbance at 530 nm)

3.2.4.6 pH of dried cocoa nib

The pH of dried cocoa beans was measured according to the method in 3.2.3.1.

3.3.4.7 Equivalent Percent Fully Brown (EB score)

The EB score was performed according to a method of Sulaiman et al. (2014). A total of 100 dried fermented beans was randomly taken and cut lengthwise from the middle using a sharp knife for maximum surface exposure of cotyledon and observed for surface colour under artificial light. Colour of the bean's cotyledon was classified based on fully brown, partly brown, partly purple, purple or slaty. Defects such as germinated and insect-infested beans were also recorded. The EB score was calculated as below:

EB score = [(1x %Fully Brown) + (0.7% x (%Partly Brown+%Partly Purple)) + (0.5x (%Fully Purple)) + (0.3x (%slaty))]

3.3.5 Volatile organic compounds (VOCs) analysis

The VOC of roasted cocoa samples (deshelled) was determined according to a modified method of Rodriguez-Campos et al. (2012) by using a fibre 50/30 um divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS) by Supelco. Cocoa

sample (2.0g) in the HS was heated at 60 °C (15 mins) using a water bath to reach equilibrium, followed by the Fiber exposition (30 mins) to the sample in the HS at 60 °C. The VOC was analysed by gas chromatography-mass spectrometry (GCMS) equipped with an Innowax capillary column (60m * 0.25 mm id * 0.25 um film thickness). The oven temperature was set at 40 °C for 5 mins, increased to 200 °C at a rate of 10 °C/mins, maintained at 200 °C for 30 mins. The carrier gas was high purity helium at 0.7 mL/mins. The splitless injection mode was 240 °C (0.5 mins). The selective mass detector was a quadrupole with an electronic impact ionization system at 70eV and 260 °C. The analysis of GCMS peaks was performed according to a modified method of Ruiz-Hernández et al. (2018) and Ren et al. (2020). General screening of the VOCs in roasted beans was by comparing the mass spectra of each compound with the MSD Chemstation software, Agilent Technology. Then, these volatile compounds were further screened where the identification of compounds was based on three criteria: (1) by comparing the mass spectra with the library of mass spectra (MSD Chemstation software, Agilent Technology); (2) by comparing the retention index with literature data and (3) the identification was confirmed by using pure external standards of the components. The relative abundance of VOCs within a profile was expressed as the total integrated area of each compound and subjected for One-way analysis of variance (ANOVA).

3.3.6 Sensory evaluation of cocoa liquor

3.3.6.1 Cocoa liquor preparation

A total of 8 samples including Ghana cocoa beans were evaluated for sensory attributes. The 8 samples were Batch 1 (PK, HT and Mix) and Batch 2 (PK, HT and MIX), control and Ghana liquors. Cocoa beans after deshelled were ground into cocoa paste by using (Panasonic food processor MK-F800SSL). Cocoa liquors were then conched for 6 h using a stone concher

(Spectra 11 Chocolate Melanger, Coimbatore, India). MasterSizer 3000 (Laser Diffraction Particle Size Analyser, Malvern Instruments Ltd., UK) equipped with a Hydro EV was used to ensure the particle size of cocoa liquor was at d_{90}-value below 30 μm (i.e. 90% of the particles in the liquor are finer than 30 μm). All cocoa liquor were kept in a sealed glass bottle and stored at 20 °C for further analysis.

3.3.6.2 Sensory evaluation

A total of 8 trained panellists from Malaysian Cocoa Board participated in the evaluation. Descriptive analysis with a scale of 0 to 10 was used to evaluate the sensory of cocoa liquor, where 0 indicates the absence or minimum intensity and 10 indicates the maximum intensity. The guideline for the description of the sensory score was based on the Cocoa of Excellence Technical Committee (2017). Ghana cocoa liquor was used as a reference sample, where the score for the reference sample was used as a basis for the scoring of flavour attributes for the other cocoa samples. The evaluated flavour characteristics were cocoa, bitter, astringent, acid, sweet, fresh fruits, browned fruits, floral, spicy, woody, nutty, roasted, dirty, meaty, over fermented, smoky, mouldy, other off flavours and overall global quality. Each sample was labelled with randomly selected 3-digits numerical code. During the evaluation of cocoa liquor, the panellists work individually in a sensory booth and they were free to describe any other taste that may be present in the samples.

3.3.7 Statistical analysis

The analysis was conducted in triplicates. All data unless otherwise stated, were analysed by using two-way Analysis of Variance (ANOVA) followed by mean comparison using Tukey test at $p \leq 0.05$ levels with Statistical Package for the Social Sciences (SPSS) version 23. The correlation analysis among TPC, TFC and antioxidant activities of cocoa beans

were analysed by the same statistical package. Pearson correlation strength was based on guides of Evans (1996) in which the absolute value of correlation coefficient could be described as: [± 0.00 ± 0.19], [± 0.20 ± 0.39], [± 0.40 ± 0.59], [± 0.60 ± 0.79], and [± 0.80 ± 1.0], which signified the very weak, weak, moderate, strong, and very strong correlations, respectively.

Statistical analysis for VOC of roasted cocoa beans was according to a modified method of Crafack et al. (2013), where the duplicate was carried out and results were expressed as an average of two fermentation batches, resulted in 4 samples (PK, HT, Mix, and control). One-way Analysis of Variance (ANOVA) was conducted to compare peak areas of the individual compounds identified in roasted cocoa beans by GCMS. Compounds showing significant variance ($p \leq 0.05$) were subjected to a post-hoc Tukey's HSD (Honestly Significant Difference) to identify the significantly different samples. For sensory evaluation of cocoa liquor, the result was an average of two batches (a total of 4 samples: PK, HT, Mix and Control) and analysed by One-way ANOVA at $p \leq 0.05$.

3.4 Results and Discussion

3.4.1 Dual culture method to determine the relationship of *P. kudriavzevii* and *H. thailandica* as a mixed starter

Dual culture agar plating was carried out to determine if there was any neutral, symbiotic or antagonistic relationship between the *P. kudriavzevii* and *H. thailandica.* The growth of *P. kudriavzevii* alone in a control plate was shown in Figure 3.1a, whereas the growth of solely *H. thailandica* was shown in Figure 3.1b. By agar plating method, the parallel streak of both yeasts (*P. kudriavzevii* and *H. thailandica)* on the same YEPD agar plate was shown in Figure 3.1c. The growth of both yeasts was observed on the same plate as showed in Figure

3.1c. The antagonistic effect was not observed as both yeasts formed colonies on the respective streaked area.

Cocoa fermentation is associated with microorganisms such as yeast which set as a pioneer species dominating the cocoa beans mass, followed by lactic acid bacteria and acetic acid bacteria (Schwan, 1998). The biodiversity of yeast, lactic acid and acetic acid bacteria were different according to the region (Delgado-Ospina et al., 2020). Several frequently reported yeast genera are *Candida, Hanseniaspora, Pichia, Rhodotorula, Sacchromyces* and *Wickerhamomyces* (Delgado-Ospina et al., 2020). A few species that are found during the drying stages are species such as *H. opuntiae, C. insectorum, P. kudriavzevii, P. sporocuriosa* and *Issatchenkia hanoiensis* (Delgado-Ospina et al., 2020). The study also reported that the growth of *Hanseniaspora* and *Pichia* genera are favourable particularly at the beginning of the fermentation process (Fernández Maura et al., 2016). Both yeast genera are acid-tolerant yeast strains and adapt well to the low pH of cocoa bean mass (pH 3.3 to 4.0) at the initial stage of fermentation (Fernández Maura et al., 2016). Hence, *Hanseniaspora* and *Pichia* genera are yeasts dominated the cocoa fermentation process.

According to Crafack et al. (2013), a high occurrence of *H. thailandica* was detected in cocoa fermentation, consisting of approximately 24%. The *P. kudriavzevii* is found in Ivorian cocoa fermentation during 24 to 48 h of fermentation (Koné et al., 2016). Both *P. kudriavzevii* and *H. thailandica* were the dominant yeasts that are commonly identified in cocoa fermentation. Based on our result, *P. kudriavzevii* did not suppress the growth of *H. thailandica* and vice versa (Figure 3.1). Both yeasts were capable to grow on the same media, signified their relationship was neutral.

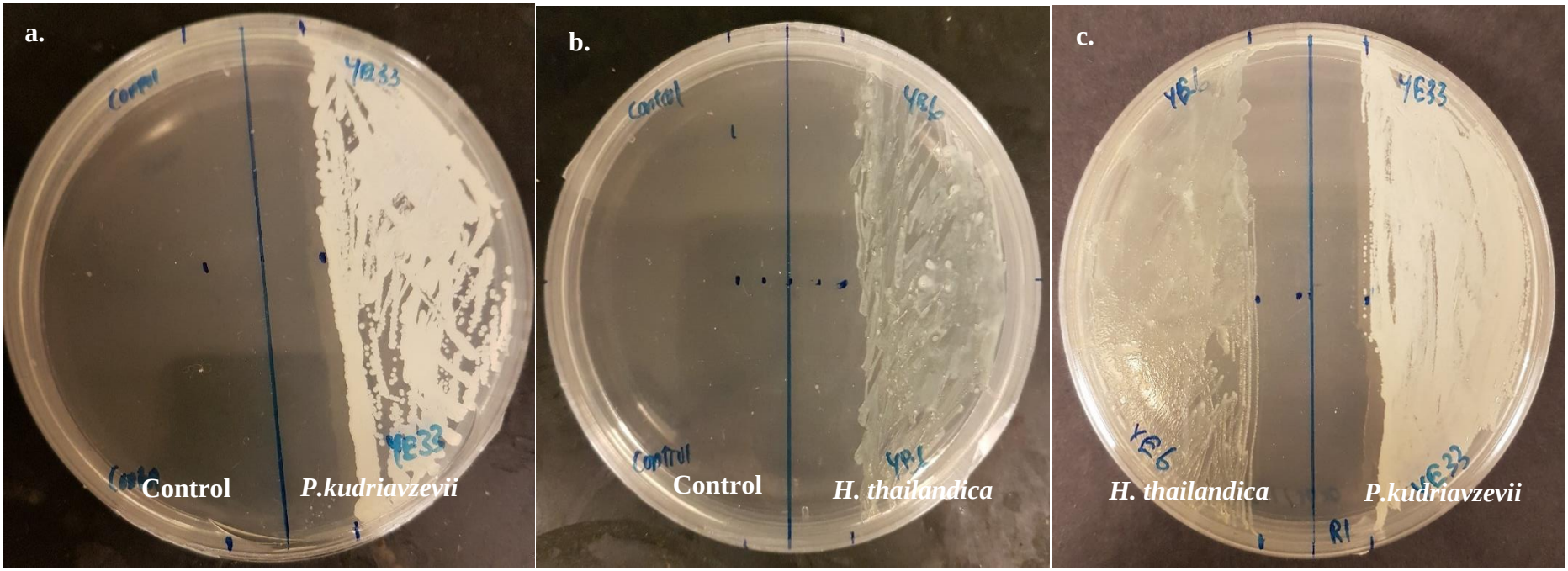


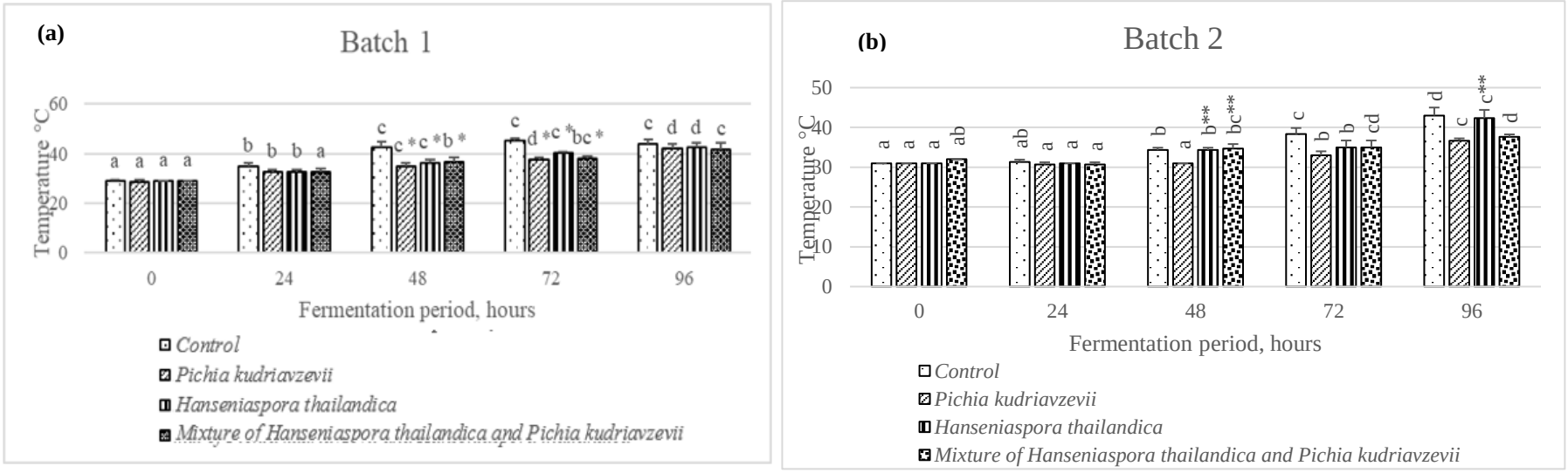
Figure 3.1 Dual culture YEPD agar plating: (a) Plate was showing the growth of *P. kudriavzevii* with control; (b) Plate was showing the growth of *H. thailandica* with control; (c) Plate was showing the growth of *P. kudriavzevii* and *H. thailandica* on the same plate

3.4.2 Cocoa fermentation

3.4.2.1 Temperature

The temperature of two fermentation batches applied with yeast starter culture increased significantly ($p \leq 0.05$) after 96 h fermentation (Figure 3.2a and Figure 3.2b). During the first 2 days of fermentation, the temperature of Batch 1 treated with yeast starter continued to increase ($p \leq 0.05$) up until 96 h. The temperature of control increased significantly ($p \leq 0.05$) until 48 h and maintained a constant temperature until 96 h without significant difference between 48 to 96 h. The same behaviour was also observed in Batch 2 where the temperature of fermentation treated with yeast and control increased significantly during the first 2 days, except fermentation added with *P. kudriavzevii.* Fermentation added with *P. kudriavzevii* only achieved high temperature (33 °C) after 72 h after which further increased until after 96 h. Control achieved higher temperature ($p \leq 0.05$) than the starter culture added fermentation from 48 until 96 h. The higher temperature of 42.33 °C was only found in fermentation added with *H. thailandica* after 96 h. The temperature of Batch 1 (28.7 °C to 45°C) and Batch 2 (30.67 °C to 43 °C) fermentations were consistent with a previous study (Schwan & Wheals, 2004).

Temperature rise is caused by energy released during the exothermic reaction of the conversion of ethanol into acetic acid by acetic acid bacteria (Nielsen et al., 2007). The production of acetic acid will diffuse into cocoa beans causing nib's acidification, which also generates heat that causes the temperature of bean mass to increase up to 50°C. However, a lower temperature range between 45 °C to 50 °C is needed to accomplish complete chocolate flavour development during the fermentation (Ho et al., 2014). In brief, the addition of yeast starter culture in both batches of fermentation achieved a temperature of 43°C and 45°C, which was needed for a complete cocoa fermentation process.



(*) indicates significant lower temperature than control ($p \leq 0.05$) comparison within the same hours at every fixed 24 h interval until 96 h based on two-way ANOVA, Tukey test ($p \leq 0.05$). Bars indicated standard error of the mean (±SEM). The small letter indicating comparison for different yeast treated fermentations, different small letter indicating significant difference ($p \leq 0.05$) between the treated fermentation, using two-way ANOVA.

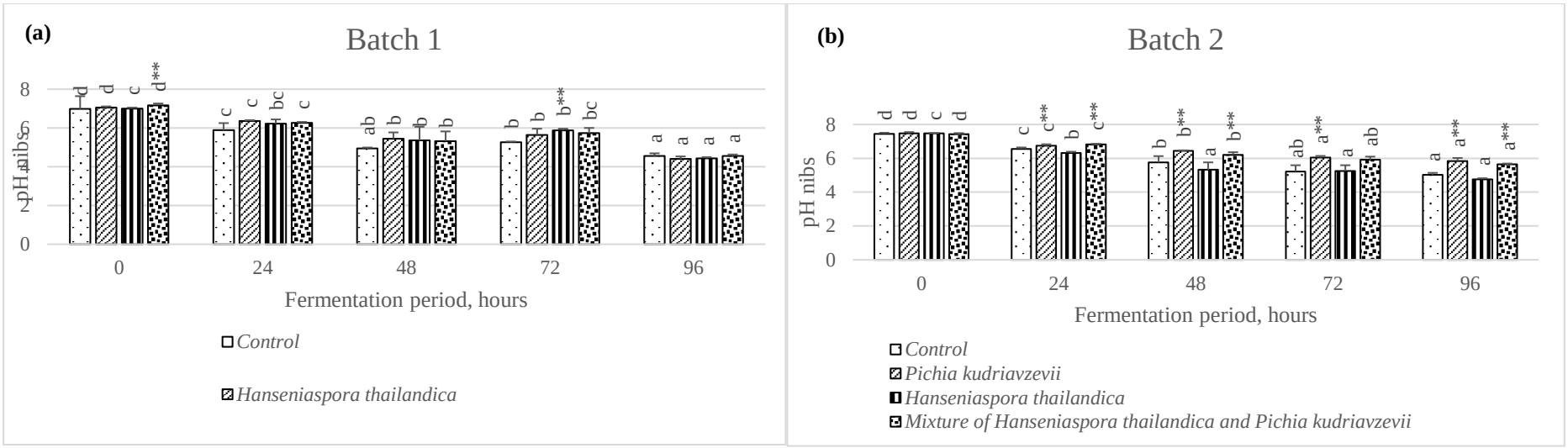
Figure 3.2 Comparison of temperature during different treated fermentation: (a) Batch 1; (b) Batch 2 at 0, 24, 48, 72 and 96 h fermentation

3.4.2.2 pH of cocoa nibs

Temperature and pH (pH nibs) are important indicators for enzyme activities and flavour development of cocoa beans (Camu et al., 2008). The pH of cocoa nibs for both fermentation batches decreased significantly ($p \leq 0.05$) after 96 h (Figure 3.3a and Figure 3.3b). During 0 h fermentation, pH of cocoa beans treated with mixed yeast starter culture was significantly higher than the control. The pH values of cocoa nibs fermented with yeast starter culture in comparison with spontaneous fermentation were not statistically significant ($p \leq 0.05$) from 24 to 96 h, except during 72 h, where the pH was found higher in beans treated with *H. thailandica*.

In Batch 2 fermentation, the pH of cocoa beans added with *P. kudriavzevii* and *H. thailandica* was significantly ($p \leq 0.05$) lower as compared to control after 24 h. The pH of cocoa beans treated with *H. thailandica* and control were also significantly lower ($p \leq 0.05$) than the other starter culture added fermentations after 48 to 96 h. The pH of cocoa nibs fermented by different yeast starter culture was ranging from pH 4.4 to 7.17 (Batch 1) and 4.76 to 7.43 (Batch 2) as previously reported by Voigt et al. (1994).

The reduction of pH nibs observed in two batches of fermentation, particularly observed at the end of fermentation was associated with nib's acidification. Nib's acidification is important for the Maillard reaction to take place (Voigt et al., 1994). It was worth to highlight that the pH of cocoa beans added with *P. kudriavzevii* and *H. thailandica* were significantly ($p \leq 0.05$) lower as compared to control after 24 h, which may be due to the diffusion of more alcohol and acetic acid into the cocoa nibs. Our study concluded that the addition of yeast starter culture in both fermentation batches managed to achieve the desired pH nibs for the successful formation of chocolate flavour precursors.



(**) indicates significant higher pH nibs than control ($p \leq 0.05$) at every fixed 24 h interval until 96 h based on two-way ANOVA, Tukey test ($p \leq 0.05$). Bars indicated standard error of the mean (±SEM). The small letter indicating comparison for different yeast added fermentations, different small letter indicating significant difference ($p \leq 0.05$) between the added fermentation, using two-way ANOVA.

Figure 3.3 Comparison of pH nibs of cocoa beans: (a) Batch 1; (b) Batch 2 at 0, 24, 48, 72 and 96 h fermentation

3.4.3 Antioxidant content of dried cocoa beans

3.4.3.1 Determination of Total Polyphenols Content (TPC)

After the fermentation process, all cocoa beans were oven-dried into dried cocoa beans. The dried cocoa beans added with *H. thailandica* had the highest TPC as compared to control ($p \leq 0.05$) (Figure 3.4a). This is consistently observed in both batches of fermentation. The TPC of the dried cocoa beans after fermented with mixed starter culture or *P. kudriavzevii* showed no significant difference compared to control. Among the two batches, there was no significant difference in terms of TPC level. Overall, the field fermentation outcome was in line with our simulation results (Ooi et al., 2020) where cocoa beans fermented with yeast showed high TPC ($p \leq 0.05$) after 96 h fermentation. The TPC of dried bean in batch 1 varied from 99.59 mg/g GAE to 174.23 mg/g GAE, which was consistent with the literature (Oracz & Nebesny, 2019). However, TPC for batch 2 of field fermentation that was ranging from 168.19 to 375.33 mg/g GAE was higher than reported in previous literature. This discrepancy could be a result of cocoa varieties and geographical regions. Previous literature reported that the raw cocoa beans of Amazon hybrid variety (Clone CCN51) from Ecuador was 1-3 folds higher than the Trinitario cultivars from Venezuela (Oracz & Nebesny, 2019). In the current study, despite all the mixed cocoa clones were obtained from a single region, different composition of clones may contribute to the variation in phenolic content. The phenolic content was far more genotype-dependent than influenced by either organic or integrated grown (Veberic, 2016).

The high TPC found in cocoa beans fermented with *H. thailandica* could be due to the release of phenolic compounds, which was bound in the cellular structure of cocoa beans (Oracz & Nebesny, 2019). The cellular structure degradation of cocoa beans, which occurs during drying would release the bound phenolic compounds, leading to the increment of phenolic content (Oracz & Nebesny, 2019). Our study suggested that *H. thailandica* were

capable of aiding the release of the bound phenolic compounds, contributing to high TPC content in dried cocoa beans. In brief, the application of yeast starter culture in fermentation produced consistent results between two batches of field fermentations. In the current study, cocoa beans fermented by *H. thailandica* as starter culture achieved the highest TPC.

3.4.3.2 Determination of Total Flavonoid Content (TFC)

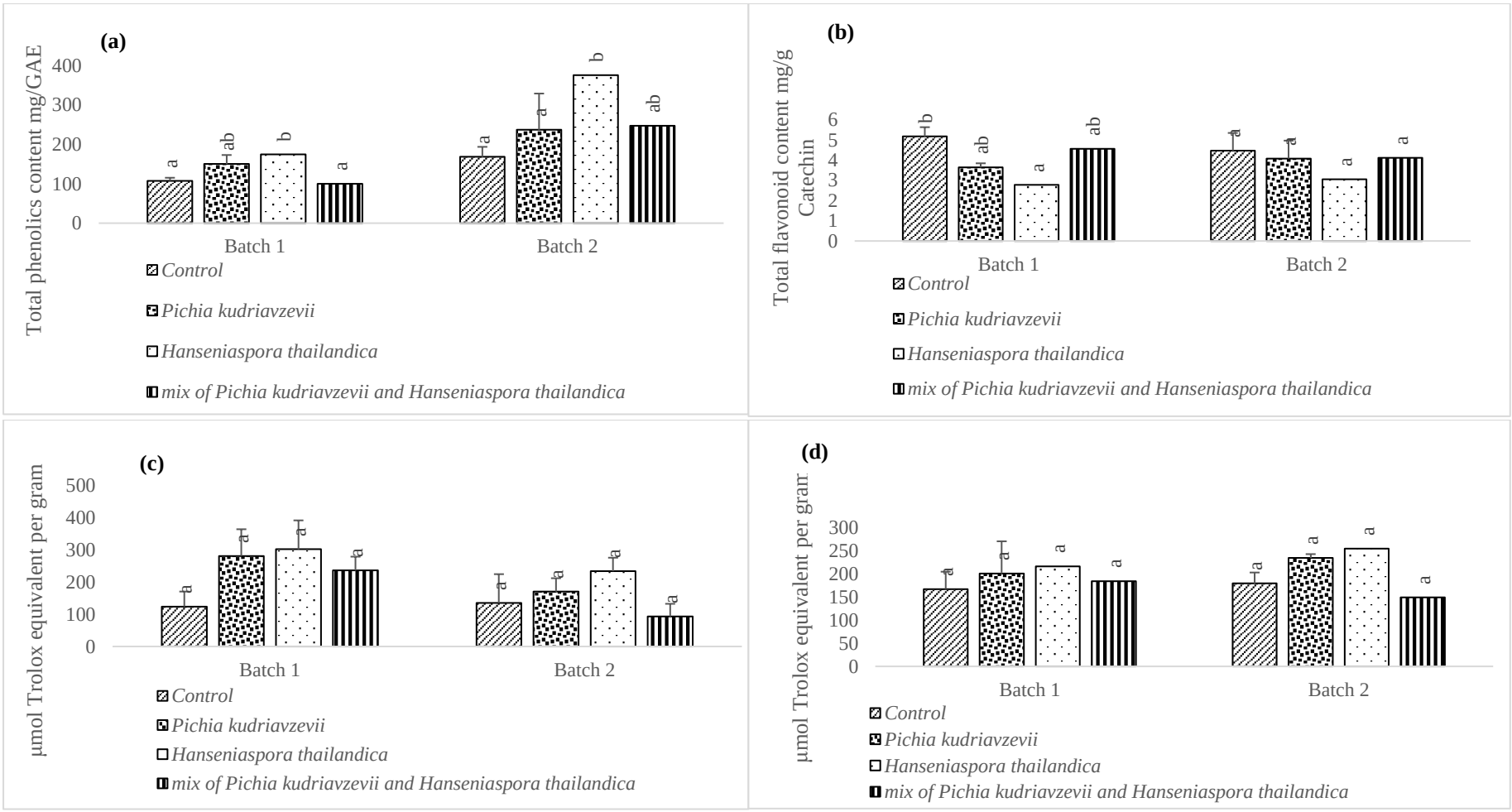
Control cocoa beans showed the highest TFC ($p \leq 0.05$) while beans added with *H. thailandica* had the lowest TFC for both batches. The values were not significant for batch 2 fermentation (Figure 3.4b). The TFC ranging from 2.77 to 5.16 mg/g Catechin, which was supported by a previous study (Fenglin et al., 2013).

