The effects of fermentation and roasting of Hass avocado seeds on colour and antioxidant activities

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Abstract

Nowadays, the food industry has paid more attention to using food by-products for conversion to value-added products. Nearly 1000 tons of avocado wastes including avocado peels, avocado seeds, wastewater, and the pulp are generated in the production of avocado oil in New Zealand each year (Anuja Nadkarn, 2017). These wastes are ideal raw materials for food use as they may have high nutritional value and be beneficial for health. Avocado by-products are utilized as food ingredients may contribute to solving the serious environmental problems caused by the disposal of processing wastes.

In this study, we wanted to investigate the influence of fermentation and roasting on colour, total phenolic content (TPC) and antioxidant capacity of New Zealand *'Hass'* cultivar avocado seeds. Avocado seeds were subjected to natural fermentation and inoculated fermentation with either *Lactobacillus plantarum* or kefir. These fermented samples were evaluated in terms of visual plate count values of lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeasts. The number of microorganisms increased significantly ($p<0.001$) over 7 days of fermentation in all samples. The colony forming units (cfu) of LAB, AAB, and yeast significantly ($p<0.001$) increased in the first five days. A statistically significant difference was observed between the day5 and day7 counts of LAB and yeast, but this increase was no more than 1 log cfu after 5 days indicating that growth had reached its stationary phase. Although TPC and antioxidant capacity significantly ($P<0.05$) decreased after fermentation of avocado seeds, fermentation with *Lactobacillus plantarum* led to significantly ($P<0.05$) higher TPC and antioxidant activities compared to kefir and naturally fermented samples.

The dried fermented avocado seeds in all three fermentation conditions (*L. plantarum,* kefir and natural fermentation) were further subjected to roasting. Roasting time and temperature were found to significantly ($p < 0.05$) affect the colour (L^*, a^* and b^* value), TPC, and antioxidant capacities. $L^*$and b^* values of roasted avocado seeds significantly ($P < 0.001$) declined with the increase of roasting temperature and time. Roasting also significantly ($P < 0.05$) increased TPC with longer roasting time. In terms of antioxidant activity, only the value of FRAP assay significantly ($P < 0.001$) rise with increasing roasting temperature and time. In addition, roasted samples fermented by *L. plantarum* represented significantly ($P < 0.001$) higher TPC and antioxidant capacity (using both CUPRAC and FRAP assays) compared to kefir and naturally fermented samples. This study demonstrated that fermentation of avocado seeds in combination with roasting could yield a powder with high TPC and good antioxidant activity, which can be potentially be incorporated in food as ingredients to enhance nutrition and confer value-added benefits.

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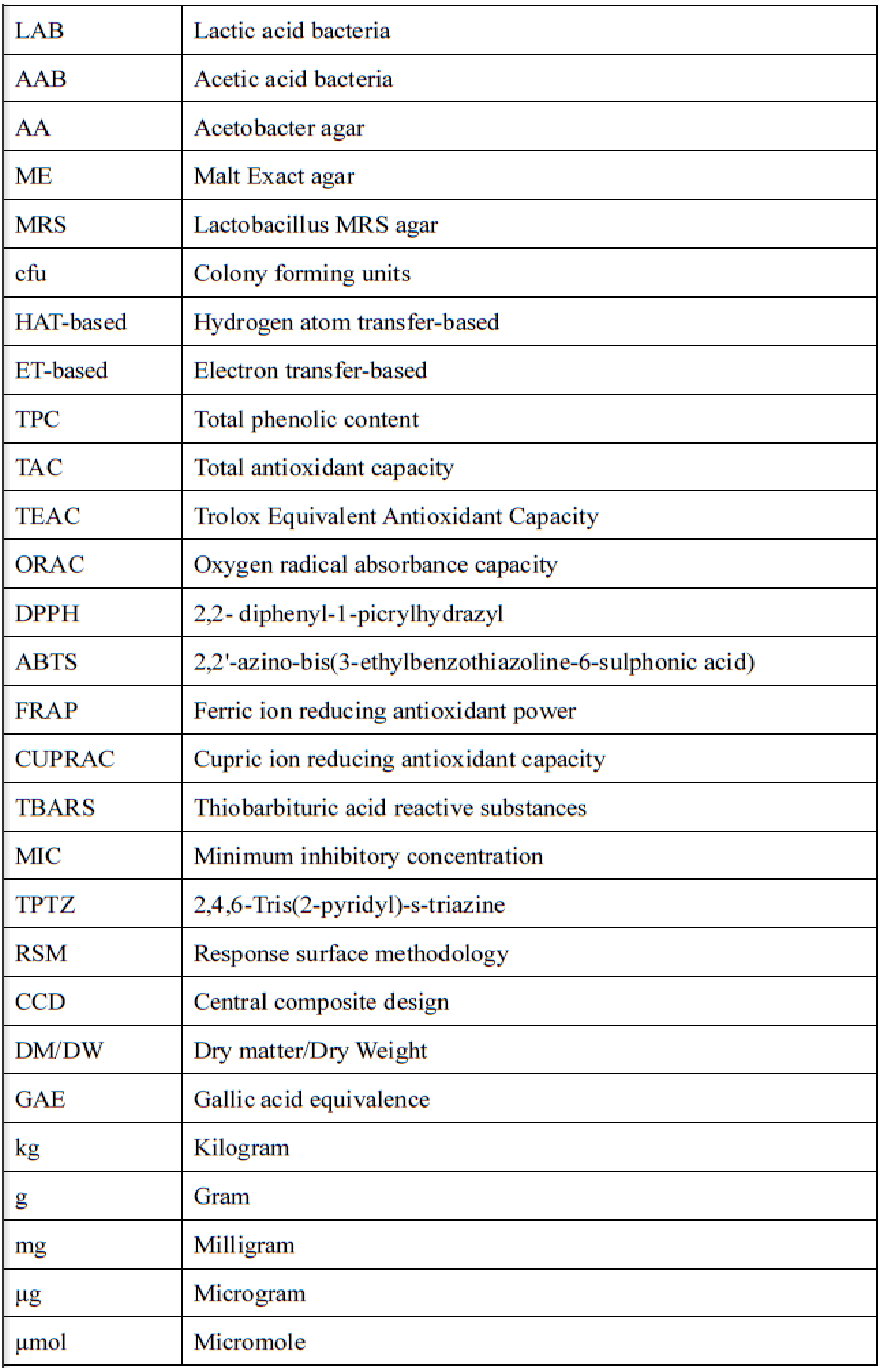
Abbreviation



LAB	Lactic acid bacteria
AAB	Acetic acid bacteria
AA	Acetobacter agar
ME	Malt Exact agar
MRS	Lactobacillus MRS agar
cfu	Colony forming units
HAT-based	Hydrogen atom transfer-based
ET-based	Electron transfer-based
TPC	Total phenolic content
TAC	Total antioxidant capacity
TEAC	Trolox Equivalent Antioxidant Capacity
ORAC	Oxygen radical absorbance capacity
DPPH	2,2- diphenyl-1-picrylhydrazyl
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
FRAP	Ferric ion reducing antioxidant power
CUPRAC	Cupric ion reducing antioxidant capacity
TBARS	Thiobarbituric acid reactive substances
MIC	Minimum inhibitory concentration
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
RSM	Response surface methodology
CCD	Central composite design
DM/DW	Dry matter/Dry Weight
GAE	Gallic acid equivalence
kg	Kilogram
g	Gram
mg	Milligram
µg	Microgram
µmol	Micromole

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1. Introduction

There is a significant increasing global trend to utilize natural resources, especially plant by-products produced in food industries. Once the edible fraction fruit like pulp is separated, the skin, peel, and seeds become by-products of food processing. Apple pomace (25–30% of total weight), banana peels (approximately 30% of fruit weight), orange peels (50–65% of total weight), and plum pomaces are the major waste products of global food manufacturing (*Kristl et al., 2011*). The polyphenols in apple pomace can vary with different extraction methods (*Ajila, et al. 2011*). *González-Montelongo et al. (2010)* found that banana peels were rich in phytochemical compounds with high antioxidant capacities, including polyphenols, and anthocyanin compounds. In addition, plum pomace was characterized by high polyphenol and flavanol contents (*Dulf, et al. 2016*), and orange peels were reported by *Hernandez, et al. (2016)* that it contained polyphenols and flavonoids. As all these plant by-products are rich sources of minerals, vitamins, as well as bioactive compounds, they are potential sources of phytonutrients.

Avocado is an important tropical fruit that is highly nutritious and benefits for health, which was characterized by abundant minerals, polyphenols and antioxidant ability (Araújo, et al. 2018). Ranade & Thiagarajan, (2015) stated that avocado pulp was used in avocado oil production due to its high lipid content. Avocado oil made of avocado pulp contains high unsaturated fatty acids content and abundant fat-soluble nutrients including β-carotene, vitamin E(alpha-tocopherol) (1.97mg/100g), vitamin K (phylloquinone) (21μg/100g), vitamins B-6 (0.29mg/100g), retinol, ascorbic acid, and thiamine (*Dreher & Davenport, 2013*), which were of importance for human health and can help the body maintain its antioxidant properties. For example, vitamin C (8.8mg/100g) can be utilized by the body to produce vitamin E and helped to reduce the oxidation of low-density lipoprotein cholesterol. Also, there was a 35% higher level of potassium (507mg/100g) in the avocado than a banana (358 mg/100g). In addition, the low content of sodium (8mg/100g) and the high content of potassium in avocado is beneficial for people who have cardiovascular diseases *(Maitera, et al. 2014)*.

Although avocado fruit is well known for its content of lipids and has been widely used to manufacture avocado oil, few studies have focused on the nutritional benefits of avocado seeds. Araújo, et al. (2018) collected avocado seeds from different cultivars and countries and found that seeds contained 1.87-9.44% moisture, 4.05- 6.7% lipid, 3.09 - 9.63% protein, 2.73-4.3% ash, and 2.19-4.24% fibre. According to *Wang, et al. (2010),* the avocado seeds had significantly more antioxidant capacities than pulp and peels. Seeds, in fact, accounted for 57% of the antioxidant capacity of the whole fruit. Also, phenolic compounds and antioxidant activities varied with different avocado cultivars. The dominant cultivar of avocado in New Zealand is *'Hass'* avocado, accounting for 90% production. *Araújo, et al. (2018)* stated that *'Hass'* avocado seeds had a higher TPC than *'Spain'* avocado. Similarly, *'Hass'* avocado seeds contained higher TPC and antioxidant components than other cultivars, according to *Wang, et al. (2010).*

The use of different extraction methods and antioxidant assays may result in different results. According to *Rodríguez-Carpena, et al. (2011b)*, the *'Hass'* pulp and *'Fuerte'* pulp extracted by 70% acetone had a higher TPC than 70% methanol and ethyl acetate extractions. Additionally, the results of antioxidant capacity may vary from antioxidant assays with different mechanisms as well as the influence of substrates and solvent system on determination methods (*Çelik, et al., 2010*). The mechanism of HAT-based assays like ORAC assays was based on the competition of peroxyl radicals between antioxidants and substrates during thermal processing (*Apak, et al. 2008*). While the mechanism of ET-based assays like CUPRAC and FRAP assays was to measure the antioxidant capacity by colour changes generated from the reduction of antioxidants.

The FRAP and CUPRAC assays used in this study have been reported by previous studies that they were influenced by the difference of determination substances. *Bean, et al. (2009)* showed that the FRAP assay had higher sensitivity to ascorbic acid than the CUPRAC assay. *Çelik, et al. (2010)* also reported that the antioxidant capacity of different antioxidant compounds (quercetin, ferulic acid and catechin) exhibited significantly different results in the ethanol and methanol extracts between using the CUPRAC assay and the FRAP assay. *Bean, et al.* (*2009*) further stated that this may be due to the chemical reaction that occurred at lower pH (pH 3.6) in FRAP assay may lead to the inhibition of reducing capacity. Furthermore, lipophilic antioxidants can only be detected by using the CUPRAC assay rather than the FRAP method, which accounted for differences between these two assays. Thus, it is important to select the right assay for valid evaluation of antioxidant properties due to the different antioxidant structures and properties (e.g. hydrophilic and lipophilic antioxidants), the solvent system (e.g. pH value or extraction methods) and different sensitivities (e.g. CUPRAC and FRAP assay).

Fermentation with microorganisms like LAB, AAB, and fungi can improve bioactive compounds in foods significantly. Previous studies have shown that polyphenol content in plum fruit significantly increased by 44.16% after fermentation with *R. oligosporus*, and increase by 35.32% with *A. niger* (*Dulf, et al. 2016*). *Rui, et al. (2017)* found that the TPC in fermented soy seeds showed a significant increase after 17.5h fermentation, compared to unfermented ones. A similar trend was found in the fermented apple pomace that the TPC increased significantly after fermentation *(Ajila, et al. 2011*). Sandhu, et al. (2016) found that TPC in all fermented wheat cultivars was significantly higher than corresponding unfermented samples. Additionally, *Zhang, et al. (2012)* also reported that total flavonoid and free phenolic acid content were significantly enhanced in fermented wheat, with a total polyphenolic content ranging from 29.37 to 54.16 and from 32.79 to 66.37 mg/g, respectively.

Roasting has been proved that it has an important effect on physiochemical properties of seeds and beans. Roasting generates volatile compounds from plant seeds (e.g. cocoa beans and coffee beans) that contribute to flavour. According to *Açar, et al. (2009)*, the effect of roasting on the total antioxidant capacity (TAC) value was associated with the decline of antioxidant capacity by degrading heat-labile constituents, or the increase of antioxidant activity by producing new antioxidant compounds. The roasted fermented coffee bean had significantly higher levels of chlorogenic acid (ranging from 14.87 to 24.60 mg/g) and total acids after fermentation *(Bressani, et al.*

2018). Jan, et al. (2019) stated that the TPC and reducing power of kalonji seeds significantly improved by pan and microwave roasting. Similarly, TPC and antioxidant activity of almond seeds significantly increase from 7.5 to 19.21 mg GAE/g after roasting (*Lin, et al. 2016)*. In the study of *Zieliński, et al. (2019)*, roasting also significantly increased the antioxidant capacity of buckwheat fermented with *L. plantarum W42* from 7.9 to 11.01 µmol Trolox/g in FRAP assay.

In contrast, *Açar, et al. (2009)* found that when roasting time was less than 30mins, the antioxidant activities of hazelnut, peanut and sunflower seeds decreased. After that, the antioxidant activities tended to rise significantly and reached the maximum value with increasing roasting time to 60 min. The initial decrease was probably due to the reduction of antioxidant compounds while the increase was observed in longer roasting time may be attributed to the generation of antioxidant products during Maillard reaction, especially in the rich-starch materials. However, the findings might have been more convincing if the author had mentioned the extraction and detecting assays of bioactive compounds. The solubility and sensitivity to solvents of antioxidant compounds made it complex to compare antioxidant capacities.

Antioxidant activities of plant by-products have gained more attention since they benefit to human health. The studies summarized above have shown that fermentation and roasting significantly improved the antioxidant activities of seeds and nuts. Hence the aim of this study was to determine the influence of fermentation on avocado seeds using food-grade microorganisms *(Lactobacillus plantarum* and kefir). Although roasting can improve antioxidant activities, results may vary with roasting temperature and time. Therefore, this study also investigated the effects of roasting time and temperature of fermented avocado seeds using the Central Composite Design (CCD) on colour and antioxidant activities.

2. Literature review

2.1 Agricultural wastes and by-products

In the case of agro-based food industries, *Kristl et al. (2011)* stated that once the edible fraction like pulp was separated, the skins, peels, and seeds left were by-products of food processing. The improper disposal of these agro-wastes can lead to environmental pollution (*Dhillon, et al. 2013)* and consume a large number of resources (*Jayathilakan, et al. 2012*). Therefore, food bio-products and agro-industrial wastes have caught global attention as potential renewable feedstocks. The food industry also aims to recycle this biomass to produce different high-value products.

2.1.1 Nutritional composition of plant by-products

Seeds, peels, pomace are the most common wastes generated while producing products like juice, jelly, oil, and beverage. These plant by-products are characterized by abundant minerals, polyphenols, and dietary fibre. *Dhillon, et al. (2013)* demonstrated that apple pomace contained abundant minerals, carbohydrates, and fibres, which has a low caloric value. In addition, apple pomaces were reported to be an ideal substrate for microorganisms to produce organic acids (*Ajila, et al. 2011*). *Achak, et al. (2009)* also reported that banana peels were highly nutritious, containing 1.33% Na, 1.05% Al, 3.22% Cl and 14.86% K. The high level of potassium made it a potential dietary supplement.

Extraction time and temperature can influence the nutrient composition of these by-products According to the study of *Hernández ‑ Carranza, et al. (2016)*, vitamin C content in orange peels significantly decreased by 20.6% of the original content when extraction time and temperature increased. Vitamin C was the highest when extracted at 20°C for 0.5h. In apple pomace, extraction in a high temperature-short time model promoted significantly increased vitamin C concentration and extraction at 60°C for 1h was found to be the best extraction condition. Vitamin C values were very low and not detectable in the banana peel.

2.1.2 Biochemical properties of plant by-products

The benefits of plant by-products include abundant flavonoids and polyphenols (*Hernández, et al. 2016; Dulf, et al. 2016*), and improvement of antioxidant capacity using ABTS (*González-Montelongo et al. 2010*), DPPH (*Ajila, et al. 2011*) and FRAP (*Hernández - Carranza, et al. 2016*) assays.

Plant by-products have been characterized by polyphenols and antioxidants. These bioactive compounds vary with plant types, solvents used for extraction and method of extractions. *Ajila, et al. (2011)* indicated that acetone extract (80%) of apple pomace resulted in a significantly higher DPPH assay result compared to 80% ethanol and 80% methanol extracts with ultrasonic-assisted extraction. *González-Montelongo et al. (2010)* further reported that 50% acetone extraction had the highest extraction efficiency for polyphenols in banana peels, compared to methanol, ethanol, and acetone extractions. *M'hiri, et al. (2015)* demonstrated that the TPC in orange peels obtained by supercritical CO_2 extraction and high-pressure extraction were significantly lower than conventional solvent extraction.

2.1.3 Utilization of plant by-products

Plant by-products contain abundant bioactive compounds and antioxidants, which has the probability to be utilized to produce value-added products (*Dhillon, et al. 2013; M'hiri, et al. 2015*) or can be used to adsorb chemicals (*Achak, et al. 2009*).

Apple pomace contained polyphenolics and were regarded as natural antioxidants (*Dhillon, et al. 2013*). *Ajila, et al. (2011)* found that TPC in fermented apple pomace was significantly higher than unfermented ones, which indicated potential applications as an antioxidant or antimicrobial. Plum kernels also contained a high proportion of unsaturated fatty acids that had benefit for cardiovascular health. *Dulf, et al. (2016)* fermented plum kernel with *R. oligosporus* for 6 days and found that polyphenol content significantly increased compared to unfermented sample. Fermentation of by-products will degrade the conjugation of most phenolic compounds linked to sugar moieties, organic acids, amines, and lipids, in order to increase their ability to function as good antioxidants. Moreover, orange peels were rich in bioactive compounds and has been used as a source of molasses and pectin in the food industry (*M'hiri, et al. 2015*). Additionally, *Achak* and other researchers *(2009)* have used banana peels as a bio-sorbent to remove chemicals in industrial wastewater (olive mill waste waters) due to its high adsorption capacity of phenolic compounds.

In conclusion, these plant by-products are beneficial as they contain flavonoids and phenolic compounds with good antioxidant capacities. Hence proper utilization of these food by-products for conversion to value-added products is fast becoming an important priority in the food industry.

2.2 Avocado pulp and avocado seed

2.2.1 Nutritional composition of avocado

Avocado is an important highly nutritious tropical fruit *(Vinha, et al. 2013, Araújo, et al 2018*) and health benefits (*Maitera, et al. 2014*), which are essentially due to its high levels of minerals (*Duarte, et al. 2016*), vitamins (*Dreher & Davenport, 2013*), and unsaturated lipid content. Avocado pulp had a significantly higher water content (70.83%) and fat content (43.5%) compared to avocado peels and seeds (*Vinha, et al. 2013*). *Dreher & Davenport, (2013)* reported that abundant vitamins were in avocado such as β-carotene, vitamins C, vitamin E (alpha-tocopherol), vitamin K (phylloquinone), vitamins B-6, retinol, ascorbic acid, thiamine. The major contribution of vitamins C and vitamins E was to benefit human health and help the body maintain antioxidant properties. For example, vitamin C (8.8mg/100g) recycled vitamin E and helped to reduce the oxidation of low-density lipoprotein cholesterol. There was also a significantly higher level of potassium (507mg/100g) in an avocado than a banana (358 mg/100g), as well as low content of sodium (8mg/100g) in avocado, which contributed to avocado consumption beneficial for cardiovascular health (*Maitera, et al. 2014*). Moreover, it has been reported by Araújo, et al. (2018) avocado seeds were regarded as an alternative starch source since they contained around 30% proportion in a whole avocado.

2.2.2 Avocado oil

In the New Zealand market, *'Hass'* is the most common avocado cultivar, accounting for nearly 95% of the local plantation. And the avocado oil production in New Zealand comprises a huge amount of global production. One of avocado oil factories, 'Olivado' manufactures around 150 metric tons of avocado oil annually, and 170000 litres of them are produced in New Zealand (*The 'Olivado' avocado oil factory, 2017*).

Ranade & Thiagarajan, (2015) stated that avocado pulp was used in avocado oil production due its high lipid content. According to *Duarte, et al. (2016)*, avocado oil made of avocado pulp consisted of fat-soluble nutrients and higher proportion of unsaturated fatty acids than other vegetable oils. These fatty acids contributed to promoting health benefits and made phytochemicals in avocado bioavailable.

2.2.3 Avocado waste

Once the avocado pulp is removed in the production of avocado oil, the peels and seeds are left as currently useless agricultural waste *(Kosińska, et al. 2012)*. It was reported that each 250ml bottle of avocado oil is obtained from 20 avocado fruits, weighing 130g each *(The 'Olivado' avocado oil factory, 2017)*. Nearly 1000 tons of avocado wastes including avocado peels, avocado seeds, wastewater, and the pulp are generated in the production of avocado oil each year (*Anuja Nadkarn, 2017*). Besides that, people in New Zealand consume nearly 25000 tons of avocados, which further generates 7500 tons of avocado wastes (16% seed and 16% peel) (*NZ Avocado industry Ltd. 2018)*. Therefore, the efficient utilization of avocado by-products is becoming increasingly important. The avocado seeds accounted for 16% of the total avocado weight and had a rich polyphenol profile with potential bioactivities (*Dabas, et al. 2013; Vinha, et al. 2013*). And the avocado peels were also reported to have higher rich polyphenols (*Rodríguez-Carpena, et al. 2011b; Calderón-Oliver, et al. 2016*) and higher antioxidant capacities than the avocado pulp. (*Wang, et al. 2010; Kosińska, et al. 2012*). Thus, characterizing the nutrients of avocado wastes may produce value-added nutritional products (e.g. phenolic compounds and natural antioxidants), and this would contribute to the development of the processed-food industries, especially avocado industries.

The phytochemical composition of avocado waste products in previous studies are summarized in Table 2.1. *Vinha, et al. (2013)* stated that the Algarvian *'Hass'* avocado seeds had significantly higher proportion of total protein (2.19%) and organic acid content (2.67%), and the lowest ash (1.29%) and moisture content (54.45%) when compared to the peels and pulp. Also, the vitamin C content of Algarvian *'Hass'* avocado was significantly higher in peels and seeds than those in the pulp, which may be associated with the higher antioxidant capacity. Similarly, *Rodríguez-Carpena, et al. (2011a)* reported that Spain *'Hass'* avocado seeds had significantly the lowest ash content and highest protein content in comparison to peels and pulp.

Nutritional composition of avocado seed varies based on where it is obtained. The Spain *'Fuerte'* seed had significantly higher amounts of total protein (2.22%) and ash content (0.83%), and the lowest moisture content when compared to the peels and pulp (*Rodríguez-Carpena, et al. 2011a*). According to *Galvão, et al. (2014)*, all three Brazilian avocado cultivars had significantly higher amounts of proteins (3.6-5.3%) in seeds compared to the pulp (1.4-2.1%) and peels (3.6-3.9%). The *'Collinson'* cultivar had significantly higher protein content in seeds and peels than those of the other cultivars. Additionally, *'Fortuna'* avocado cultivar contained lower lipid, protein, and ash contents in seeds than those in the *'Collinson'*, and *'Barker'* cultivars. These significant differences between avocado seeds are normal since the composition of avocado during its development that is not only limited to cultivars but also the edaphoclimatic conditions of the region of avocado production, ripeness and storage conditions.

2.2.4 The biochemical properties of avocado peel and seed

Although avocado fruit is well known for its content of lipids and is widely used to manufacture avocado oil, few studies have focused on the biochemical properties of avocado seeds, such as TPC and antioxidant ability of avocado seeds (Table2.1).

Bioactive compounds

Both the peels and the seeds of avocado fruit are rich in phenolic compounds. Based on the comparison among eight cultivars of avocado, *'Hass'* avocado contained the significantly highest TPC in pulp and seeds than non-*'Hass'* cultivars (*Wang, et al. 2010*). In addition, seeds in all cultivars contained the significantly highest TPC, followed by peels, and pulp that had the lowest. Similarly, the study of *Vinha, et al.*

(2013) showed that the Algarvian *'Hass'* avocado seeds had significantly the highest levels of total polyphenols and flavonoids when compared to peels and pulp. Moreover, *Rodríguez-Carpena, et al. (2011a)* also reported that Spain *'Hass'* seeds had significantly higher polyphenols than pulp. With other avocado cultivars, similar findings can be observed that the seeds of *'Simmonds'* cultivar had the significantly highest TPC in the comparison to pulp for the avocados tested (*Wang, et al. 2010*).

Some studies have shown that the TPC of avocado peels are significantly higher than that in avocado seeds. *Calderón-Oliver, et al. (2016)* found that the TPC in Mexico avocado peels was significantly higher than seeds. In terms of flavonoids, Mexico avocado peel extracts had significantly higher content (10.9 mg QER/g) than the seeds. *Rodríguez-Carpena, et al. (2011b)* further demonstrated that *'Fuerte'* peels had the significantly highest TPC, followed by *'Hass'* peels whereas *'Hass'* seeds were the lowest. This was further confirmed by *Kosińska, et al. (2012)* who found that avocado peels in *'Shepard'* and *'Hass'* cultivars contained significantly higher TPC than seeds, while *'Hass'* avocado peels exhibited the significantly highest TPC than those in *'Shepard'* variety.

Antioxidant capacity

The DPPH (*Wang, et al. 2010*), ABTS (*Kosińska, et al. 2012*), FRAP (*Calderón-Oliver, et al. 2016)* and CUPRAC (*Rodríguez-Carpena, et al. 2011a*) assays are commonly used to determine antioxidant capacity. The bioactive compounds in avocado contribute to the high antioxidant capacity (*Vinha, et al. 2013*). *Wang, et al. (2010)* reported that the antioxidant capacity of avocado seeds accounted for 57% of a whole avocado while peels contributed to 38%. The author further stated that seeds had the significantly highest antioxidant capacity using the DPPH assay, followed by peels whereas pulp was the lowest in all cultivars tested. *Rodríguez-Carpena, et al. (2011a)* stated that seeds of both *'Hass'* and *'Fuerte'* had significantly higher antioxidant capacity than pulp.

However, avocado peels exhibited significantly higher antioxidant activity than seeds in some studies (*Calderón-Oliver, et al. 2016*). According to *Kosińska, et al. (2012)* avocado peels exhibited significantly higher antioxidant capacity than seeds in

Australian *'Hass'* and *'Shepard'* cultivars using the ABTS assay. FRAP results also showed that Mexico avocado peels exhibited significantly higher antioxidant capacity than seeds (*Calderón-Oliver, et al. 2016*).

2.2.5 Utilization of avocado by-products

Previous studies reported that the phytochemicals properties and antioxidant capacity of avocado seeds and peels make it useful as a natural antioxidant source (*Rodríguez-Carpena, et al. 2011b*) that can be used to protect some diabetic rat tissues (*Ezejiofor, et al. 2013*), and to inhibit the growth of some microorganisms (*Leite, et al. 2009*).

In accordance with the study of *Rodríguez-Carpena, et al. (2011b),* the addition of avocado byproduct extracts played an important role in the colour of raw pork patties. L* and b* values represented a less reduction in raw pork patties with avocado peel and seed extracts added while a* value increased significantly during 15 days of cold storage, compared to unadded ones. In addition, the *'Hass'* seeds and peels resulted in significantly higher a* value of raw pork patties than *'Fuerte'* variety (peels and seeds) and control. The results indicated that the discolouration of raw pork patties was inhibited by antioxidants in avocado by-product extracts during cold storage. Furthermore, the raw pork parties with avocado by-product extracts exhibited significantly lower TBARS values during chilled storage, which was due to inhibition of lipid oxidative reactions *(Rodríguez-Carpena, et al. 2011b*). In terms of protein oxidation, raw pork patties containing *'Hass'* avocado seed and peel extracts had significantly lower oxidation products of certain amino acids than the control at day 15, which meant that the addition of avocado by-product extracts inhibited the protein oxidation of raw pork patties (Rodríguez-Carpena, et al. 2011b). Therefore, avocado seeds and peels could be used as natural antioxidants due to their significant antioxidant capacity as they can efficiently inhibit lipid and protein oxidative reactions.

In 2013, *Ezejiofor* and colleagues reported that three weeks of daily, blood glucose levels of diabetic rats treated by oral avocado seed aqueous extracts was significantly reduced in comparison to diabetic control ones. During the 7 days of experimental period *(Ezejiofor, et al. 2013)*, diabetic control rats decreased the body weight, while a significant rise in body weight occurred in rats treated with avocado seed aqueous extracts. The administration of avocado seed aqueous extracts corrected the loss in body weight and significantly restored these levels towards normal. These results indicated that the aqueous avocado seed extracts presented the anti-diabetic properties and contributed to protect tissues like pancreas, kidneys, and liver. This may result from mineral elements and phytochemicals contained in the avocado seeds. Minerals played an important role in regulating key enzymes, which were important in the formation of glucose and diabetes can be managed by using existing glucose more effectively as well as reduced hyperglycaemia by increasing glucose metabolism.

Leite and others *(2009)* have found that hexane avocado seed extracts showed the significantly lowest MIC (Minimum inhibitory concentration) of yeast (*Candida sp., Cryptococcus neoformans, and Malassezia pachydermatis*), followed by methanol avocado seed extracts, whereas the control MIC was the highest. The antibacterial effect of both hexane and methanol avocado seed extracts inhibited yeast growing, with the significantly highest antifungal effects were found in the hexane extracts of the avocado seeds (*Cardoso, et al. 2017*). *Leite, et al. (2009)* further stated that various bioactive components (e.g. flavonoids, tannins) were found in avocado seed methanol and hexane extracts. Therefore, avocado seeds have been used to inhibit microorganisms growing that can improve food safety and prolong food shelf life.

Table 2.1 Nutritional composition and biochemical properties of avocado byproducts.

Avocado byproducts	**Extraction methods**	**Nutritional composition**	**Biochemical properties**	**References**
Avocado peels	1. Dried for 120 h in the ambient conditions of pressure and temperature (25 °C) 2. Oven-dried at 60 °C by 24 h; freeze dried for 24 h 3. Methanol extracts	Not reported	*Total phenolic compounds (mg GAE/100g DW)* Avocado dried peel had significantly the highest total phenolic compounds (1252.31 mg GAE 100 g−1 DW) compared to raw peel. *Total flavonoids (mg QE 100 g−1 DW)* There was no significant difference between fresh and dried peels. *FRAP assay (µmol $FeSO_4$/g)* Oven drying significantly concentrated avocado peel antioxidant activity. Avocado dried peel had significantly the highest antioxidant capacities using the FRAP assay (441.83 µmol $FeSO_4$/g), compared to raw avocado peel.	*Morais, et al. (2015)*
Avocado pulp, skin, and seed from	Not reported	*Nutrition composition* The protein content and lipid content in avocado pulp of the three cultivars, *'Fortuna' 'Collinson', and 'Barker'* were significantly	Not reported	*Galvão & Nigam (2014)*

'Fortuna' 'Collinson', and 'Barker' cultivars		different between the cultivars. In comparison to the pulp and peel, the seeds had significantly higher amounts of proteins. Seed and peel of the *'Collinson'* cultivar had significantly higher protein content than other cultivars. The lipid, protein, and ash contents in the seed of the fruits of the *'Fortuna'* cultivar were lower and significantly different than those of the *'Collinson', and 'Barker'* cultivars. The seed oil of *'Collinson'* cultivar contained the significantly the lowest (30.8% of total fatty acids) content of SFA, and significantly high concentrations of 9,12-octadecadienoic (23.9 to 29.4% of total fatty acids) and 9,12,15-octadecatrienoic (9.9 to 18.3% of total fatty acids) fatty acids.		
Mexico 'Hass' avocado pulp	Not reported	*Vitamins* Avocados were one of the few foods that contained significant levels of both vitamins C and E. *Minerals*	Not reported	*Dreher & Davenport. (2013)*

		Avocados were naturally very low in sodium and contained significantly high potassium. *Lipids* Avocado oil contained a monounsaturated fatty acids (MUFA)-rich fruit oil with 71% MUFA, 13% polyunsaturated fatty acids (PUFA), and 16% saturated fatty acids (SFA). *Sugars* Avocado contained significantly less sugar compared to other fruits and the primary sugar found in avocados is a unique seven-carbon sugar called D-mannoheptulose and its reduced form, perseitol.		
Florida avocado from different cultivars (*'Slimcado'*, *'Simmonds'*, *'Loretta'*, *'Choquette'*, *'Booth 7'*,	Vortex and sonication extraction with acetone/water/ acetic acid (70:29.7:0.3, v/v/v)	Not reported	*Total phenolic content (mg GAE /g)* For all cultivars, seeds contained the significantly highest total phenolic content whereas the pulp had the lowest. '*Hass'* avocado seed contained significantly higher phenolic content than all the non-Hass cultivars. Among the Florida avocados tested, *'Simmonds'* had the significantly highest phenolic content in the seeds.	*Wang, et al. (2010)*

'Booth 8', 'Tonnage') and Mexican *'Hass'* avocado			*Antioxidant capacity (ORAC and DPPH assay)* For all cultivars, seeds contained the significantly highest antioxidant capacities, followed by the peel, and pulp had the lowest. *'Hass'* avocado seeds contained significantly higher antioxidant capacities than all the non-Hass cultivars. Among the Florida avocados tested, *'Simmonds'* had the significantly higher phenolic content in the seeds and peels when compared to others. The antioxidant capacity determined by DPPH assay was about half of the values determined by the ORAC assay.	
Algarvian *'Hass'* avocado	Water extraction by homogenization at 40 °C	*Nutrients* Avocado pulp had a significantly higher water content (70.83%), fat content (43.5%) than avocado peel and seed. The seed had significantly higher amounts of total protein (2.19%) and lowest ash content. Vitamin C content was significantly higher in avocado peel and seed than in pulp. *Organic acid content* In the case of the mature Algarvian avocado,	*Total phenolics* The avocado seed exhibited the significantly highest levels of total phenolics when compared to peel and pulp. *Total flavonoids* The avocado seed exhibited the significantly highest levels of total flavonoids when compared to peel and pulp. *Carotenoids*	*Vinha, et al. (2013)*

		the seed has significantly higher acidity than the skin or pulp.	The skin of the fruit had the highest carotenoid content since this tissue is usually the fraction where these phytochemicals are concentrated.	
Brazilian avocado pulp	Enzyme-assisted aqueous extraction	*Nutrients* Avocado has four times more nutritional value than any other fruit except for banana, containing significant levels of fat-soluble vitamins. Avocado significantly stands out on potassium levels (339mg /100g) when compared to other fruits. In comparison to other vegetable oils, avocado oil is characterized by having significantly high levels of monounsaturated fatty acids (oleic and palmitoleic acids), low polyunsaturated fatty acids (linoleic acid), and relatively high levels of saturated fatty acid (palmitic and stearic acids).	*Phenolic compounds* Avocado contained substantial amounts of bioactive compounds such as phytosterols, especially in the lipid fraction, and the main phytosterol was β-sitosterol.	*Duarte, et al. (2016)*
Fully ripened *'Hass' and 'Fuerte'* from a local supermarket	Extracted in ethyl acetate; acetone/water (70:30 v/v); or methanol/water (70:30 v/v)	*Nutrition* Spain 'Fuerte' seed had significantly higher amounts of total protein (2.22%) and ash content (0.83%) and the lowest moisture content when compared to the peel and	*Total Phenolic Content (mg GAE/100 g dry mater)* *'Hass'* and *'Fuerte'* seeds had significantly higher phenolic concentrations than pulp. Acetone extracts exhibited the significantly	*Rodríguez-Carpena, et al. (2011a)*

in Spain		pulp.	highest value in seed and peel when compared to methanol and ethyl acetate extracts in both varieties. The avocado peels and seeds had significantly higher amounts and a larger variety of phenolic compounds than the avocado pulp. *CUPRAC Assay (mmol Trolox/g fresh matter)* *'Hass'* and *'Fuerte'* seeds had significantly higher phenolic concentrations than pulp. Acetone extracts of peels and seeds were the most significantly efficient followed by the methanol and ethyl acetate extracts. *'Fuerte'* peel and seed extracts had significantly higher antioxidant activity compared to the *'Hass'* extracts.	
'Hass' and *'Fuerte'* avocado	acetone/water (70:10 v/v)	Nor reported	*Total Phenolic Content (mg GAE/g dry mater)* Significant differences were found between the two varieties *('Hass' vs. 'Fuerte')* and avocado materials (peel vs. seed). *'Fuerte' Peel (F-P)* extracts had the significantly highest polyphenol contents, followed by *'Hass' peel (H-P), 'Fuerte' Seed (F-S)* and the lowest in the extracts from	*Rodríguez-Carpena, et al. (2011b)*

			'Hass' seed (H-S). *DPPH Assay (mmol Trolox/g fresh matter)* *'Fuerte'* peel and seed extracts had significantly higher antioxidant activity compared to the *'Hass'* extracts.	
Avocado peels and Seeds of Two Varieties (*'Shepard'* & *'Hass'*) from Australia	Extracted with 80% methanol at a solid to solvent ratio 1:8 in a thermostatic shaking water bath	Nor reported	*Total Phenolic Content (mg CE/g DW)* The results of TPC revealed significant variation in the TPC of avocado byproduct both with respect to the fruit *component (peels or seeds) and the variety.* Avocado peels in the two cultivars contained the significantly higher TPC than seed while *'Hass'* avocado peel exhibited the significantly highest TPC. *ABTS assay (mmol Trolox/g DW)* Avocado peels exhibited significantly higher antioxidant activity than seed in the two cultivars. *'Hass'* peel exhibited significantly higher antioxidant activity than that of the *'Shepard'* variety. *ORAC (mmol Trolox/g DW)*	*Kosińska, et al. (2012)*

			Avocado peels exhibited significantly higher antioxidant activity than seed in the two cultivars. *'Hass'* peel exhibited significantly higher antioxidant activity than that of the *'Shepard'* variety.	
Mexico avocado byproducts: seed and peel	Samples were boiled with water and stirred by a magnetic stirrer for 30 min and was then filtered using filter paper	*Moisture content (%)* Avocado seed (55.4%); Avocado peel (74.99%); Avocado pulp (76.45%) (Not reported ANOVA results)	*Total Phenolic Content (mg GAE/g extract)* Avocado peels contained the significantly higher TPC than seed. *Total Flavonoids (mg QER/g extract)* Avocado peels contained the significantly higher TFC than seed. *ORAC (mg Trolox equivalents/g extract)* Avocado peels exhibited significantly higher antioxidant activity than seed. *FRAP (mg Trolox equivalents/g extract)* Avocado peels exhibited significantly higher antioxidant activity than seed.	*Calderón-Oliver, et al. (2016)*

2.3 Microorganisms

Microorganisms play an important role in the manufacture of fermented foods. Previous studies have shown that fermentation can influence the composition and bioactive compounds in foods. The fermentation process with microorganisms in food can increase nutritional value like increasing proteins and minerals (*Oliveira, et al. 2017*), degrade antinutrients like glycinin and β-conglycinin in by-products (*Medeiros, et al. 2018)* and increase polyphenols and antioxidants (*Zhang, et al. 2012; Dhiraj & Kalidas 2002; Dulf, et al. 2016*). Thus, several common food-grade microorganisms used in the food fermentation (Lactic acid bacteria, Acetic acid bacteria, yeast, and fungi) in previous studies have been concluded in Table 2.2, in order to present the influence on physiochemical properties of fermented food.