The difference of TFC could be associated with the *Theobroma cacao* L. varieties and regions of cultivation, climate conditions, postharvest manipulations and storage conditions where all these parameters may affect the antioxidant properties of cocoa beans (Oracz & Nebesny, 2016). In a similar study, yeast fermented coffee extracts show a lower TFC as compared to control (Kwak et al., 2018). The aforementioned phenomenon is a result of fermentation that is more effective in producing soluble phenolic compounds compared to flavonoids (Kwak et al., 2018). In respect to that, the minimum TFC detected in dried beans might be related to the higher phenolic compounds that were produced during the fermentation. It was also reported that different yeast species or strains can produce significant differences in antioxidant activity (TPC and TFC) in fermented green coffee beans (Haile & Kang, 2019a). Thus, we suggested that *H. thailandica* and *P. kudriavzevii* have more influence on TPC than TFC. Overall, the addition of yeast starter culture during fermentation showed consistent results between the two fermentation batches.

3.4.3.3 Determination of DPPH and 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ABTS Free Radical Scavenging Activities

There was no significant difference ($p \leq 0.05$) in DPPH and ABTS content of cocoa bean between the two batches of fermentation. (Figure 3.4c and Figure 3.4d). The DPPH and ABTS content of cocoa beans from field fermentation ranging from 93.33 to 301.23 μmol/g TE and 149.16 to 255.06 μmol/g TE, were consistent with the literature (Batista et al., 2016).

The degradation of highly thermolabile phenolic compounds during heat treatment contributes to the decrement of antioxidant activity (Oracz & Nebesny, 2016). The variation in antioxidant capacity of the cocoa cultivars could be related to the differences in the level of polyphenols and other antioxidants. Besides, factors such as different growing conditions of cocoa fruit, time of harvest and drying condition of cocoa beans could cause the variation in antioxidant capacity (Oraz & Nesbeny, 2016). Antioxidant activities of cocoa beans added with yeast starter cultures during fermentation were in accordance with a previous study (Ooi et al., 2020).



Small alphabet indicates the comparison of different yeast added fermentations within the same batch, different small letter indicating a significant difference between the added fermentation within the same batch at ($p \leq 0.05$) based on two-way ANOVA, Tukey test ($p \leq 0.05$). Bars indicated standard error of the mean (±SEM)

Figure 3.4 Antioxidant content of dried cocoa beans: (a) Total phenolics content (mg/g GAE); (b) Total flavonoid content (mg/g Catechin); (c) DPPH content of beans (µmol/g TE); (d) ABTS content of beans (µmol/g TE) of beans added with *Hanseniaspora thailandica, Pichia kudriavzeviii* and mixture of both species for two batches of fermentation

3.4.3.4 Correlation between TPC, TFC and antioxidant activities

Table 3.1 showed that very weak negative correlations among DPPH radical scavenging activity and TPC were observed in control and sample fermented with *P. kudriavzevii.* The current finding is similar to a study of Oraz & Nesbeny (2016), who also observed no correlation among DPPH radical scavenging activity and TPC. This phenomenon could be explained by the antioxidant capacity of cocoa extract, which may relate to the presence and interaction of bioactive components (methylxanthines) and products of Maillard reaction (Oraz & Nesbeny, 2016), other than phenolic compounds. Strong negative to a very weak positive or negative correlation between phenolic compounds (TPC and TFC) and ABTS were observed in all chocolates (Table 3.1). This had indicated the possible degradation of antioxidant capacity of phenolics at high temperature during fermentation (43°C and 45°C) (section 3.4.2.1). The finding was in accordance with a similar study where the antioxidant capacity of phenolics compounds could be destroyed at high extraction temperature of 25°C (Thoo et al.,2013). Thus, the degradation of antioxidant capacity of phenolics may lead to inconsistency correlation between TPC, TFC and ABTS.

It is also worth to highlight that strong to very strong negative correlations between DPPH and TPC ($r = -0.705$, $r = -0.830$) were observed in samples fermented with *H. thailandica* and Mix (Table 3.1). A very strong negative correlation between DPPH and TFC ($r = -0.821$) was also observed in samples fermented with *P. kudriavzevii* ($p \leq 0.05$) (Table 3.1), suggested that these phenolic and flavonoid compounds could not be completely accountable for the free radical scavenging activity of the studied fermented samples. The very weak to moderate ($r = -0.009$ to 0.598) correlations between DPPH and TFC were observed in all the other samples, suggesting that flavonoids did not work through the electron transfer action (DPPH free radical scavenging). The current findings were in accordance with previous

observations, where positive, negative and weak correlations were reported between TPC, TFC and antioxidant capacities of cereal and cocoa (Dulf et al., 2017; Oracz & Nebesny, 2016; Oracz & Zyzelewicz, 2019). This phenomenon could be associated with the degree of polymerization during certain stages of fungal growth (Dulf et al., 2017). The increment in degree of polymerization would improve the free radical scavenging activity of the phenolic compounds (Dulf et al., 2017). In a similar study, treatment of certain yeast is capable of releasing the insoluble bound phenolic acids in wheat bran, leading to high antioxidant properties (Moore et al., 2007). In brief, these correlation analyses suggest that TPC and TFC in the samples could probably work through other mechanisms，other than DPPH and ABTS (electron transfer reactions). The phenolics and flavonoids compounds may not be the main constituents that led to the antioxidant activities of the fermented samples.

Table 3.1 Pearson correlation coefficients (r) among TPC, TFC and antioxidant activities of cocoa beans fermented spontaneously (control), fermented with *P. kudriavzevii, H. thailandica* and mixture of two yeasts

Sample	**Control**		***P.kudriavzevii***		***H.thailandica***		**Mix**	
	DPPH (μmol/g TE)	**ABTS** (μmol/g TE)	**DPPH** (μmol/g TE)	**ABTS** (μmol/g TE)	**DPPH** (μmol/g TE)	**ABTS** (μmol/g TE)	**DPPH** (μmol/g TE)	**ABTS** (μmol/g TE)
TPC (mg/g GAE)	-0.169	-0.333	-0.090	-0.037	-0.705	0.188	-0.830* ($p<0.05$)	-0.687
TFC (mg/g catechin)	-0.009	0.126	-0.821* ($p<0.05$)	-0.443	0.079	-0.038	0.055	0.295

Correlation significant at * $p \leq 0.05$

3.4.4 Fermentation index, pH of the dried cocoa bean and Cut test/ Equivalent Percent Fully Brown Score (EB score)

Fermentation index measures the degree of fermentation based on the brownness formed in the cocoa beans (Nsor-Atindana et al., 2012). Both fermentation batches produced well-fermented beans ranging from 1.12 to 1.35 (Figure 3.5a). However, control in Batch 1 showed under-fermented beans after 96 h.

There was no significant difference ($p \leq 0.05$) in pH of dried beans between control and treated beans (Figure 3.5b). In contrast, treated beans of batch 2 had a higher pH ($p \leq 0.05$) than control. Based on comparison among batches, the pH of dried beans added with *H. thailandica* and mixture of the 2 yeast species from Batch 2 was the highest ($p \leq 0.05$) (Figure 3.5b). The pH range of dried beans of the current study (pH 5.31 to 6.80) was higher than the reported in previous literature (pH 4.5 to 5.5) (Guehi et al., 2010).

Table 3.2 shows the percentage of EB score ranging from 98.3% to 100% was an indication of complete fermentation. The lowest EB score was found in batch 1 control and batch 2 sample added with mix yeast starter culture. The scores were not statistically different between control and treated beans ($p \leq 0.05$).

The under-fermented control beans in batch 1 may be associated with the clone variety, which requires a longer period of fermentation. According to Bimont et al. (2017), fermentation duration is dependent on cocoa variety. For instance, Trinitario and Nacional clones require 3 and 5 days fermentation, respectively (Bimont et al., 2017). Fermentation degree is a measure of oxidation of anthocyanins in cocoa beans and a result of a reduction of polyphenols concentration (Hernández-Hernández et al., 2016). In the current study, dried cocoa beans

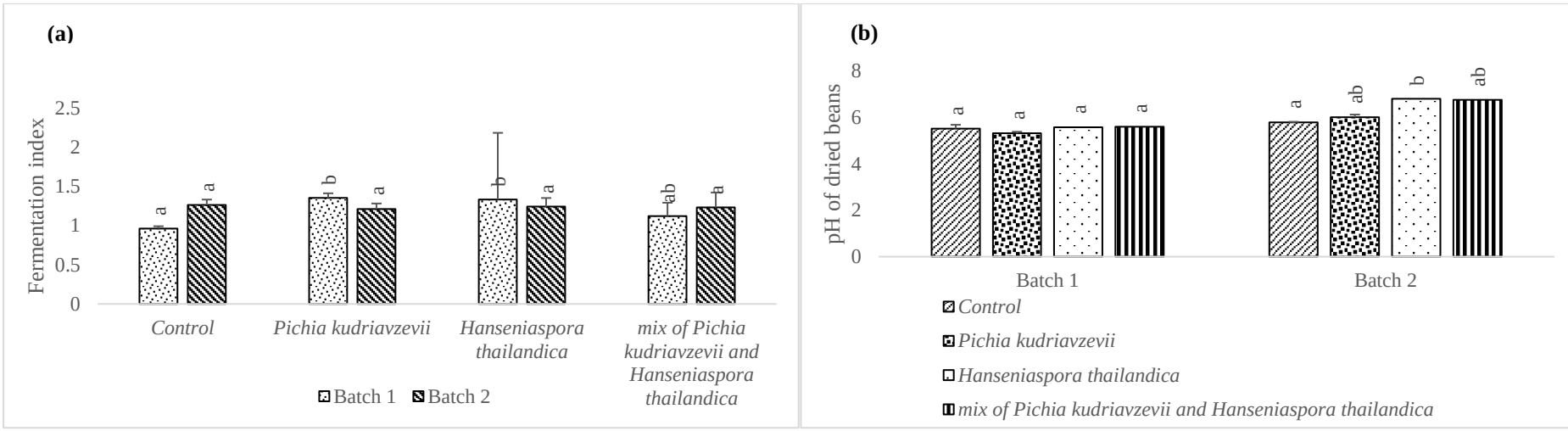
added with *H. thailandica* achieved ideal fermentation index despite high TPC was also produced in the beans.

The pH of dried cocoa beans of the current study was lower (pH 5.31 to 6.75) as compared to a study using 20% or 30% inoculum consisting of yeast, lactic acid bacteria & acetic acid bacteria, where high pH 5.9 to 7.2 and low fermentation index (poorly fermented) beans were reported (Sandhya et al., 2016). This could be due to the usage of different clones of cocoa pods from different geographical regions. Based on the EB score and fermentation index, the application of yeast starter cultures in both batches of fermentation produced less acidic and well-fermented cocoa beans.

Table 3.2 Equivalent Percent Fully Brown Score (%) of Batch 1 and 2

Sample	**Batch 1 (%)**	**Batch 2 (%)**
Control	98.3±2.08[a]	100±0.00[a]
P. kudriavzevii	99.3±1.15[a]	99±1.00[a]
H. thailandica	99.7±0.58[a]	100±0.00[a]
Mix of *P. kudriavzevii* and *H. thailandica*	100±0.00[a]	98.3±2.08[a]

Small alphabet indicates the comparison of different yeast treated fermentations within the same batch, different small letter indicating a significant difference between the added fermentation within the same batch at ($p \leq 0.05$) based on two-way ANOVA, Tukey test ($p \leq 0.05$). Bars indicated standard error of the mean (±SEM)



Small alphabet indicating comparison of the same added fermentation among 2 batches, different small letter indicating a significant difference between the same added fermentation among 2 batches at ($p \leq 0.05$) based on two-way ANOVA, Tukey test ($p \leq 0.05$). Bars indicated standard error of the mean (±SEM)

Figure 3.5 (a) Fermentation index; (b) pH of dried beans produced from fermentation added with *Hanseniaspora thailandica, Pichia kudriavzeviii* and mixture of both species in two batches of fermentation

3.4.5 Analysis of the volatile profile of roasted cocoa beans

Generally, a total of 130 volatile compounds were identified in the roasted cocoa bean by comparing the mass spectra of each compound with the MSD Chemstation software (Table 3.3a). After comparing the retention index of individual compound with the literature data and external standard, a total of 18 volatile compounds were identified in the roasted cocoa bean. They were categorized into 8 groups consisted of acids (5 groups), alcohols (2 groups), aldehydes (2 groups), ketones (2 groups), esters (2 groups), terpenes (1 group), pyrazines (3 groups), and terpenes alcohol (1 group).

The volatile ketone such as acetophenone has a floral odour. Sample added with yeast starter cultures such as *H. thailandica* (0.28%), *P. kudriavzevii* (0.37%) and a mixture of both yeasts (0.57%) had no significant difference as compared to control (0.38%). Esters represent the second most important chemical group in the cocoa product (Rodriguez-campos et al., 2012). The 2- phenyl ethyl acetate, which gives floral odour is one of the positive flavour notes in cocoa aroma (Rodriguez-campos et al., 2012). It is desirable to have a high concentration of 2-phenylethyl acetate. This compound was detected in samples added with yeast *H. thailandica* (0.20%), *P. kudriavzevii* (0.34%) and a mixture of both yeasts (0.14%). There was no significant difference among samples added with yeast and control (0.42%).

The terpenes alcohol compounds especially trans-Linalool oxide gives rise to sweet and floral aroma notes to chocolate (Ascrizzi et al., 2017). This compound was only detected in the sample added with *P. kudriavzevii* (0.04 %). It was absent in control and other samples added with a yeast starter culture.

Pyrazines are the flavour compounds that represent roasted, burnt and nutty notes. They present in foods processed under high temperature and low-humidity condition. They are a

product of Maillard reaction (Kongor et al., 2016). Pyrazine such as tetramethyl-pyrazine confers nutty, chocolate and roasted notes to cocoa. The tetramethyl-pyrazine content of samples in descending order was control (8.89%) followed by sample added *P. kudriavzevii* (8.37%), mixed yeast starter sample (7.45%) and *H. thailandica* (7.28%). The tetramethyl-pyrazine derives from fermentation process instead of the subsequent heat treatments (Ascrizzi et al., 2017). We suggested that the sample added with a single yeast starter culture influenced the VOC concentrations of cocoa liquor. Flavour compounds such as trans-Linalool oxide (sweet and floral aromas) were only uniquely detected in sample added with PK, signifying the presence of unique flavour in sample fermented with a selected yeast starter.

Table 3.3a General screening of volatile organic compounds (VOCs) identified in the roasted cocoa beans fermented with *P. kudriavzevii* (PK), *H. thailandica* (HT), a mixture of PK and HT (Mix) and control. Fill box indicates presence and open box indicates absence of the compound

No.	VOCs	Control	HT	PK	MIX	Odour descriptor*
Aldehydes						
1	Acetaldehyde					fruity: pungent ethereal aldehydic fruity
2	2-methylpropanal					aldehydic: fresh aldehydic floral green
3	Pentanal					almond, malt, pungent
4	3 methyl 1 butanal					ethereal aldehydic chocolate peach fatty
5	Heptanal					Green
6	3-hydroxy-butanal					chocolate: pungent cocoa musty green malty bready
7	3-(methylthio)-propanal					foul and persistent odour.
8	Benzaldehyde					almond like odour
9	Benzeneacetaldehyde					Honey, floral rose, sweet, powdery, fermented, chocolate with a slight earthy nuance
10	α-Ethylidenbenzeneacetaldehyde					Musty, sweet narcissus cortex beany honey cocoa nutty radish
11	Butanal					chocolate: pungent cocoa musty green malty bready
12	alpha.-(2-methylpropylidene)Benzeneacetaldehyde					sweet cocoa nutty rose powdery chocolate
13	Dodecanal					aldehydic: soapy waxy aldehydic citrus green floral
14	Octadecanal					oily
15	5-Methyl-2-phenyl-2-hexenal					aldehydic bitter cocoa nut skin green sweet chocolate fruity butyric; cocoa hexenal
16	Tetradecanal					waxy; fatty waxy amber incense dry citrus peel musk
Acids						
17	Acetic acid, methyl ester					Ethereal sweet fruity
18	Acetic acid					Sour, astringent, vinegar

No.	VOCs	Control	HT	PK	MIX	Odour descriptor*
19	2-methyl propanoic acid					acidic: pungent acidic cheesy vinegar
20	4-oxo-pentanoic acid					sweet caramel acidic acetoin buttery (caramellic)
21	Acetic acid, 2-methylpropyl ester					sweet fruity ethereal banana tropical (fruity)
22	3-methyl-butanoic acid methyl ester					fruity: strong apple fruity pineapple
23	2-methyl propanoic acid					acidic sour cheese dairy buttery rancid
24	Butanoic acid					cheesy: sharp acetic cheese butter fruit
25	3-methyl-butanoic acid ethyl ester					fruity: fruity sweet apple pineapple tutti frutti
26	3-methyl-butanoic acid					cheesy
27	3-methyl-pentanoic acid					sour cheese fresh fruity (flavour); animal sharp acidic cheesy green fruity (animal odour)
28	2-methyl-hexanoic acid					cheesy; fruity cheese oily fatty lard
29	2-methyl-pentanoic acid					cheesy; buttery flavour
30	Pentanoic acid, methyl ester					fruity: sweet green fruity apple pineapple nutty
31	2-methyl-butanoic acid					acidic
32	2-hydroxy-4-methyl-pentanoic acid, methyl ester					sweet fruity musty
33	3-methyl-2-Butenoic acid					vegetable: musty potato tomato earthy vegetable creamy
34	Pentanoic acid					Cheesy; Acidic and sharp, cheese-like, sour milky, tobacco, with fruity nuances
35	3-hydroxy-butanoic acid, ethyl ester,					fruity; fruity green grape tropical apple skin
36	Heptanoic acid					cheesy: rancid sour cheesy sweat
37	Hexanoic acid					cheesy: sour fatty sweat cheese
38	Hexanoic acid, ethyl ester					fruity: sweet fruity pineapple waxy green banana
39	2-methyl-propanoic acid, 2-methylbutyl ester					fruity: fruity ethereal tropical banana

No.	VOCs	Control	HT	PK	MIX	Odour descriptor*
40	2-methyl-propanoic acid, 3-methylbutyl ester					fruity: fruity waxy apricot pineapple green banana
41	Acetic acid, hexyl ester					fruity; fruity green apple banana sweet
42	3-oxo-butanoic acid, ethyl ester					Fresh fruity green apple fatty (Fruity)
43	Propanoic acid, ethyl ester					fruity: sweet fruity rum juicy fruit grape pineapple
44	Butanoic acid, ethyl ester					fruity: fruity pineapple cognac
45	2-Hexenoic acid					fruity; powerful fruity sweet warm herbal;(flavour) acidic cheesy fruity sweet *flavour
46	2-methyl-propanoic acid, ethyl ester					Sweet, etherial and fruity with pungent, alcoholic, fusel and rummy nuances
47	Heptanoic acid, ethyl ester					fruity; fruity pineapple cognac rum wine
48	Benzoic acid methyl ester					min phenolic wintergreen almond floral cananga
49	3-methyl-butanoic acid, 2-methylbutyl ester					fruity: herbal fruity earthy cheese apple green
50	3-methyl-butanoic acid, 3-methylbutyl ester					fruity: sweet fruity green ripe apple jammy tropical
51	Octanoic acid, methyl ester					fatty waxy rancid oily vegetable cheesy
52	Pentanoic acid, ethyl ester					fruity: sweet fruity apple pineapple green tropical
53	Acetic acid, phenylmethyl ester					floral
54	Benzoic acid					balsamic
55	Diethyl-acetic acid					ethereal fruity sweet weedy green; sweet, grape, apple and rum-like
56	Benzoic acid, ethyl ester					Minty
57	Octanoic acid					fatty
58	Benzene acetic acid, methyl ester					honey; Sweet, floral, fruity, honey and spice like
59	Octanoic acid, ethyl ester					Waxy

No.	VOCs	Control	HT	PK	MIX	Odour descriptor*
60	Butanoic acid, 1-methylethyl ester					faint odour
61	Butanoic acid, ethyl ester					fruity pineapple
62	Benzeneacetic acid, ethyl ester					floral: sweet floral honey rose balsam cocoa
63	4-oxo-pentanoic acid					caramellic: sweet caramel acidic acetoin buttery
64	Benzeneacetic acid					Sweet taste at low concentrations and has a rose-like odour
65	Propanoic acid, 2-phenylethyl ester					floral: floral red rose fruity honey balsam storax
66	Phenethyl acetate					Floral
67	Butanoic acid, hexyl ester					green: green sweet fruity apple waxy soapy
68	Benzenepropanoic acid, ethyl ester					hyacinth rose honey fruity rum (Floral)
69	n-Decanoic acid					fatty
70	2-Propenoic acid, 3-phenyl-, ethyl ester, (E)-					balsamic
71	Dodecanoic acid, methyl ester					waxy: waxy soapy creamy coconut mushroom
72	Benzeneacetic acid, 2-methylpropyl ester					chocolate: sweet floral honey chocolate amber
73	Dodecanoic acid, ethyl ester					sweet waxy fruity apple grape oily brandy
74	Tetradecanoic acid					waxy (mystic acid- fatty acid)
75	Butanoic acid, 3-hydroxy-, methyl ester					apple:mild fruity green apple winey
76	Tetradecanoic acid, ethyl ester					waxy
77	Decanoic acid, ethyl ester					waxy: sweet waxy fruity apple grape oily brandy
Ketones						
78	3-hydroxy 2 butanone					sweet buttery creamy dairy milky fatty
79	4-methyl-2(3H)-Thiazolethione					nutty, green
80	Butyrolactone					creamy oily fatty caramel

No.	VOCs	Control	HT	PK	MIX	Odour descriptor*
81	Acetophenone					floral
82	Acetic acid, octyl ester					Floral
83	3,7-dimethyl-2,6-octadienyl ester, pentanoic acid					floral: rose fruity pineapple
84	n-Hexadecanoic acid					waxy
85	Decanoic acid, ethyl ester					waxy
86	2-phenylethyl ester benzoic acid					floral: soft rose balsam honey floral
87	Benzoic acid, hexyl ester					sweet floral aromatic
88	Benzoic acid, 2-methylpropyl ester					balsamic
89	Hexanoic acid, 2-phenylethyl ester					floral: sweet honey floral waxy woody sweaty green banana pineapple
90	Dodecanoic acid					fatty
Alcohols						
91	2-pentanol					Green, mild green
92	2-heptanol					citrus
93	3-methyl-2-Butanol					Fruity
94	3-methyl-2-Pentanol					Pungent, fusel, cognac and wine, cocoa, with green fruity
95	3-methyl-1-Butanol					fusel, alcoholic, pungent, etherial, cognac, fruity, banana and molasses
96	1-amino- 2-Propanol					fishy
97	2-Ethyl-1-butanol, methyl ether					Alcoholic, sweet musty alcoholic
98	2,3-Butanediol					butter
99	2-pentanol, acetate					weak odour of banana; herbal
100	3-methyl-1-Butanol, acetate					sweet fruity banana solvent
101	1,3-Butanediol					none
102	2,2-dimethyl-1-Butanol					Roasted
103	4-methyl-2-Pentanol					pungent: pungent alcohol

No.	VOCs	Control	HT	PK	MIX	Odour descriptor*
104	Benzyl alcohol					odour: floral (floral rose phenolic balsamic) Flavour: Fruity (chemical fruity cherry almond balsamic bitter)
105	3-bromo-2-butanol, acetate					Ethereal sweet fruity
106	.alpha.-methyl-benzenemethanol					floral: floral earthy green honeysuckle
107	Phenylethyl Alcohol					Sweet, floral, fresh and bready with a rosey honey nuance
108	.alpha.-methyl-benzeneethanol					floral-weak rose
Esters						
109	2,3-Butanedioldiacetate					none
110	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate					floral: floral rose lavender green waxy (lavender)
Monoterpenes						
111	Beta-myrcene					spicy
Pyrazines						
112	2,3-dimethyl-pyrazine					Hazelnut, roasted, baked
113	1,1-dimethyl-hydrazine					ammonia like odour
114	Trimethyl-pyrazine					cooked flavour
115	2-ethenyl-6-methyl pyrazine					potato: roasted potato
116	Tetramethyl-pyrazine					Nutty, chocolate, roasted, tea.
117	2,3,5-Trimethyl-6-butylpyrazine					nutty: nutty nut skin earthy powdery cocoa baked potato roasted peanut hazelnut musty
Nitrogen compounds						
118	Benzonitrile					almond-like odour
Phenols						
119	Phenol					sweet and tarry
120	2-methoxy- phenol					fruity, pineapple
121	2-methyl-5-(1-methylethyl)-phenol					spicy: spice woody camphor thymol

No.	VOCs	Control	HT	PK	MIX	Odour descriptor*
122	4,4'-(1-methylethylidene) bis phenol					mild phenolic
Alkane						
123	Tetradecane					mild waxy
124	Pentadecane					waxy
Oxygenated						
125	Linalool oxide trans					floral: floral herbal earthy green
Others						
126	1,4-Butanediamine					foul odour of putrefying flesh
127	1,6-Octadien-3-ol, 3,7-dimethyl-					floral: citrus floral sweet bois de rose woody green blueberry
128	Indole					(Animal) pungent, floral, slightly naphtha and mothball like with a faecal and animalic musty character
129	Thiazole					at 0.10 % in propylene glycol. pyridine nutty meaty
130	2,3,5-Trimethyl-6-ethylpyrazine					nutty, musty, powdery cocoa, potato and musty

*Obtained from literatures.

Table 3.3b Further screening of volatile organic compounds (VOCs) identified in the roasted cocoa beans fermented with *P. kudriavzevii* (PK), *H. thailandica* (HT), a mixture of PK and HT (Mix) and control

Group	Retention time (min)	Compound	Odour descriptors[a]	Mean GC-MS peak area x 10^5				Kovats Retention Index[b]	References
				Control	PK	HT	Mix		
Acids	2.544	Acetic acid	Sour, astringent, vinegar	24.75±5.06[a]	31.94±15.6[a]	32.65±15.7[a]	26.4±17.8[a]	660-662	Rodriguez-Campos et al. (2012)
	8.141	3-methyl-Butanoic acid	Rancid, cheese	3.21±3.00[a]	4.51±3.90[a]	5.05±3.98[a]	3.29±2.31[a]	817-888	Ascrizzi et al. (2017)
	8.555	2-methyl- Butanoic acid	Rancid, cheese	0.75±1.13[a]	1.23±1.65[a]	1.48±1.49[a]	1.35±1.14[a]	832-894	Ascrizzi et al. (2017)
	16.734	n-Decanoic acid	Rancid, fatty	0.02±0.00[a]	0.01±0.02[a]	0.01±0.02[a]	0.01±0.01[a]	1362-1402	Rodriguez-Campos et al. (2012)
	19.207	Dodecanoic acid	Metal	0.04±0.04[a]	0.06±0.04[a]	0.06±0.04[a]	0.05±0.04[a]	1549-1580	Rodriguez-Campos et al. (2012)
Sum				**28.77**	**37.75**	**39.25**	**31.10**		
Aldehyde	3.227	3-methyl-butanal	Malty, chocolate	0.02±0.04[a]	0.00±0.00[a]	0.00±0.00[a]	0.05±0.11[a]	646-657	Rodriguez-Campos et al. (2012)
	18.441	5-Methyl-2-phenyl-2-hexenal		0.07±0.05[a]	0.10±0.08[a]	0.14±0.05[a]	0.08±0.09[a]	1483	Tuenter, et al. (2020)
Sum				**0.09**	**0.10**	**0.14**	**0.13**		
Ketones	3.635	3-hydroxy- 2-Butanone		1.19±1.60[a]	0.65±0.35[a]	0.43±0.43[a]	1.37±0.33[a]	707-720	Tuenter, et al. (2020)
	12.201	Acetophenone	Must, flower, almond, sweet	0.38±0.05[a]	0.37±0.23[a]	0.28±0.11[a]	0.57±0.38[a]	1068	Crafack et al. (2014)
Sum				**1.57**	**1.02**	**0.71**	**1.94**		
Esters	15.097	Ethyl phenylacetate	Fruity, sweet	0.30±0.47[a]	0.34±0.38[a]	0.10±0.12[a]	0.11±0.09[a]	1252	Rodriguez-Campos et al. (2012)

	17.184	n-Butyl benzoate		0.09±0.10[a]	0.06±0.11[a]	0.06±0.13[a]	0.11±0.13[a]	1377	Tuenter, et al. (2020)
Sum				**0.39**	**0.40**	**0.16**	**0.22**		
Alcohols	4.156	3 methyl-1-butanol	Malty, bitter, chocolate	0.94±1.88[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	730-760	Rodriguez-Campos et al. (2011)
	5.735	2,3-butanediol	Buttery	20.99±5.72[a]	12.24±4.83[a]	19.95±12.48[a]	10.17±2.74[a]	782-819	Rodriguez-Campos et al. (2012)
Sum				**21.93**	**12.24**	**19.95**	**10.17**		
Pyrazines	9.229	2,3-dimethyl-Pyrazine	Caramel, cocoa	0.10±0.03[a]	0.12±0.04[a]	0.18±0.10[a]	0.12±0.07[a]	911-920	Ascrizzi et al. (2017)
	12.565	Tetramethyl-pyrazine	Roasted, green, coffee, cocoa	8.89±3.81[a]	8.37±1.75[a]	7.28±1.91[a]	7.45±2.79[a]	1086-1087.3	Ascrizzi et al. (2017)
	13.790	2,3,5-Trimethyl-6-ethylpyrazine	Earthy, peanuts, cocoa, roasted nuts	0.13±0.04[a]	0.08±0.13[a]	0.11±0.05[a]	0.11±0.04[a]	none	Ascrizzi et al. (2017)
Sum				**9.12**	**8.57**	**7.57**	**7.68**		
Terpenes	10.748	β-Myrcene	Spicy	0.08±0.12[a]	0.13±0.03[a]	0.00±0.00[a]	0.09±0.19[a]	992	Tuenter, et al. (2020)
Sum				**0.08**	**0.13**	**0.00**	**0.09**		
Terpenes alcohol	14.006	trans-Linalool oxide		0.00±0.00[a]	0.01±0.02[a]	0.00±0.00[a]	0.00±0.00[a]	1091	Ascrizzi et al. (2017)
Sum				**0.00**	**0.01**	**0.00**	**0.00**		

[a] Flavor notes reported.
[b] Obtained of literature.
Small alphabet indicates the comparison of different yeast added fermentations, different small letter indicating a significant difference between the added fermentation at ($p \leq 0.05$) based on one-way ANOVA, Tukey test ($p \leq 0.05$). Bars indicated standard error of the mean (±SEM).

3.4.5.1 Principal Component Analysis (PCA)

The score plot in Figure 3.6 shows a clear separation of cocoa beans added with *H. thailandica* (HT), *P. kudriavzevii* (PK), a mixture of 2 yeasts (Mix) and control. The first two principal components (PCs) explain the maximum variations where 45.3% and 40.6% of date variance was observed for PC1 and PC2, respectively. This analysis included 8 VOC groups (alcohols, esters, pyrazines, acetic acid, aldehydes, ketones, terpenes and terpenes alcohol groups). A clear separation of roasted cocoa beans added with HT, PK, Mix or control was observed in score plot (Figure 3.6), indicating a difference in quality and quantity of the developed volatiles among these samples.

Based on the positive side in PC1, control or sample added with HT was highly influenced by volatile compound groups such as ketones, esters, pyrazines, terpenes and terpenes alcohol. Those volatile compounds shared common flavour attributes such as sweet, fruity, almond, nutty, spicy and floral. The negative side of PC1 showed that acids, alcohols and aldehydes volatile compounds groups were highly influenced by sample added with PK and Mix. These compounds conferred flavour notes such as sour, astringent, rancid, malty and chocolate. It indicated that roasted cocoa beans of PK and Mix had high concentrations of these volatile compounds.

For the positive side of PC2, sample added with PK or HT was highly influenced by volatile compounds such as acids, aldehydes, esters, terpenes and terpenes alcohol. These compounds are responsible for sour, astringent, rancid, cheese, bitter, malty, chocolate, fruity, sweet, roasted and nutty notes. For the negative side of PC2, samples added with control and mix cultures were highly influenced by alcohol, pyrazines, and ketones compounds, which conferred to roasted nuts, cocoa, malty and chocolate flavours, respectively. It also signified

that roasted cocoa beans added with control and mixed cultures had high concentrations of these volatile compounds.

Overall, the PCA findings showed that VOC profiles of cocoa fermentation added with *H. thailandica, P. kudriavzevii* or mixture of the two yeast starter cultures were distinctly different from the spontaneous fermentation.

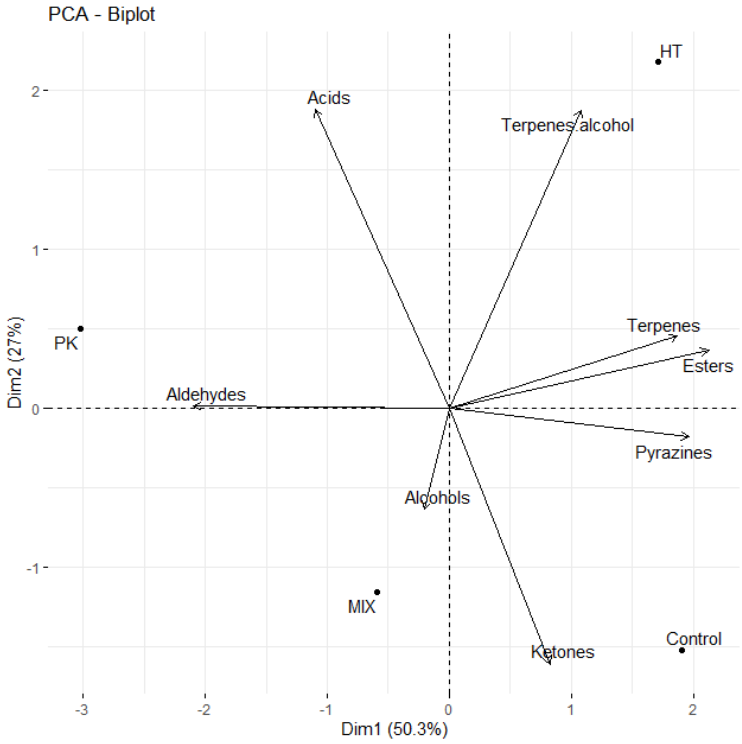


Figure 3.6 Principal Component Analysis (PCA) biplot of the chemical classes of the roasted cocoa beans fermented with *P. kudriavzevii* (PK), *H. thailandica* (HT), a mixture of PK and HT (Mix) and control

3.4.6 Sensory evaluation of cocoa liquor

Figure 3.7 and Table 3.4 showed the sensorial differences of cocoa liquors produced with different starter cultures based on 19 descriptors. Overall, the sensory profile of cocoa liquor produced with different yeast starter culture was distinctively different as compared to control and Ghana samples ($p \leq 0.05$). Generally, Ghana cocoa liquor can be characterized as being sweet with cocoa and roasted flavours. Control cocoa liquor was characterized as having

cocoa note with considerably bitter and astringent flavours, with the absent of sweet and spicy flavours. Sample added with mixed culture produced cocoa liquor that was sweet, spicy with high in bitterness and astringent flavours. Sample added with *P. kudriavzevii* produced cocoa liquor that was regarded as cocoa, sweet, spicy with least bitterness and least astringency compared to control ($p \leq 0.05$). Sample added with *H. thailandica* also produced sweet, spicy notes with least astringent cocoa liquors as compared to control ($p \leq 0.05$).

In terms of cocoa flavour, Ghana sample had the highest score whereas the sample added with *H. thailandica* or mixed cultures, had the lowest score of cocoa flavour as compared to control ($p \leq 0.05$). The cocoa flavour is associated with volatile alcohols, ketones and pyrazines, as measured by GCMS (Table 3.3). Sample added with mixed culture had the highest score of bitterness whereas sample added *P. kudriavzevii* had the lowest score of bitterness as compared to control ($p \leq 0.05$). Bitterness is highly associated with the polyphenols of cocoa liquors (John et al., 2019). Despite sample added with *H. thailandica* recorded the highest TPC in dried cocoa beans, however its chocolate produced was not very bitter. The bitterness reduction of HT chocolate could be due to the loss of polyphenols from the cocoa beans particularly during the roasting process (Rocha et al., 2017).

Ghana sample had the highest score of roasted flavour as compared to control. Sample added with mixed starter culture had the lowest score of roasted flavour than control ($p \leq 0.05$). Roasted flavour is associated with the pyrazine group whereby it is particularly influenced by tetramethyl-pyrazine compound (Table 3.3) (Rodriguez-campos et al., 2012). Sample added with mixed culture had the highest score of astringency ($p \leq 0.05$). Sample added with *P. kudrivzevii* or *H. thailandica* had the lowest score of astringency as compared to control ($p \leq 0.05$). Astringency is conferred by acid compounds (Table 3.3) (Ascrizzi et al., 2017). Interestingly, all the samples expressed a low intensity of acid flavour with no significant

difference among them ($p \leq 0.05$). The acid flavour is highly associated with high concentrations of acetic acid (Table 3.3).

It was noted that sweet flavour was detected in all the samples but absent in cocoa liquor of spontaneous fermentation. The sweet flavour is highly associated with terpenes alcohol compounds such as trans-Linalool oxide (Toker et al., 2020). Trans-Linalool oxide compound was not detected in control by GCMS (Table 3.3), which is in accordance with the current sensory result. Uniquely, a very low intensity of spicy flavour was only detected in samples added with yeast starter cultures. The spicy flavour is highly associated with Beta-myrcene compound (Table 3.3) (Toker et al., 2020). Flavour attributes such as fresh fruits, browned fruits, floral, woody, nutty and other off flavour were not statistically different as compared to control ($p \leq 0.05$). Other off-flavour attributes such as dirty, meaty, mouldy, smoky and over fermented notes were considerably absent in all the cocoa liquors. Based on the result, it was suggested that the VOC profile was correlated with most of the sensory perceptions.

From the overall quality attributes (Table 3.4), Ghana sample was highly preferred by trained panels, followed by sample added with *P. kudriavzevii,* cocoa liquor from spontaneous fermentation and sample added with mixed culture. Sample added with *H. thailandica* was the least preferred by trained panels (Table 3.4). The addition of yeast species as starter culture in fermentation modulated the flavour profile of the cocoa liquor. Flavour attributes such as sweet and spicy notes were the unique flavours that were only detected in cocoa liquor samples produced using beans fermented with yeast starter cultures.

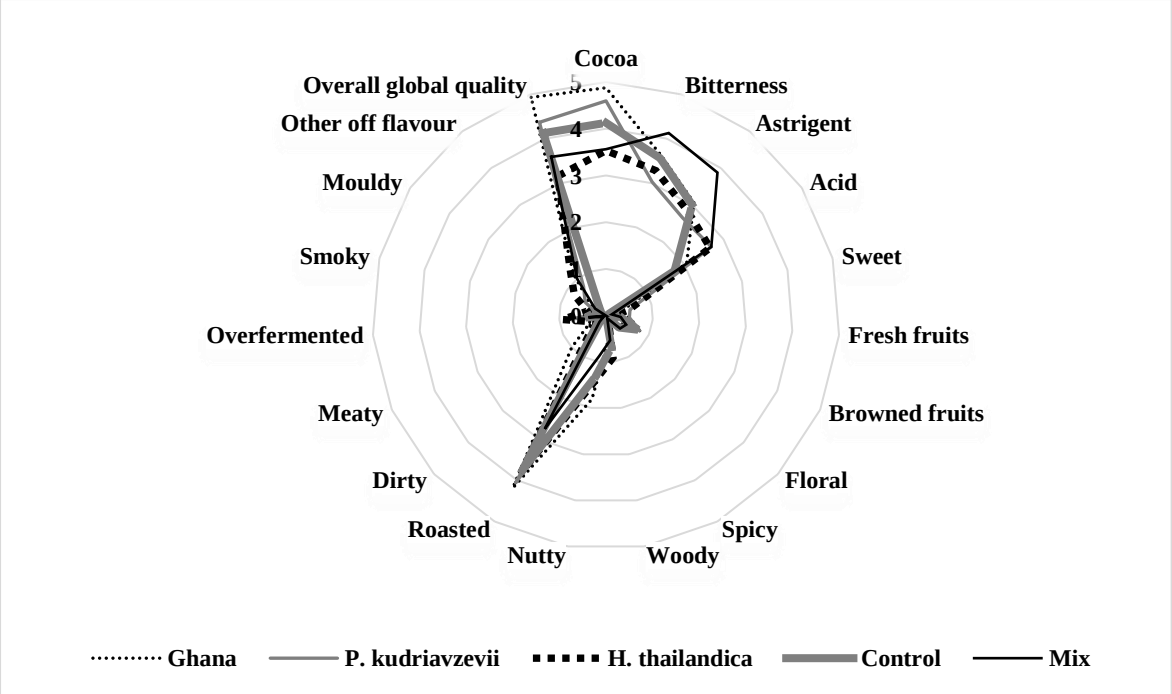


Figure 3.7 Sensory profiles of the cocoa liquor produced from Ghana, cocoa beans spontaneously fermented (control) and cocoa beans added with yeast starter cultures (PK, HT and Mix). The centre of the diagram corresponds to the lowest flavour intensity and perimeter to the highest flavour intensity

Table 3.4 Sensory profiles of the cocoa liquor produced from Ghana cocoa beans, control (cocoa beans that were spontaneously fermented) and cocoa beans added with yeast starter cultures

Sample	Cocoa	Bitterness	Astringent	Acid	Sweet	Fresh fruits	Browned fruits	Floral	Spicy	Overall global quality
Ghana	4.88 ± 1.13^{b}	3.63 ± 0.88 ^{ab}	3.06 ±0.9 ^{ab}	2.06 ±0.94^{a}	0.31 ±0.88^{a}	0.5 ±0.71^{a}	0.81±0.75^{a}	0.25±0.46^{a}	0 .00 ±0.00^{a}	4.94 ±0.94^{b}
P. kudriavzevii	4.59 ± 1.00^{ab}	3.03 ± 0.81^{a}	2.66 ±0.91^{a}	2.69 ±1.14^{a}	0.53 ±0.88^{a}	0.5 ±0.73^{a}	0.78±0.55^{a}	0.38±0.59^{a}	0.06 ±0.25^{a}	4.38 ±1.27^{ab}
H. thailandica	3.53 ± 1.27^{a}	3.28 ± 0.91^{ab}	2.84 ±0.72^{a}	2.66 ±1.26^{a}	0.5 ±1.14^{a}	0.13 ±0.39^{a}	0.63±0.72^{a}	0.13±0.34^{a}	0.09 ±0.38^{a}	3.16 ±1.17^{a}
Control	4.13 ± 1.09^{ab}	3.56 ± 0.98 ^{ab}	3 ±0.68 ^{ab}	1.75 ±0.73^{a}	0.00 ±0.00^{a}	0.31 ±0.57^{a}	0.75±0.63^{a}	0.38±0.50^{a}	0.00 ±0.00^{a}	4.13 ±0.99^{ab}
Mix	3.56 ± 0.93^{a}	4.13 ± 0.85b	3.88 ±0.94 ^{b}	2.69 ±1.01^{a}	0.13 ±0.50a	0.31 ±0.63a	0.47±0.74^{a}	0.41±0.82^{a}	0.06 ±0.25^{a}	3.59 ±1.65^{ab}
Sample	**Woody**	**Nutty**	**Roasted**	**Dirty**	**Meaty**	**Over fermented**	**Smoky**	**Mouldy**	**Other off-flavours**	
Ghana	0.44 ±0.73^{a}	1.81±0.96^{a}	4.18 ±0.92^{b}	1.00 ±1.93 ^{b}	0.38 ±0.74^{a}	0.38 ±1.06^{a}	0.25 ±0.46^{a}	0.38 ±1.06^{a}	1.13±1.89^{a}	
P. kudriavzevii	0.66 ±1.19^{a}	1.25±1.00^{a}	4.06 ±1.01^{ab}	0.00 ±0.00^{a}	0.13 ±0.34^{a}	0.69 ±1.01^{a}	0.19 ±0.40^{a}	0.53 ±1.06^{a}	0.78±1.02^{a}	
H. thailandica	0.91 ±1.19^{a}	1.41±1.10^{a}	3.81 ±1.03^{ab}	0.41 ±1.20^{ab}	0.31 ±1.01^{a}	0.94 ±1.06^{a}	0.41 ±0.76^{a}	0.81 ±1.47^{a}	1.25±1.73^{a}	
Control	0.69 ±1.29^{a}	1.31±1.03^{a}	3.75 ±1.37^{ab}	0.25 ±0.45^{ab}	0.00 ±0.00^{a}	0.44 ±0.79^{a}	0.25 ±0.45^{a}	0.13 ±0.34^{a}	0.25±0.45^{a}	
Mix	0.53 ±0.96^{a}	0.88±1.12^{a}	2.75 ±1.54^{a}	0.19 ±0.54^{ab}	0.06 ±0.25^{a}	0.38 ±0.89^{a}	0.00 ±0.00^{a}	0.28 ±0.77^{a}	1.00±1.76^{a}	

Small alphabet indicates the comparison of different yeast added fermentations, different small letter indicating a significant difference between the added fermentation at ($p \leq 0.05$) based on one-way ANOVA, Tukey test ($p \leq 0.05$). Bars indicated standard error of the mean (±SEM)

3.5 Conclusion

This study determined the influence of *H. thailandica* and *P. kudriavzevii* in the cocoa field fermentation. It was highly reproducible as consistent results (temperature, pH of nibs, and antioxidant contents) were observed in the two batches of fermentation. Based on the correlation analysis, the current study suggested that phenolics and flavonoids compounds may not be the main constituents that led to the antioxidant activities of the fermented samples. The use of *H. thailandica* in fermentation produced cocoa beans with improved TPC and well fermentation index (1.24 and 1.33). Based on the PCA, the VOC profiles of fermentation added with *H. thailandica, P. kudriavzevii* and mixture of these two yeast starter cultures were noticeably different from spontaneous fermentation. Our sensory result showed that the overall acceptance of cocoa liquor samples added with yeast starter culture were comparable to Ghana cocoa liquor. Sensory attributes such as sweet and spicy notes were the unique flavours that were only found in the sample added with yeast starter cultures. We concluded that the inoculation of yeast in fermentation influenced the organoleptic and antioxidant properties of cocoa beans. The quality of cocoa beans produced was in line with guidelines of the Malaysian Cocoa Board (MS 2672:2017).

Chapter 4

Work presented in this chapter was submitted to *Journal of the Science of Food and Agriculture* as an article entitled “Volatile organic compounds and sensory profile of dark chocolates made with cocoa beans fermented with *Pichia kudriavzevii* and *Hanseniaspora thailandica*”.

4 Physicochemical properties, volatile organic compounds and sensory profile of dark chocolates made with Malaysian cocoa beans fermented with selected yeast starters

4.1 Introduction

Cocoa (*Theobroma cacao* L.) is the crucial material used in chocolate production. The quality of cocoa beans is determined by its aroma, which is formed by volatile compounds (Castro-Alayo et al., 2019). Spontaneous cocoa fermentation without any starter culture often produces acidic, off-flavour and over-fermented beans (Schwan & Wheals, 2004). Thus, controlled fermentation by using different types of microorganisms in fermentation has been a research interest. Several studies had proposed the use of starter culture in fermentation in order to produce the uniform quality of cocoa beans. The addition of *Saccharomyces cerevisiae* in fermentation accelerated the fermentation process and produced chocolate with different volatile and sensory profiles (Menezes et al., 2016). Our lab scale fermentation results found that *H. thailandica* and *P. kudriavzevii* resulted in cocoa beans with higher TPC as compared to the spontaneous fermentation (Ooi et al., 2020). Study on the influence of yeast starter during cocoa fermentation in Malaysia is still limited. Thus, it is our research aims to determine the antioxidant content, flavour and sensory characteristics of chocolates produced using beans fermented with these selected yeast starters.