2.3.1 *Lactobacillus sp.*

Lactic acid bacteria (LAB) used in the fermentation of food products were concluded in Table 2.2, based on previous studies. Generally, LAB counts significantly increase in the first five days over 7 days of fermentation and are either stable or show a decreasing trend due to lack of nutrients (*Palani, et al. 2016; Tosukhowong, et al. 2011)*. LAB produces lactic acid during fermentation and decreases pH, which contributes to ideal growing conditions for LAB that in turn inhibits the growth of other bacteria (*Tosukhowong, et al. 2011; Sengun, et al. 2009*). Furthermore, cultivation of LAB on plant (e.g. soy bean seeds and white radish juice) can significantly influence bioactive compounds and nutrients in food *(Rui, et al. 2017; Kuda, et al. 2010*).

Microbial counts

Sauerkraut is produced by spontaneous fermentation utilizing the natural microbiota, especially LAB. According to the study of *Palani, et al. (2016),* the number of LAB was extremely low (<10 cfu/g) at the beginning of spontaneous fermentation. LAB significantly increased at the onset of fermentation and reached the highest counts at Day 5 over 9 days of fermentation. *Xiong, et al. (2012)* further reported that LAB was first detected after 12h and increased significantly in the first 2 days when sauerkraut was fermented with LAB for 7 days, resulting in a maximum value at Day 2. The increase of LAB counts at the start of fermentation may be the production of lactic acid during fermentation drop the pH to some extent and indicated the presence of sufficient nutrients to support LAB growth. In the spontaneous fermentation of cocoa bean, *Fleet & Zhao (2018)* reported that LAB was first detected at 24 h, after which it significantly increased to 10^6 cfu/g at 72h, and then significantly decreased to about 10^3 cfu/g from 120h to 144h of fermentation, probably due to lack of nutrients. *Lefeber, et al. (2011)* reported the initial population of LAB of the spontaneously fermented cocoa bean to be 3.30 log cfu/g, which may be due to *L. fermentum* and *L. plantarum* that have been already present in the Australian cocoa bean before fermentation. The LAB counts increased and reached a maximum at 72h and followed by a reduction at the end of fermentation (144h) (ANOVA results not reported).

Tosukhowong, et al. (2011) reported that the initial LAB counts in pork fermented with *L. plantarum* to be 6.22 log cfu/g, which significantly increased in the first four days over 7 days of fermentation and reached a maximum at day 4 with 9.01 log cfu/g in the fermented pork samples. The *Enterobacteriaceae* in fermented samples decreased significantly from 10^6 to less than 10^2 cfu /g within four days. The anaerobic environment also contributed greatly significant growing conditions for LAB and was likely to inhibit of growth of other organisms like *Enterobacteriaceae* by organic acids, which were produced during the fermentation process. *Sengun, et al. (2009)* showed a similar trend in Turkish fermented cereal fermented with LAB. The highest CFU/g values of LAB were obtained at day2 during 3 days of fermentation, while the *Enterococcus spp.* was inhibited to below 1 log cfu/g during 3 days of spontaneous fermentation (ANOVA results not reported).

Chemical composition

Lactobacillus sp. can significantly influence bioactive compounds and antioxidant activities in food (Table 2.2). *Rui, et al. (2017)* found that the increase in TPC of fermented soybean seeds may be associated with the growth of inoculated *L. plantarum B1-6*. The number of *L. plantarum B1-6* significantly increased and reached a maximum value at 17.5h over a 30h-fermentation and TPC also significantly increased at that point, compared to unfermented soy seeds. *Palani, et al. (2016)* further showed that the increase of bioactive compounds in fermented sauerkraut was also associated with the growth of LAB. The significantly higher LAB counts on day 5 contributed to the formation of ascorbigen and indole-3-carbino, which increased significantly and reached maximum values at day 9 of fermentation, compared to unfermented samples. Moreover, the results in *Kuda, et al. (2010)* showed that LAB strains significantly increased the O_2^- scavenging capacity of fermented daikon juice from 45% to 98% in comparison to the unfermented sample. *Widyastuti & Febrisiantosa, (2014)* reported that goat milk yoghurt fermented with *L. plantarum, L. Brevis*, etc. resulted in a significantly higher level of exopolysaccharide than unfermented one.

However, the study by *Hashemi, et al. (2017)* indicated that a significant decrease in TPC of the lemon juice fermented by *L. plantarum* for 48h, which was mainly attributed to phenolic compounds metabolism enhanced by *L. plantarum. Filannino, et al. (2015)* found that all *L. plantarum* strains significantly decreased the concentration of phenolic acids of cherry juice and broccoli puree during fermentation for 24h by about 70% when compared to the unfermented samples. Moreover, a similar finding was reported by *Othman, et al. (2009)* that both inoculated and spontaneous *(L. plantarum)* fermentation of Chétoui olive for 70 days led to 32–58% reduction of TPC and the 50–72% reduction of antioxidant capacity, compared to the unfermented ones (ANOVA results not reported). *Othman, et al. (2009)* further explained that these phenolic compounds were metabolized by starter strains *(L. plantarum).*

2.3.2 Fungi and Yeast

Most fungi and yeast are suitable microorganisms for solid-state-fermentation, as they are optimally active at very low water activity. Few studies report on the microbial counts of fungi. Hence mainly yeast counts will be discussed. The effects of fungi or yeast fermentation on the chemical composition of plant by-products in terms of polyphenol content (*Dulf, et al. 2016; Zhang, et al. 2012*), antioxidant capacity (*Sandhu,* *et al. 2016; Ajila, et al. 2011*) as well as other bioactive compounds (*Bressani, et al. 2018; Ribeiro, et al. 2017)* are summarized in Table 2.2.

Microbial counts

Ribeiro, et al. (2017) fermented coffee beans with yeasts *Saccharomyces* cerevisiae for 12 days. Counts of *S. cerevisiae CCMA 0200* significantly increased in the first 24h and reached the highest value of 5.9 log cells/g at 24h. While *S. cerevisiae CCMA 0543* counts increased significantly and reached a peak of 7.1 log cells/g at 48h. *Evangelista, et al. (2014)* reported that the microbial counts of coffee samples inoculated with yeast was also significantly higher than control but the fermentation days with the highest number varied from the yeast species. For example, *Candida parapsilosis* in non-washed coffee beans increased to a maximum value at day 12, while *S. cerevisiae UFLA YCN724* significantly increased to 6.8 log cfu/ml at day 4 over 14 days of fermentation (*Evangelista, et al. 2014*). In addition, *Fleet & Zhao (2018)* used several yeast combinations (yeast+AAB and yeast+LAB+AAB) to ferment cocoa beans over 144h. No yeast was detected within 48h and then the counts significantly increased to a maximum at 72h during spontaneous fermentation. For cocoa beans only fermented by yeast, all inoculated yeast significantly increased to the highest counts 10^8 cfu/g during the next 24 h and non-significantly decreased until the end of the fermentation (144h). For the cocoa beans fermented by yeast+AAB and yeast+LAB+AAB, the highest counts of all the yeast species at 24h were significantly less than other fermentations without acetic acid bacteria (yeast only and yeast +LAB). And there was a significant dying off in yeast species (except for *Hanseniaspora guilliermondii*) from 96h to 144h when compared to the fermentation without the presence of AAB over the fermentation of 144h. These data indicated that there was a negative effect of AAB on the growth and survival of yeasts.

Chemical composition

The effects of fungi fermentation on the chemical composition of plant by-products have been widely investigated in previous studies (Table 2.2). *Dulf et al. (2016)* reported that polyphenol content in plum pomace significantly increased after fermentation with *R. oligosporus* and *Aspergillus niger* for 14 days when compared to unfermented ones. *Zhang, et al. (2012)* further reported that TPC in fermented wheat with *Cordyceps militaris* for 21 days was significantly higher than unfermented samples with all three extractions (70% acetone, 70% ethanol and water). Furthermore, the TPC results of fermented wheat extracted by 70% acetone was significantly the highest, followed by 70% ethanol, whereas water extract was the lowest. Similarly, *Sandhu, et al. (2016)* stated that TPC of wheat fermented by *Aspergillus awamorinakazawa* increased significantly over 6 days of fermentation, compared to unfermented samples. TPC was significantly the highest at day 4 of fermentation of wheat WHD-943 cultivar (3598 μg GAE/g). The enhancement of TPC might be related to microbe-induced liberation of polyphenols from the destruction of the plant cell (*Dulf, et al. 2016*). *Sandhu, et al. (2016)* found that enzymes (e.g. β-glucosidase enzyme) may also contribute to liberating bound polyphenols that improved TPC.

Antioxidant capacity has been found to effected by fermentation. DPPH values of plum pomace fermented by *R. oligosporus* and *A. niger* significantly increased by 35.40% and 27.70% respectively at day 6 of fermentation, compared to the unfermented samples (*Dulf, et al. 2016*). Similar results were found in wheat fermented by *Cordyceps militaris (Zhang, et al. 2012)* that fermented wheat exhibited a significantly higher Fe^{2+}-chelating ability than the unfermented wheat. *Sandhu, et al. (2016)* found that the significantly highest values of fermented wheat with *Aspergillus awamorinakazawa* in ABTS assay and DPPH assay were obtained at day 5 of fermentation, compared to unfermented samples.

Fermentation with yeast or fungi can improve nutrient content (*Bressani, et al. 2018; Ribeiro, et al. 2017)* and degrade antinutrients in plant by-products (*Medeiros, et al. 2018; Rui, et al. 2017*). *Ribeiro, et al. (2017)* found that some organic acid concentrations (e.g. acetic and succinic acids) increased significantly in the coffee beans fermented by *S. cerevisiae* in comparison to unfermented ones. Moreover, *Bressani, et al. (2018)* found that total acids significantly higher in coffee beans fermented with *S. cerevisiae* for 400h, compared to the uninoculated ones. Besides that, fermentation of soybean meal with *Bacillus sp.* significantly increased the crude protein content by 8.27% and total soluble protein concentrations, compared the unfermented ones (*Medeiros, et al. 2018*). And the author further stated that fermentation with *Bacillus sp.* may help to degrade antinutrients in soybean meal. *Rui, et al. (2017)* also reported that in vitro protein digestibility of soybean seeds fermented with *Lactobacillus plantarum B1-6* improved significantly and had a maximum rate of 83.9% after fermentation for 17.5 h, compared to unfermented samples.

2.3.3 Kefir

kefir is a combination of three types of microorganisms that generally includes acetic acid bacteria (AAB), LAB and yeasts. No researches have examined the fermentation of plant by-products with kefir. A combination of LAB+AAB+yeast is usually used in the fermentation of cocoa beans to improve antioxidant capacity (*Malbaša, et al. 2011*), and increase organic acids that contribute to generate in flavour compounds (*Bortolini, et al. 2016; Lefeber, et al. 2010b)* (Table 2.2).

Microbial counts

Lefeber, et al. (2011a) reported that the community dynamics of spontaneously fermented cocoa beans over 144h involved the growth of yeasts, LAB, and AAB. The initial level of the LAB counts was 10^3 cfu/g, and then the LAB increased significantly to a maximum after 72 h. The yeast counts significantly increased from 3.30 to 7.73 log cfu/g after 72h. No colonies of AAB were found in fermented cocoa beans at the beginning of fermentation. However, after 72h, the AAB counts significantly increased and reach the highest 9.08 log cfu/g. This may be because AAB requires air and can only grow when the quantity of oxygen was sufficient for the oxidation of ethanol by AAB. The similar growing trends of microorganisms was also observed in the findings of *Papalexandratou, et al (2013)* who studied spontaneous fermentation of cocoa beans over 120h. The initial counts of LAB of 5.8 log cfu/g significantly increased to a maximum after 30h of fermentation. The community of yeast then significantly increased until a maximum population at 35h of fermentation and then declined significantly to less than 10^2 cfu/g at 120h of fermentation. Moreover, the number of AAB started to increase significantly from 30h and reached the highest counts at 35h of fermentation. This can be explained by the commensalism that occurred during the early stage of fermentation as yeast provided alcohol to AAB for further oxidation.

In another spontaneous fermentation of cocoa bean (*Fleet & Zhao, 2018*), no colonies of LAB and AAB were observed at the start of natural fermentation. At 144 h of natural fermentation, the communities of both LAB and AAB significantly increased to maximum values at 72h. With the use of mixed cultures (yeast +LAB+AAB and yeast +AAB) over 144h cocoa bean fermentation, all the yeast species (except for *Hanseniaspora guilliermondii*) significantly increased in the early stage (0-72h) and significantly decreased from 96h to the end of fermentation (144h). This may be due to the interactions between yeast and AAB. The antagonism of AAB to yeast growth was observed with further fermentation (96h-140h). Acetic acid produced by AAB reduced the pH value to below the normal physiological values, and directly affected transporting enzymatic activities, such as enolase, a key enzyme of glycolysis, resulting in inhibition of yeast growth. The results in the study of *Krusong & Vichitraka, (2010)* in the fermented pineapple vinegar were similar. The yeast *H. guilliermondi* grew significantly fast in the first 24h after inoculating AAB into the fermented pineapple vinegar. Two days later, the yeast started to grow less and died off significantly until the end of fermentation (day10). And *Krusong & Vichitraka, (2010)* further explained that AAB provided nothing to yeast and the antagonistic effect of AAB to yeast was that the accumulating acetic acids produced by AAB caused an antagonistic effect on the growth of yeast.

Chemical composition

It has been reported that antioxidants increased after fermentation with mixed cultures (LAB+AAB+yeast). *Malbaša, et al. (2011)* stated that an increase in antioxidant capacity was observed in all fermented black and green tea (1: AAB+ *Zygosaccharomyces sp.*; 2: AAB+ *Saccharomyces cerevisiae*; 3 kombucha (control)) after three days of fermentation in comparison to unfermented tea. The results of antioxidant capacity can vary from different substrates and starter cultures. Furthermore, the highest antioxidant capacity using the DPPH assay of black tea was obtained with the fermentation of *S. cerevisiae* + AAB, compared to other cultures. And green tea fermented by kombucha (control) indicated the highest antioxidant activity (ANOVA results not reported) *(Malbaša, et al. 2011)*. In terms of total acids, the author stated that the total acids content increased with increased antioxidant activities.

Organic acids produced in the fermentation had a vital contribution in improving the flavour of cocoa bean. *Bortolini, et al. (2016)* further stated that the production of organic acids like acetic acid and lactic acid may be related to LAB, AAB and yeast. *Fleet & Zhao (2018)* reported that acetic acid and lactic acid had significantly higher concentrations in LAB- or AAB-fermented cocoa beans than samples without them. Lactic and acetic acids acted as flavour precursor molecules and initiated biochemical reactions in the cocoa bean fermentation *(Lefeber, et al. 2010b*). Higher levels of acetic acid and lactic acid may contribute to the formation of cocoa flavour precursors like amino acids and short-chain peptides (*Bortolini, et al. 2016*), which explained that why cocoa beans inoculated with LAB, AAB and yeast had better quality.

It is evident that yeast, LAB, and AAB are useful for fermentation as they can increase nutritional content like proteins and vitamin C, degrade antinutrients like glycinin and β-conglycinin, as well as improve TPC and antioxidant ability. However, although most fermentation with microorganisms can significantly improve the phenolic compounds, differences do exist. Some results from previous studies indicated that microorganisms could also metabolize phenolic compounds to decrease antioxidant capacity but depended on the microorganism species and the substrates, which needs to be further examined in the future study.

Table 2.2 Microorganisms used in the fermentation of plant and plant byproduct.

Substrates	**Microorganisms**	**Time &Temp**	**Preparations**	**Detection results**	**References**
Lemon juice	*L. plantarum LS5*	Incubated at 37°C for 48h	1. samples were determined during every hour 2. samples stored at 4°C for 28 days after fermentation	*Lactic acid bacteria (LAB)(cfu/g)* The cell counts of the *L. plantarum LS5* significantly increased during fermentation (37°C for 48h) and significantly decreased after storage (4°C for 28 days). *Citric acid content (g/L)* Citric acid content was reduced significantly during the fermentation by *L. plantarum*, being 13.2 g/L within 48 h of fermentation. *Total phenolic content* There was a significant decrease of total phenolic compounds in lemon juice fermented by *L. plantarum* after fermentation of 48h. *Antioxidant capacity* There was a significant increase in the results of DPPH scavenging and FRAP assays in lemon juice after fermentation of 48h.	*Hashemi, et al. (2017)*
Soy bean seed	*Lactobacillus plantarum B1-6*	Incubated at 37°C for different	1. 12h prior to treatments in boiling water (uncooked and	*Lactic acid bacteria (LAB)(cfu/g)* The addition of 2 g/100 g sucrose significantly improved the growth of *L. plantarum B1-6* relative to the cultures	*Rui, et al. (2017)*

		durations (6, 15, 24, and 30 h)	cooked for 1, 5, 10, and 15 min) 2. Sterilized at 121°C for 20 min 3. Supplement: sucrose (0, 1, 2, 3, and 4 g/100 g) 4. Extracted with 10-fold dilution of 80% ethanol at 50°C for 4 h	without sucrose supplement. The growth of *L. plantarum B1-6* was significantly affected by fermentation time and the highest value was 8.12 log cfu/ml at 17.5h fermentation. *Proteins (g/100 g)* Compared with unfermented soy seeds, no significant changes were observed in lipid and ash contents in soy seeds, whereas protein content significantly increased from 33.10 to 34.19 g/100 g after 17.5h solid-state fermentation. *Total phenolic content (µg GAE/g DW)* Compared with unfermented soy seeds, those that underwent fermentation for 17.5 h showed significant improvement in total phenolic content. *In vitro protein digestibility (%)* Compared with unfermented soy seeds, in vitro protein digestibility improved significantly during fermentation, reaching the maximum rate of 83.9% at 17.5 h of fermentation.	
White cabbage heads	*Lactic acid bacteria*	Incubated at aerobic fermentation	1. Mixed well with 0.9% salt before fermentation 2. Dried in a freeze	*Lactic acid bacteria (LAB)(cfu/g)* Numbers of LAB were extremely low (<10) at the start of the fermentation and then it significantly increased	*Palani, et al. (2016)*

(sauerkraut)		for 9 days at 19–20°C	dryer after fermentation 3. Used 70% aqueous methanol extraction	to the highest count of 1.92 *10^8 cfu/g at Day 5. (ANOVA results not reported). *pH value* The initial pH of the sauerkraut was 6.0 and it significantly decreased to 4.0 after 7 days' fermentation. *Bioactive compounds (Conc in µmol/100 g FW)* Ascorbigen and Indole-3-Carbinol increased significantly from Day 5 to Day 9. At the end of fermentation (Day 9), ascorbigen was significantly higher content of 13.0µmol/100 g FW, and Indole-3-Carbinol reached a maximum with 4.52µmol/100 g FW when compared to unfermented ones.	
Chinese sauerkraut	*Lactic acid bacteria*	Incubated at ambient temperature (20-25 °C) for 7 days	1. Garlic (4%), Chinese prickly ash (1.5%), hot red peppers (2%) and ginger (2%).	*Lactic acid bacteria (LAB)(log cfu/g)* LAB was first detected after 12h and the load increased significantly in the first 2 days, resulting in a maximum value (7.9 log cfu/g) at Day 2. Then a significant decrease was found at the end of fermentation (day7) to 5.7 log cfu/g in comparison to day2. *pH value* The mean pH value significantly declined from 6.02 at the beginning of fermentation to 3.55 at day 4.	*Xiong, et al. (2012)*

				Titratable acidity(as % Lactic acid) There is a significant increase in titratable acidity from 0.01 to 0.57% on each day till the 5.5th day, after which the rise was not significant.	
Thai traditional fermented pork in Nham (A, B, C, D in different treatments)	*Lactobacillus plantarum BCC 9546*	Incubated for 7 days at 30 ± 1 °C and 50 ± 2% relative humidity	1. Pork samples: 60% ground pork meat +40% cooked pork rind 2. Add 0.4% sucrose, 1.9% salt, 4.3% cooked rice, 0.2% erythorbate, 0.2% trisodium phosphate, 0.2% monosodium glutamate, 2% whole bird chilli, 0.01% potassium nitrite and 4.3% garlic	*Lactic acid bacteria (LAB) (log cfu/g)* LAB counts significantly increased in the first four days from 6.22 to 8.99 log cfu/g in fermented pork samples and values after 4 days showed no significance. *Enterococci (log cfu/g)* The initial counts of *Enterobacteriaceae* in fermented samples were 6.55 cfu /g that decreased significantly to less than 10^2 cfu /g within four day. *pH value* The pH values of pork samples decreased significantly from pH 6.4 (day0) to 4.35 (day7) after the fermentation of 7 days.	*Tosukhowong, et al. (2011)*
Turkish fermented cereal-Tarhana	*Lactobacillus spp.*	Incubated at 30 °C and 42 °C for 3-5 days		*Lactic acid bacteria (LAB) (log cfu/g)* The highest CFU/g values of LAB were obtained during the first 2 days of fermentation. Later in the fermentation the LAB counts were either stable or showed a decreasing trend probably reflecting the start of nutrient depletion (ANOVA results not reported).	*Sengun, et al. (2009)*

				Enterococcus spp. (log cfu/g) The community of *Enterococcus spp.* is inhibited to below 1 log cfu/g with the increase of LAB with 3 days spontaneous fermentation (not reported ANOVA results).	
White radish juice	*lactic acid bacteria (LAB)*	Incubated at 15°C for 7 days	The 50% (w/w) juice was autoclaved at 115 °C for 15 min	*O_2^- scavenging capacity (%)* LAB strains significantly increased O_2^- scavenging capacity of fermented daikon juice.	*Kuda, et al. (2010)*
Wheat (*Triticum aestivum* Linn.)	*Cordyceps militaris*	Incubated at (25±2) °C for 21 days	1. Autoclaved at 121 °C, 20 min after fermentation 2. Substrate: moisture content 45 % and pH =6.0 3. Three extraction methods: water, 70 % ethanol and 70 % acetone	*Total polyphenolic content (mg GAE/g)* Total phenolic content in fermented wheat was significantly higher than unfermented samples for all three extractions. The 70% acetone extraction showed the significantly highest total polyphenolic content, followed by 70% ethanol, and water extract that had the lowest. *Total flavonoid content (rutin equivalent mg/g)* The 70% ethanol extraction showed the significantly highest total flavonoid content, followed by 70% acetone, and the lowest with water extraction. Total flavonoid content of fermented wheat only in the water extract are significantly higher than unfermented ones.	*Zhang, et al. (2012)*

				DPPH scavenging activity (%) Water extracts of fermented wheat at a concentration higher than 0.3 mg/mL exhibited significantly higher antioxidant activities compared to unfermented ones. *Fe^{2+}-chelating ability (%)* It was found that fermented wheat extract exhibited a significantly higher Fe^{2+}-chelating ability than did the respective extract of the unfermented wheat.	
Different cultivars of wheat	*Aspergillus awamorinakazawa*	Incubated for 6 days at 30°C	1. Dried in an oven at 60 °C for 24 h 2. Extract with 4 ml acidified methanol (HCl/methanol/water, 1:80:10, v/v/v) at room temperature (25°C for 2 h	*Total phenolic content (µg GAE/g)* For all cultivars, TPC increased significantly during fermentation with *Aspergillus awamorinakazawa* in comparison to their corresponding non-fermented counterparts. The significantly highest TPC 3598 µgGAE/g was obtained at day4 in fermented wheat WHD-943 cultivar. *DPPH· scavenging activity (%)* For all cultivars, DPPH· scavenging activity of fermented wheat increased significantly in comparison to their corresponding non-fermented counterparts. Days of fermentation had a significant effect on DPPH· scavenging activity and the highest value was on day 5 with 64.2%. *ABTS scavenging activity (µmol/g)*	*Sandhu, et al. (2016)*

				The highest ABTS scavenging activity of fermented wheat with *Aspergillus awamorinakazawa* was observed on day 5 of fermentation, which exhibited a significant increase from 7.2 to 81.8 µmol/g compared to unfermented samples. Total flavonoids content*(µg CE/g)* Fermentation increased TFC significantly and reached maximum on day 5 of fermentation compared to unfermented samples.	
Plum pomace	*Aspergillus niger, Rhizopus oligosporus*	Incubated at 30°C for 14 days	1. Extract with (hydrochloric acid/methanol/ water in the ratio of 1:80:19) at 40 °C for 30 min in an ultrasonic bath	*Polyphenol content (mg/g DW)* The amounts of phenolics increased significantly until the maximum yields was achieved at day6. Plum by-products fermented with *R. oligosporus* significantly increased polyphenols (from 679.80 mg/g DW to 980.00 mg/g DW) compared to unfermented ones. Plum by-products fermented with *Aspergillus niger* significantly increased polyphenols by 30.15% (from 1295.31 mg/g DW to 1685.96 mg/g DW) compared to unfermented samples. *Total flavonoid content (QE/100 g DW)* Fermented plum by-products showed significantly increase in flavonoid contents until the maximum yields	*Dulf, et al. (2016)*

				were reached at day6. A significant increase to 809.42 mg QE/100 g DW- by *R. oligosporus*, and 755.52 QE/100 g DW- by A. niger were foiund when compared to unfermented semples.. *DPPH radical inhibition capacity (RIC)(%)* The value of DPPH assay in fermented plum pomace significantly increased by 35.40% (for SSF with *R. oligosporus*) and 27.70% (for SSF with *A. niger*) by day 6 compared to the start of fermentation.	
Solid -state-fermented apple pomaces	*P. chrysosporium*	Incubated at 37 ± 1°C for 14 days	1. Treated with inducers Copper sulphate (2 mM), veratryl alcohol (2 mM) and Tween-80 (0.1%) 2. Different solvents such as water; 60% ethanol; 70% ethanol, 80% ethanol; 60% acetone; 70% acetone and 80% acetone; 60% methanol; 70% methanol and 80% methanol 3. Ultrasonic assisted	*Polyphenol content* The phenolic content was significantly higher in fermented apple pomace than unfermented apple pomace. In the case of microwave-assisted extraction, the polyphenol content increased significantly from 40 to 60°C. As for ultrasonic assisted extraction, the polyphenol concentration increased significantly with the increase in temperature from 30–40 °C. *DPPH assay (µg DW sample)* The antioxidant capacity was significantly higher in fermented apple pomace than unfermented apple	*Ajila, et al. (2011)*

			extraction and microwave-assisted extraction	pomace. Acetone extract (80%) of both apple pomace and fermented apple pomace exhibited higher and significant inhibitory activity against DPPH radical with ultrasonic assisted extraction when compared to other solvents.	
Soybean meal	*Bacillus cereus, Bacillus subtilis and Bacilus amyloliquefacien,*	Incubated at 42 °C for 48 h without any agitation	1. Sterilized by mild heating	*Protein content* Total soluble protein concentrations in the soybean meal fermented with *Bacillus cereus* and *Bacillus subtilis* were significantly increased after 24 h solid-state fermentation compared to the unfermented control. Fermentation with *Bacillus sp.* significantly increased crude protein content by 8.27% when compared to the unfermented. *Antinutrients* fermentation with *Bacillus sp.* may help to degrade antinutrients in soybean meal including breaking down larger soy protein to 35 kDa and under eliminating antigenicity against glycinin and β-conglycinin.	*Medeiros, et al. (2018)*
Coffee inoculated with yeast	*Yeasts (S. cerevisiae CCMA 0543)*	Incubated at room temperature	1. Sun- dried to obtain 11% moisture 2. Samples (100 g) were	*S. cerevisiae CCMA 0543 (log cells/g)* During fermentation, treatments inoculated with *S. cerevisiae* CCMA 0543 and CCMA 0544 exhibited a	*Bressani, et al. (2018)*

starter cultures		for 400h	collected every 48 h 3. Different treatments: Inoculate yeast suspension directly (D) 4. Yeast suspension homogenized with coffee cherries and left for 16 h (B)	larger yeast population compared to their respective control. *Total acids (g/Kg)* Compared to uninoculated coffee beans, the significantly highest total acid was shown in fermented coffee beans for *S. cerevisiae* CCMA 0543 (D) treatment, which reached 23.84 g/Kg total acids after fermentation for 400h. There was a significant loss of chlorogenic acid in roasted coffee beans when compared to unroasted ones.	
Coffee (*Ouro Amarelo &Mundo Novo*) fermentation	*Yeast* *Saccharomyces cerevisiae (CCMA 0200 and CCMA 0543*	Incubated at 28°C for 12 days	1. Dried when the beans reached a moisture content of 11–12% 2. Samples were collected at 0, 24, 48 and 284 h	*Yeast* The inoculum significantly increased the population of *S. cerevisiae* compared to control. The coffee variety significantly influenced *Saccharomyces cerevisiae* populations counts. *'Mundo Novo'* coffee beans fermented with *S. cerevisiae CCMA 0200* and *S. cerevisiae CCMA 0543* had significantly higher population counts than *'Ouro Amarelo'* coffee beans *Carbohydrates and organic acids* There was a significant difference between coffee varieties (*Ouro Amarelo & Mundo Novo*) for carbohydrates and organic acids, except for malic acid.	*Ribeiro, et al. (2017)*

				The acetic and succinic acid concentrations increased significantly only in coffee bean of the *Ouro Amarelo* variety inoculated with *S. cerevisiae* CCMA 0543 (1.86 and 5.78 mg/g, respectively) compared to unfermented samples. The *S. cerevisiae* CCMA 0543 yeast was the most suitable coffee inoculants compared to *S. cerevisiae* CCMA 0200 yeast.	
Coffee beans	*S.cerevisiae* *Candida parapsilosis and Pichia guilliermondii*	Incubated at 28 °C for 14 days	Drying under the sun until 11-12% moisture content reached.	*Yeast (YEPG media) (log cfu/ml)* Total yeast counts showed no significant difference during the fermentation of washed and non-washed coffee cherries inoculated with different strains.	*Evangelista , et al. (2014)*
Malaysian cocoa bean Spontaneous fermentation	*Hanseniaspora opuntiae, Saccharomyces cerevisiae, Lactobacillus fermentum, and Acetobacter pasteurianus*	Incubated at 25-35 °C for 10h	1. Thoroughly rinsed with water under high pressure 2. Sun-drying for 5-7 days 3. Samples were collected after 0, 6, 12, 24, 30, 36, 48, 54, 60, 72, 84, 96, and 120 h 4. Box 1 (30.0-35.0 °C) and Box 2 (24.5-30.0 °C) fermentations	*Yeast* (*log CFU/g*) The yeast counts significantly increased until a maximum population of 7.0 log cfu/g during the first 12 and 36 h of the Box 1 (30.0-35.0 °C) and Box 2 (24.5-30.0 °C) fermentations, respectively. *Lactic acid bacteria (LAB)* (*log CFU/g*) LAB counts significantly increased during the first 30-36 h of both Box 1 (30.0-35.0 °C) and Box 2 (24.5-30.0 °C) fermentations and reached the highest counts 9.0 log cfu/g.	*Papalexandratou, et al. (2013)*

				Acetic acid bacteria (AAB) (log CFU/g) The AAB counts significantly increased after 6 h of the Box 1 (30.0-35.0 °C) fermentation, reaching a maximum of 7.5 log CFU/g after 72 h. The AAB counts significantly increased after 30 h of the Box 2 (24.5-30.0 °C) fermentation, reaching a maximum of 7.5 log CFU/g after 84 h.	
Cocoa bean Spontaneous fermentation	Lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeast	Incubated at 28 °C for 144h	1. Anaerobic fermentation during the first two days, and later fermentation with oxygen 2. Sundried on coverable platforms for approximately seven to ten days 3. Samples were collected at 24, 48, 72, 96, 120, and 144 h of fermentation	*Lactic acid bacteria (LAB) (log CFU/g)* The LAB counts increased significantly to a maximum of 9.13 log cfu/g after 72 h. *Yeast (log CFU/g)* The yeast counts significantly increased from 3.30 to 7.73 log cfu/g after 72h. *Acetic acid bacteria (AAB) (log CFU/g)* No colonies of AAB are found in fermented cocoa beans at the beginning of fermentation. After 72h, the AAB counts significantly increased and reached the maximum of 9.08 log cfu/g.	*Lefeber, et al. (2011)*
Australian cocoa bean fermentation	*Yeast, LAB, AAB*	Incubated at 25 °C (0–12 h), 30 °C (12–24	1. Dry at 30 °C and relative humidity 70 % for 5 days	*Natural fermentation* No colonies of LAB and AAB are observed at the start of natural fermentation. During the natural fermentation	*Fleet & Zhao, (2018)*

		h), 35 °C (24–36 h), 40 °C (36–48 h), 45 °C (48–72 h) and 48 °C (72–144 h)		of 144 h, the community of LAB and AAB significantly increases and reaches a peak of 5.8 log cfu/g and 6.8 log cfu/g respectively at 72h. *Fermentation of mixed cultures* All the yeast species in cocoa beans fermented by yeast+LAB+AAB significantly increased during the first 24 h to maximum. There is no significant difference until 120h and then yeast counts in cocoa beans fermented by yeast+LAB+AAB significantly decrease until the end of fermentation (144h). *Ethonal (mg/g)* No ethanol was found in unfermented nibs, but ethanol increased significantly during fermentation. There was no significant difference for all inoculated fermentations, but ethanol concentration in naturally fermented beans was significantly lower than inoculated samples. *Glycerol production (mg/g)* The glycerol production was similar for all inoculated fermentations but its concentration in these beans was significantly higher by about 2–3 fold than that in naturally fermented cocoa beans.	

				Sugars (mg/g) Glucose and fructose in cocoa beans increased significantly after fermentation, compared to unfermented ones. But the differences were not statistically significant between inoculated beans and naturally fermented beans. *Organic acid (mg/g)* The concentration of lactic acid and acetic acid were significantly higher in bean fermented in the presence of LAB and AAB than uninoculated cocoa beans.	
Kombucha beverage	*Yeast, AAB kombucha culture*	Incubated at 28 °C for 10 days	1. Added 7% solution of sucrose 2. SC1: *S. cerevisiae* + AAB, SC2: *Zygosaccharomyces sp.* + AAB, Control: kombucha culture	*Antioxidant activity (%)* A significant increase of the antioxidant capacity was observed in all cultivation mixtures (SC1, SC2 and control) after three days of fermentation (average value 47.7%). The highest average antioxidant capacity of black tea was obtained with the fermentation of *S.cerevisiae* + AAB when compared to other cultures. And green tea fermented by kombucha (control) indicates the highest antioxidant activity (ANOVA results not reported). *Vitamin C (mg/l)*	*Malbaša, et al. (2011)*

				There was a correlation between the highest values of antioxidant activities and the quantity of vitamin C in some series of samples (ANOVA results not reported). *Total acids content (g/L)* The total acids content increased with increased antioxidant activities. Black tea samples fermented with *S. cerevisiae* +AAB that had the highest antioxidant capacity using the DPPH assay also had the highest total acids content (ANOVA results not reported).	

2.4 Extraction methods and antioxidant assays

Biologically, antioxidants play their health-beneficial roles via transferring a hydrogen (H) atom or an electron (e−) to reactive species, thereby deactivating them. Antioxidant activity assays imitate this action. There are two classifications of antioxidant detection methods including electron transfer (ET)- and hydrogen atom transfer (HAT)-based assays. The HAT-based assays like ORAC assay measure the antioxidant capacity by the competition of peroxyl radicals generated from azo compounds between antioxidant and substrate during thermal processing (*Apak, et al. 2008*). Most of these assays are kinetic-based, meaning that they are more concerned with the rate rather than thermodynamic conversion efficiency of the radical reaction with the antioxidant. While the ET-based assays like CUPRAC, Folin-Ciocalteu, ABTS, and FRAP assays measure the antioxidant capacity by colour changes generated from the reduction of antioxidants. The degree of colour change is correlated to the concentration of antioxidants in the sample. Additionally, antioxidant structure, solvent system and properties may have effect on the mechanisms of ET- and HAT-based assays (*Çelik, et al., 2010*). The differences are attributed to the use of different solvents (*Ajila, et al. 2011; González-Montelongo, et al. 2010; M'hiri, et al. 2015*), extraction temperature and time (*Hernández‐Carranza, et al. 2016; Ajila, et al. 2011*), substrates (*Hernández‐Carranza, et al. 2016*) and antioxidant assays (*Çelik, et al. 2010; Bean, et al. 2009*).