The chocolate making processes and sensorial characteristics of chocolate are often determined by the rheological properties (Cahyani et al., 2019). The rheological properties decide the manufacturing processes needed for obtaining final chocolate with desired product texture and melting characteristics (Cahyani et al., 2019). Thereby, the current research also studied the rheological, hardness and melting properties of chocolates produced using beans fermented with selected yeast starters. This information would serve as a foundation for chocolate production whereby it could be used to assess the manufacturing techniques applied

in the chocolate making process. To the best of our knowledge, the study on the rheological and melting properties, antioxidant properties, VOC and sensory of chocolates produced using cocoa beans fermented with selected yeast starter culture in Malaysia has few literatures. Hence, this study determined the rheological and melting properties, antioxidant properties, VOC and sensory profile of chocolates produced using beans fermented with *H. thailandica*, *P. kudriavzevii* or mixture of the two yeasts.

4.2 Materials and methods

4.2.1 Materials

Dried cocoa beans were obtained from previous work (Ooi et al., 2020) in which cocoa beans (PBM 123, BR 25, MCBC 1 and MCBC 8 obtained from two farms in Pahang, Malaysia) were fermented with yeast starters (*Hanseniaspora thailandica* (MH979675) (HT), *Pichia kudriazevii* (MH979681) (PK) and a mixture of these two yeasts (Mix)). The yeasts were isolated from a spontaneous cocoa bean fermentation and identified as previously reported in Ooi et al. (2020). Well-dried cocoa beans (moisture content of 7.5%) were oven roasted according to Sulaiman et al. (2014) at 145 ºC, 30 mins, deshelled and used in chocolate production. Ghana chocolate was produced using Ghana cocoa beans, which were purchased from Pastry Pro Pte Ltd. These beans were roasted and a commercial product which involved spontaneous fermentation. The Ghana chocolate was used as a reference for comparison of sensory profile.

4.2.2 Chocolate production

A total of 8 samples were produced which were known as Batch 1 (PK, HT and Mix) and Batch 2 (PK, HT and MIX), control and Ghana liquors. Chocolate production was according to a modified method of Ho et al. (2014). Cocoa liquor was produced from cooled

roasted nibs. Each sample was produced by mixing 70% cocoa liquor with 30% of sugar (Gula Prai fine granulated sugar, Malaysia). The mixture was conched for 6 h at approximately 74 ˚C using a stone concher (Spectra 11 Chocolate Melanger, Coimbatore, India). After conching, the liquid chocolate was tempered manually in a metal bowl and chocolate melter. Chocolate was heated up to 45 ˚C to melt all the fat crystals, then cooled to 27 ˚C and finally heated to 33 ˚C. Then, chocolate was poured into a mould, cooled at 4 ˚C for one hour. After cooling, the harden chocolate was packed and sealed in a bag and store at 4 ˚C for further analysis (Rheological measurement (viscosity), melting properties, hardness, cocoa fat analysis, determination of TPC, TFC, and DPPH free radical scavenging activities, analysis of polyphenols and methylxanthines by HPLC-DAD, VOCs, and sensory analysis). The particle size distribution of the final chocolate was measured according to a method of Żyżelewicz et al. (2016) by using MasterSizer 3000 (Laser Diffraction Particle Size Analyser, Malvern Instruments Ltd., UK) equipped with a Hydro EV to ensure the particle size of cocoa liquor was at d_{90}-value of below 30 μm (i.e. 90% of the particles in the liquor are finer than 30 μm). Control chocolate was produced using cocoa beans of spontaneous fermentation (fermentation process with indigenous microorganisms present in the cocoa fruit).

4.2.3 Rheological properties

Rheological properties of the molten chocolates were measured according to a modified method of Aidoo et al. (2015), by using rheometer MCR 302 (Modular Compact Rheometer, Anton Paar Ltd., Austria) equipped with a cup and bob geometry (CC27). Chocolate samples were heated in an oven (Memmert, Germany) at 45 ˚C prior to analysis. The temperature of the stage was set at 40 ˚C to prevent solidification of the sample during analysis. Approximately 20 mL of sample was transferred to the cup and measurement was taken with a bob and a gap of 1 mm. The sample was equilibrated at 40 ˚C for 15 mins prior to measurement, followed by

pre-shearing at 5 s^{-1} for 5 mins. Shear stress was measured with increasing shear rate from 2 s^{-1} to 50 s^{-1} and held at 50 s^{-1} for 5 mins, then decreased from 50 s^{-1} to 2 s^{-1}, following parameters of the International Office of Cocoa, Chocolate and Confectionery (IOCCC) software program (Rheocompass 1.20, Anton Paar Ltd., Austria). Data were fitted to the Casson model to obtain yield stress and viscosity.

4.2.4 Melting properties

Melting properties of chocolate were measured by using a Differential Scanning Calorimeter (DSC Pyris 4000 DSC, Perkin Elmer Ltd., US) equipped with nitrogen gas flow rate of 20 mL/mins, according to a modified method of Aidoo et al. (2015). Onset temperature (T_{onset}), endset temperature (T_{end}), maximum peak temperature (T_{max}) and enthalpy of melting (ΔHmelt) were calculated using the DSC software (Pyris Series DSC 8500, Perkin Elmer Ltd., US). Melting index was calculated as T_{end} - T_{onset}.

4.2.5 Hardness

The hardness of chocolate was measured by Texture Analyser (TA-XT plus, Stable Microsystems Ltd., UK) with a load cell of 500 N and a P/2N needle probe as described by Aidoo et al. (2015). The hardness was measured from five different locations of chocolate for each replicate. The mean values and standard deviations were calculated.

4.2.6 Cocoa fat analysis

The fat content of chocolate was determined by Soxhlet extraction according to AOAC Official Method 963.15.

4.2.7 Sample extraction for antioxidant contents of chocolates

Sample extraction was performed following a modified method of Ioannone et al. (2015).

4.2.7.1 Determination of Total Polyphenols Content (TPC)

TPC of the chocolate sample was performed following the method of Ioannone et al. (2015) and reported as milligram gallic acid equivalents per gram (mg/g GAE) of defatted chocolate.

4.2.7.2 Total Flavonoid Content (TFC)

TFC of the chocolate sample was conducted following a modified method of Jia et al. (1999). The absorbance of the sample was measured against a blank at 510 nm. Catechin (Sigma Aldrich, USA) was used as a standard and results were expressed as milligram Catechin equivalents per gram of defatted chocolate (mg/g Catechin).

4.2.7.3 DPPH Free Radical Scavenging Activities

The DPPH free radical scavenging activity of chocolate was determined according to a modified method of Zzaman & Yang (2013). Absorbance was read at 515 nm using the microplate reader (Tecan, Switzerland). Ascorbic acid was used as a standard. Ethanol mixed with DPPH was used as a blank, while water was used as a control. The scavenging effect was determined based on the percentage of DPPH free radical scavenging activity and expressed as micromole of Trolox per gram of defatted chocolate.

4.2.8 Analysis of polyphenols and methylxanthines by High Performance Liquid Chromatography with UV-Vis Detection (HPLC-DAD)

4.2.8.1 Sample preparation

Removal of a lipid fraction from chocolate and extraction of polyphenols were according to a modified method of Hammerstone et al. (1999). Defatted chocolate was dissolved in 90% (vol/vol) water plus 2% (vol/vol) acetic acid (pH 2.5) and 10% (vol/vol) acetonitrile, placed in an ultrasonic bath for 10 min, and filtered with a 0.45 μm cellulose filter. Standards were treated under the same conditions at 200 and 400 mg L^{-1}.

4.2.8.2 HPLC analysis of Polyphenols and methylxanthines

Chocolate samples were analysed for epicatechin, catechin, theobromine and caffeine by HP 1200 series HPLC (Hewlett-Packard, CA), according to a modified method of Camu et al. (2008). 10 μL of samples were analysed by using HPLC ZORBAX Eclipse Plus C18 (4.6 x 250 mm, I.D. 5 micron, Agilent, US). UV detection at 274 nm and 322 nm were applied for epicatechin and catechin; 254 nm was applied for theobromine and caffeine. The mobile phase, at a flow rate of 1.0 mL min^{-1}, consisted of water plus 2% acetic acid (eluent A, HPLC grade, ≥ 98%, Merck, US) and acetonitrile (eluent B, HPLC grade, Fisher Scientific, US), with the following gradient: 0.0 min, 10% A and 90% B; 20.0 min, 85% A and 15% B; 26.0 min, 85% A and 15% B; 27.0 min, 85% A and 15% B; 30.0 min, 20% A and 80% B; 33.0 min, 0 % A and 100 % B; 36.0 min, 20% A and 80% B. Quantification of (-)-epicatechin and (+)-catechin were conducted by using external standard solutions of epicatechin (52 mg L^{-1}) and catechin (22 mg L^{-1}). Results were expressed as milligram components per gram of chocolate product.

Quantification of theobromine and caffeine were conducted by standard solutions of theobromine (0.08 mg mL^{-1}) and caffeine (0.02 mg mL^{-1}).

4.2.9 Volatile organic compounds (VOCs) analysis

The VOC of chocolate samples were determined according to a modified method of Rodriguez-Campos et al. (2012) by using a fibre 50/30 um divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS) by Supelco. Chocolate sample (2.0g) in the Headspace (HS) was heated at 60 °C (15 mins) using a water bath to reach equilibrium, followed by the fibre exposition (30 mins) to the sample in the HS at 60 °C. The analysis of GCMS peaks was performed according to a modified method of Ruiz-Hernández et al. (2018) and Ren et al. (2020). General screening of the VOCs in chocolate sample was by comparing the mass spectra of each compound with the MSD Chemstation software, Agilent Technology. Then, these volatile compounds were further screened where the identification of compounds was based on three criteria: (1) by comparing the mass spectra with the library of mass spectra (MSD Chemstation software, Agilent Technology); (2) by comparing the retention index with literature data and (3) the identification was confirmed by using pure external standards of the components. The relative abundance of VOCs within a profile was expressed as the total integrated area of each compound and subjected for One-way analysis of variance (ANOVA).

4.3.0 Sensory evaluation

Sensory analysis of the chocolates was carried out by 8 trained panel members of the Malaysian Cocoa Board. Descriptive analysis with a scale of 0 to 10 was used, where 0 indicates the absence or minimum intensity and 10 indicates the maximum intensity. The guideline for the description of the sensory score was based on the Cocoa of Excellence

Technical Committee (2017). Ghana cocoa liquor was used as a reference sample, where the score for the reference sample was used as a basis for the scoring of flavour attributes for the other cocoa samples. The evaluated flavour descriptors were cocoa, bitter, astringent, acid, sweet, fresh fruits, browned fruits, floral, spicy, woody, nutty, roasted, dirty, meaty, over fermented, smoky, mouldy, other off flavours, snap, hardness, melting properties and overall global quality. Each sample was labelled with randomly selected 3-digits numerical code. During the evaluation, the panellists work individually in a sensory booth and they were free to describe any other taste that may present in the samples.

4.3.1 Statistical analysis

The analysis was conducted in triplicates. All data unless otherwise stated, were analysed by using two-way Analysis of Variance (ANOVA) followed by mean comparison using Tukey test at $p \leq 0.05$ levels with Statistical Package for the Social Sciences (SPSS) version 23. Correlation analysis among rheological properties, hardness, cocoa fat and melting index were performed by SPSS package. The correlation analysis among TPC, TFC and antioxidant activities of chocolates were also analysed by the same statistical package. Pearson correlation strength was based on guides of Evans (1996) suggested for the absolute value of correlation coefficient could be described as: [$\pm 0.00 \pm 0.19$], [$\pm 0.20 \pm 0.39$], [$\pm 0.40 \pm 0.59$], [$\pm 0.60 \pm 0.79$], and [$\pm 0.80 \pm 1.0$], which signified the very weak, weak, moderate, strong, and very strong correlations, respectively.

For VOC analysis of chocolate, the duplicate was carried out and results were expressed as an average of two batches, resulted in 5 samples (PK, HT, Mix, control and Ghana). One-way Analysis of Variance (ANOVA) was conducted to compare peak areas of the individual compounds identified in chocolate sample by GCMS. Compounds showing significant variance ($p \leq 0.05$) were subjected to a post-hoc Tukey's HSD (Honestly Significant Difference)

to identify the significantly different samples. For sensory evaluation, the result was an average of two batches (a total of 5 samples: PK, HT, Mix, control and Ghana) and analysed by one-way ANOVA.

4.4 Results and Discussion

4.4.1 Rheological properties

Figure 4.1 showed that chocolate produced using cocoa beans fermented with different yeast starter cultures had different viscosities. The viscosity of chocolate produced using cocoa beans fermented with PK was the highest ($p \leq 0.05$) whilst viscosity of chocolate produced using Ghana cocoa beans was the lowest ($p \leq 0.05$). There was no significant difference among the viscosity of chocolates produced using cocoa beans of control, HT and Mix. In the current study, Casson viscosity of 3.84 to 7.82 Pa.s (Figure 4.1) was consistent with the literature (Żyżelewicz et al., 2018).

The viscosity of chocolate is highly associated with the cocoa butter content, where increasing cocoa butter results in a decrement of viscosity (Gao et al., 2015). The low viscosity of Ghana chocolate could be related to the presence of high cocoa fat content in the Ghana beans (Table 4.1). Chocolate with a high viscosity cannot be used for enrobing or coating for smoother and thinner chocolates (Afoakwa et al., 2008). This is because highly viscous chocolate would form skirting on the bottom edge of a confectionery, leading to unpleasant product appearance (Sundara et al., 2014). As such, chocolate produced using beans fermented with PK which possessed high viscosity was not ideal for the enrobing process.

Highly viscous chocolate requires more power to pump during mixing and it also leads to sticky mouth-feel (Walker, 2009; Żyżelewicz et al., 2018). The high viscosity of PK chocolate could make it less popular in terms of sticky mouthfeel as shown in the sensory result

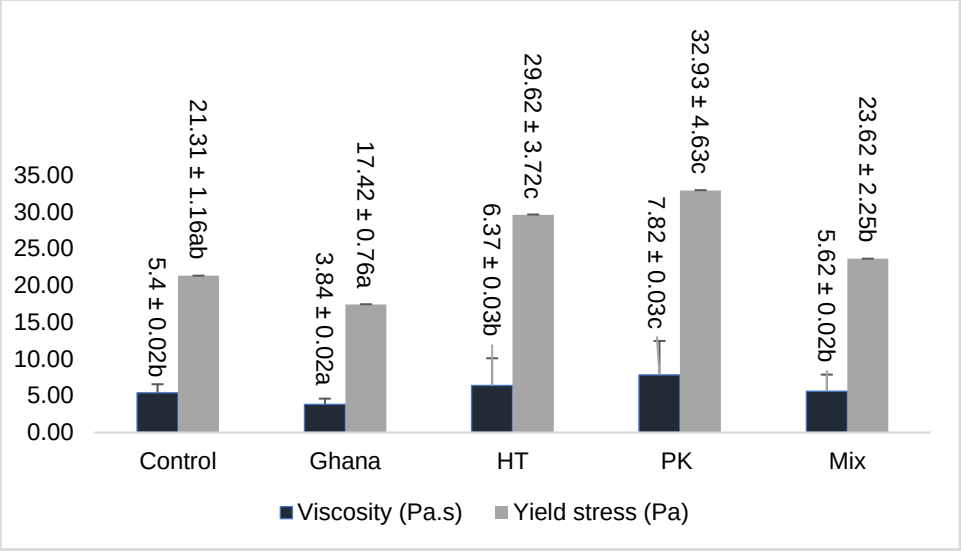
(Figure 4.3 and Table 4.7). In brief, chocolate produced using cocoa beans fermented with PK would require more force to move through the pipeline; unsuitable for chocolate coatings application and could be less desirable in terms of mouthfeel.

Casson yield stress is important for sensory quality and it determines the stress required to make chocolate begin to flow (Aidoo et al., 2015). Yield stress of chocolates produced using cocoa beans fermented with PK or HT was the highest ($p \leq 0.05$) whilst yield stress of chocolate produced using Ghana cocoa beans was the lowest ($p \leq 0.05$). In the current study, the yield stress of 17.42 to 32.93 Pa was consistent with the literature (Aidoo et al., 2015).

Low yield stress is typically observed in chocolate with high fat content, where the fat would enable the solid particles to move and aid in chocolate flow (Graef et al., 2011). This was consistent with the observation of low yield stress observed in Ghana chocolate, where a high cocoa fat content was recorded (Table 4.1). A previous study found that sample with a lower fat (25-35%) and particle sizes of 18-25 μm had exhibited high yield stress (408.80 to 32.37Pa), and the cause was associated with the high particle-to-particle interactions (Afoakwa et al., 2008). In the current study, dark chocolates with high cocoa solids (70%) without the addition of lecithin and milk were produced. The high yield stress observed in chocolate produced using beans fermented with HT (29.62 Pa) or PK (32.93 Pa), where high cocoa fat content was recorded could be a result of the proportion and interaction of small particles (and their specific surface area) within the chocolate liquor, where similar high particle-to-particle interactions were occurring in the chocolate liquor.

Different yield stress values are required for different practical aspects (Stauffer, 1998). For instance, a high yield stress value is required to prevent decoration from collapsing; whereas a lower yield value is needed for moulding (Stauffer, 1998). The texture and strength of a product are closely associated with yield stress (Sad, 2008). We suggest that chocolate

produced using beans fermented with PK or HT could be suitable for couverture application due to the high yield stress. Chocolates (Control, Ghana and Mix chocolates) with lower yield stress could ease the moulding process as the flowy chocolate can be injected into the mould.



Results are the mean ± S.D. Mean values followed by the different small alphabet indicates a significant difference between the chocolate produced from beans after yeast added fermentation ($p \leq 0.05$) based on one-way ANOVA, Tukey test ($p \leq 0.05$).

Figure 4.1 Casson viscosity (Pa.s) and Casson yield stress (Pa) of chocolate produced using Ghana beans, beans fermented with yeast starter (HT, PK and Mix) and spontaneously fermented beans (Control)

4.4.2 Melting properties

The onset temperature (T_{onset}), peak temperature (T_{max}), end temperature (T_{end}) and enthalpy (ΔHmelt) were recorded in Table 4.1. Peak onset is the temperature at which a specific crystal starts to melt; peak temperature is at which melting rate is the greatest; whereas end temperature is the end of melting, completion of liquefaction (Aidoo et al., 2015). All the samples displayed similar properties, at which there was no significant difference of onset temperature (T_{onset}), peak temperature (T_{max}) and end temperature (T_{end}) among them ($p \leq 0.05$). However, chocolate produced using cocoa beans fermented with HT had the highest enthalpy of melt (25.11 J/g); whereas chocolate produced using control beans had the lowest value (15.49 J/g) ($p \leq 0.05$). The T_{onset}, T_{max} and T_{end} values ranged between 17.22 to 23.79 ˚C, 20.60

to 27.34 ˚C and 23.84 to 30.68 ˚C, respectively and ΔHmelt values changed between 15.49 and 25.11 J/g. These findings were similar with previous literature (Oba et al., 2017).

The DSC melting properties (Table 4.1) was in accordance with the melting properties of sensory evaluation (Fig. 4.3). Melting properties can be influenced by crystal size, polymorphs, particle size and fat content (Tan & Kerr, 2018). The particle size of cocoa butter in the present study was below 30 μm. The variation of melting temperature in this study could be due to the fat content of the fermented cocoa beans. The study reported that chocolate with higher fat content and finer particles would require a longer heating time to be melted (Tan & Kerr, 2018). This was consistent with the high enthalpy of melt and melting temperature observed in chocolate produced using beans fermented with HT, where high fat content and finer particles (19.3 μm) were observed. Control chocolate which exhibited the low cocoa fat and low enthalpy of melt (Table 4.1) also showed that a lower fat content would require a shorter heating time to be melted. The current study suggested that the high enthalpy of melt in chocolate produced using beans fermented with HT could probably be associated with the higher fat content in the beans. However, further study is required to better understand the influence of yeast starter on the cocoa fat composition of cocoa beans and its resulted chocolate.

Table 4.1 Melting properties and cocoa fat of chocolates produced using Ghana beans, beans fermented with yeast starter (HT, PK and Mix) and spontaneously fermented beans (Control)

Chocolate type	Melting properties					
	T_{onset} (˚C)	T_{max} (˚C)	T_{end} (˚C)	(ΔHmelt) (J/g)	Cocoa fat (%)	Particle size (μm)
Control	17.22 ± 0.17^{a}	20.60 ± 0.10^{a}	23.84 ± 0.56^{a}	15.49 ± 2.46^{a}	70.25 ± 5.71^{a}	15.8 ± 0.06^{a}
Ghana	17.59 ± 0.20^{a}	21.09 ± 0.06^{a}	24.18 ± 0.01^{a}	19.41 ± 1.99^{ab}	83.28 ± 3.02^{b}	22.0 ± 0.15^{c}
HT	23.79 ± 7.26a	27.08 ± 6.99^{a}	30.31 ± 6.75^{a}	25.11 ± 6.64^{b}	75.39 ± 7.32^{ab}	19.3 ± 0.25^{b}
PK	23.39 ± 7.81^{a}	26.93 ± 7.70^{a}	29.73 ± 7.23^{a}	19.74 ± 6.96^{ab}	81.30 ± 3.95^{b}	26.1 ± 0.10^{d}
Mix	23.75 ± 7.65^{a}	27.34 ± 7.45^{a}	30.68 ± 6.53^{a}	19.96 ± 4.67^{ab}	73.79 ± 7.24^{ab}	28.1 ± 0.21^{e}

Results are the mean ± S.D. Mean values followed by the different small alphabet in the same column indicate a significant difference between the chocolate produced from beans after yeast added fermentation between the batches at ($p \leq 0.05$) based on one-way ANOVA, Tukey test ($p \leq 0.05$).

4.4.3 Relationships between rheological, textural and melting properties of chocolates

Based on the correlation coefficients, very strong correlations between yield stress and viscosity ($r = 0.981$ to 0.998, $p \leq 0.01$) were observed in chocolates produced using beans of control, Ghana, HT or PK, indicated that the two parameters were highly associated and could be used to estimate the rheological behaviour of chocolate during chocolate processing (Table 4.2). These findings were supported by a previous study of Afoakwa et al. (2008). Yield stress is closely associated with particle-particle interaction and specific surface area of particles (Servais et al., 2003). Viscosity is linked with the shape, volume fraction of particles, particle size distribution and viscosity of continuous phase (Servais et al., 2003). The increasing of particle sizes would result in a decrement of yield stress and viscosity (Afoakwa et al. 2008), signified both yield stress and viscosity are dependent on the particle size distribution. Strong to very strong correlations among viscosity, yield stress and fat were found in chocolates produced using beans of Ghana, HT or PK, indicating the cocoa fat contents were strongly associated with the yield stress and viscosity of the chocolates. The high correlations among viscosity, yield stress and fat as recorded in PK or HT chocolate suggested both parameters (viscosity and yield stress) could be used independently to evaluate the rheological properties of dark chocolates produced using beans fermented with PK or HT.

The strong relationship between viscosity and hardness of Mix or PK chocolate ($r = 0.883$, $p \leq 0.05$; $r = 0.688$) showed that the textural (hardness) and rheological (viscosity) properties could be applied to predict the processing behaviours during the dark chocolate making (Table 4.2). The relationship between rheological and textural properties are strongly correlated with the melting index of chocolate (Afoakwa et al., 2008). Strong to very strong correlations among viscosity, yield stress and melting index were observed in control, PK or

Mix chocolate (Table 4.2), suggested that both rheological parameters (viscosity and yield stress) could be used to predict the melting index of chocolate. The strong correlations signified the high inter-relationship among viscosity, yield stress and melting index. This was correlating with the previous finding where yield stress and viscosity were associated with the melting index of dark chocolate (Afoakwa et al., 2008). The stability of fat crystal during tempering and cooling temperature is closely related to the melting behaviour of the chocolates (Afoakwa et al., 2008). In brief, the high inter-relationship between yield stress and viscosity observed in chocolates produced using control, Ghana, HT or PK, suggested both the rheological parameters could be used for manufacturing control of chocolate production.