Differences exist with various extraction methods, extraction time and temperature used in antioxidant assays. In the study by *Ajila, et al. (2011)*, fermented apple pomace extracted with 80% acetone and ultrasonic assisted extraction had significantly higher antioxidant ability using DPPH assay than other combinations (e.g. ethanol and water). *González- Montelongo, et al. (2010*) further reported that 50% of acetone had the highest efficiency in the extraction of polyphenols and antioxidant capacity in banana peels, compared extracts of other solvents (methanol, ethanol, acetone). *M'hiri, et al. (2015)* studied three extraction methods and found that microwave-assisted extraction significantly increased the TPC of orange peels compared to the conventional solvent extraction. With increasing microwave power ranged from 100 to 200 W, there was a significant increase in TPC of orange peel powder. As for supercritical CO_2 extraction, the results showed that the TPC and TFC were significantly lower than samples obtained using conventional solvent extraction. The TPC in high-pressure extraction of orange peels was significantly lower than while TFC was significantly higher than the conventional solvent extraction. Furthermore, *Ajila, et al. (2011)* found that the TPC of both fermented and unfermented apple pomaces increased significantly in the microwave-assisted extraction, with increasing temperature from 40 to 60°C and increasing time from 5 to 10 min. As for ultrasonic assisted extraction, the polyphenol concentration increased significantly with the increasing temperature from 30 to 40°C and time from 20 to 30 min in fermented apple pomace. In addition, *Hernández - Carranza, et al. (2016)* compared different extraction conditions of three fruit by-products. For banana peels, the best extraction condition of flavonoids was at 60°C for 0.5 h. The increase in aqueous extraction time and temperature significantly increased TPC, with the best extraction condition at 60°C for 12 h. For TPC and TFC, the best extraction conditions for apple pomace was obtained at 40°C for 6 h and 40°C for 12h, respectively. As for orange peels, the significantly highest values of TPC and TFC were obtained with extraction at 60°C for 12 h and 40°C for 1 h respectively.

Antioxidant assays used to assess antioxidant activities of plant by-products include DPPH, FRAP (*Hernández, et al. 2016*) and ABTS (*González, et al. 2010*) assays. *Hernández, et al. (2016)* stated that aqueous extraction time of 1h at 40°C resulted in the significantly highest value of DPPH assay in banana peels, while only extraction temperature significantly affected the results of FRAP assay with the best condition at 60°C for 0.5 h. With apple pomace, both extraction time and temperature significantly affected the results, with the best condition at 60°C for 1 h for DPPH assay and 40°C for 3 h for FRAP assay. Additionally, the results of *González-Montelongo et al. (2010)* indicated that the antioxidant capacity of banana peels in *'Gruesa'* cultivar increased significantly in the DPPH and ABTS assays at 120min with increasing extraction temperature from 25 to 55°C.

2.5 Drying and Roasting

Unlike other heating methods, drying and roasting are commonly used with foods such as nuts and seeds. Previous studies have reported that drying methods are used to control the moisture content of fermented samples for safe and easy storage. *Evangelista, et al. (2014)* exposed coffee fruits (both fresh and fermented) on cement patio until they reached 11–12% moisture content. Similarly, *Lefeber, et al. (2011)* employed sun-drying in spontaneous cocoa bean fermentations as a pre-treatment before roasting. In addition, drying and roasting are necessary processing methods to develop flavour precursors generated from cocoa bean fermentation.

Drying and roasting of beans and seeds can influence the functional properties and chemical composition as seen in Table 2.4. *Jan et al. (2019)* reported that Kalonji seed flour roasted by pan and microwave had significantly higher water and oil absorption capacity than unroasted ones. *Lin, et al. (2016)* reported that the roasted almond kernel resulted in significantly higher oil yields than unroasted kernel.

Drying and roasting can also increase TPC and antioxidant activities of avocado peels. *Rotta et al. (2015)* further reported that dehydrated avocado peels contained significantly higher TPC than fresh peels. Results of FRAP and DPPH assays showed that drying significantly increased antioxidant capacity of avocado peels. Similarly, *Morais, et al. (2015)* showed that avocado dried peels exhibited significantly higher TPC and antioxidant capacities using the FRAP assay, compared to raw avocado peels. According to *Jan, et al. (2019)*, roasting significantly increased the TPC, antioxidant ability using DPPH assay of microwave-roasted and pan-roasted Kalonji seed flour, compared to unroasted samples.

Roasting also contributes to the improvement of the TPC and antioxidant capacity of beans, nuts and kernels. *Bressani, et al. (2018)* reported that the roasting time and degree of roasting significantly influenced trigonelline concentration of coffee beans. *Lin, et al. (2016)* found that the amounts of the antioxidants in roasted almond kernels significantly rise with increasing temperature and roasting duration and reached a maximum at 200 °C and 20 min. Total antioxidant capacity (TAC) values were used to evaluate the antioxidant capacity of nuts and beans in *Açar, et al. (2009)*. However, the author found that the TAC values of nuts and beans significantly decreased within 10 min of roasting at 150 °C. The author explained that the decrease in TAC values at the start of roasting was probably associated with the destruction of antioxidant components. With prolonged roasting time, TAC values increased significantly for most pulses (borlotti bean, black bean, giant lentils, chickpea and yellow soybean), cashew nut, as well as pinenut, and reached a maximum value at 60 min of roasting. The increase in TAC with longer roasting time may contribute to releasing bound antioxidants and forming Maillard reaction products with higher antioxidant capacity, especially in the rich-starch materials (e.g. chickpea, cashew nut).

The studies summarized above have shown that fermentation and roasting significantly improved the antioxidant activities of seeds and nuts. Hence the aim of this study was to determine the difference between unfermented and fermented avocado seeds by using food-grade microorganisms (*Lactobacillus plantarum* and kefir) for inoculation. Moreover, this study also investigated the effects of roasting on the colour, TPC and antioxidant activities of avocado seeds with the Central Composite Design (CCD). This study aimed to determine how fermentation of avocado seeds and subsequent roasting influences antioxidant capacity. The results of antioxidant capacity were measured by ET-based assays as CUPRAC and FRAP assays, which were easily and diversely applicable in conventional laboratories and relatively insensitive to a number of parameters adversely affecting certain reagents such as DPPH, i.e., air, sunlight, humidity, and pH, to a certain extent.

Table 2.4 Changes in chemical composition and properties of fermented plant byproducts with drying and roasting.

Plant byproducts	**Preparations**	**Nutritional composition**	**Biochemical properties**	**Reference**
Coffee inoculated with yeast starter cultures		Not reported	*S. cerevisiae CCMA 0543 (log cells/g)* During fermentation, treatments inoculated with *S. cerevisiae* CCMA 0543 and CCMA 0544 exhibited a larger yeast population compared to their respective control. *Total acids (g/Kg)* Compared to uninoculated coffee beans, the significantly highest total acid was shown in fermented coffee beans for *S. cerevisiae* CCMA 0543 (D) treatment, which reached 23.84 g/Kg total acids after fermentation for 400h. There was a significant loss of chlorogenic acid in roasted coffee beans when compared to unroasted ones.	*Bressani, et al. (2018)*
Avocado peels	1. Dried for 120 h in the ambient conditions of pressure and temperature (25 °C) 2. Oven-dried at 60 °C by 24 h; freeze dried for 24 h 3. Methanol extracts	Not reported	*Total phenolic compounds (mg GAE/100g DW)* Avocado dried peel resulted in the significantly highest total phenolic compounds (1252.31 mg GAE 100 g−1 DW) compared to raw peel. *Total flavonoids (mg QE 100 g−1 DW)* There was no significant difference between fresh peels and dried peels. *FRAP assay (μmol $FeSO_4$/g)*	*Morais, et al. (2015)*

			Oven drying significantly concentrated avocado peel antioxidant activity. Avocado dried peel had the significantly highest antioxidant capacities using the FRAP assay (441.83 µmol $FeSO_4$/g), compared to raw avocado peel.	
Roasted pulses, nuts and seeds	1. Roasted in electrical oven at 150 °C for 10, 30 and 60 min	*Moisture content (%)* The moisture content significantly decreased below 3% after a roasting time of about 30 min in all samples.	*Total antioxidant capacity* The results indicated that TAC values of nuts significantly decreased within 10 min of roasting at 150 °C. This decrease was statistically significant for walnut, hazelnut and pistachio. The increase in TAC after 30 min was significant for soybean varieties and seeds, but not for bean varieties and nuts except hazelnut and pistachio. With prolonged roasting time, TAC values tended to increase for many samples and reached the maximum level at the end of 60 min of roasting.	*Açar, et al. (2009)*
Almond (Prunus dulcis) kernel	1. No fermentation 2. Roast in a forced air convection oven at 150, 180 and 200 °C for either 5, 10 or 20 min 3. 80% ethanol	*Oil yield (%)* Roasted almond kernel (except that roasted at 150 °C for 5 min) had significantly higher oil yields than the unroasted sample.	*Total phenols (mg GAE/g of dried extract)* The levels of total phenolics in the extracts from the almond kernels roasted at 180°C for 20 min and 200 °C for 20 min were significantly higher than extracts prepared from the raw samples.	*Lin, et al. (2016)*

	extraction	Almond kernels roasted at 180 °C for 20 min had the highest oil yield compared with the others.	*Total flavonoids (mg CE/g of dried extract)* Compared to the raw almond kernel, ethanolic extracts of samples roasted at 180°C for 20 min and 200 °C for 20 min had significantly higher total flavonoid. *Antioxidant capacity* Almond kernels roasted at 200 °C for 20 min had the highest antioxidant activities and browning index; followed by samples roasted at 180 °C for 20 min compared to unroasted samples. Almond kernels roasted with higher temperature and longer duration had significantly higher amounts of the antioxidant constituents that also had stronger antioxidant activity.	
Kalonji (Nigella sativa) seed flour	1. Pan-roasted at 190 °C with constant stirring for 150–180s 2. Microwave oven-roasted for 120–150 s at 900W	*Water absorption capacity (WAC)(%)* Both pan- roasted and microwave roasted samples significantly increased the WAC, compared to unroasted samples. *Oil absorption capacity (OAC)(%)* The OAC of roasted kalonji seed flour was found to be significantly higher than the untreated samples.	*Total phenolic content ((mg GAE)/g)* Roasting significantly increased the TPC in case of microwave roasted and pan roasted samples when compared to untreated ones. *DPPH radical scavenging activity (%)* Roasting of sample resulted in a significant increase in antioxidant activity. However, microwave roasted sample had higher antioxidant activity compared to pan roasted sample.	*Jan, et al. (2019)*

			Reducing power (%) Roasting of samples significantly increased the reducing power in comparison to unroasted samples.	

3. Materials and methods

3.1 Materials: avocado seeds

The *'Hass'* cultivar avocados used in this study were harvested from July through November of 2018 in New Zealand. Seeds from ripe avocado were obtained from a local avocado oil industry, Olivado Ltd, located in Waipapa, Kerikeri, New Zealand. During cold refined avocado oil processing, avocado fruit by-products were generated when the avocado fruits were separated by machine into peels, seeds, and pulp. The seeds were obtained and stored in a freezer (-17 °C) for further usage.

3.2 Fermentation of ground avocado seed particles

Prior to fermentation, 3kg (approximately 160 seeds) were crushed by the crusher (Fuller® Tool, US). After crushing, the skins of the ground avocado seed particles were removed and the samples were broken down into homogeneous ground particles, using a laboratory grinding mill (Polymix System PX-MFC 90D, Malaysia). The ground avocado seed particles of about 0.5-1mm in size were placed in plastic petri dishes and were subjected to fermentation. The ground avocado seed particles were divided into three batches for natural fermentation and fermentation with either *Lactobacillus plantarum* or kefir. Each fermentation batch contained 900g (approximately 50 seeds) of the ground samples.

L. plantarum (Fonterra Research Centre, Palmerston North, New Zealand) and kefir (Body Ecology TM, CA, USA) were inoculated into MRS broth (Fort Richard company, New Zealand) one day prior to inoculation. Three batches of ground avocado seed particles subjected to each of the three different fermentation conditions: i) 1% *Lactobacillus plantarum* (Fonterra Research Centre, Palmerston North, New Zealand), ii) 1% kefir (Body Ecology TM, CA, USA) and iii) natural fermentation. After inoculation, the *L. plantarum* fermented samples were incubated in a carbon dioxide incubator (Thermo Fisher Scientific, USA) (37 ± 2°C) over seven days of fermentation, in order to create a relatively anaerobic condition for growth of *L. plantarum*. kefir and naturally fermented samples were incubated in an incubation room (37 ± 2°C) for seven days. Each batch was fermented in triplicates.

3.3 Drying of fermented ground avocado seed particles

After fermentation, fermented avocado seeds were further subjected to roasting. Fermented ground avocado seed particles were firstly dried in plastic petri dishes without cover at 60 °C for 48 h in a desiccator (Harvest Maid Deluxe FD1000). Dried avocado seed samples were kept in a desiccator until the roasting process was carried out.

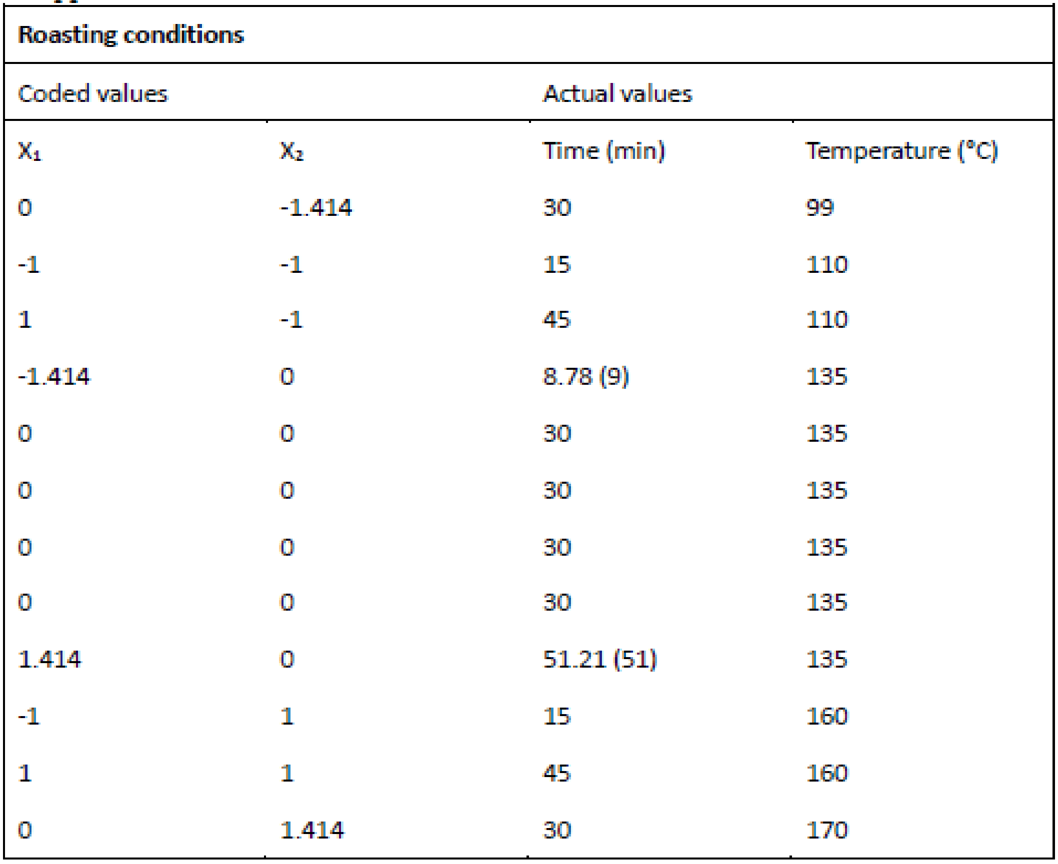
3.4 Experimental design for roasting

After drying, all triplicate samples of dried fermented avocado samples were subjected to roasting. The weight of samples used for roasting was 720g. The roasting process was carried out using an oven (Magellano Digital, PIRON, Italy). A central composite design (CCD) was used to investigate the effects of roasting dried fermented ground avocado seed particles using a two-factor five-point pattern (*Spada, et al. 2017*). The two factors were roasting time (15–45 min) and roasting temperature (110–160 °C), testing at five points with four repetitions at central one giving a total of 12 treatments (*Krysiak, et al. 2013*). The coded and actual values of the 12 treatments using the two factors (roasting temperature and time) are shown in Table 3.1. Each trial was carried for the three dried fermented samples (*L. plantarum,* kefir and naturally fermentation) and their triplicates. Six variables (responses) namely, L*, a* and b* values for colour, TPC, as well as CUPRAC and FRAP assays for antioxidant capacity determinations were evaluated in this study. Response surface methodology (RSM) was used to evaluate the effect of roasting conditions on dried fermented ground avocado seed particles, in terms of colour, TPC and antioxidant capacity. The data obtained were analysed using the Minitab v. 17 Statistical Software (Minitab Inc., Coventry, UK). An equation Y (Y=f (X_1, X_2) for each variable, in terms of two independent factors X_1 (roasting temperature) and X_2 (roasting time) was assumed. The quadratic function was used in order to approximate the function f:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_{11} X_1^2 + B_{22} X_2^2 + B_{12}X_1 X_2$$

where B_0, B_1, B_2, B_{11}, B_{22}, B_{12} were the corresponding regression coefficients and X_1, X_2 were two coded factors for roasting time (min) and temperature (°C), respectively. The equations were shown in Appendix 1.

Table 3.1 Experimental design showing coded and actual values of roasting temperature and time used in the central composite design (CCD) employed. Two factors (roasting temperature and time) were coded as X1 and X2, respectively and the equations showing the quadratic polynomial model that was fitted to each response were shown in Appendix 1.



Roasting conditions			
Coded values		Actual values	
X_1	X_2	Time (min)	Temperature (°C)
0	-1.414	30	99
-1	-1	15	110
1	-1	45	110
-1.414	0	8.78 (9)	135
0	0	30	135
0	0	30	135
0	0	30	135
0	0	30	135
1.414	0	51.21 (51)	135
-1	1	15	160
1	1	45	160
0	1.414	30	170

3.5 Production of roasted fermented avocado seed powder

After roasting, the roasted dried fermented ground avocado seed particles were cooled to 25°C and further used a mortar and pestle to ground into powder. The roasted fermented avocado seed powder was stored in food-grade plastic bags in a freezer (-17°C) for chemical analysis.

3.6 Microbial analysis

According to previous studies *(Xiong, et al. 2012; Lefeber, et al. 2011; Fleet & Zhao 2018)*, fermentation was carried out for 7 days under three fermentation conditions (*Lactobacillus plantarum*, kefir and natural fermentation). In (terms of microbial analysis, the fermented ground avocado seed particles were collected at 37°C on 0, 2, 5 and 7 days of fermentation from each fermentation treatment carried out (*L. plantarum*, kefir and natural fermentation). The inoculated ground seed particle samples at day 0 under each fermentation condition were the unfermented samples. The number of microflorae on culture medium was evaluated by plate counting. Briefly, 1g of the fermented ground avocado seed particles was diluted aseptically with 9 mL of peptone water to prepare a series of seven dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}). After that, the diluted samples (0.1ml) were spread on specific growth media and plate counts were determined on a daily basis over 7 days of fermentation. In total triplicate experiments were carried out for the three fermentation conditions (*L. plantarum*, kefir and natural fermentation). For each treatment carried out, duplicated determinations of plate counts were carried out.

For *L. plantarum* fermented samples, the Lactobacillus MRS agar (Fort Richard company, New Zealand) was used to determine microbiological growth. With the kefir and naturally fermented samples, Lactobacillus MRS agar, Acetobacter agar, and Malt Exact agar (Fort Richard company, New Zealand) were employed to determine microbiological growth. The microflora on Lactobacillus MRS agar was enumerated after anaerobic incubation under CO_2 at 37°C for 7 days. The microflora on Acetobacter agar and Malt Exact agar were enumerated after aerobic incubation at 37°C for 7 days. The results of plate counts were expressed as log cfu/g.

3.7 Chemical analysis

3.7.1 Chemicals

Methanol, acetone, Folin-Ciocalteu reagent, sodium carbonate (Na_2CO_3), $CuCl_2$, Neocuproine (Nc), ammonium acetate (NH_2Ac), HCl, $FeCl_3$, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), sodium acetate trihydrate, glacial acetic acid, ascorbic acid and gallic acid were obtained from Sigma-Aldrich (Sigma-Aldrich, Australia) for the determination of TPC and antioxidant capacity. All chemical analysis described in the following sections (3.7.2 – 3.7.5) were carried out on both fermented ground avocado seed samples and roasted fermented avocado seed powder.