Table 4.2 Correlation analysis between rheological, textural, cocoa fat and melting parameters of chocolates produced from spontaneous fermentation (control), Ghana beans and beans added with HT, PK and Mix

		Viscosity	**Hardness**	**Yield stress**	**Melting index**	**Cocoa fat**
Control	**Viscosity**	1	0.212	0.994**	0.855*	-0.803
	Hardness	0.212	1	0.109	-0.285	-0.706
	Yield stress	0.994**	0.109	1	0.908*	-0.73
	Melting index	0.855*	-0.285	0.908*	1	-
Ghana	**Viscosity**	1	-0.296	.998**	-.953**	1.000**
	Hardness	-0.296	1	-0.335	0.078	-0.316
	Yieldstress	.998**	-0.335	1	-.934**	1.000**
	Melting index	-.953**	0.078	-.934**	1	-
HT	**Viscosity**	1	-0.652	.991**	-0.654	.908*
	Hardness	-0.652	1	-0.667	0.65	-0.341
	Yield stress	.991**	-0.667	1	-0.697	.900*
	Melting index	-0.654	0.65	-0.697	1	-
PK	**Viscosity**	1	0.688	.981**	0.746	.859*
	Hardness	0.688	1	0.686	0.779	0.75
	Yield stress	.981**	0.686	1	.812*	.914*
	Melting index	0.746	0.779	.812*	1	-
MIX	**Viscosity**	1	.883*	-0.775	-0.654	-0.476
	Hardness	.883*	1	-0.742	-0.608	-0.219
	Yield stress	-0.775	-0.742	1	.946**	-0.073
	Melting index	-0.654	-0.608	.946**	1	-

** Correlation is significant at the 0.01 level

* Correlation is significant at the 0.05 level

4.3.4 Antioxidant of chocolates

4.3.4.1 Determination of Total Polyphenols Content (TPC), Total Flavonoid Content (TFC), DPPH Free Radical Scavenging Activities

Table 4.3 showed that there was no significant difference of TPC between chocolates produced using cocoa beans fermented with yeast starter culture and spontaneous fermentation. Only chocolate produced using beans fermented with Mix had the highest TPC as compared to control ($p \leq 0.05$). The value of TPC in chocolate was consistent with the literature (Ramli et al., 2001). According to a previous work conducted by Ooi et al. (2020), the highest TPC content was found in dried cocoa beans fermented with HT. However, the TPC of chocolate produced using beans fermented with HT was not correlated with the TPC of dried cocoa beans, suggesting the loss of polyphenols content during the chocolate processing.

In terms of TFC, there was also no significant difference between the chocolates produced using beans fermented with yeast starter culture and spontaneous fermentation ($p \leq 0.05$) (Table 4.3). The range of TFC (23.61 to 36.84 mg/g Catechin) in the current study was higher than reported in the literature (Cheng et al., 2009). Additionally, control chocolate had the highest DPPH free radical scavenging activity among all the chocolates ($p \leq 0.05$). Ghana chocolate had the lowest DPPH free radical scavenging activity ($p \leq 0.05$). The value of DPPH free radical scavenging activity of the current study (29.8 to 42.91 μmol/g TE) was consistent with the literature (Hu et al., 2016).

Evaporation of volatile polyphenols that usually occurs during conching (70 to 80 ˚C) is also one of the key factors that lead to polyphenols reduction (Albak & Tekin, 2016; Barišić et al., 2019). The high TPC found in chocolate produced using beans fermented with Mix could

probably due to the biotransformation of polyphenols during fermentation. According to Xu et al. (2019), microbial hydrolyzation of polyphenols leads to more bioactive compounds being released during fermentation and ultimately contribute to the enhancement of antioxidant activity. The present study proposed that the high TPC of chocolate produced using beans fermented with Mix could be due to the liberation of polyphenols from the intracellular of cocoa beans by microbial fermentation.

4.3.4.2 Analysis of Flavanols by High Performance Liquid Chromatography with UV-Vis Detection (HPLC-DAD)

Flavanols such as (+)-catechin, (-)-epicatechin and methylxanthines (theobromine and caffeine content) of chocolates were shown in Table 4.3. The chocolate produced using beans of spontaneous fermentation had the highest (+)-catechin content among all the chocolates ($p \leq 0.05$). Meanwhile, the lowest (+)-catechin content was found in chocolate produced using beans fermented with HT ($p \leq 0.05$). The values of (+)-catechin (0.017 to 0.079 mg/g) were consistent with the literature (Żyżelewicz et al., 2016).

The (-)-epicatechin content of control chocolate was the highest. Whereas, the lowest (-)-epicatechin content was found in Ghana chocolate ($p \leq 0.05$). The (-)-epicatechin contents of chocolates produced using beans fermented with yeast starters were lower than control but higher than Ghana chocolate ($p \leq 0.05$). The current study demonstrated that (-)- epicatechin contents of all the chocolates were higher than the (+)-catechin contents, in line with previous literature (Cheng et al., 2009).

The high (+)-catechin and (-)-epicatechin contents in control chocolate could be related to the presence of non-fat cocoa solids (NFCS) (Cheng et al., 2009). NFCS is an indication of higher phenolic content (Cheng et al., 2009). In this study, all cocoa powder and liquor except

for Ghana samples did not go through alkalization. It is known that alkalization reduced the antioxidant properties and flavanol contents in cocoa powder (Cheng et al., 2009). Commercial chocolate usually uses alkalized cocoa powder and thus having lower polyphenols content (Cheng et al., 2009). As such, control chocolate produced from cocoa beans which were non-alkaline would have the highest (+)-catechin and (-)-epicatechin contents. Besides, the low (-)-epicatechin and (+)-catechin contents of chocolates produced using beans fermented with yeast starters could be associated with the reduction of epicatechin and soluble polyphenol content, which occurred during fermentation. The usage of yeast starter in fermentation is reported to influence the polyphenols content during fermentation (Ooi et al., 2020). During fermentation, proanthocyanidins would reduce through the hydrolysis of tannins and procyanidins in order to produce monomeric epicatechin compounds. The reduction of tannins contents also includes the reduction of procyanidins B2, B5, C1 in cocoa beans after the fermentation (Dang & Nguyen, 2019). Therefore, the current study suggested that the usage of yeast starter during fermentation was among the factors, which led to the low (-)-epicatechin and (+)-catechin contents of chocolates produced.

Furthermore, processes such as drying, fermentation and roasting could be the other factors that lead to some loss of (-)-epicatechin and (+)-catechin in cocoa (Hurst et al., 2011). According to Hurst et al. (2011), heat-related epimerization from (-)-epicatechin to (-)-catechin occurred in cocoa beans during various processing steps. The present study proposed that the addition of yeast starter during fermentation influenced the antioxidant content of fermented cocoa beans. Nevertheless, the final antioxidant content of chocolate was still largely influenced by various processing of cocoa beans, especially during the drying and roasting stages.

In this study, theobromine content of chocolate produced using beans fermented with PK was the highest as compared to control and Ghana chocolates ($p \leq 0.05$). While the caffein content of chocolate produced using beans fermented with HT was the highest among all samples ($p \leq 0.05$) (Table 4.3). Both control and Ghana chocolates had shown lower theobromine and caffeine content ($p \leq 0.05$). The range of theobromine (0.0004 to 0.5335 mg/g Theobromine) and caffeine (0.010 to 0.098 mg/g Caffeine) content were similar with a previous study (Camu et al., 2008).

Caffeine and theobromine are methylxanthines recognized as bioactive compounds of cacao, where theobromine constitutes about 4% on a fat free basis and caffeine content is about 0.2% (Maleyki & Ismail, 2008). However, very low theobromine was found in the control and Ghana chocolates. This could be due to the activity of theobromine-degrading filamentous fungi during fermentation (Copetti et al., 2011). The previous study also reported *P. kudriavzevii* and *Hanseniaspora uvarum* have an antagonistic or inhibitory effect against the proliferation of fungi (Horváth et al., 2019; Apaliya et al., 2018), which could probably lead to the lack of bio-detheobromination activity by the same filamentous fungi against the treated samples (Table 4.3). This postulation, however, remains to be further confirmed.

The high theobromine and caffeine content of chocolates produced using beans fermented with yeast starters had shown benefits to human consumption. For instance, studies show that theobromine can suppress cough in human without the side effects (Franco et al., 2013). The high caffeine and theobromine content of chocolates produced using beans fermented with PK or HT could be supported by a similar study (Jin et al., 2014). According to Jin et al. (2014), two mutants *Saccharomyces cerevisiae* are capable to produce more theobromine and caffeine. In relation to that, the current study had screened and selected naturally isolated yeast starters in previous work (Ooi et al., 2020). Hence, the type of yeast

applied during fermentation influenced the methylxanthines content of cocoa beans and subsequently the produced chocolate. In the current study, HT or PK is recommended as a potential yeast starter to improve the content of methylxanthines (caffeine and theobromine) in cocoa beans.

Table 4.3 Total phenolic content (TPC) (mg/g GAE), total flavanoid content (TFC) (mg/g Catechin), DPPH free radical scavenging activity (DPPH) (μmol/g TE), (+)-catechin, (-)-epicatechin, theobromine and caffeine contents (mg/g) of chocolates produced using spontaneous fermented and yeast added cocoa beans

	TPC (mg/g GAE)	**TFC (mg/g Catechin)**	**DPPH (μmol/g TE)**	**(+)-Catechin (mg/g)**	**(-)-Epicatechin (mg/g)**	**Theobromine (mg/g)**	**Caffeine (mg/g)**
Control	55.1 ± 0.82^{a}	36.84 ± 1.57^{b}	42.91 ± 9.39^{b}	0.079 ± 0.000^{c}	6.969 ± 0.001^{c}	0.0004 ± 0.000^{a}	0.011 ± 0.000^{a}
Ghana	55.63 ± 0.89^{a}	24.54 ± 8.25^{a}	29.8 ± 3.65^{a}	0.020 ± 0.000^{b}	0.100 ± 0.000^{a}	0.0282 ± 0.002^{a}	0.010 ± 0.000^{a}
H. thailandica	54.62 ± 1.36^{a}	23.61 ± 2.72 ^{a}	35.69 ± 3.18^{ab}	0.017 ± 0.001^{a}	1.303 ± 1.165^{b}	0.2266 ± 0.116^{b}	0.098 ± 0.086^{b}
P. kudriavzevii	54.41 ± 2.9^{a}	23.81 ± 7.41^{a}	34.08 ± 6.27^{ab}	0.018 ± 0.002^{ab}	0.935 ± 0.702^{ab}	0.5335 ± 0.217^{c}	0.083 ± 0.050^{ab}
Mixture	64.61 ± 3.84^{b}	33.29 ± 5.72^{ab}	36.16 ± 6.44^{ab}	0.020 ± 0.003^{b}	1.496 ± 0.219^{b}	0.2857 ± 0.050^{b}	0.030 ± 0.005^{ab}

Results are the mean ± S.D. Mean values followed by the different small alphabet in the same column indicate a significant difference between the chocolate produced from beans after yeast added fermentation between the batches at ($p \leq 0.05$) based on one-way ANOVA, Tukey test ($p \leq 0.05$).

4.3.4.3 Correlation analysis between TPC, TFC, (+)-catechin, (-)-epicatechin, theobromine, and caffeine with antioxidant activity

Polyphenols (epicatechin, catechins, TPC, TFC and DPPH) and methylxanthines (theobromine and caffeine) were studied for their correlation coefficients (Table 4.4). Based on the correlation between TPC and TFC, chocolates produced using beans fermented spontaneously (control) and beans fermented with PK recorded very strong ($r = 0.923$, $p \leq 0.01$) and strong ($r = 0.723$) correlations, respectively. Finding in the current study was supported with a previous study whereby the antioxidative activity was improved through fermentation (Hur et al., 2014), where a very strong correlation between TPC and TFC was observed in the control chocolate. Fermentation would breakdown the plant cell walls to liberate or induce the production of bioactive compounds, leading to higher TPC in the fermented product (Hur et al., 2014). Hence, the observed antioxidant activity of the present study could be due to the presence of polyphenolic compounds.

Chocolate produced using beans fermented with HT had a weak positive correlation between TPC and DPPH ($r = 0.336$), but strong correlations between (+)- catechin and TPC ($r = 0.716$), (-)-epicatechin and TPC ($r = 0.708$), and (+)- catechin and TFC ($r = 0.617$). This indicated that the addition of HT in fermentation may result in the production of catechin and epicatechin that contribute to TPC and TFC. The positive correlation between TPC, TFC and antioxidant activities observed in HT or PK chocolate was highly associated with microbial fermentation. Fungi are capable of producing bioactive compounds which could contribute to the total amount of antioxidants available (Hur et al., 2014). The TPC and TFC contents were reported to increase during coffee fermentation added with yeast (Haile & Kang, 2019a). During coffee fermentation, the yeast starter is found to release proteolytic enzymes, which

hydrolyze complexes of phenolic into more biologically, simple and soluble-free active phenol forms (Haile & Kang, 2019a). The releasement and bioavailability of bound flavanoid components during fermentation could also lead to a strong correlation of TFC observed in chocolate produced using beans fermented with HT or PK.

However, strong to very strong negative correlations between (-)-epicatechin and TPC ($r = -0.874$, $p \leq 0.05$); (+)- catechin and TFC ($r = -0.648$); and (-)-epicatechin and TFC ($r = -0.907$, $p \leq 0.05$) were observed in chocolate produced using beans fermented with PK, indicating catechin and epicatechin may not be the compounds that were produced during fermentation with PK. The negative correlations between (-)-epicatechin and TPC; (+)- catechin and TFC; (-)-epicatechin and TFC of PK chocolate were found to signify (+)-catechin or (-)-epicatechin was not the one that was responsible for the TPC or TFC. Similarly, the negative correlation between TPC and DPPH of Mix chocolate ($r = -0.717$) could be likewise. Certain fermentations were reported to have a negative effect on antioxidant activity (Hur et al., 2014). For instance, olive fermentation processing has noted a decrement of antioxidant value due to the loss of phenolic (Hur et al., 2014). The antioxidant activity would decrease as a result of phenolic loss during the degradation of phenolic compounds (Hur et al., 2014). Hence, the usage of PK or Mix starter in fermentation could exert a similar effect on the fermented beans and the resultant chocolate. The in-depth biochemistry of the liberation, production and loss of these polyphenolic compounds during microbial fermentation would require further research and were beyond the scope of the current study.

The negative correlations between TPC and TFC ($r = -0.981$, $p \leq 0.01$), and TPC and DPPH ($r = -0.923$, $p \leq 0.01$) observed in Ghana chocolate signified that flavonoid content did not correspond to TPC; while phenolic compounds may not be responsible for the measured DPPH activities. The correlations between TPC and DPPH of all chocolates except chocolate produced using beans fermented with HT were inconsistent, where very strong to very weak

negative correlations were observed (Table 4.4). The different correlations between TPC and DPPH; TPC and TFC observed in other chocolates could be associated with the types of fermented beans. The TPC of cocoa beans varies depending on cocoa clones and geographical regions (Nebesny, & Oracz, 2016). In the current study, the reason responsible for the weak relationship observed between TPC and DPPH, and between TPC and TFC remained unknown.

The chocolate produced using spontaneously fermented beans (control) showed a moderate positive correlation between the (+)- catechin and DPPH (r = 0.597) (Table 4.4). The rest of the chocolates recorded inconsistent correlations between the (+)- catechin and DPPH; (-)-epicatechin and DPPH, signifying multiple factors that might affect the antioxidant activity (Table 4.4). The inconsistent correlation between (+)-catechin and TFC; (-)-epicatechin and TFC, and (-)-epicatechin and DPPH could be attributed to factors such as pH shifts during the fermentation. The study reported that anthocyanins are stable at low pH, the scavenging capacity of anthocyanins would increase when an increase in pH (Hur et al., 2014). The stability of catechin is also greatly dependent on pH, where catechin is not stable in alkaline solutions (Hur et al., 2014). Epicatechin is also reported to be unstable at pH above 5 (Pico et al., 2019). Control chocolate was the only sample which showed a weak positive correlation between (+)-catechin and DPPH, and this signified that the addition of yeast starters could have influenced the availability of (+)-catechin and (-)- epicatechin contents. In the current study, beans fermented with yeast starter were less acidic, where a pH of 5.31 to 6.80 was observed (refer to section 3.4.4). Thus, the less acidic beans produced using yeast starters in the present study could influence the availability of catechin and epicatechin in cocoa beans and the resulted chocolate.

A very strong correlation between DPPH and theobromine was found in chocolate made of Ghana beans (r = 0.873) (Table 4.5). The chocolate produced using beans fermented with PK had weak correlations between DPPH and theobromine (r = 0.301); DPPH and

caffeine (r = 0.310). Inconsistent correlations between DPPH and theobromine; DDPH and caffeine were recorded in all the other samples. This indicated theobromine and caffeine were not directly linked with the antioxidant activity. The weak correlations between DPPH and theobromine; DPPH and caffeine that observed in chocolates produced using beans fermented with PK were supported by a similar study (Gebeyehu, 2015). Some studies reported that the caffeine contents and antioxidant activities in coffee varieties are not proportional despite the highest value of caffeine content is observed in the sample (Gebeyehu, 2015). Other polyphenolic acids such as chlorogenic acids, ferulic acid, syringic and coumaric acids are highly contributing to the antioxidant behaviour (Gebeyehu, 2015). In the current study, the very weak negative correlation of DPPH and caffeine observed in chocolate produced using beans fermented with PK could be due to the presence of other polyphenolic acids, other than caffeine.

The genetic variant of cocoa clones used could also affect the theobromine content. Studies found that the build-up of theobromine and caffeine in Trinitario variety are in the late phase of seed development and the higher purine alkaloid content of the seed is found in Criollo cocoa seed (Pereira-Caro et al., 2013). Evidence showed that alkaloid caffeine, theobromine and caffeine exhibit antioxidant properties by producing hydroxyl radicals in the inhibition of oxidative DNA (Azam et al., 2003). However, in the current study, there was lack of correlation between theobromine and DPPH of cocoa beans, indicating a possible degradation mechanism of methylxanthines may occur during the yeast fermentation, where caffeine may be degraded by yeast into theophylline, paraxanthine or 1,3,7-methyluric acid rather than theobromine. The study reported that caffeine can be degraded into theobromine, theophylline, paraxanthine and 1,3,7-methyluric (Jokić et al., 2018). Theobromine can also be degraded into 3-methylxanthine and 7-methylxanthine (Jokić et al., 2018). The degradation mechanism of methylxanthines is complex and thus, the influence of yeast on the methylxanthines content would require a further

study to have a better understanding on the conversion of methylxanthines during the yeast fermentation.

The present study observed a positive influence of yeast starter (PK) on the caffeine content of chocolate produced (Table 4.5). This is in accordance with findings of previous literature where the usage of yeast starter such as *S. cerevisiae* in fermentation influenced the caffeine content of the cocoa beans produced (Batista et al., 2016). Based on the correlation coefficients, the current study suggested that the antioxidant activities of chocolates produced using beans fermented with HT or PK could be highly associated with the TPC and TFC (specifically (+)- catechin and (-)-epicatechin) contents. The use of HT or PK starter resulted in the production of less acidic beans, which in turn could affect the (+)- catechin and (-)-epicatechin contents in cocoa beans.

Table 4.4 Pearson correlation coefficients (r) among TPC, TFC and antioxidant activities of chocolates produced from spontaneous fermentation (control), Ghana beans and beans added with HT, PK and Mix

Sample	Control					Ghana					HT				
	TPC (mg/g GAE)	**TFC (mg/g Catechin)**	**DPPH (µmol/g TE)**	**(+)-Catechin (mg/g)**	**(-)-Epicatechin (mg/g)**	**TPC (mg/g GAE)**	**TFC (mg/g Catechin)**	**DPPH (µmol/g TE)**	**(+)-Catechin (mg/g)**	**(-)-Epicatechin (mg/g)**	**TPC (mg/g GAE)**	**TFC (mg/g Catechin)**	**DPPH (µmol/g TE)**	**(+)-Catechin (mg/g)**	**(-)-Epicatechin (mg/g)**
TPC (mg/g GAE)	1	0.923**	-0.173	-0.308	0.489	1	-0.981**	-0.923**	0.396	0.178	1	0.361	0.336	0.716	0.708
TFC (mg/g Catechin)	0.923**	1	0.219	-0.072	0.511	-0.981**	1	0.980**	-0.484	-0.268	0.361	1	0.468	0.617	0.385
DPPH (µmol/g TE)	-0.173	0.219	1	0.597	0.068	-0.923**	0.980**	1	-0.555	-0.349	0.336	0.468	1	-0.057	-0.180
(+)-Catechin (mg/g)	-0.308	-0.072	0.597	1	-0.437	0.396	-0.484	-0.555	1	-0.308	0.716	0.617	-0.057	1	0.870*
(-)-Epicatechin(mg/g)	0.489	0.511	0.068	-0.437	1	0.178	-0.268	-0.349	-0.308	1	0.708	0.385	-0.180	0.870*	1
Sample	**PK**					**Mix**									
	TPC (mg/g GAE)	**TFC (mg/g Catechin)**	**DPPH (µmol/g TE)**	**(+)-Catechin(mg/g)**	**(-)-Epicatechin (mg/g)**	**TPC (mg/g GAE)**	**TFC (mg/g Catechin)**	**DPPH(µmol/g TE)**	**(+)-Catechin (mg/g)**	**(-)-Epicatechin (mg/g)**					
TPC (mg/g GAE)	1	0.723	-0.628	-0.483	-0.874*	1	0.535	-0.717	-0.249	-0.329					
TFC (mg/g Catechin)	0.723	1	-0.085	-0.648	-0.907*	0.535	1	-0.254	-0.535	-0.599					
DPPH (µmol/g TE)	-0.628	-0.082	1	-0.185	0.369	-0.717	-0.254	1	-0.434	-0.347					
(+)-Catechin (mg/g)	-0.483	-0.648	-0.185	1	0.714	-0.249	-0.535	-0.434	1	0.988**					
(-)-Epicatechin (mg/g)	-0.874*	-0.907*	0.369	0.714	1	-0.329	-0.599	-0.347	0.988**	1					

** Correlation is significant at the 0.01 level; *Correlation is significant at the 0.05 level

Table 4.5 Pearson correlation coefficients (r) among DPPH, theobromine and caffeine contents of chocolates produced from spontaneous fermentation (control), Ghana beans and beans added with HT, PK and Mix

Sample/antioxidants	**DPPH (µmol/g TE)**				
	Control	**Ghana**	**HT**	**PK**	**Mix**
Theobromine (mg/g)	**-0.211**	**0.873**	**-0.084**	**0.301**	**-0.408**
Caffeine (mg/g)	**-0.119**	**-0.555**	**-0.087**	**0.310**	**-0.405**

4.3.5 Analysis of volatile profile of chocolates

Based on the general screening of VOCs, a total of 29 volatile compounds were detected in the chocolate samples (Table 4.6a) However, further screening only recorded a total of 12 volatile compounds in Ghana chocolate, control chocolate and chocolates produced using beans fermented with PK, HT and Mix. They were grouped into pyrazines (1 group), acids (2 groups), others (2 groups), alcohols (3 groups) and aldehyde (4 groups). The chocolate produced using beans fermented with PK, HT, Mix and control showed various volatile compounds (Table 4.6b) with specific yeast starter (Crafack et al., 2014).

Aldehyde was the most predominant group of volatile compounds detected in the chocolates. It is the key compounds for good cocoa flavour (Rodriguez-campos et al., 2012). Pentanal, hexanal, octanal and nonanal were the major aldehyde compounds detected in chocolates. There was no significant difference among the concentration of pentanal, hexanal and octanal. However, chocolate produced using beans fermented with yeast starters had higher nonanal compounds as compared to control and Ghana chocolates ($p \leq 0.05$). This compound confers tallowy and soapy-fruity flavours (Crafack et al., 2014). Aldehyde compounds are formed commonly by Strecker degradation of free amino acids particularly during roasting (Aprotosoaie et al., 2016). The current finding was in accordance with a previous study where high nonanal contents were also found in pineapple wine produced from *S. cerevisiae* strain BV818 and CECA (Lin et al., 2018). It was suggested that the high nonanal compounds detected in chocolates produced using beans added with yeast were highly associated with the yeast metabolism during cocoa fermentation.

Alcohols represented the second most abundant volatile compounds found in the chocolate. High alcohol contents are favourable as they contribute to cocoa products with

flowery and candy notes (Rodriguez-campos et al., 2012). Alcohol compounds such as 3 methyl-1-butanol was only detected in control with no significant difference among all the chocolate samples ($p \leq 0.05$). This compound confers malty, bitter and chocolate flavours (Rodriguez-Campos et al., 2012). Moreover, 1-Pentanol was another alcohol compound that detected in control, Ghana chocolate and chocolate produced using beans fermented with HT. This compound deliberated flowery and sweet, indicating chocolates produced using beans fermented with PK and Mix had a low intensity of such flavour attributes. Besides, phenylethyl alcohol, which conferred flowery flavour was only detected in chocolates produced using beans fermented with yeast starters. This compound is also highlighted in cocoa fermentation inoculated with *Kloeckera apiculata* and *Saccharomyces cerevisiae* (Rodriguez-campos et al., 2011). Alcohol contents are closely associated with the alcohol metabolic activities of yeast strains (Lin et al., 2018). The study also reported the use of yeast strains during fermentation are contributing to higher alcohols in wine (Perrusquía-Luévano et al., 2019). In relate to that, current study suggested the application of yeast starter in fermentation had influenced certain types of alcohol contents.