3.7.2 Polyphenol extraction

TPC determination was carried out on both unfermented (day 0) and fermented ground avocado seeds that further underwent roasting. Unfermented and fermented ground avocado seed particles or roasted fermented avocado seed powder (0.1g) was weighed in a centrifuge tube and extracted with 4ml 50% methanol. The centrifuge tubes were homogenized using a disperser (IKA, T 25 digital ULTRA-TURRAX®) at 14000rpm for 90 seconds and then kept for one hour. After that, the centrifuge tubes were centrifuged using a centrifuge (Eppendorf, Centrifuge 5810R, South Pacific) at 1500rpm for 15mins and the supernatant liquid from the top was extracted and transferred into a 10ml volumetric flask. The extraction was repeated with 70% acetone and the steps described above after the solvent extraction step was repeated. Deionized water was added to the same volumetric flask containing methanol and acetone extracts and made up to the 10ml mark. After that, the extract (1ml) was transferred to another 10ml volumetric flask and diluted to the 10ml mark using distilled water. The extracts were stored in a freezer at -17°C until further analysis.

3.7.3 Estimation of TPC

The determination of TPC of both fermented avocado seeds and roasted fermented avocado seed powder were determined by Folin-Ciocalteu assay *(Wang, et al. 2010).* Gallic acid (0.1g) was dissolved and made up to volume in a 100 ml volumetric flask, equivalent to a 1g/L stock solution. A series of standard solutions were prepared with equivalent concentrations of 80, 40, 20, 10, 5, 2.5, 0 mg/L by using the stock solution and distilled water and then measured the absorbance at 765 nm to generate a standard curve.

The sample extracts (1ml) obtained in Section 3.7.2 was mixed with 500ul Folin–Ciocalteu reagent in a glass vial and kept for 5 mins. Then added 1.5ml of 20% Na_2CO_3

and the solution was incubated for two hours in the dark (a foil was used to cover the glass vials). Absorbance at 765 nm was measured with a Ultrospec 2100 Pro spectrophotometer (Amersham Pharmacia Biotech, UK). The TPC in the avocado seed extracts was expressed as mg gallic acid equivalents (GAE)/g of samples extracted weight. All the determinations were performed in triplicates.

3.7.4 Antioxidant analysis

In this study, we used the CUPRAC and FRAP assays, which were both electron transfer (ET)- based assays, to measure the antioxidant capacity as a result of reduction of antioxidants. The degree of colour change was correlated to the concentration of antioxidants in the sample. Because 1) These methods were easily and diversely applicable in conventional laboratories using standard colorimeters rather than necessitating sophisticated equipment and qualified operators. 2) The CUPRAC reagent was more stable and accessible than other chromogenic reagents (e.g., ABTS, DPPH). And the redox reaction giving rise to a coloured chelate of Cu(I)-Nc was relatively insensitive to a number of parameters adversely affecting certain reagents such as DPPH, i.e., air, sunlight, humidity, and pH, to a certain extent. 3) FRAP and CUPRAC assay had different sensitivities to specific antioxidants. e.g. FRAP assay had higher sensitivity to ascorbic acid while lipophilic antioxidants only can be detected by CUPRAC assay. 4) The redox reaction that occurred at different pH values. CUPRAC assay was carried out at pH 7 buffer as opposed to the acidic conditions (pH 3.6) of FRAP. And physiological pH 7 conditions may enhance the reactions of antioxidant compounds.

3.7.5 CUPRAC assay

The CUPRAC (cupric reducing antioxidant capacity) assay was employed to determine antioxidant capacity of sample extracts based on *Apak, et al. (2007)*. In this assay, ascorbic acid (0.1g) was dissolved and made up to volume in a 100 ml volumetric flask, equivalent to a 1g/L stock solution. A series of standard solutions were prepared using a 1g/L ascorbic acid stock solution. The final concentrations of the standard solutions were 80, 40, 20, 10, 5, 2.5, and 0 mg/L and then measured the absorbance at 450 nm to generate a standard curve.

1 ml sample extracts obtained as described in Section 3.7.2 or distilled water was mixed with 1 ml $CuCl_2$ (0.01M), 1 ml NH_4AC (1 M, pH7), 1 ml Neocuproine (Nc)(0.075 M), and 0.1 ml deionized water to yield a total volume of 4.1 ml. The extracts were incubated for 5 min at room temperature, and the absorbance value was measured at 450 nm with a Ultrospec 2100 Pro (Amersham Pharmacia Biotech, UK) spectrophotometer. The CUPRAC values of the avocado seed extracts were expressed as mg ascorbic acid equivalent/g of sample. All the determinations were performed in triplicates.

3.7.6 FRAP assay

The ferric reducing antioxidant power (FRAP) assay was applied to carry out antioxidant capacity sample extracts as described by *Morais, et al. (2015*). Ascorbic acid (0.1g) was dissolved in some water and then made up to volume in a 100 ml volumetric flask to make a 1g/L stock solution. A series of standard solutions were prepared using a 1g/L ascorbic acid stock solution. The final concentrations of the standard solutions were 80, 40, 20, 10, 5, 2.5, and 0 mg/L and then measured the absorbance at 593 nm to generate a standard curve.

The FRAP reagent was obtained by mixing 10ml 300 mM Acetate Buffer, 1ml 10 mM TPTZ (dissolved in 10ml 40 mM HCl) and 1ml 20 mM $FeCl_3$ together and then kept at 36°C water bath. After that, freshly prepared FRAP reagent (2 ml) was added into 0.1ml of the sample extracts (obtained in Section 3.7.2) (or distilled water). Then distilled water (0.9 ml) was added in a glass vial, mixed it with sample extracts and left to rest for four minutes. Finally, the absorbance value of the solution was measured at 593 nm with a Ultrospec 2100 Pro (Amersham Pharmacia Biotech, UK) spectrophotometer. The FRAP values of avocado seed extracts were expressed as mg ascorbic acid equivalent/g of sample. All the determinations were performed in triplicates.

3.8 Colour analysis

The colour of samples was determined by using a Nix Pro Colour Sensor (Nix Sensor Ltd., Canada) only for the roasted fermented avocado seed powders. The powders were mixed thoroughly, and all the determinations were performed in triplicates. The results of colour analysis were expressed as L*, a*, and b* values of roasted fermented ground avocado seed powders.

3.9 Statistical Analysis

For microbial analysis, we enumerated the microorganisms on 0, 2, 5 and 7 days of fermentation from each fermentation treatment carried out (*L. plantarum*, kefir and natural fermentation). The mean value of the plate counts of LAB, yeast and AAB from fermented samples under each fermentation treatment on the different days were determined. A two-way ANOVA was used to evaluate the results of plate counts. The two variables were fermentation days (0, 2, 5, and 7 days) and culture (*L. plantarum*, kefir and natural fermentation). Results were analysed by using Minitab v. 17 Statistical Software (Minitab Inc., Coventry, UK) using a two-way ANOVA.

In terms of chemical analysis, the TPC, as well as CUPRAC and FRAP antioxidant assays of both unfermented (day 0) and fermented ground avocado seed samples were carried out. Minitab v. 17 Statistical Software (Minitab Inc., Coventry, UK) was used to evaluate the results and carry out a two-way ANOVA. The two independent variables examined were fermentation (with or without fermentation) and culture (*L. plantarum*, kefir and natural fermentation). The significances ($p < 0.05$) of parameters were expressed by the p-value and represented by the * symbol ($p^{***} < 0.001$; $p^{**} < 0.01$; $0.01 <= p^{*} < 0.05$; $p >= 0.10$). Post-hoc comparisons were performed using the Tukey's HSD test to evaluate whether the differences between means were significant ($P< 0.05$) or not.

All analytical determinations of roasted avocado seed powders were carried out in triplicates. The data of L*, a* and b* values for colour, TPC, as well as CUPRAC and FRAP antioxidant assays of samples were determined by a two-way ANOVA in the Minitab v. 17 Statistical Software (Minitab Inc., Coventry, UK). The generated two-way ANOVA table included regression coefficients of all linear, quadratic, and interaction terms for two variables (temperature and time) and one covariate (culture). The significance ($p < 0.05$) of all parameters (temperature, time and culture) were expressed by the F value and represented by the * symbol (p*** < 0.001; p** < 0.01; 0.01 <= p* < 0.05; p >= 0.10). In addition, main effect plots, interaction plots and contour plots were generated and the main effect and interactions of two variables (temperature and time) and one covariate (culture) were also determined. These plots displayed the effects of roasted avocado seed powders on TPC, colour and antioxidant activities.

4. Results and discussion

4.1 Microbial analysis

Fermentation of ground avocado seed particles was carried out by allowing natural fermentation (without inoculation) and by inoculating with either *L. plantarum* or kefir. Results show that microorganisms grew in all avocado seed samples that include the inoculated and the naturally fermented ones. The number of colony-forming units (cfu) increased significantly ($p<0.001$) as the fermentation time increased in all samples. The cfu of LAB (Table 4.1), AAB (Table 4.2), and yeast (Table 4.3) significantly ($p<0.001$) increased in the first five days when compared to the unfermented (day 0) samples and reached maximum at that point. The differences were no more than 1 log cfu between the day5 and day7 counts of LAB and yeast, indicating that the growth had reached its stationary phase.

Table 4.1 The number of lactic acid bacteria cells in fermented avocado seeds over 7 days of fermentation.

	Fermentation time (Day)(FT) (Log cfu/g)				**F-value**		
Culture	0	2	5	7	Culture	FT	Culture*FT
L. plantarum (MRS)	7.24 ± 0.24^{Ad}	7.66 ± 0.08^{Ac}	8.63 ± 0.26^{Aa}	8.46 ± 0.33^{Ab}	491.21 ***	1199.04 ***	335.22 ***
Kefir (MRS)	5.56 ± 0.5^{Bd}	7.64 ± 0.09^{Bc}	8.83 ± 0.04^{Ba}	8.15 ± 0.04^{Bb}			
Natural fermentation (MRS)	$<2^{Cd}$	5.35 ± 0.07^{Cc}	9.23 ± 0.11^{Ca}	8.65 ± 0.05^{Cb}			

The colony forming units (cfu) values are represented as means ± standard deviations. Values with a different letter are significantly different ($p<0.05$) according to the Tukey's HSD (Honestly Significant Difference) test. Different uppercase superscripts (A, B, C) represent a statistically significant effect within the column and lowercase superscripts (a, b, c) indicate significant differences across each row. * symbol represents p value (p*** < 0.001; p** < 0.01; 0.01 <= p* < 0.05; p >= 0.10).

Table 4.1 summarizes the growth of lactic acid bacteria (LAB) with kefir, *L. plantarum* and natural fermentation of avocado seeds affected by fermentation over 7 days. The LAB community in all three fermentations significantly increased after 5 days of fermentation when compared to the unfermented samples (day 0 samples), and maximum growth occurred after 5 days of fermentation ($p<0.001$). The increase in LAB counts during the fermentation was probably due to the anaerobic environment that was gradually formed (*Xiong et al. 2012*) and indicated the presence of sufficient nutrients to support LAB growth. Subsequently, the lack of increase of no more than 1 log cfu after 5 days indicated that LAB growth had reached its stationary phase, which may be due to that LAB were fastidious microorganisms and they required more nutrients (e.g. amino acids, carbohydrates) for their growth *(Liu, et al. 2016)*. In this study, unfermented samples (day 0 samples) of naturally fermented avocado seeds showed no detectable LAB ($<10^2$ cfu/g) at first 24h and then significantly increased. Similar observations can be observed for cocoa beans *(Fleet & Zhao 2018)* and sauerkraut *(Palani, et al. 2016)* that the number of LAB was extremely low ($<10^2$ cfu/g) at the beginning of spontaneous fermentation and significantly increased during fermentation. Interestingly, the highest numbers of LAB in the natural fermentation were significantly higher than those in fermentations inoculated with *L. plantarum* or kefir. The results of natural fermentation suggested that there may be natural LAB microflora present on the avocado seeds and that the incubation conditions favoured their growth *(Zhang, et al. 2012)*.

Table 4.2 The number of acetic acid bacteria cells in fermented avocado seeds over 7 days of fermentation.

	Fermentation time (Day)(FT) (Log cfu/g)				**F-value**		
Culture	0	2	5	7	Culture	FT	Culture*FT
Kefir (AA)	$<2^{Bc}$	6.51 ± 0.36^{Bb}	7.51 ± 0.10^{Ba}	7.59 ± 0.14^{Ba}	110.69 ***	5604.49 ***	176.54 ***
Natural fermentation (AA)	$<2^{Ac}$	5.35 ± 0.10^{Ab}	9.39 ± 0.09^{Aa}	9.11 ± 0.05^{Aa}			

The colony forming units (cfu) values are represented as means± standard deviations. Values with a different letter are significantly different ($p<0.05$) according to the Tukey's HSD (Honestly Significant Difference) test. Different uppercase superscripts (A, B, C) represent a statistically significant effect within the column and lowercase superscripts (a, b, c) indicate significant differences across each row. * symbol represents p value (p*** < 0.001; p** < 0.01; 0.01 <= p* < 0.05; p >= 0.10)

The counts of acetic acid bacteria (AAB) in avocado seeds fermentations with the kefir starter cultures are summarized in Table 4.2. No colonies ($<10^2$ cfu/g) of AAB were found on Acetobacter agar (AA) for the day0 samples (unfermented samples) of kefir and natural fermentation. The colonies of AAB were not detected until 48h which was consistent with the study of *Lefeber, et al. (2011)* that no colonies of AAB were found in spontaneously fermented cocoa beans at the beginning of fermentation. *Lefeber, et al. (2011)* further explained that AAB requires oxygen and could only grow when the quantity of oxygen was sufficient to provide for the oxidation of ethanol by AAB. Subsequently, the numbers of AAB in both kefir and naturally fermented samples increased significantly from day2 until day7 of fermentation when compared to the unfermented samples (day 0 samples). This may be associated with the sufficient nutrients for AAB and their oxidation of ethanol produced by yeasts during fermentation *(Fleet & Zhao, 2018)*. Furthermore, the population of AAB in the naturally fermented avocado seeds (9.39 log10 cfu/g) was statistically significantly higher than kefir samples (7.51 log cfu/g). This may be due to the mutual interactions between the acetic acid bacteria and other microorganisms, which were naturally present in the avocado seeds that could enhance the growth of AAB *(Papalexandratou, et al. 2013).*

Table 4.3 The number of yeast cells in fermented avocado seeds over 7 days of fermentation.

	Fermentation time (Day)(FT) (Log cfu/g)				**F-value**		
Culture	0	2	5	7	Culture	FT	Culture*FT
Kefir (ME)	5.44 ± 0.42^{Ad}	7.13 ± 0.07^{Ac}	8.85 ± 0.05^{Aa}	7.84 ± 0.32^{Ab}	396.86 ***	1739.03 ***	473.56 ***
Natural fermentation (ME)	$<2^{Bd}$	5.31 ± 0.04^{Bc}	9.35 ± 0.11^{Ba}	9.10 ± 0.04^{Bb}			

The colony forming units (cfu) values are represented as means± standard deviations. Values with a different letter are significantly different ($p<0.05$) according to the Tukey's HSD (Honestly Significant Difference) test. Different uppercase superscripts (A, B, C) represent a statistically significant effect within the column and lowercase superscripts (a, b, c) indicate significant differences across each row. * symbol represents p value ($p^{***} < 0.001$; $p^{**} < 0.01$; $0.01 <= p^{*} < 0.05$; $p >= 0.10$).

Table 4.3 shows the yeast counts of fermented avocado seeds with kefir and natural fermentation. In this study, only unfermented samples (day 0 samples) of naturally fermented avocado seeds showed no detectable yeasts (<10^2 cfu/g) and the numbers of yeast first detected at 48h. Subsequently, the yeast count kept increasing significantly during fermentation and reached a maximum on day 5 (8.85 in kefir samples and 9.35 log cfu/g in the natural samples). The similar findings were reported by *Fleet & Zhao, (2018)* that no yeast was detected within 48h in spontaneously fermented cocoa beans and then the counts significantly increased for both spontaneous and inoculated fermentation. This increase in yeast counts during the fermentation may be due to the anaerobic environment that was gradually formed and indicated the presence of sufficient nutrients to support yeast growth (*Bressani, et al. 2018*). In this fermentation, the lack of increase of no more than 1 log cfu after 5 days indicated that yeast growth had reached its stationary phase. This was probably due to the interactions between the yeast and other microflora *(Krusong & Vichitraka, 2010)* or the domination of other microflora like AAB during fermentation processing *(Fleet & Zhao, 2018).* Moreover, the community of yeast on day5 of fermentation in the kefir samples was statistically lower than in the naturally fermented samples. This may be related to the accumulation of acetic acids produced by AAB reduced the pH value to below the normal physiological values, and directly affected transport or enzymatic activities, such as enolase, a key enzyme of glycolysis, resulting in inhibition of yeast growth *(Krusong & Vichitraka, 2010).*

In this study, the number of LAB, AAB, and yeast in avocado seeds significantly ($P<0.05$) increased over 7 days of fermentation in all three fermentation conditions (*L. plantarum,* kefir and natural fermentation) in comparison to unfermented (day 0) ones. Similarly, *Fleet, et al (2018)* reported that the numbers of LAB, AAB, and yeast in spontaneously and inoculated fermented inoculated cocoa beans significantly increased after fermentation for 5 days when compared to the unfermented (day 0) ones. In comparison to inoculated fermentation with kefir and *L. plantarum* samples, naturally fermented avocado seeds had statistically higher microbial concentration after 5 days of fermentation. This probably indicated that some microorganisms were naturally present in the avocado seeds and that the fermentation conditions supported their strong growth. Also, there might be some mutual interactions occurring between these microorganisms that were favourable for their growth *(Papalexandratou, et al. 2013; Krusong & Vichitraka, 2010)*.

4.2 Chemical analysis

4.2.1 Phenolic content and antioxidant activities of fermented avocado seeds

Table 4.4 Phenolic content and antioxidant capacity of fermented avocado seeds.

	Phenolic content (mg GAE/g)		**CUPRAC (mg ascorbic acid equivalent/g)**		**FRAP (mg ascorbic acid equivalent/g)**		**P-value**
Bacteria Species (BS)	Without fermentation	Fermentation for 5 days	Without fermentation	Fermentation for 5 days	Without fermentation	Fermentation for 5 days	Fermentation
L. plantarum	20.56±5.28^{Aa}	16.92±3.65^{Bab}	61.69±12.98^{Aa}	50.01±13.46^{Bab}	15.40±4.66^{Aa}	10.43±2.63^{Bab}	***
Kefir	22.46±1.50^{Aa}	11.99±1.91^{Bbc}	64.35±5.71^{Aa}	31.61±4.11^{Bbc}	16.14±0.79^{Aa}	6.73±0.96^{Bbc}	***
Natural fermentation	22.25±0.84^{Aa}	4.37±0.64^{Bc}	64.09±2.52^{Aa}	15.39±1.99^{Bc}	16.02±1.33^{Aa}	3.56±0.26^{Bc}	***

The values were represented as mean ± standard deviation. Values with a different letter are significantly different ($p<0.05$) according to the Tukey's HSD (Honestly Significant Difference) test. Different lowercase superscripts (a, b) indicate significant differences within the row in each chemical assay. Different uppercase superscripts (A, B) indicate significant differences within the column in each chemical assay. * symbol represents p value (p*** < 0.001; p** < 0.01; 0.01 <= p* < 0.05; p >= 0.10).

In this study, the microbial counts of *L. plantarum*, kefir and naturally fermented avocado seeds were significantly the highest from day 5 until day 7 of fermentation. It was reported by *Kachouri, et al. (2015)* that the highest antioxidant capacity using DPPH assay and antioxidant activity of *L. plantarum LAB 1* increased with the increase of colony-forming unit (cfu) and resulted in the highest cfu of *L. plantarum LAB 1*. TPC and antioxidant analysis of fermented avocado seeds were further carried out on day 5 samples as we found the number of colony-forming units (cfu) increased significantly and reached a peak at day 5 for all three fermentation conditions. Differences were no more than 1 log cfu between the day 5 and day7 counts of microflora indicating that the growth of microorganisms had reached its stationary phase.

Table 4.4 shows the changes in TPC and antioxidant capacities of fermented avocado seeds inoculated with *L. plantarum* and kefir, as well as samples that were naturally fermented. The results indicated that the TPC and antioxidant capacities of both inoculated (*L. plantarum* and kefir) and naturally fermented avocado seeds significantly ($P < 0.001$) decreased compared to unfermented (day 0) samples. Similarly, both spontaneously and inoculated fermented Chétoui olives resulted in 32–58% reduction in TPC and 50–72% reduction in antioxidant activity, compared to unfermented samples *(Othman et al. 2009)*. Additionally, *Gan, et al. (2017)* found that the reduction of TPC was observed in some naturally fermented beans and LAB-fermented beans, compared to unfermented samples, suggesting the metabolism of polyphenols happened both in spontaneous and inoculated fermentation.

In terms of TPC and antioxidant capacities, there were no significant differences as expected between the unfermented (day 0) samples under all three fermentation conditions in each chemical assay. However, fermented avocado seeds under all three fermentation conditions were significantly ($P<0.05$) different from each other. TPC and antioxidant capacities of samples inoculated by *L. plantarum* were significantly ($P<0.05$) the highest than kefir fermented samples. The TPC and antioxidant activity of naturally fermented samples were the significantly lowest. These changes will be discussed in detail in the following sections.

Total phenolic content (TPC)

As seen in Table 4.4, a significant (P <0.001) decrease was observed in the TPC of fermented avocado seeds that were inoculated with *L. plantarum* and kefir, and naturally fermented samples compared to unfermented (day 0) avocado seeds. This decrease was probably related to the polyphenols were metabolized and degraded into other compounds by enzymes such as β-glucosidase that produced by microbe like LAB (Gan, et al. 2017). *Filannino, et al. (2015)* also that showed all strains of *L. plantarum* significantly decreased the concentration of phenolic acids of cherry juice and broccoli puree during fermentation for 24h by about 70% compared to the unfermented samples. Similarly, *Hashemi, et al. (2017)* demonstrated a significant decrease in TPC in lemon juice fermented by *L. plantarum* for 48h in comparison of unfermented samples. They further explained that the decreasing trend was mainly attributed to phenolic compounds that were metabolized by *L. plantarum*. Moreover, the lower TPC of fermented avocado seed samples in our study may be associated with oxidation reactions during fermentation. Similar results were observed in fermented rambutan seeds *(Mehdizadeh, et al. 2015)* that the TPC of was significantly reduced by 59% after 10 days of spontaneous fermentation, compared to unfermented rambutan seeds. This reduction was attributed to the oxidation of polyphenols by polyphenol oxidase.

After fermentation, a significantly (P<0.05) higher value of TPC was observed in the fermented avocado seeds inoculated with *L. plantarum* (16.92 mg GAE/g), followed by kefir (11.99 mg GAE/g), and naturally fermented avocado seeds (4.37 mg GAE/g). The fact that fermented avocado seeds inoculated with *L. plantarum* contained the significantly highest TPC may be related to enzymatic hydrolysis of polymerized phenolic compounds and microbe-induced destruction of the plant cell wall that can subsequently result in the release of polyphenols. Besides that, the naturally fermented avocado seeds contained the significantly lowest TPC, which was probably due to the presence of other microorganisms naturally present in the substrates that can enhance the metabolism of polyphenols during fermentation. The finding in this study was also consistent with the study of *Othman et al. (2009)* that the TPC in spontaneously fermented green olives, black olives and varicoloured olives resulted in a higher reduction of TPC compared to fermented olives inoculated by *L. plantarum* (ANOVA results not reported in this study).

Antioxidant capacity

Fermentation significantly (P <0.001) decreased the antioxidant capacity for both inoculated and naturally fermented avocado seeds in comparison to unfermented (day 0) samples. Similarly, *Othman, et al. (2009)* found that both spontaneous and inoculated *(L. plantarum)* fermentation of Chétoui olive for 70 days led to a decrease in antioxidant activity by 50-72% compared to unfermented samples (ANOVA results not reported). This reduction in antioxidant capacity was not only related to the decrease of related antioxidant enzyme activities in the fermented samples *(Gan, et al. 2017)*, but also attributed to the metabolism and microbial degradation of antioxidants into other lower antioxidant activity compounds *(Svensson, et al 2010; Othman et al. 2009)*. Furthermore, *Filannino, et al. (2015)* reported that *L. plantarum* strains significantly decreased the concentration of phenolic acids of cherry juice and broccoli puree during fermentation for 24h by about 70% compared to unfermented samples. They explained that this decrease was related to the consumption of protocatechuic acids, caffeic acids by *L. plantarum* during fermentation. *Svensson, et al (2010)* also found that the phenolic acids and flavonoids significantly decreased in red sorghum fermented by LAB compared to unfermented samples. They further suggested that the decrease was likely due to the LAB degrading and metabolizing antioxidants such as ferulic acids, and caffeic acids during fermentation. Besides that, the interactions between polyphenols and/or other components present in plant extracts may also influence the results of TPC and antioxidative capacity. In the spontaneous fermentation of tea, *Kim, et al (2011)* found that the loss in the transformation from tea catechins to theaflavins contributed to the lower antioxidant capacity of fermented tea than unfermented samples.

After fermentation, the antioxidant capacities of fermented avocado seeds inoculated by *L. plantarum*, kefir and natural fermentation were significantly (P<0.05) different from each other. Fermented avocado seeds inoculated by *L. plantarum* had the had significantly (P<0.05) the highest antioxidant capacity (CUPRAC and FRAP assays), followed by kefir fermented samples, while naturally fermented samples had significantly (P<0.05) the least antioxidant capacity. Similarly, *Đorđević, et al. (2010)*

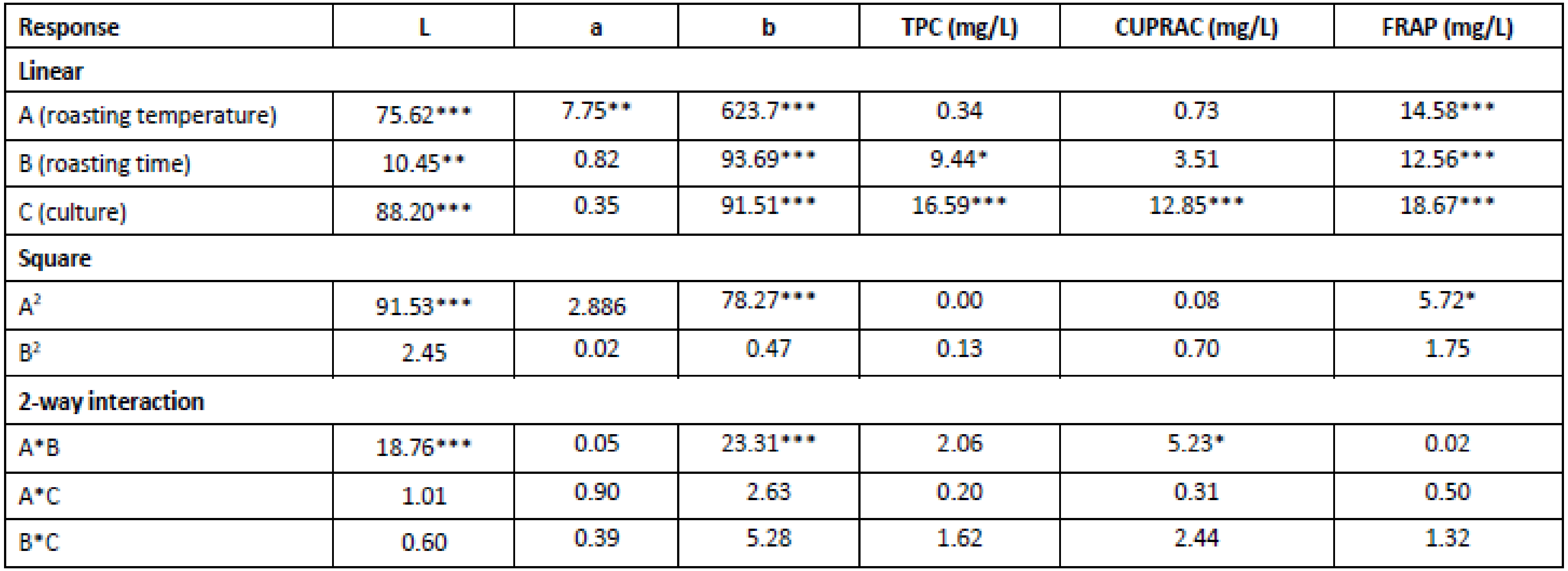
demonstrated that cereal samples fermented with *L. rhamnosus* had higher antioxidant activity than fermented samples with *S. cerevisiae*. In this study, fermented avocado seeds inoculated with *L. plantarum* had the significantly higher antioxidant capacity than kefir and naturally fermented samples. This higher result of *L. plantarum* may be associated with the destruction of the plant cell wall that subsequently resulted in the release of bound antioxidants *(Gan, et al. 2017)*. Another possibility of this result may attribute to plant-based LAB themselves have antioxidant activity *(Hur, et al. 2014)*. Similar results were observed in another two strains of *L. fermentum* and *Streptococcus thermophilus* that they had significant antioxidative activity against superoxide dismutase (SOD) by decreasing SOD oxidative stress *(Kullisaar, et al. 2002)*.

Naturally fermented avocado seeds contained the significantly lowest antioxidant capacity and this may be related to the presence of other microorganisms naturally present in the substrates that can enhance the metabolism of antioxidant phenolic compounds during fermentation. *Chinma, et al. (2014)* reported that the naturally fermented rice bran flour had significantly lower antioxidant capacity than those fermented by yeast. *Othman, et al. (2009)* further reported that TEAC values of spontaneous fermentation in green, varicoloured and black olives showed a higher reduction compared to inoculated fermentation (*L. plantarum*) of samples (ANOVA results not reported).

Results in this study indicated that the TPC and antioxidant capacity of avocado seeds significantly decreased after fermentation in all three fermentation conditions (*L. plantarum*, kefir and natural fermentation). This might be related to the metabolism of polyphenols that happened both in spontaneous and inoculated fermentation *(Svensson, et al 2010)*. In addition, the decrease may also be associated with oxidation reactions during fermentation *(Mehdizadeh, et al. 2015)*. Significantly higher values of TPC and antioxidant capacity were observed in the fermented avocado seeds inoculated with *L. plantarum*, followed by kefir, and naturally fermented avocado seeds. This may be attributed to the fact that *L. plantarum* is associated with the destruction of the plant cell wall that can subsequently result in the release of bound antioxidants *(Rui, et al. 2016; Gan, et al.2017)*. In addition, it may be that plant-based LAB themselves have antioxidant activity *(Hur, et al. 2014)*.

4.2.2 Total phenolic content (TPC) and antioxidant activities of roasted avocado samples

Table 4.5 The two-way ANOVA of the regression models and regression coefficients for parameters used in the roasting of fermented avocado seeds. (All the fermented seed have been fermented for 5 days, dried and roasted.) A = Roasting temperature, B = Roasting time, C = Culture. * symbol represents p value (p*** < 0.001; p** < 0.01; 0.01 <= p* < 0.05; p >= 0.10).



Response	L	a	b	TPC (mg/L)	CUPRAC (mg/L)	FRAP (mg/L)
Linear						
A (roasting temperature)	75.62***	7.75**	623.7***	0.34	0.73	14.58***
B (roasting time)	10.45**	0.82	93.69***	9.44*	3.51	12.56***
C (culture)	88.20***	0.35	91.51***	16.59***	12.85***	18.67***
Square						
A^2	91.53***	2.886	78.27***	0.00	0.08	5.72*
B^2	2.45	0.02	0.47	0.13	0.70	1.75
2-way interaction						
A*B	18.76***	0.05	23.31***	2.06	5.23*	0.02
A*C	1.01	0.90	2.63	0.20	0.31	0.50
B*C	0.60	0.39	5.28	1.62	2.44	1.32

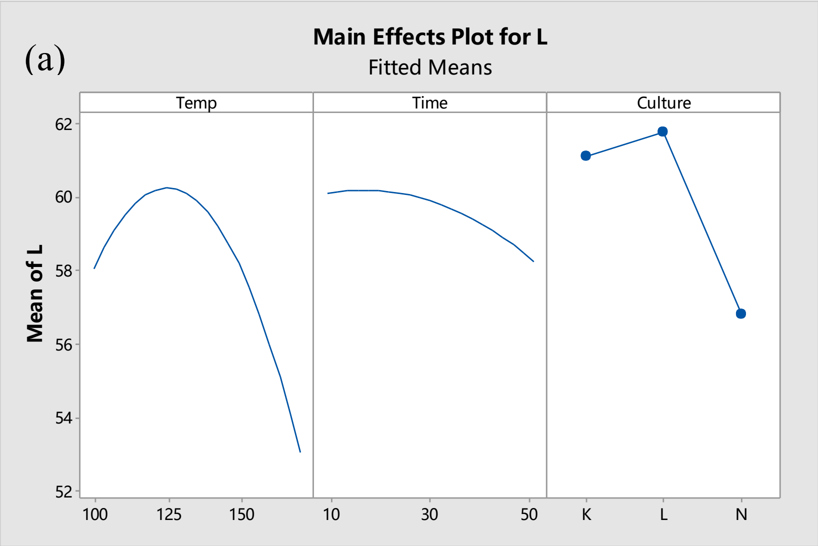
The two-way ANOVA of the regression models and regression coefficients for six variables are presented in Table 4.5, indicating each response (L*, a*, b*, TPC, and antioxidant capacity) was influenced by roasting time and temperature during roasting processing of fermented avocado seeds. The larger F-values and the smaller numerical p values in the estimated regression coefficients of fermented avocado seeds signify that the related regression coefficients were highly significant (* symbol represents p-value and p*** < 0.001; p** < 0.01; 0.01 <= p* < 0.05; p >= 0.10). The regression equations for colour values (L*, a* and b*), TPC, and antioxidant capacity in this study are summarized in Appendix 1.

The culture in Table 4.5 refers to naturally fermented, and kefir and *L. plantarum* fermented avocado seeds. As seen in Table 4.5, there were highly significant ($p < 0.001$) parameters in L*- and b*-value models, including linear roasting temperature, linear roasting time, linear culture, quadratic roasting temperature, and temperature–time interaction. As for a* value model, only the linear roasting temperature parameter was significant ($p < 0.01$), while there was no significance in other linear or quadratic terms. Based on the TPC model, the linear roasting time and linear culture parameters were significant ($p < 0.05$). Meanwhile, the linear culture and temperature–time interaction parameters were significant ($p < 0.05$) in the CUPRAC assay model. In addition, the linear roasting temperature, linear roasting time, linear culture, and quadratic roasting temperature parameters were significant ($p < 0.05$) for the FRAP assay model. The results in this study showed that roasting significantly influenced on the colour and antioxidant capacity of roasted fermented avocado seeds, which were consistent with the results reported on the roasting of fermented rambutan seeds *(Chai, et al. 2019). Spada, et al. (2017)* further demonstrated that roasting time and temperature were important factors contributing to the TPC of both unfermented and fermented jackfruit seeds.

4.2.3 Main effect plots and interaction plots

The two-way ANOVA results were obtained from the regression models and regression coefficients in Table 4.5. As for colour, roasting temperature, roasting time and culture had significantly influenced L* and b* values of the roasted fermented avocado seeds, while roasting temperature only had a significant main effect on a* value. In terms of TPC, roasting time and culture significantly influenced the TPC of roasted fermented avocado seeds. Additionally, the type of culture used for fermentation had a significant main effect on CUPRAC assay results, while roasting temperature, roasting time and culture had significant main effects on FRAP assay results. Furthermore, the interactions between roasting temperature and time were only significant in terms of L*, b* and CUPRAC assay results. Therefore, only the significant main factors and interactions will be further discussed using the main effect and interaction plots based on the two-way ANOVA model that was used in this study (Table 4.5).

L* value



(b)

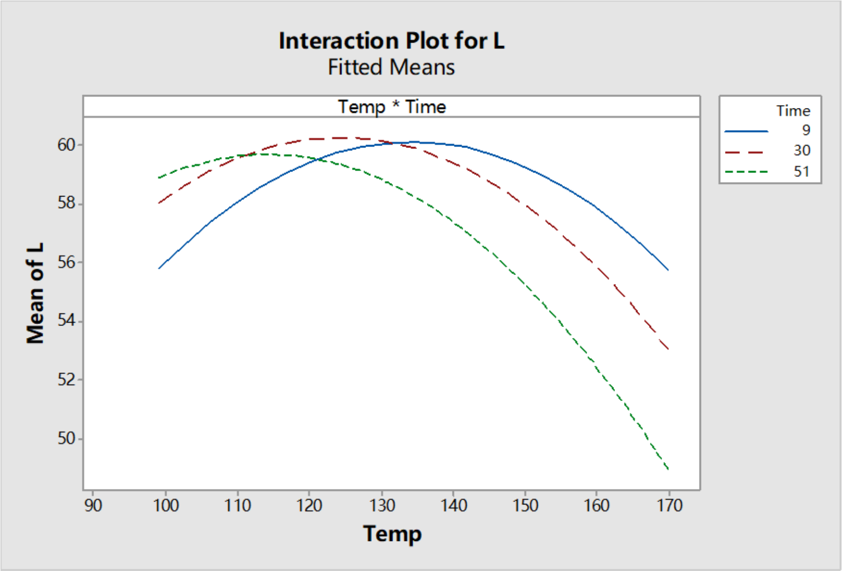


Figure 4.1 The factorial plots for L* value of roasted fermented avocado seeds (a) the main effect plot, (b) the interaction plot.

It can be seen from Figure 4.1(a) that the main effect plots for the factors of roasting time, temperature, and culture, had significant (P <0.001) main effects on the L* value of avocado seeds. With increasing roasting temperature, the L* value of roasted fermented avocado powder significantly increased up to 60.4 at 125°C, and then declined with increasing temperature from 130°C to 170°C. Similarly, in roasted cocoa bean *(Zzaman & Yang, 2014)*, hazelnuts *(Ozdemi, et al., 2000)* and corn kernels *(Chung et al., 2014),* the lightness of roasted samples initially increased and then significantly decreased with roasting at higher temperatures. The denaturation of protein decreased in moisture content *(Ozdemi, et al. 2000)*, and the concentration of oil particles surrounding the protein matrix *(Zzaman & Yang 2014)* could attribute to the increase in L* value (lightening) of roasted samples at the start of roasting. Moreover, the finding that L* value significantly decreased with increase in roasting time (9 to 51min) was consistent with other studies that investigated the effect of roasting on fermented rambutan seeds *(Chai, et al. 2019)*, fermented cocoa beans *(García-Alamilla, et al. 2017)* and roasted peanut kernels *(Bagheri, et al. 2018)*. This decrease in L* value may be due to that the browning reaction that generated Maillard reaction products (brown nitrogenous polymers and melanoidins) was favoured at higher temperatures and longer duration, which are responsible for dark colour formation *(Chai, et al. 2019)*. In terms of culture, roasted fermented avocado seeds inoculated with *L. plantarum* had the highest L* value compared to kefir and naturally fermented samples. The highest L* value in *L. plantarum* samples might be associated with the metabolism of LAB that may change pH values that in turn influenced the formation of Maillard reaction products *(Hur, et al. 2014)*, which were responsible for colour formation.