Acetic acid was found in all the chocolate samples, except for Ghana chocolate (Table 4.6). There was also no significant difference among all the samples ($p \leq 0.05$). This compound confers sour, astringent and vinegar flavours (Rodriguez-Campos et al., 2012). Another acid such as dodecanoic acid was also detected in all the samples except for chocolates produced using beans fermented with HT. This acid confers metal flavour (Rodriguez-Campos et al., 2012). Generally, the production of acids in cocoa beans are related to microorganism during the fermentation and would reduce during the drying and roasting process (Ascrizzi et al.,

2017). Our study showed that microorganisms-aided fermentation produced chocolate samples with higher acid as compared to control and Ghana chocolates.

Overall, flavour volatiles in dark chocolate matrices were highly associated with the starter culture activity and the flavour precursors produced during fermentation. Our study showed that starter cultures modulated the volatiles profile of chocolates. Nevertheless, in depth study is required in order to better understand the metabolic pathway of each single yeast used during fermentation. The sensorial profile of the chocolates would be discussed in section 4.3.6.

Table 4.6a General screening of volatile organic compounds (VOCs) identified in the chocolates produced from beans fermented with *P. kudriavzevii* (PK), *H. thailandica* (HT), a mixture of PK and HT (Mix) and spontaneously fermented beans (control). Fill box indicates presence and open box indicates absence of the compound.

No	**VOCs**	**Control**	**Ghana**	**PK**	**HT**	**Mix**	Odour*
1.	Acetic acid	■		■	■	■	Sour, astringent, vinegar
2.	3-methyl-butanal		■				ethereal aldehydic chocolate peach fatty
3.	Pentanal	■	■	■	■	■	almond, malt, pungent
4.	3-hydroxy- 2-Butanone	■					chocolate: pungent cocoa musty green malty bready
5.	3 methyl-1-butanol	■					Malty, bitter, chocolate
6.	1-Pentanol	■	■		■		Flowery, sweet
7.	2,3-butanediol	■			■		butter
8.	Hexanal	■	■	■	■	■	Green
9.	3-methyl-Butanoic acid	■					cheesy
10.	2-methyl- Butanoic acid	■					acidic
11.	2,3-dimethyl-Pyrazine	■					Caramel, cocoa, sweet, baked
12.	β-Myrcene						spicy

No	VOCs	Control	Ghana	PK	HT	Mix	Odour*
13.	Trimethyl- Pyrazine						
14.	Octanal						Green, fresh fruit
15.	Benzyl Alcohol						odour: floral (floral rose phenolic balsamic) Flavour: Fruity (chemical fruity cherry almond balsamic bitter)
16.	Benzeneacetaldehyde						Honey, floral rose, sweet, powdery, fermented, chocolate with a slight earthy nuance
17.	Acetophenone						floral
18.	Tetramethyl-pyrazine						Roasted, green, coffee, cocoa
19.	Nonanal						
20.	Phenylethyl Alcohol						Flowery, spicy, honey-like, rose
21.	Decanal						Sweet, roasted, cocoa, rhum-like
22.	Ethyl phenylacetate						Pineapple
23.	n-Decanoic acid						fatty

No	Compound	Control	Ghana	PK	HT	Mix	Odour*
24.	n-Butyl benzoate						
25.	Pentadecane						waxy
26.	5-Methyl-2-phenyl-2-hexenal						aldehydic bitter cocoa nut skin green sweet chocolate fruity butyric; cocoa hexenal
27.	Dodecanoic acid						Metal
28.	Heptadecane						Perfume
29.	Caffeine						Bitter

*Obtained of literature.

Table 4.6b. Further screening of volatile organic compounds (VOCs) identified in the chocolates produced from beans fermented with *P. kudriavzevii* (PK), *H. thailandica* (HT), a mixture of PK and HT (Mix) and spontaneously fermented beans (control)

No.	Group	Retention time (min)	Compound	Odour descriptors[a]	Mean GC-MS peak area x 10^5					Kovats Retention Index[b]	References
					Control	Ghana	PK	HT	Mix		
1	**Acid**	2.458	Acetic acid	Sour, astringent, vinegar	4.96±9.91[a]	0.00±0.00[a]	4.33±8.25[a]	0.27±0.55[a]	1.52±1.32[a]	600-662	Rodriguez-Campos et al. (2012)
10		19.137	Dodecanoic acid	Metal	0.06±0.07[a]	0.09±0.19[a]	0.10±0.21[a]	0.00±0.00[a]	0.03±0.06[a]	1549-1580	Rodriguez-Campos et al. (2012)
	Sum				5.02	0.09	4.43	0.27	1.55		
2	**Aldehyde**	3.460	Pentanal	Pungent, bitter, malt, almond	0.01±0.01[a]	0.01±0.01[a]	0.02±0.04[a]	0.14±0.17[a]	0.03±0.05[a]	698-702	Crafack et. al. (2014)
5		6.075	Hexanal	Green	2.94±3.92[a]	1.78±2.34[a]	3.05±3.05[a]	5.76±9.53[a]	2.23±1.82[a]	800-805	Crafack et. al. (2014)
6		10.961	Octanal	Orange peel, oily, fatty, soapy	0.09±0.18[a]	0.16±0.18[a]	0.32±0.24[a]	0.52±0.43[a]	0.13±0.15[a]	981-1006	Crafack et. al. (2014)
8		12.779	Nonanal	Tallowy, soapy-fruity	0.31±0.39[a]	0.22±0.30[a]	0.65±0.50[ab]	1.31±0.57[b]	0.5±0.09[ab]	1089-1105	Crafack et. al. (2014)
	Sum				3.35	2.17	4.04	7.73	2.89		
3	**Alcohols**	5.304	3 methyl-1-butanol	Malty, bitter, chocolate	0.01±0.02[a]	0.00±0.00a	0.00±0.00a	0.00±0.00a	0.00±0.00a	730-760	Rodriguez-Campos et al. (2012)
4		5.305	1-Pentanol	Flowery, sweet	0.03±0.04 [a]	0.01±0.01[a]	0.00±0.00[a]	1.13±1.79 [a]	0.00±0.00[a]	762-779	Crafack et. al. (2014)
9		12.986	Phenylethyl Alcohol	Flowery	0.00±0.00[a]	0.00±0.00[a]	0.17±0.20 [a]	0.18±0.13 [a]	0.45±0.53 [a]	1113	Rodriguez-Campos et al. (2012)

	Sum				0.04	0.01	0.17	2.31	0.45		
7	**Pyrazines**	12.520	Tetramethyl-pyrazine	Roasted, green, coffee, cocoa	1.12 ±2.03[a]	0.07±0.11 [a]	0.34 ±0.29[a]	0.46±0.34 [a]	0.38±0.45 [a]	1086-1087.3	Ascrizzi et al. (2017)
	Sum				1.12	0.07	0.34	0.46	0.38		
11	**Others**	20.729	Heptadecane		0.04±0.05 [a]	0.06±0.08 [a]	0.02±0.04 [a]	0.00±0.00[a]	0.07±0.05 [a]	none	Menezes et. al. (2016)
12		22.779	Caffeine		0.11±0.13 [a]	0.21±0.42 [a]	0.90±0.71 [a]	1.18±1.32 [a]	0.86±0.34 [a]	1842	Menezes et. al. (2016)
	Sum				0.15	0.27	0.92	1.18	0.93		

[a] Flavor notes reported.

[b] Obtained of literature.

Small alphabet indicates the comparison of different yeast added fermentations, different small letter indicating a significant difference between the added fermentation at ($p \leq 0.05$) based on one-way ANOVA, Tukey test ($p \leq 0.05$). Bars indicated standard error of the mean (±SEM)

4.3.5.1 Principal Component Analysis (PCA)

The score plot presented in Figure 4.2 explains 87.2% of the total variation between the 5 chocolate samples. The first two principal components (PCs) explained the maximum variation where 56.8% and 30.4% were observed for PC1 and PC2, respectively. A clear separation of chocolates produced using cocoa beans fermented with yeast starters, control and Ghana chocolates was shown in Figure 4.2, indicating a difference in quality and quantity of the volatile compounds formed among these chocolates.

Based on the positive side on PC1, chocolate sample produced using beans fermented with Mix or HT was closely associated by VOC groups such as aldehydes, alcohols and others. Those volatile compounds had flavour attributes of pungent, bitter, chocolate, malty, flowery, fatty, fruity and sweet. On the negative side of PC1, control, Ghana and PK samples were closely related to volatile compounds of acids and pyrazines. This indicated chocolates produced using control beans, Ghana beans and beans fermented with PK had a high concentration of volatile compounds, which conferred flavour attributes of sour, astringent, metal, roasted and cocoa.

On the positive side of PC2, volatile groups such as pyrazines, alcohols, aldehydes and acids were correlated with control, HT or PK samples. Therefore, chocolate produced using control beans and beans fermented with HT or PK had a high concentration of these volatile compounds, which deliberated flavour attributes of pungent, bitter, chocolate, malty, flowery, fatty, fruity, sweet, sour, astringent, metal, roasted and cocoa. Based on the negative side of PC2, only Ghana and Mix samples were observed, indicating there were less prominent flavour characteristics in these chocolates. In summary, the PCA result showed that VOC profiles of

chocolates produced using beans fermented with HT, PK and Mix yeast starters were prominently different from control and Ghana chocolates.

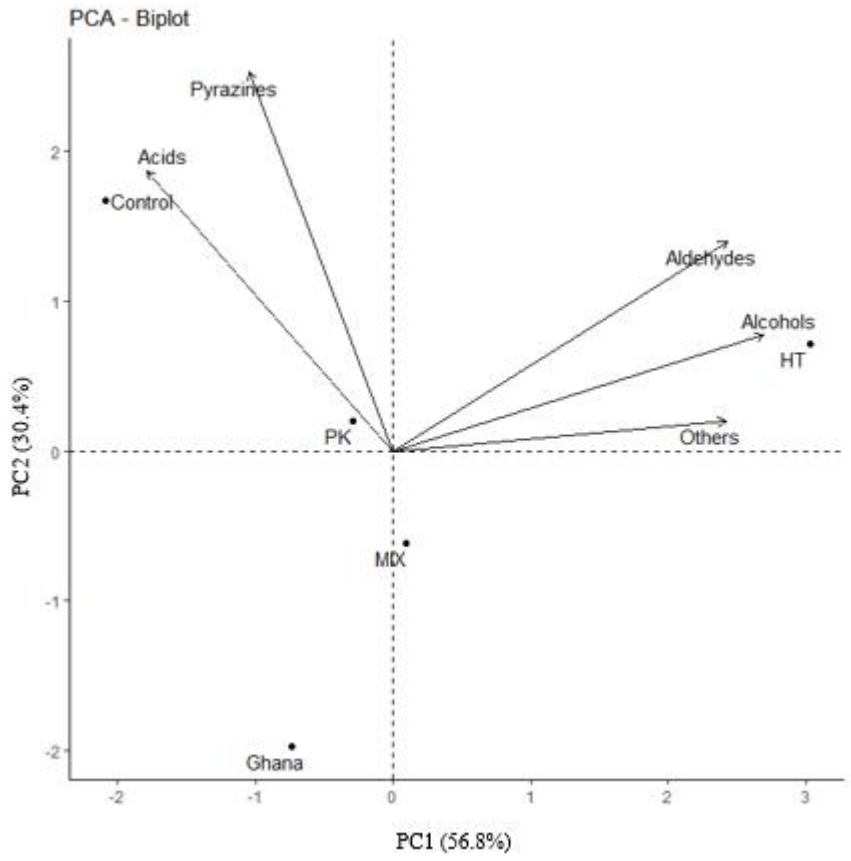


Figure 4.2 Principle Component Analysis (PCA) biplot of the chemical classes of the chocolates produced using spontaneously fermented beans (control), Ghana beans (Ghana) and beans fermented with *P. kudriavzevii* (PK), *H. thailandica* (HT), a mixture of PK and HT (Mix)

4.3.6 Sensory evaluation of chocolates

The sensory profile of control and Ghana chocolates were similar with each other and distinctly different as compared to chocolates produced using beans fermented with HT, PK and Mix (Figure 4.3, Table 4.7). In general, Ghana chocolate was characterized as having the least bitterness, astringency, acid, woody taste, low snap and quick melting as compared to chocolates produced using cocoa beans fermented with yeast starters ($p \leq 0.05$). Control chocolate was characterized as having a low cocoa taste, sweetness, roasted tastes and had low scores for hardness, snap and melting property ($p \leq 0.05$). The chocolate produced using cocoa

beans fermented with PK was characterized as having high cocoa, acid, woody and roasted tastes ($p \leq 0.05$). This chocolate had a good snap and high melting point as compared to all the other chocolates ($p \leq 0.05$). The chocolate produced using beans fermented with HT was characterized as having the low cocoa taste but more towards acidic, sweet and roasted tastes. It also had a good snap and high melting point (score of 4.91) as compared to control (score of 2.56) and Ghana (score of 1.97) chocolates ($p \leq 0.05$). The chocolate produced using cocoa beans fermented with Mix had high bitterness, astringency and sweetness ($p \leq 0.05$). This chocolate also possessed good snap, hardness and melting property ($p \leq 0.05$).

Based on the sensory result, there was no significant difference in all the descriptors among the chocolate samples, except for bitter, astringent, acid, sweet, woody, roasted, snap, hardness and melting property ($p \leq 0.05$). Chocolates produced using beans fermented with yeast starters were more bitter and astringent than control and Ghana samples ($p \leq 0.05$). This outcome was in accordance with the finding of antioxidant contents, where the bitterness in chocolates was associated with the presence of alkaloids such as theobromine and caffeine (Table 4.3). A previous study also reported that dominant bitterness and astringency were detected in chocolate produced by inoculated fermentation (Batista et al., 2016).

The chocolate produced using beans fermented with PK the most acidic whereas Ghana chocolate was the least acidic ($p \leq 0.05$). The chocolate produced using beans fermented with Mix had the sweetest taste; while chocolate produced using control beans had the least sweetness ($p \leq 0.05$). These findings were supported by a former study where chocolate produced from beans inoculated with *Saccharomyces cerevisiae* is noted with a significant perception of sweet and sour (Menezes et al., 2016).

Based on the overall sensory attributes (Table 4.7), chocolate produced using cocoa beans fermented with Mix was the most preferred by trained panels, followed by chocolate

produced using beans fermented with HT, PK, and Ghana chocolate. The chocolate produced using control beans was the least preferred by trained panels. Overall, the addition of yeast species in fermentation modulated the flavour profile, especially for flavour attributes such as bitterness, astringency, acidic and sweetness of the resulted chocolates.

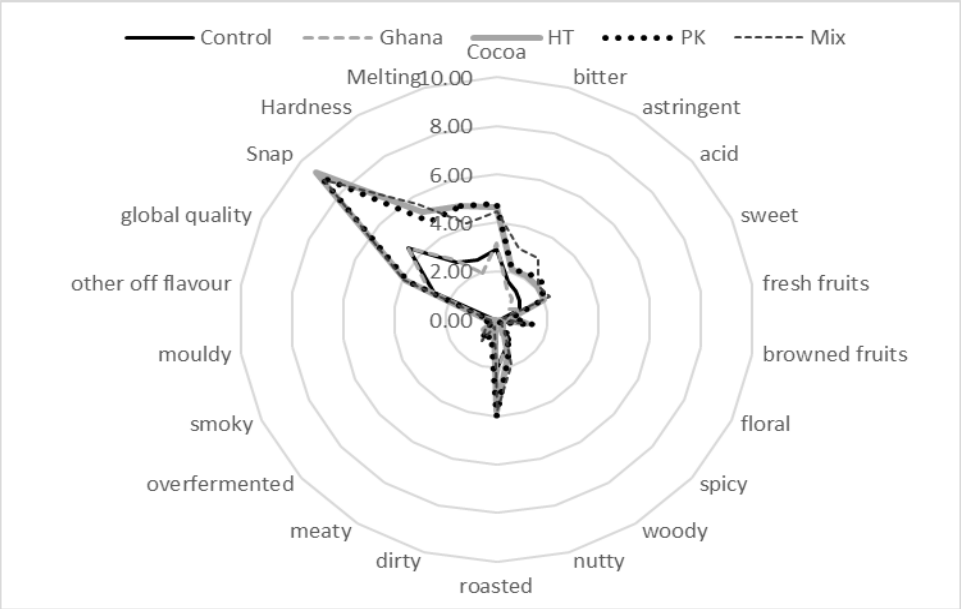


Figure 4.3 Sensory profiles of the chocolates produced using Ghana, control (spontaneously fermented beans) and cocoa beans fermented with PK, HT and Mix. The centre of the diagram corresponds to the lowest flavour intensity and perimeter to the highest flavour intensity

Table 4.7 Sensory profiles of the chocolates produced using Ghana cocoa beans, control (spontaneously fermented beans) and cocoa beans fermented with PK, HT and Mix

Sample	Cocoa	bitter	astringent	acid	sweet	fresh fruits	browned fruits	floral	spicy	woody	nutty
Control	2.91 ± 3.08^{a}	1.63 ± 1.81^{a}	1.38 ± 1.52^{ab}	1.16 ± 1.30^{ab}	0.97 ± 1.16^{a}	0.22 ± 0.41^{a}	0.53 ± 0.74^{a}	0.19 ± 0.44^{a}	0.13 ± 0.34^{a}	0.94 ± 0.27^{ab}	1.25 ± 1.45^{a}
Ghana	3.16 ± 3.33^{a}	1.34 ± 1.43^{a}	1.06 ± 1.14^{a}	0.66 ± 0.81^{a}	1.03 ± 1.20^{ab}	0.31 ± 0.60^{a}	0.56 ± 0.73^{a}	0.34 ± 0.60^{a}	0.13 ± 0.34^{a}	0.00 ± 0.00^{a}	1.16 ± 1.39^{a}
HT	4.66 ± 1.04^{a}	2.16 ± 0.81^{ab}	2.13 ± 0.97^{abc}	2.09 ± 1.07^{bc}	2.00 ± 1.10^{bc}	0.59 ± 0.88^{a}	1.19 ± 1.12^{a}	0.09 ± 0.27^{a}	0.31 ± 0.79^{a}	0.50 ± 0.82^{ab}	1.50 ± 1.15^{a}
PK	4.81 ± 1.00^{a}	2.16 ± 0.83^{ab}	2.25 ± 0.91^{bc}	2.34 ± 1.06^{c}	1.94 ± 0.70^{abc}	0.59 ± 0.92^{a}	1.47 ± 1.04^{a}	0.28 ± 0.58^{a}	0.13 ± 0.34^{a}	0.81 ± 1.22^{b}	1.66 ± 0.93^{a}
Mix	4.47 ± 0.96^{a}	3.06 ± 0.98^{b}	3.00 ± 1.05^{c}	2.16 ± 1.18^{bc}	2.28 ± 0.91^{c}	0.28 ± 0.52^{a}	1.00 ± 1.08^{a}	0.38 ± 0.74^{a}	0.25 ± 0.61^{a}	0.63 ± 0.94^{ab}	1.97 ± 0.90^{a}
Sample	**roasted**	**dirty**	**meaty**	**Over-fermented**	**smoky**	**mouldy**	**other off flavour**	**global quality**	**Snap**	**Hardness**	**Melting**
Control	2.06 ± 2.21^{a}	0.19 ± 0.75^{a}	0.16 ± 0.63^{a}	0.19 ± 0.40^{a}	0.13 ± 0.50^{a}	0.25 ± 0.58^{a}	0.13 ± 0.50^{a}	2.69 ± 2.94^{a}	4.50 ± 4.72^{a}	2.81 ± 2.98^{a}	2.56 ± 2.71^{ab}
Ghana	2.31 ± 2.47^{ab}	0.06 ± 0.25^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.06 ± 0.25^{a}	0.00 ± 0.00^{a}	0.44 ± 1.26^{a}	2.91 ± 3.17^{a}	4.50 ± 4.77^{a}	2.94 ± 3.08^{ab}	1.97 ± 2.15^{a}
HT	3.78 ± 1.11^{bc}	0.28 ± 0.68^{a}	0.68 ± 0.17^{a}	0.66 ± 1.01^{a}	0.00 ± 0.00^{a}	0.13 ± 0.34^{a}	0.53 ± 0.81^{a}	3.88 ± 1.13^{a}	9.28 ± 1.03^{b}	5.31 ± 0.73^{c}	4.91 ± 0.86^{c}
PK	4.00 ± 1.06^{c}	0.67 ± 1.01^{a}	1.01 ± 0.25^{a}	0.53 ± 0.88^{a}	0.13 ± 0.29^{a}	0.56 ± 1.03^{a}	0.44 ± 0.89^{a}	3.75 ± 0.86^{a}	9.00 ± 1.32^{b}	4.81 ± 0.73^{bc}	4.88 ± 0.70^{c}
Mix	3.78 ± 1.08^{bc}	0.31 ± 0.70^{a}	1.08 ± 0.27^{a}	0.56 ± 0.96^{a}	0.13 ± 0.34^{a}	0.16 ± 0.35^{a}	0.44 ± 0.91^{a}	3.97 ± 1.42^{a}	8.81 ± 1.56^{b}	5.66 ± 0.96^{c}	4.13 ± 0.83^{bc}

Small alphabet indicates the comparison of different yeast added fermentations, different small letter indicating a significant difference between the added fermentation at ($p \leq 0.05$) based on one-way ANOVA, Tukey test ($p \leq 0.05$). Bars indicated standard error of the mean (±SEM)

4.4 Conclusion

Based on the rheological properties, both viscosity and yield stress of chocolates produced using beans of control, Ghana, HT or PK could be useful for rheological prediction in the chocolate making process. Chocolates produced by using cocoa beans fermented with PK or HT could be suitable for couverture application due to the high yield stress value obtained. The high enthalpy of melt of chocolate produced using cocoa beans fermented with HT was possibly associated with the higher fat content presented in the beans. Based on the correlation analysis, the use of HT or PK starter influenced the fermentation where less acidic beans were produced, and these could affect the (+)- catechin and (-)-epicatechin contents in cocoa beans. Based on PCA, the VOC profiles of chocolates produced using cocoa beans inoculated with HT, PK and Mix were noticeably different from Ghana and control chocolates. The addition of yeast species during fermentation modulated the flavour profile, especially bitterness, astringency, acidic and sweetness of the resulted chocolates. Bitterness and astringency were more intense in chocolates produced using beans fermented with yeast starters, which could be highly linked with the methylxanthine contents (theobromine and caffeine). Overall, the addition of certain yeast starter culture in cocoa fermentation influenced the rheological and melting properties, antioxidant content, VOC and sensory profiles of chocolate produced. *H. thailandica* or *P. kudriavzevii* is recommended as a potential yeast starter to improve the content of methylxanthines (caffeine and theobromine) in the cocoa product. We would like to conclude that the usage of yeast starter in fermentation would open an alternative approach to the production of chocolate with a unique sensory profile that is deemed natural without the addition of artificial flavouring agents.

Chapter 5

5 General conclusions and Future work

5.1 General conclusions

Yeast species is precedence to the quality of the final product in cocoa bean fermentation. The usage of starter culture in fermentation had produced cocoa beans with various sensory profiles. The present study was designed to evaluate the effect of isolated yeast as starter culture in cocoa simulation media based on total polyphenols content, total flavonoid content, fermentation index and total soluble solids of cocoa beans produced. Based on the lab screening study, *H. thailandica, P. kudriavzevii* and a mixture of the two yeasts were selected and applied in the field fermentation (20 kg of cocoa beans). Next, the effect of these selected yeast species on the fermentation parameters (pH of pulps, pH of nibs, total soluble solids, fermentation index, cut test and pH of dried nibs) and antioxidant contents (TPC, TFC, DPPH and ABTS scavenging activities) of fermented beans produced from 2 batches of field fermentation were investigated. The volatile organic compounds (VOC) of roasted beans and the sensory acceptance of cocoa liquor produced were also studied. Chocolates produced from using these cocoa beans were studied for their physicochemical properties, antioxidant contents (TPC, TFC and DPPH), polyphenols ((-)-epicatechin and (+)-catechin), methylxanthines (theobromine and caffeine), VOC and sensory profiles.

Firstly, naturally-existing cocoa yeasts identified in cocoa fermentation were evaluated by using a cocoa simulation media and screening were based on fermentation and antioxidant parameters. The current findings showed that the application of selected yeast starter culture in cocoa fermentation produced cocoa beans with higher total polyphenols and flavonoid content compared to control at certain fermentation periods. We observed that with the addition of selected yeast starter culture (*H. thailandica, P. kudriavzevii, H. opuntiae, Wickerhamomyces* species and *S. cerevisiae)*, the fermentation process proceeded efficiently in sugar metabolism.

P. kudriavzevii (Genbank number: MH979681) and *H. thailandica* (Genbank number: MH979675) were potential yeast species in modulating the antioxidant activities of the dried cocoa beans. These two yeasts were recommended for field application (consisting of 20 kg beans).

In the second phase, the influence of *H. thailandica* and *P. kudriavzevii* in the cocoa field fermentation on fermentation parameters and antioxidant properties were determined. The application of yeast starter in fermentation was highly reproducible as consistent trends (temperature, pH of nibs, and antioxidant contents) were observed in the two fermentation batches. The usage of *H. thailandica* in fermentation improved the TPC of cocoa beans with fermentation index of 1.33 and 1.24. The VOC profiles of fermentation added with *H. thailandica, P. kudriavzevii* and mixture of these two yeast starter cultures were noticeably different from spontaneous fermentation. Our sensory result showed that the overall acceptance of cocoa liquor samples produced using beans added with yeast starter culture were comparable to Ghana cocoa liquor. Sensory attributes such as sweet and spicy notes were the unique flavours that were only found in cocoa liquors produced using beans added with yeast starter cultures (PK, HT or Mix).

Lastly, chocolates produced using cocoa beans obtained from field fermentation were analysed for their physicochemical properties, antioxidant content, VOC and sensory profiles. Both rheological parameters (viscosity and yield stress) of chocolates produced using beans of control, Ghana, HT or PK could be useful for rheological prediction in the chocolate making process. Fat content exhibited the strongest effect on the variability in the rheological properties of chocolates produced using beans fermented with yeast starters (PK, HT or Mix), followed by particle size. The high enthalpy of melt of chocolate produced using cocoa beans fermented with HT was possibly associated with the higher fat content presented in the beans. The usage

of HT or PK during fermentation influenced the methylxanthines content of cocoa beans and subsequently the sensory of chocolate produced. In the current study, HT or PK is recommended as a potential yeast starter to improve the content of methylxanthines (caffeine and theobromine) in cocoa beans. The VOC profiles of chocolates produced using cocoa beans inoculated with HT, PK and Mix were noticeably different from Ghana and control chocolates. Sensorial attributes such as bitterness, astringency, acidic and sweetness were modulated by the addition of yeast species during fermentation. Bitterness and astringency attributes were more intense in chocolates produced using beans fermented with yeast starters, which were highly associated with the presence of methylxanthines (theobromine and caffeine contents). Overall, the addition of yeast starter culture in cocoa fermentation influenced the rheological and melting properties, antioxidant content, VOC and sensory profiles of chocolate produced.

5.1.1 Overall conclusions

In the present study, field applications by using *P. kudriavzevii* (MH979681), *H. thailandica* (MH979675) and a combination of the two yeasts as starter cultures in field application (20 kg beans) were complied with guidelines of Malaysian Cocoa Board (MS 2672:2017). The usage of HT was recommended in fermentation, as the improved TPC and well fermentation index of cocoa beans were obtained. The VOC profiles of cocoa liquors and chocolates produced using beans fermented with HT, PK and Mix were distinctively different from Ghana and control samples. The chocolate produced using cocoa beans fermented with PK or HT could be suitable for couverture application due to the high yield stress value obtained. These chocolates were suitable for decoration purpose due to more mechanical strength and ease of handling. Mix chocolate with lower yield stress could be applied in the moulding process as the machine can easily pump the chocolate mixture into mould and air pockets can also be removed easily. Mix chocolate was most preferred by trained panels of

Malaysian cocoa board, whereas control chocolate was the least preferred. Chocolates produced using beans added with yeast starter were also more bitter and astringent than control. These potential yeast starters are recommended for the cocoa fermentation and could be applied according to its purpose served. A mix yeast starter is more suitable to produce chocolate with sensory attributes of sweet, bitter and astringent tastes. Whereas, PK yeast starter is more suitable to produce chocolate with high cocoa and acidic tastes. HT starter is ideal to produce chocolate with low cocoa but more acidic and sweet tastes. Current research pointed out the importance of reliably improved cocoa beans fermentation by using the yeast starter culture, which can deliver fermented dry cocoa beans with a unique taste in a reproducible way.

The novelty of the present study lies in the usage of selected yeast starter in cocoa field fermentation to improve the antioxidant content of cocoa beans and resulted chocolates. The present study provides insights into the antioxidant activities, aroma and sensory of the cocoa beans and chocolates produced after the addition of selected yeast starter culture. Even when the different batch of fermentation was carried out, the usage of yeast starter managed to produce cocoa beans which complied with guidelines of Malaysian Cocoa Board (MS 2672:2017). We concluded that the inoculation of yeast in fermentation influenced the organoleptic and antioxidant properties of cocoa beans. The usage of yeast starter can overcome the sensory variability which typically seen in the spontaneous cocoa bean fermentation.

5.2 Suggestions for future work

The usage of yeast starter in fermentation is recommended as it enables the production of chocolates with improved antioxidant content and unique chocolate flavour regardless of the season. The usage of yeast starter would help to improve the crop values of cocoa beans

produced by cocoa smallholders by producing less acidic cocoa beans. This would also open an alternative approach to the production of chocolate with a unique sensory profile.

Future research is needed to better understand the roles of these yeasts and their biochemical reactions within the cocoa bean. Metagenomic Next Generation Sequencing (mNGS) can be carried out to obtain a thorough understanding of the yeast microbial succession involved during the cocoa fermentation in Malaysia. It is also suggested to determine the metabolic pathway of the yeasts involved during fermentation. The in-depth study of the role of yeast with cocoa polyphenols and cocoa proteins are important information that would help to gain a better understanding of the yeast involves in cocoa fermentation.

The application of other yeast starter culture in fermentation is also recommended to gather more insight into the flavour, sensory information and knowledge on the chemical composition of cocoa beans. X-ray diffraction (XRD) can be performed in the future to confirm the crystal in the chocolate produced using beans fermented with yeast starters. A comprehensive study on the influence of fat content on flavour and sensory properties of chocolates produced using beans fermented with yeast starter is recommended, as this information will help to ensure the production of quality cocoa beans.

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Appendices

Appendix A (Chapter 2) Publication during enrolment

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Influence of selected native yeast starter cultures on the antioxidant activities, fermentation index and total soluble solids of Malaysia cocoa beans: A simulation study

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ABSTRACT

Antioxidant activity of cocoa beans is often influenced by drying and roasting stages. In this study, 13 naturally-existing yeast strains were isolated and used as a starter culture and the resulted antioxidant properties of cocoa beans were determined by total polyphenols content (TPC), 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and total flavonoids content (TFC). The isolated yeasts were identified via sequencing using universal primers ITS 1 and 4. Results revealed that the 13 naturally-existing yeast strains were *Pichia kudriavzevii* (MH979676, MH979680, MH979681, and MH979677), *Hanseniaspora thailandica* (MH979675), *Hanseniaspora* species (MH979678), *Wickerhamomyces* species (MH979679), *Saccharomyces cerevisiae* (MH979683), *Hanseniaspora opuntiae* (MH979684) and *Candida quercitrusa* (MH979685, MH979687, MH979686 and MH979682). Yeasts were selected based on the phylogenetic analysis, where each species of different genus (except *Candida* genus) was used as a starter culture. Dried cocoa beans inoculated with isolates (*Hanseniaspora thailandica, Pichia kudriavzevii, Hanseniaspora opuntiae, Hanseniaspora* species, *Wickerhamomyces* species and *Saccharomyces cerevisiae)* contained TPC, TFC and DPPH ranging from 21.82 to 69.81 mg/g Gallic acid (GAE), 1.68–6.33 mg/g Catechin and 113.85 to 328 μmol/g Trolox (TE), respectively. It is noted that there was no significant change of the antioxidant activity between isolates at 24-h to 120-h fermentation. Based on the current study, *Hanseniaspora thailandica* and *Pichia kudriavzevii* are the potential starter cultures that result in cocoa beans with higher antioxidant content ($p < 0.05$) compared to natural fermentation.

1. Introduction

The rising popularity towards dark chocolate is stimulated by a growing interest in healthy living. Cocoa contains flavonoids (antioxidants) which are beneficial to improve cardiovascular health, blood flow and cognitive functions (Katz, Doughty, & Ali, 2011). Cocoa beans undergo fermentation to give rise to the chocolaty flavour of the cocoa beans. Cocoa fermentation involved microbes such as yeast which dominates the process, followed by lactic acid bacteria, and acetic acid bacteria that grow on the mucilaginous pulp of the cocoa beans. During fermentation, alcohols and acetic acid produced by microbes diffuse into the cotyledon of cocoa beans, leading to biochemical reactions within the bean, thus producing the chocolate precursors (Misnawi, 2008).

Yeasts have been used as starter culture in fermentation. Fermentations inoculated with yeasts such as *Saccharomyces cerevisiae* and *Pichia kluyveri* prone to produce beans with coffee, sour and bitter flavour chocolate (Batista, Ramos, & Ribeiro, 2015). Crafack et al. (2013) also reported that cocoa fermentation inoculated with mixed inoculum (*Kluyveromyces marxianus* KM16-6/*Lactobacillus fermentum* L18/*Acetobacter pasteurianus* A149) has been shown to produce high level of bitter-sourness and astringent chocolate with the lowest score of sweetness and general liking. There is no specific study on the influence of yeast as a starter culture on the antioxidant properties of cocoa bean. However, wine yeast (*Saccharomyces cerevisiae)* is well known to cause decrease in the phenolic content of wines (Caridi, Cufari, Lovino, Palumbo, & Tedesco, 2004). In a study using different strains of *Saccharomyces cerevisiae* strain Sc2659 was reported to not only metabolized sugar (brix) faster than strain Sc1483, it also produced wine with significantly higher values of colour intensity, total polyphenols and monomeric anthocyanins compared to strain Sc1483 (Caridi et al., 2004). These preliminary results had shown interesting

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correlations between yeast strains used for winemaking and phenolic composition of wine, indicating that yeast strain behaviour may modify chromatic properties, phenolic profile and antioxidant power of wine (Caridi et al., 2004).

Despite existing literature has demonstrated the potential of yeast starter culture in controlled fermentation to improve the flavour of cocoa beans; there is a lack of literature showing how such fermentation would impact the antioxidant activities of the cocoa beans. To the best of our knowledge, this is the first study on determining the antioxidant activities of cocoa beans after inoculated with a single native yeast species as starter culture using a simulation medium. The present research aims to determine the antioxidant activities of cocoa beans inoculated with starter cultures consisting of isolated yeast species from Malaysia. This simulation study provides insight on the antioxidant activities of dried cocoa beans inoculated with yeast starter cultures.

2. Materials and methods

2.1. Isolation of naturally-existing yeasts isolates

The spontaneous cocoa bean fermentation was performed to obtain naturally-existing yeast isolates. Yeast isolates after identification were then applied as a starter culture using a cocoa simulation medium. Cocoa shallow box fermentation ($31 \times 31 \times 31$ cm^3) with 20 kg of wet cocoa beans was used for yeast isolation according to method of Bariah, Ibrahim, and Yang (2014). The cocoa fermentation process was carried out for five days and the turning process of cocoa beans was completed at 72 h of cocoa fermentation. Shallow cocoa box was firstly sterilized with 99.9% ethanol prior to loading of cocoa beans. The cocoa beans were filled up to approximately 29 cm compared to the height of the box. The box was covered with gunny sack once the beans were loaded. Mixed cocoa clones such as PBM 123, BR 25, MCBC 1 and MCBC 8 were bought from Malaysian Cocoa Board Jengka, Pahang for the fermentation process.

Sample collection was conducted according to method by Ooi, Sepiah, and Bariah, (2016). Samplings were conducted by randomly collecting (at 0, 6, 24, 48, 72, 96 and 120 h fermentation) cocoa beans from different points of the box. Samples were mixed and kept in a sterile polybag followed by storage in a refrigerator at −20 °C for laboratory and microbial analysis.

2.2. Isolation of yeast

Yeast isolates were obtained from the collected samples by bean swab method as suggested by Ooi et al. (2016). The bean swab method was carried out where cocoa bean was respectively swabbed on the yeast extract media and incubated at 25 °C for yeast growth. Serial dilution was conducted according to Pereira et al. (2017) where 25 g of cocoa beans with pulp was added to 225 mL saline-peptone water (v/v 0.1% peptone (Merck, Germany), v/v 0.8% NaCl (Merck, Denmark), followed by homogenization using a stomacher at a normal speed for 5 min.

Yeast extract media was used for yeast isolation. The media consisted of glucose 50 g (Fisher Scientific, UK), yeast extract 3.0 g (Becton, Dickinson and Company, France), KH_2PO_4 0.1 g (Hamburg Chemicals, UK), NaCl 0.1 g (Merck, Denmark), $CaCl_2$ 0.013 g (Friedemann Schmidt, Australia), distilled water 1 L and agar 15 g (Becton, Dickinson and Company, France). Media was adjusted to pH 5.5 before autoclaved (Hirayama Hiclave HVE-50, Japan) at 121 °C for 15 min. Yeast cultures were incubated at 30 °C for 48–72 h. Yeast isolates were selected for pure culture preparation and identification by molecular technique. Purified yeast isolates were preserved in 25% glycerol at −20 °C (Hamburg Chemicals, UK) as stock culture.

2.3. Identification of yeast isolates via DNA sequencing

2.3.1. gDNA extraction from pure cultures

Genomic DNA (gDNA) extraction protocol was according to Ooi et al. (2016). Colonies of yeast from the pure yeast colony after three days of growth at 30 °C were picked by using sterilized tooth picks and placed into the Eppendorf tube filled with 200 μL of lysis buffer solution (Triton X-100 10 mL, SDS 5 g, NaCl 2.922 g and Tris HCl 0.7882 g at pH 8.0, EDTA 0.1461 g at pH 8.0). The tube was heated in 70 °C water bath for 3 min and kept frozen for 3 min in −80 °C freezer. The previous step was repeated once followed by addition of a Chloroform (J.T. Baker, UK): isoamyalcohol (analytical UNIVAR reagent, Australia) mix (24:1, v:v). Tubes were then centrifuged (13000 rpm, 10 min) and new aqueous phase was then transferred to a tube. Two volumes of absolute cold ethanol (R&M chemicals, UK) was added to the aqueous phase and then the mixture was incubated overnight at −20 °C. The tube was then spun at 13000 rpm for 10 min. Supernatant was discarded and pellet was air dried under the air flow laminar for 10 min. 50 μL of Tris-EDTA (TE) buffer was added and the tube was incubated at −20 °C.

2.3.2. PCR analysis

Primers ITS 1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS 4 (5′-TCCTCCGCTTATTGATATGC-3′) were used for DNA amplification. Amplification of yeast was performed in a final volume of 50 μL containing 17.45 μL of PCR master mix direct load (Biotech rabbit), 4 μL of DNA template and 28.55 μL of deionized water. For the PCR reaction, initial denaturation was conducted at 94 °C for 4 min, followed by 29 cycles of denaturation at 94 °C for 2 min, annealing at 55 °C for 2 min, extension at 72 °C for 2 min and lastly final extension at 72 °C for 10 min followed by cooling at 10 °C for infinity. PCR was performed using a thermo cycler (MJMini BIO-RAD, US). PCR product was then loaded and separated by electrophoresis in 1% (w/v) agarose gels in 1x Tris/Borate/EDTA (TBE) buffer at 80 V, 120 A for 40 min before viewing by gel imaging viewer. The PCR products were stored at −20 °C before purification and sequencing.

2.3.3. BLAST search for sequencing information

The sequencing information was edited and aligned by using Chromas Pro (version 1.7.4) and then analysed by Basic Local Alignment Search Tool (BLAST) through the National Centre for Biotechnology Information (NCBI) to identify the closest yeast species to the yeast isolates.

2.4. Fermentation of cocoa beans with yeast starter culture

Cocoa media was prepared in the laboratory according to modified method of Pereira, Miguel, Ramos, & Schwan, 2012 in Erlenmeyer flasks. 500 mL of cocoa pulp media was placed in 500 mL of Erlenmeyer flasks to be used as a medium. The cocoa pulp media was added with 17 g/L of fructose (Hamburg Chemicals, UK), 25 g/L of glucose (Fisher Scientific, UK), 10 g/L of citric acid (Fisher Scientific, UK), 5 g/L of yeast extract (Becton, Dickinson and Company, France), 5 g/L of peptone (Merck, Germany) and 20% (w/v) fresh cocoa seeds.

Yeast species was used as starter culture in fermentation after species identification. Yeasts were selected based on the phylogenetic outcome, where each species of different genus was used as a starter culture. The yeast culture was prepared according to method modified from Ooi et al. (2016). Each yeast species was firstly cultured in 1.5 mL YEPD broth consisting of peptone (Merck, Germany) (1%), dextrose (Fisher Scientific, UK) (2%), and yeast extract (Becton, Dickinson and Company, France) (0.3%). The cultures were incubated overnight (12 h) at 30 °C (Binder, Germany). The number of yeast cells in suspension was counted by hemocytometer in cells/ml (Hirschmann, Germany). Suspension of the culture with concentration of 10^6 cells mL^{-1} was prepared by diluting the suspension with distilled water. Yeast starter culture with 1.5 mL culture was mixed with 500 mL of

cocoa pulps media in an Erlenmeyer flask and incubated at 30 °C. Control was a spontaneous fermentation process with indigenous microorganisms present in the cocoa fruit. Samples were collected every 24 h until 120 h for analysis. Samples were then oven dried at 38 °C until 6.5% moisture content was obtained.

2.5. Antioxidant activities

2.5.1. Sample extraction

Sample extraction was conducted according to modified method of Ioannone et al. (2015). Ground sample (1.5 g) was defatted 3 times by extracting with 10 mL of hexane and dried under fume hood for 20 min. 1 g of dried and defatted material was extracted with 5 mL of 70:29.5:0.5 acetone/water/acetic acid (v/v/v) by mixing for 1 min in a vortex mixture and sonication for 10 min at room temperature. The mixture was spun at 2100 *g* for 10 min and then filtered with Whatman filter paper no 4. The filtered cocoa extract was used for all chemical analysis.

2.5.2. Total polyphenol content

The total polyphenol content of the samples was determined according to method of Ioannone et al. (2015). Sample extract was firstly diluted in ratio of 1:999 μL methanol (J.T. Baker, UK), then 20 μL of diluted sample was taken to mix with 100 μL of Folin-Ciocalteau's reagent (diluted in 1:10 with water) (Merck, USA), followed by 75 μL of 10% (w/v) sodium bicarbonate (Na_2CO_3, Friendemann Schmidt, Australia) (Hamburg Chemicals, UK) solution was added in the 96 wells plate. Colour was allowed to develop in the dark for at least 2 h. Absorbance of the sample was measured at 740 nm by using an ultraviolet–visible (UV–vis) spectrophotometer, (PerkinElmer Lambda 25, USA). Standard curve to determine total polyphenol content was prepared using standard Gallic acid (Sigma Aldrich, USA) according to modified method of Singleton and Rossi (1965). Standard Gallic acid (Sigma Aldrich, USA) samples of 10 known concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.00 mg/mL were prepared. Total polyphenol content of sample was reported as milligram gallic acid equivalents (GAE) per gram defatted cocoa.

2.5.3. Determination of DPPH free radical scavenging activity

The DPPH free radical scavenging activity of cocoa samples was according to the modified method of Zzaman and Yang (2013). 100 μL of diluted sample was added to 200 μL of 2,2 diphenyl-1-pic-rylhydrazyl (DPPH) (Sigma Aldrich, USA). The mixture was then left to react for 30 min at room temperature in the dark. After incubation, absorbance was read at 515 nm using the microplate reader (Tecan, Switzerland). Ascorbic acid was used as a standard. Ethanol mixed with DPPH was used as a blank while water was used as a control. The scavenging effect was determined based on percentage of DPPH free radical scavenging activity using the equation below and expressed as micromole of Trolox per gram of defatted cocoa.

$$\%\ \text{DPPH radical scavenging activity} = \frac{\text{Absorbance of control} - \text{absorbance of sample}}{\text{Absorbance of control}} \times 100\%$$

2.5.4. Total flavonoid content

Total flavonoid content was performed according to modified method of Zhishen, Mengcheng, and Jianming (1999). The total flavonoid content was measured using spectrophotometric method. 0.25 mL of sample was mixed with 3 mL of deionized water and 0.3 mL of 5% $NaNO_2$ (R&M chemicals, UK). The mixture was then incubated at room temperature for 5 min. The mixture was then added with 1.5 mL of 2% aluminium chloride hexahydrate ($AlCl_3{\cdot}6H_2O$) (R&M chemicals, UK) and kept at room temperature for 5 min. Then, 2 mL of 1 mol/L sodium hydroxide (NaOH) (R&M chemicals, UK) was added. The flask was filled up to 10 mL with deionized water. The total flavonoid content was measured for its absorbance against a prepared reagent blank at 510 nm. Catechin (Sigma Aldrich, USA) was used as a standard and results are expressed as milligram catechin equivalents per gram of sample (mg CE/g).

2.5.5. Total soluble solids

Total soluble solids content of the fermented cocoa pulp for samples was measured by using Digital Hand-held Pocket Refractometer PAL-1 (Atago PAL-1, Japan) where a small amount of the sample was placed on the refractometer to get reading. The cocoa pulp was firstly separated from its nib and placed into a Falcon tube. Distilled water was added volume per volume (v/v) into the tube and vortexed. The amount of total soluble solids content of pulp was recorded in Brix unit.

2.6. Fermentation index

Fermentation index of the fermented samples was determined according to modified method of Gourieva and Tserrevitinov (1979). The cocoa nibs were ground with blender (Panasonic MX-798S, Malaysia) into powder form. 0.5 g of cocoa powder was weighed by using a weight balance and put into a 100 mL of volumetric flask. Then, a mixture of methanol (J.T Bakers, UK) and hydrochloric acid (J.T Bakers, UK) (97:3) was added into the volumetric flask. Mixture of methanol and hydrochloric acid (97: 3) was used to penetrate the cocoa bean structure to degrade the pigment (polyphenol). The mixture was shaken and kept in refrigerated condition overnight. The samples were filtered using Whatman filter paper No.4. The absorbance of the filtrates was measured at wavelengths of 460 nm and 530 nm by using UV–visible spectrophotometer (PerkinElmer Lambda 25, USA).

The ratio between absorbance 460 nm and 530 nm was used to measure the degree of fermentation of the cocoa beans. Fermentation index (FI) was calculated as follow:

Fermentation index (FI) = (Absorbance at 460 nm)/ (Absorbance at 530 nm)

According to Bariah et al. (2014), fermented beans has FI value of >1, over-fermented beans has FI value of >1.6, whereby unfermented beans has FI value of <1.

2.7. Statistical analysis

Triplicates of analysis was performed. The data were analysed using one- and two-way Analysis of Variance (ANOVA) followed by mean comparison using Turkey test at $p \leq 0.05$ levels with Statistical Package for the Social Sciences (SPSS) version 23. The phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis (MEGA X).

3. Results and discussion

3.1. Isolation and identification of yeast species

In this study, 13 yeast isolates were obtained from the mucilaginous pulp. The DNA extraction followed by polymerase chain reactions (PCR) and subsequent sequencing of the gene of 13 isolates led to the identification of 7 yeast species comprising of 5 genera namely: *P. kudriavzevii, H. thailandica, C. quercitrusa, S. cerevisiae, H. opuntiae, H.* species, and *W.* species (Table 1 and Fig. 1).

Based on the phylogenetic tree obtained (Fig. 1), *H. thailandica, H. opuntiae, H.* species and *S. cerevisiae* were closely related to each other. While, *C. quercitrusa* and *W.* species were distant genus with the previous groups mentioned. *P. kudriavzevii* isolate 43, 7, 27 and 33 were from the same genus and were highly similar with each other based on the bootstrap value (Fig. 1). In the present study, *P. kudraivzevii* isolate 33 and isolate 43 were arbitrarily selected within isolates 43, 7, 27 and 33, *H. thailandica, S. cerevisiae, H. opuntiae, H.* species and *W.* species

Table 1
Identification of yeast and their accession number deposited in NCBI gene bank.

Yeast strain code	Accession number obtained from NCBI gene bank	Yeast identity
43	MH979676	*Pichia kudriavzevii*
6	MH979675	*Hanseniaspora thailandica*
27	MH979680	*Pichia kudriavzevii*
42	MH979685	*Candida quercitrusa*
47	MH979687	*Candida quercitrusa*
1	MH979686	*Candida quercitrusa*
33	MH979681	*Pichia kudriavzevii*
7	MH979677	*Pichia kudriavzevii*
4	MH979682	*Candida quercitrusa*
32	MH979683	*Saccharomyces cerevisiae*
46	MH979684	*Hanseniaspora opuntiae*
48	MH979678	*Hanseniaspora species*
49	MH979679	*Wickerhamomyces species*

were used as a starter culture in a cocoa pulp simulation medium in subsequent study.

In this study, the predominant yeast species identified during the cocoa fermentation was similar with previous literatures. Previous literatures reported that major yeast species found in a cocoa fermentation was ranging from 6 to 10 (Kone et al., 2016; Nielson, Hoonholt, Tano-Debrah, & Jespersen, 2005). The high diversity of yeast was previously reported by several studies (Nielson et al., 2005; Lefeber, Papalexandratou, Gobert, Camu, & Vuyst, 2012; Arana-Sanchez et al., 2015). Our finding was similar with finding reported by Kone et al. (2016) where yeasts such as *S. cerevisiae, P. kudriavzevii, C. tropicalis* and *W. anomalus* were reported. Furthermore, Ho, Zhao, and Fleet, (2015) also reported that *P. kudriavzevii, S. cerevisiae, Saccharomycopsis crataegensis,* and *Hanseniaspora guilliermondii* were the predominant yeasts during cocoa fermentation. The current study concluded that *P. kudriavzevii* and *C. quercitrusa* were the most prevalent species as both species were the most identified from the total yeast isolates. The diversity of yeast communities is influenced by factors such as the use of starter cultures, type of cocoa, ripeness of cocoa pod, and postharvest treatments (Kone et al., 2016; Schwan & Wheals, 2004). *C. quercitrusa* is excluded as a starter culture as they are well known for causing disease such as Candidemia (Westblade et al., 2015).

3.2. Determination of total polyphenols content

In this study, the phenolic compounds of dried cocoa beans ranging from 21.82 ± 6.15 to 69.81 ± 38.23 mg/g GAE after 120 h fermentation (Fig. 2). There were no overall significant changes throughout 120 h fermentation, however, it was found that cocoa beans fermented with *P. kudriavzevii* isolate 33 had higher phenolic compounds than control ($p \leq 0.05$) at 24 h fermentation period while cocoa beans inoculated with *H. opuntiae* and *H. thailandica* had higher phenolic compounds than control ($p \leq 0.05$) at 96 h fermentation (Fig. 2).

This study showed a lower phenolic content in dried cocoa beans compared to previous literature. The phenolic content of raw cocoa beans in Malaysia was previously reported to vary from 71.42 to 82.68 mg/g GAE (Hii, Law, Suzannah, Misnawi, & Cloke, 2010). The lower range of TPC value found in the present study could be related to the usage of cocoa pods from a combination of various cocoa clones. It was reported that the difference in phenolic compounds was dependant on cocoa varieties and geographical regions. For instance, the raw cocoa beans seeds (Amazon hybrid variety Clone CCN51) from Ecuador was proven to contain 1–3 folds higher total phenolic content than the Trinitario cultivars from Venezuela (Oracz & Nebesny, 2016). In this study, despite all the mixed cocoa clones were obtained from a single region, clones contribute to various phenolic contents. Phenolic content was far more genotype-dependent than influenced by either organic or integrated grown (Veberic, 2016).

Phenolic compounds are secondary metabolites present in plants and can scavenge free radicals based on their electron donor ability (Haile & Kang, 2019). The high phenolic compounds found in cocoa beans fermented with *H. opuntiae, H. thailandica* and *P. kudriavzevii* Ye 33 could possibly be due to the release of the phenolic compounds, which were bound in the cellular structure of cocoa beans. These phenolic compounds are initially bound with sugar, reducing their availability to the organisms. During fermentation, organisms are capable in hydrolysing complexes of phenolics into simple, soluble-free phenols which are readily absorbed, leading to an increase in the phenolic content of cocoa seeds (Haile & Kang, 2019).

The cellular structure degradation of cocoa beans, which occurs during cocoa bean drying would also release the bound phenolic compounds, leading to the increment of phenolic content (Oracz & Nebesny, 2016). In the current study, we suggest that both yeast species and cellular structure degradation during drying may aid in releasing the bound phenolic compounds.

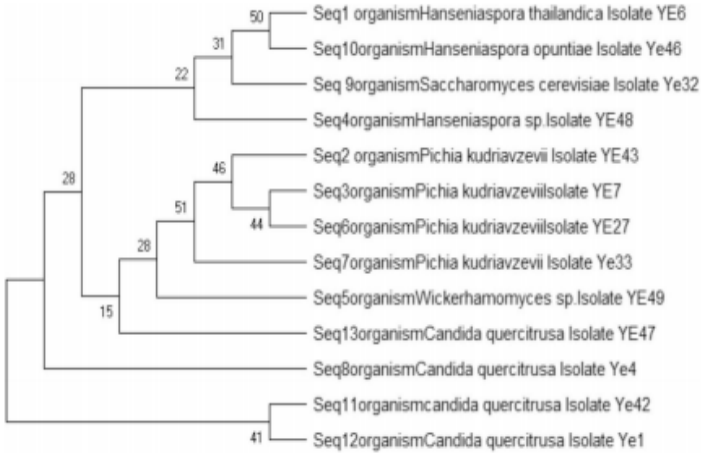
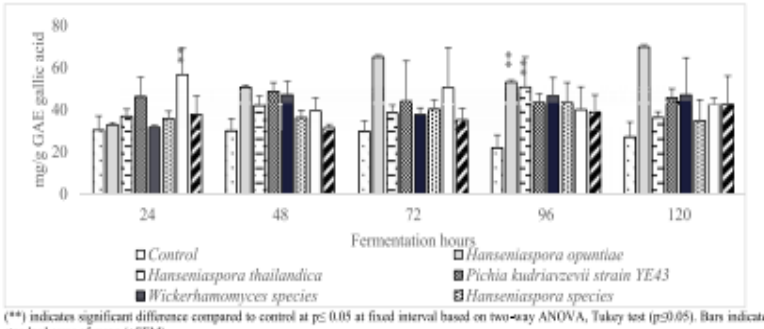
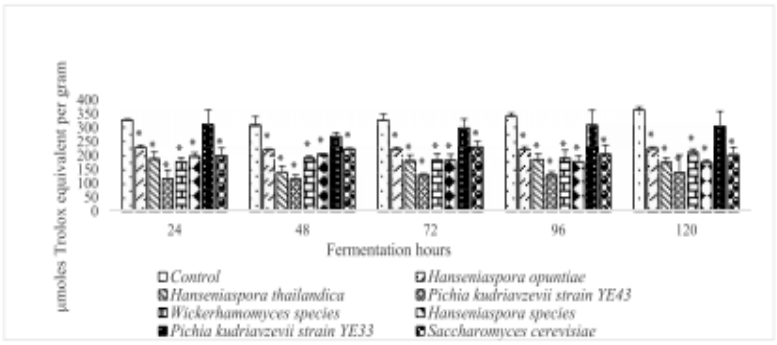


Fig. 1. Phylogenetic tree generated consisted of 13 yeast species associated with cocoa fermentation by using a Maximum Parsimony analysis, conducted in MEGA X.



(**) indicates significant difference compared to control at p≤ 0.05 at fixed interval based on two-way ANOVA, Tukey test (p≤0.05). Bars indicated standard error of mean (±SEM)

Fig. 2. Comparison of total polyphenols content (mg/g GAE Gallic acid) of cocoa beans at 24, 48, 72, 96 and 120 h fermentation.



(*) Indicates significant difference compared to control at p≤ 0.05 at fixed interval based on two-way ANOVA, Tukey test (p≤0.05) between the treated fermentation. Bars indicated standard error of mean (±SEM)

Fig. 3. Comparison of DPPH content (µmoles/g TE) of cocoa beans at 24, 48, 96 and 120 h fermentation.

3.3. Determination of DPPH free radical scavenging activity

In this study, we also examined the effect of yeast starter culture towards the DPPH free radical scavenging activity of cocoa beans. The current study showed that the DPPH free radical scavenging activity ranging from 113.85 ± 15.73 to 328 ± 20.04 µmol/g Trolox (TE) after 120 h fermentation. There was no change of DPPH free radical scavenging activity after 120 h fermentation. Nevertheless, the present research showed that all cocoa beans inoculated with yeast starter cultures except for *P. kudriavzevii* isolate 33 had lower DPPH free radical scavenging activity (at $p \leq 0.05$) than control at each fermentation hour (Fig. 3).

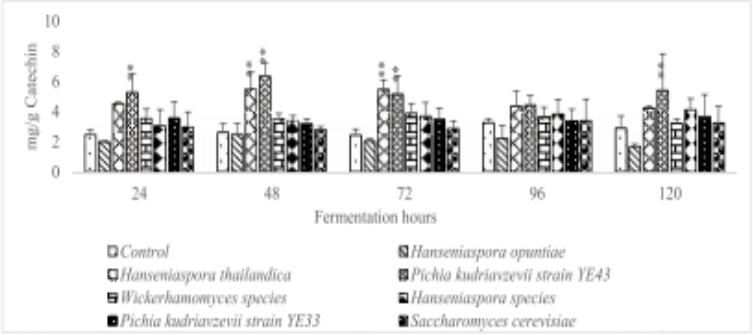
The current DPPH free radical scavenging activity of cocoa beans was lower compared to literature. Former literature showed that the DPPH free radical scavenging activity of cocoa beans was recorded at 323.8–1307.07 µmol/g TE dry weight (Oracz & Nebesny, 2016). A study carried out by (Batista, Ramos, Dias, Pinheiro, & Schwan, 2016) had also found that there was an increment of DPPH free radical scavenging activity during fermentation which was contrary to the finding of the present study. The difference in findings could be related to the used of cocoa variety. The DPPH free radical scavenging activity of cocoa beans is also highly dependent on the composition of cocoa cultivars used. Various clones are known to possess different level of polyphenols and antioxidants. Furthermore, the type of growing conditions in growing the cocoa fruit, harvesting time and drying condition applied to the cocoa beans would also contribute to the variation in antioxidant capacity (Batista et al., 2016; Oracz & Nebesny, 2016).

Moreover, the species of yeast involved in fermentation is known to affect antioxidant concentration and activity in table olive, green coffee, cocoa bean and wine (Sharma, Singh, & Sawant, 2012; Batista et al., 2016; Haile & Kang, 2019; D'Antuono et al., 2018). In a similar study where local, commercial and wild yeast strains were used as a starter culture in wine fermentation, the yeast strains were found to influence the parameters of the wine produced, including antioxidant activity (Sharma et al., 2012). In the present study, it was found that apart from cocoa beans inoculated *P. kudriavzevii*, a lower DPPH free radical scavenging activity was recorded for all other groups. In cocoa fermentation, enzymatic hydrolysis reaction causes polyphenols within the beans to be prone towards polymerization and the formation complexes with proteins, which in turn decreases the solubility of the polyphenols and therefore their antioxidant activity (Misnawi, 2008). Enzymatic hydrolysis in fermentation is a microbial directed process, and it is thus suggested that the species of yeast involved could have influenced this process, and therefore produced the low DPPH free radical scavenging activity figures (Misnawi, 2008). However, the specific reasons why *P. kudriavzevii* did not show the same behaviour as other yeast starter cultures remains unclear and could be the subject for further study.

3.4. Total flavonoid content (TFC)

The TFC of cocoa beans found in the present study ranging from 1.68 to 6.33 mg/g Catechin (Fig. 4). There was no significant change ($p \leq 0.05$) in TFC of cocoa beans for all the fermentations after 120 h fermentation. Overall, the TFC of cocoa beans inoculated with *P. kudriavzevii* isolate 43 was significantly higher ($p \leq 0.05$) than control except at 96 h fermentation (Fig. 4). The TFC of cocoa beans inoculated with *H. thailandica* was also significantly higher ($p \leq 0.05$) than control at 48 and 72 h fermentation.

The range of TFC recorded in the present study was supported by



(**) indicates significant higher TFC than control at p≤ 0.05 at every fixed 24 h interval until 120 h based on two-way ANOVA, Tukey test (p≤0.05) between the treated fermentation. Bars indicated standard error of mean (±SEM)

Fig. 4. Comparison of total flavonoid content (mg/g Catechin) of cocoa beans at 24, 48, 96 and 120 h fermentation.

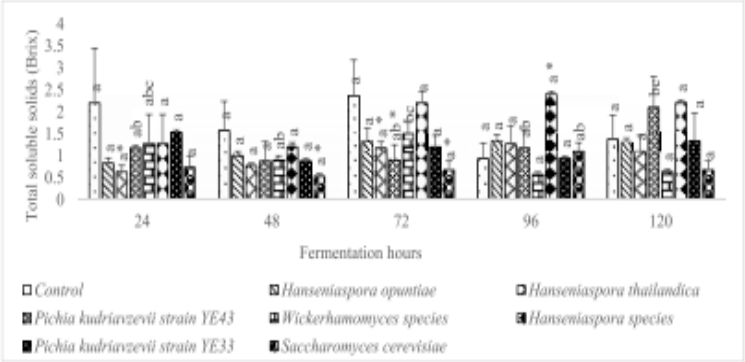
previous literatures. It was reported that the TFC was 8.33 mg/g epicatechin of raw cocoa powder and 3.50 mg/g to 12.62 mg/g epicatechin of dried cocoa bean (Zzaman & Yang, 2013; Fenglin et al., 2013). Based on a similar research carried out by Haile and Kang (2019), it was stated that there was no significant change ($p \leq 0.05$) in TFC of coffee bean produced from control and those coffee beans inoculated with yeast between different fermentation hours. Nonetheless, previous literature showed that the TFC was improved in fermented coffee added with yeast at 24 h fermentation (Haile & Kang, 2019). Yeast was claimed to be effective in increasing the number of flavonoids in coffee extracts during coffee fermentation (Haile & Kang, 2019; Kwak, Jeong, & Kim, 2018). This was in line with the present finding where cocoa beans inoculated with yeast as a starter culture produced high flavonoids content as compared to control. The increment of flavonoids content might be due to conversion of insoluble phenolic compounds into soluble flavonoids during fermentation (Haile & Kang, 2019; Kwak et al., 2018). Therefore, we suggested that both *P. kudriavzevii* and *H. thailandica* improve the conversion of insoluble phenolic compounds into soluble flavonoids which eventually produced high flavonoids cocoa beans.

3.5. Total soluble solids

During the five days fermentation, the total soluble solids varied from 0.53 to 2.4 Brix. There was no changes in total soluble solids of cocoa beans for all fermentations after 120 h (Fig. 5). The low total soluble solids of cocoa beans compared to control ($p \leq 0.05$) were observed at several fermentations specifically: fermentation added with *H. thailandica* at 24 and 72 h; fermentation added with *S. cerevisiae* at 48 and 72 h; and fermentation added with *P. kudriavzevii* isolate 43 at 72 h. On the contrary, the total soluble solids of cocoa beans were higher than control (at $p \leq 0.05$) in fermentation added *Hanseniaspora* species at 96 h.

The range of total soluble solids of cocoa beans recorded in the current study was lower than literature. Previous literature reported that the total soluble solids of cocoa beans added with yeast starter cultures was 0.57–4.37 Brix (Ooi et al. (2016)). The difference of total soluble solids of cocoa beans measured could be influenced by the losses of cocoa sweating during fermentation. During fermentation, microorganisms would secrete pectolytic enzymes to break down mucilage pulp forming by-product such as cocoa bean sweating. Cocoa sweating contains high concentrations of sugar, pectin and organic acids (Kong, Noraniza, Roselina, Yaya, & Hasanah, 2018). The loss of cocoa sweating could contribute to the lower value of total soluble solids measured in present study.

Furthermore, Moreira et al. (2017) also stated that the microbial inoculation fastened the sugar consumption in the first 24 h fermentation, which is in line with the current finding where cocoa beans fermented with selected yeasts showed lower total soluble solid contents than control. Moreover, total soluble solids of cocoa beans would also decrease as the fermentation prolonged due to loss of citrate acid and metabolism of pulp by yeast (Ho, Zhao, & Fleet, 2015). Different microbial composition was reported to affect the cocoa bean fermentation, where pulp was less metabolized during the fermentation with the addition of only acetic acid bacteria. It was also found that fermentation with *Saccharomyces cerevisiae* and *Lactobacillus plantarum*



(*) Indicates significant difference of TSS as compared to control at p≤ 0.05 at every fixed 24 h interval until 120 h. Small letter (a) in the figure indicates comparison within the same species where different small letter indicates significant difference (p≤0.05) based on two-way ANOVA, Tukey test (p≤0.05). Bars indicated standard error of mean (±SEM)

Fig. 5. Comparison of total soluble solids content (Brix) of cocoa beans at 24, 48, 96 and 120 h fermentation.

Table 2
Comparison of fermentation index of cocoa beans at 24, 48, 96 and 120 h fermentation.

Hrs	Control	H.opuntiae	H. thailandica	*P. kudriavzevii* strain YE43	*Wickerhamomyces* species	*Hanseniaspora* species	*P. kudriav-zevii* strain YE33	*S. cerevisiae*
0	1.69 ± 0.35abA	1.08 ± 0.27abA	0.89 ± 0.14aAB	1.13 ± 0.41abA	1.63 ± 0.33abB	0.94 ± 0.06abA	1.35 ± 0.39abA	1.16 ± 0.68abA
24	1.14 ± 0.07 aA	1.8 ± 0.84 aA	1.03 ± 0.19aAB	1.05 ± 0.19 aA	0.89 ± 0.27 aA	1.55 ± 0.38 aA	1.06 ± 0.09 aA	1.12 ± 0.44 aA
48	1.3 ± 0.12abA	1.35 ± 0.13bA	0.72 ± 0.01 aA	1.11 ± 0.36abA	1.05 ± 0.14abAB	1.25 ± 0.22abA	1.47 ± 0.23bA	1.11 ± 0.26abA
72	1.29 ± 0.18 aA	0.98 ± 0.22 aA	0.98 ± 0.12aAB	1.28 ± 0.21 aA	0.78 ± 0.08 aA	1.15 ± 0.30 aA	1.51 ± 0.54 aA	1.28 ± 0.48 aA
96	1.19 ± 0.31 aA	1.14 ± 0.09 aA	1.07 ± 0.10 aB	1.41 ± 0.17 aA	1.31 ± 0.16aAB	1.38 ± 0.13 aA	1.23 ± 0.35 aA	1.23 ± 0.69 aA
120	1.74 ± 0.47 aA	1.17 ± 0.30 aA	0.98 ± 0.14aAB	1.57 ± 0.36 aA	1.29 ± 0.33aAB	1.13 ± 0.06 aA	1.4 ± 0.43 aA	1.11 ± 0.31 aA

Capital letter (A) in the table indicates comparison within the same species where different capital letter indicates significant difference ($p \leq 0.05$) at different fermentation hours; small letter (a) indicating comparison for different yeast treated fermentations, different small letter indicating significant difference based on two-way ANOVA, Tukey test ($p \leq 0.05$) between the treated fermentation. Bars indicated standard error of mean (± SEM).

had significant ($p \leq 0.05$) lower sugar content while the addition of mixed microorganisms (yeast, lactic acid bacteria and acetic acid bacteria) in fermentation improved the pulp degradation (Kresnowati & Febriami, 2015). This simulation finding suggests that the addition of selected yeast species (*H. thailandica, P. kudriavzevii, H. opuntiae, W.* species and *S. cerevisiae)* aids in the breakdown of total soluble solids more efficiently.

On the other hand, the high total soluble solids of cocoa beans observed in fermentation added with *Hanseniaspora* species after 4 days fermentation could be due to the increment in releasing soluble solids such as glucose and fructose from the cocoa fruit pulp (Kong et al., 2018). Some yeast is capable in producing invertase enzyme which hydrolyses sucrose into glucose and fructose (Kong et al., 2018). It is postulated that *Hanseniaspora* species could be a potential species in producing such invertase enzyme during fermentation, leading to a higher total soluble solids content of cocoa beans produced.

3.6. Fermentation index

Fermentation index measures the degree of fermentation based on the brownness formed in the cocoa beans. Over-fermented bean has fermentation index of 1.6 while under-fermented beans has fermentation index of less than 1.0. It indicates the completeness of the fermentation (Nsor-Atindana, Zhong, & Mothibe, 2012). Generally, there was no significant difference in fermentation index of cocoa beans fermented with and without yeast starter culture (Table 2). All cocoa beans were well fermented after 96 h fermentation. However, cocoa beans produced from control was over-fermented (>1.6) at 120 h (Table 2). Beans treated with *H. thailandica* were under-fermented at 0, 48, 72 and 120 h whereas beans treated with *W.* species were under-fermented at 24 and 72 h (Table 2).

All cocoa beans were well fermented after 96 h fermentation which were consistent with previous literature reported by Pereira et al. (2017). According to Ho, Fleet, and Zhao (2018), fermentation added with selected yeasts produced dried beans that were fully fermented. This is consistent with the present study where fully fermented beans were obtained from fermentation added with selected yeasts. The degree of fermentation in cocoa beans is due to the diffusion of polyphenols during fermentation, followed by oxidation and reduction with other cellular compounds, which then turning into brown colour in cocoa beans (Hernandez, Lopez-Andrade, Ramirez-Guillermo, Ramirez, & Perez, 2016; Kresnowati & Febriami, 2015). Polyphenol oxidase involved in oxidation reaction by catalysing o-diphenol to o-quinone leading to the brown colour formation in cocoa beans (Hernandez et al., 2016). The polyphenol oxidase works best at 42–45 °C which usually achieves on day three of fermentation (Caligiani, Cirlini, Palla, Ravaglia, & Arlorio, 2007). This further corresponded to the formation of well-fermented beans which obtained after 72 h fermentation. Subsequently, over-fermented beans which detected in control after 120 h fermentation were undesirable as it would lead to off-flavours. If the fermentation continues for a long duration, growth of unwanted molds and bacteria can generate off-flavours (Caligiani et al., 2007). Moreover, unfermented cocoa beans are also suggested not desirable as they have dark grey colour and more astringent (Caligiani et al., 2007). Both over-fermented and under-fermented beans would give rise to a loss of flavour in the final product after subsequent cocoa processing. Based on our finding, it is suggested to perform yeast fermentation up to 96 h in order to avoid over-fermentation as well as the production of unfermented beans.

4. Conclusion

Overall, the present study provides insights of the antioxidant activities of the cocoa beans produced after the addition of selected yeast starter culture. The current findings showed that the application of selected yeast starter culture in cocoa fermentation produces cocoa beans with higher total polyphenols and flavonoid content compared to control at particular fermentation periods. Certain yeast fermentation of cocoa beans causes bound phenolic compounds to be released which results in higher phenolic content of cocoa seeds. We observed that with the addition of selected yeast starter culture (*H. thailandica, P. kudriavzevii, H. opuntiae, W.* species and *S. cerevisiae)*, the fermentation process proceeded efficiently in sugar metabolism. These findings suggested that *P. kudriavzevii* (MH979681) and *H. thailandica* (MH979675) are potential yeast species in modulating the antioxidant activities of the dried cocoa beans. Future study is needed to assess the effect of using these two yeast starter cultures in field condition, on the antioxidant activities of cocoa beans, sensory and antioxidant properties of resultant chocolate.

Author contributions section

Ooi Teng Sin: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Visualization, Writing-Original draft preparation, **Adeline Ting Su Yien:** Review and Resources, **Siow Lee Fong:** Supervision, Writing- Reviewing and Editing, Resources and Funding acquisition.

Declaration of competing interest

All authors have seen and approved the final version of the manuscript being submitted. The article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere. The Authors have no conflict of interest.

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Appendix B

Table A1. Summary for morphological description of yeast isolates from a spontaneous cocoa fermentation

Number	**Yeast isolates**	**Descriptions**			
		Colour	**Shape**	**Margin**	**Elevation**
1.	Yeast isolate 1	White	Circular	Entire	Convex
2.	Yeast isolate 2	White	Irregular	Undulate	Flat
3.	Yeast isolate 3	White	Circular	Entire	Raised
4.	Yeast isolate 4	White	Circular	Entire	Convex
5.	Yeast isolate 5	White	Irregular	Lobate	Flat
6.	Yeast isolate 6	White	Irregular	Undulate	Raised
7.	Yeast isolate 7	White	Circular	Entire	Raised
8.	Yeast isolate 8	White	Irregular	Undulate	Umbonate
9.	Yeast isolate 9	White	Irregular	Filiform	Raised
10.	Yeast isolate 10	White	Circular	Entire	Raised
11.	Yeast isolate 11	White	Irregular	Filiform	Flat
12.	Yeast isolate 12	Brownish white	Circular	Entire	Umbonate
13.	Yeast isolate 13	White	Circular	Entire	Raised