Two-way ANOVA results in Table 4.5 showed that roasting temperature and time interaction was significant (P <0.001). We can observe from the interaction plot in Figure 4.1 (b) that there were non-parallel lines indicating significant interactions occurring between roasting temperature and time. At the start of roasting at 99°C, L*value of roasted fermented avocado seeds increased with increasing roasting time. However, with increasing roasting temperature between 135°C to 170°C, L* value decreased with increasing roasting time. This finding was supported by results reported in previous studies *(Zzaman & Yang, 2014; Chai, et al. 2019; Ozdemi, et al. 2000)* that non-enzymatic browning generated from the Maillard reaction may produce brown nitrogenous polymers and melanoidin products.

a* value

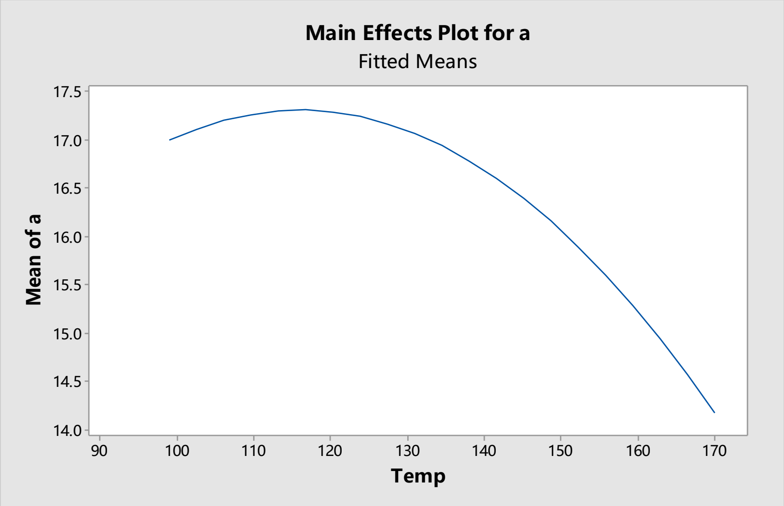


Figure 4.2 The main effect plot of roasting temperature on a* value of roasted fermented avocado seeds.

The main effect of roasting temperature on a* value is summarized in Figure 4.2. In accordance with the two-way ANOVA results shown in Table 4.5, only roasting temperature had a significant (P < 0.01) main effect on a* value (redness) of roasted fermented avocado seeds, showing a curvature with a maximum a* value at around 120°C that then significantly decreased in the range of 120 to 170°C. *García-Alamilla, et al. (2017)* stated that roasting temperature and time showed a negative nonlinear effect on $a*$ value of roasted fermented cocoa beans, which also showed an increase and then a decrease in a* value. The formation of brown pigments from Mallard reaction could attribute to increasing a* value *(Chung, et al. 2014; Somporn et al. 2011),* while the destruction of pigment during fermentation *(García-Alamilla, et al. 2017)* and roasting *(Somporn et al. 2011)* may decrease the a*value.

b* value

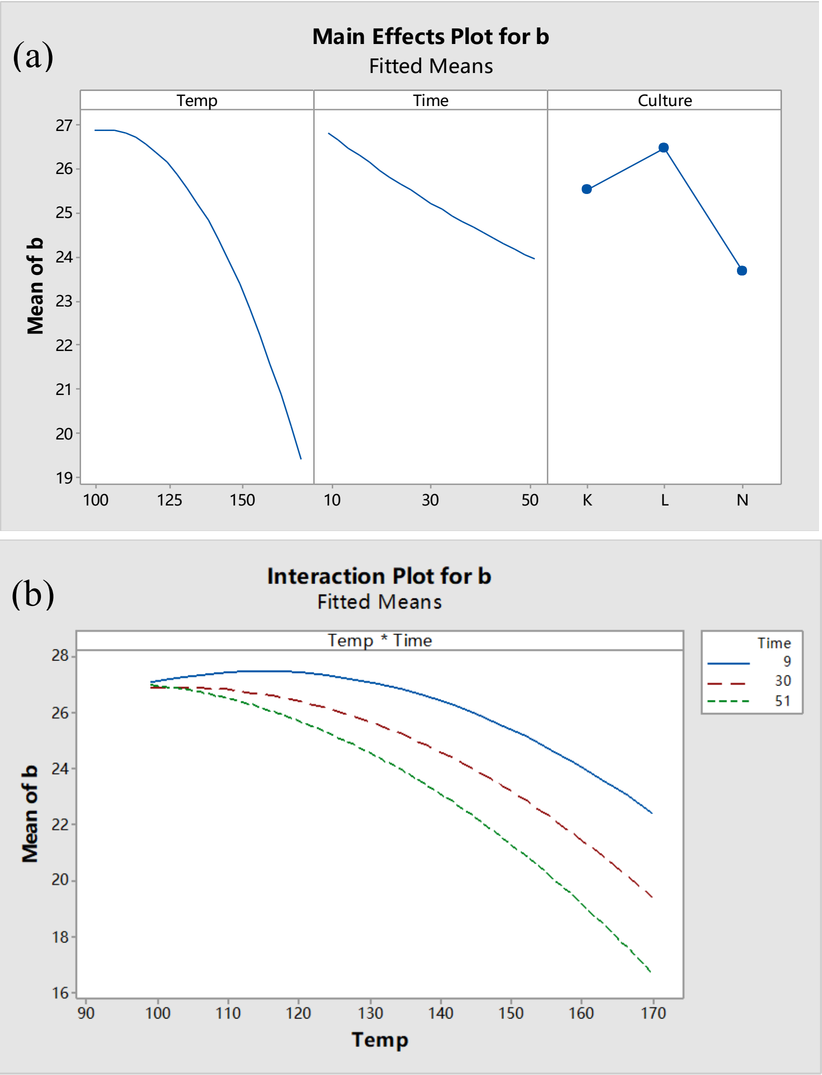


Figure 4.3 The factorial plots for b* value of roasted fermented avocado seeds (a) the main effect plot, (b) the interaction plot.

The main effects of b* value are shown in Figure 4.3 (a). Roasting temperature, time and inoculated cultures had significant (P <0.001) main effects on b* value, according to the two-way ANOVA results (Table 4.5). The highest b* value of roasted fermented avocado seeds was evident at low roasting temperature and short roasting. After that, the b* value decreased with increasing roasting temperature and time in the range of 110-170°C and 9-50min. Similarly, *García-Alamilla, et al. (2017)* reported there was a negative linear effect of roasting temperature on $b*$ value of roasted fermented cocoa beans. The authors explained that this decrease in b*value was probably due to the degradation of pigments present in cocoa beans during fermentation and roasting. In terms of culture, roasted avocado samples inoculated with *L. plantarum* had the highest b* value, compared to kefir and naturally fermented samples. This higher result may be associated with organic acids produced by LAB that decreased pH and subsequently enhanced of formation of Maillard reaction products *(Hur, et al. 2014)*.

The interaction plot of roasting temperature and time on a * value is shown in Figure 4.3 b. Two-way ANOVA results (Table 4.5) showed significant (P <0.001) interactions between roasting time and temperature. The highest b* value was obtained at 115°C and thereafter the b* value kept decreasing with the increase of roasting temperature in the range of 99-170°C, with b*values decreasing more with increasing roasting time. Similarly *Chung, et al. (2013)* reported a decrease in b* value of roasted corn kernels with increasing roasting temperature and time. They explained that the decrease in b*value was probably due to the destruction of pigments present in roasted seeds during fermentation and roasting processing.

Results of colour analysis in this study showed that the L* value of roasted fermented avocado powder significantly decreased with increasing roasting time from 130°C to 170°C. This decrease in L* value may be due to the fact that the browning reaction generated Maillard reaction products (brown nitrogenous polymers and melanoidins), which was favoured at higher temperatures and over a longer duration. As for a* and b* values, only roasting temperature showed a significant negative non-linear effect on $a*$ value of roasted fermented avocado seeds, while b* value significantly decreased with increasing roasting temperature and time. This can be probably due to the destruction of pigment during fermentation and roasting that may decrease the a* and b* values.

TPC (Total phenolic content)

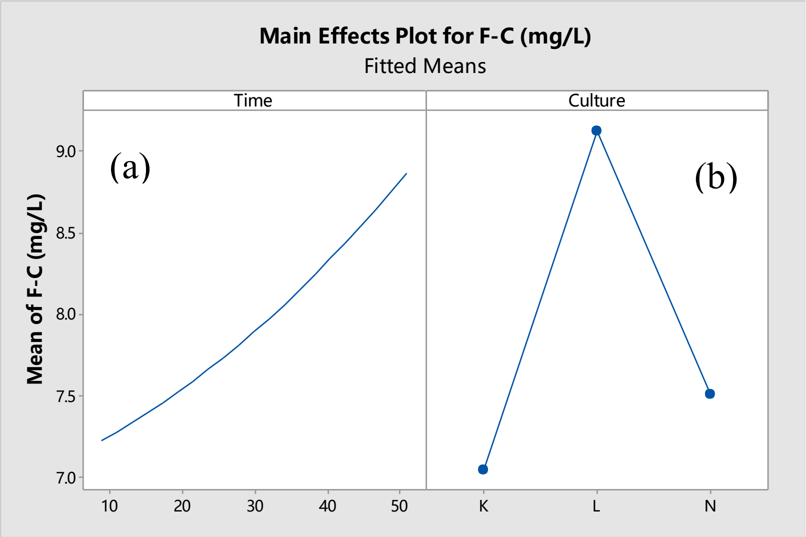


Figure 4.4 The main effect plots for TPC of roasted fermented avocado seeds (a) roasting time, (b) culture.

The main effect plots of TPC are shown in Figure 4.4, which indicated that the main effect for roasting time and inoculated cultures on phenolic compounds of roasted fermented avocado seeds. According to the two-way ANOVA results (Table 4.5), the main effect of roasting time (Figure 4.4a) and culture (Figure 4.4b) on phenolic compounds was significant ($P < 0.05$). TPC of roasted fermented avocado seeds significantly increased with increasing roasting time (Figure 4.4a). This was in accordance with results reported by the study of *Lin, et al. (2016)* who reported that TPC of almond kernels increased significantly with increasing roasting time from 5 to 20 mins at 150°C, 180°C, and 200°C. The increase in TPC in roasted seeds may be due to that thermal processing that enhanced the release of bound polyphenols into free forms and the formation of higher antioxidant activity products, which may improve the TPC *(Lin et al. 2016; Jan, et al. 2019)*. Similarly, increased TPC was observed with increasing roasting temperature and time for roasted kalonji seeds *(Jan, et al. 2019)*. In addition, roasted avocado samples inoculated with *L. plantarum* had the highest TPC (Figure 4.4b) compared to kefir and naturally fermented samples. This high TPC may be related to LAB-induced liberation of bound polyphenols by enzymes (e.g. tannase, glucosidase) or hydrolysis *(Hur, et al. 2014)*, which can increase TPC.

CUPRAC assay

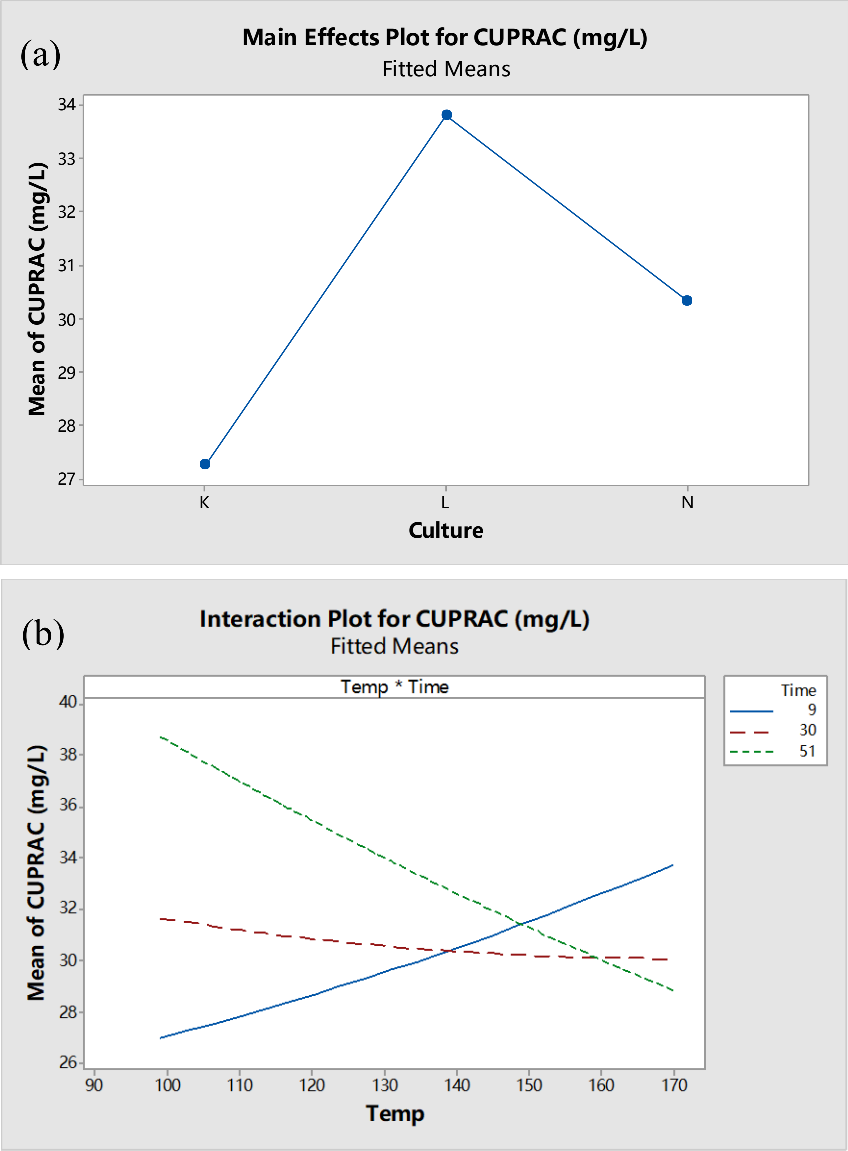


Figure 4.5 The factorial plots for CUPRAC assay of roasted fermented avocado seeds (a) the main effect plot, (b) the interaction plot.

Figure 4.5a shows that the main effect plot for culture factors with roasting of fermented avocado seeds. According to the two-way ANOVA results (Table 4.5), the main effect of culture on CUPRAC assay results was significant ($P < 0.001$). The results demonstrated that the antioxidant capacity of roasted fermented avocado seeds inoculated by *L. plantarum* was significantly the highest in comparison to kefir and naturally fermented samples. The effects of LAB on CUPRAC results may be attributed to the liberation of bound polyphenols into free radicals by enzymatic hydrolysis during fermentation, as well as the antioxidant potential of *L. plantarum* itself, which had antioxidative activity against superoxide dismutase (SOD) *(Kullisaar, et al. 2002)*.

Interaction between roasting temperature and time (Figure 4.5b) on CUPRAC assay results was significant (P <0.05) as seen in Table 4.5. At the start of roasting for 51 and 30 min, there was a decrease in CUPRAC assay results with increasing roasting temperature. Similar findings were observed in roasted fermented cocoa beans *(Ioannone, et al. 2015)* where a decrease in antioxidant capacity (FRAP assay) was observed as roasting time and temperature increased. This decline was probably due to the reduction of antioxidant compounds like flavanols and proanthocyanins. Besides that, *Arlorio et al., (2008)* reported that the potential reaction between the phenolic compounds and proteins may also contribute to the decrease in antioxidant capacity. However, at a low roasting time of 9 min, CUPRAC assay results increased with increasing temperature. This increase may be associated with the release of bound antioxidant components or formation of higher antioxidant activity compounds *(Lin, et al. 2016)*. Additionally, *Jan, et al. (2019)* stated that roasted kalonji seeds presented higher antioxidant activity (DPPH assay) at a high temperature-short time (HTST) conditions (over 180°C and 3 min) since that condition generated more melanoidin pigments compared to normal roasting. In addition, *Ioannone, et al. (2015)* found that the high temperature-short time (HTST) roasting better preserved the polyphenols content than others. This may explain the lowest value of CUPRAC assay in 51min samples that the roasting processing with higher temperature and longer time may cause higher polyphenol loss.

FRAP assay

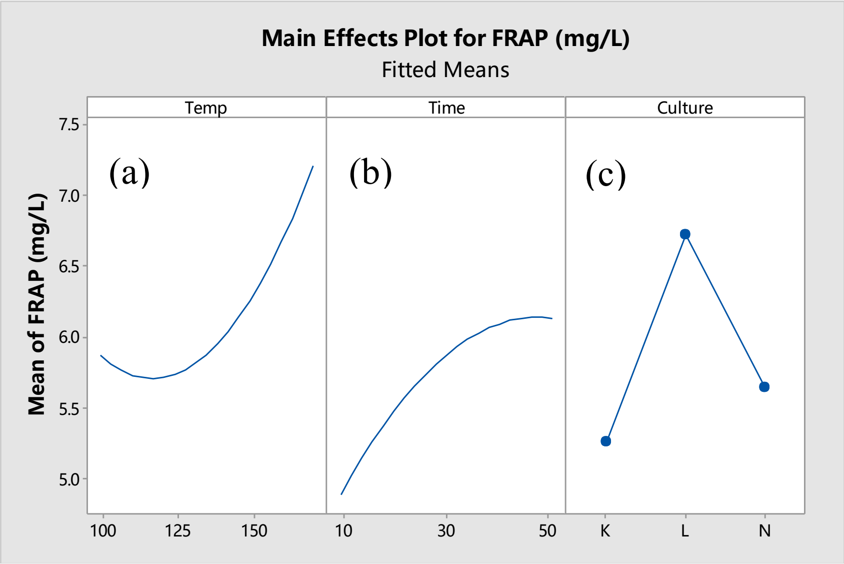


Figure 4.6. The main effect plots for FRAP assay of roasted fermented avocado seeds (a) roasting temperature, (b) roasting time, (c) culture.

The main effect plots for results of the FRAP assay are displayed in Figure 4.6. The two-way ANOVA results from Table 4.5 indicated that all three main effects for roasting temperature, roasting time and inoculated culture factors on antioxidant capacity of roasted fermented avocado seeds were significant (P <0.001). In Figure 4.6a, FRAP assay values decreased with increasing roasting temperature up to 120°C and then increased with increasing roasting temperature, reached a maximum at 170°C. Similarly FRAP assay values decreased and then increased during roasting processing during roasting of fermented cocoa beans *(Ioannone, et al. 2015)*. The initial decrease may be due to the reduction of antioxidant polyphenonls, and the later increase may be related to liberation of bound antioxidant polyphenonls with higher roasting temperature.

Moreover, with increased roasting time, the FRAP assay values increased as well as seen in Figure 4.6b. Similarly, *Açar, et al. (2009)* demonstrated that increased roasting time at 150°C, increased total antioxidant capacity (TEAC) values significantly for most pulses (borlotti bean, black bean, giant lentils, chickpea, and yellow soybean), cashew nut, as well as pinenut, and reached a maximum level at the end of 60 min of roasting. The authors suggested that the increase in TEAC with longer roasting time was likely due to the release of bound antioxidant components and generation of new antioxidants through Mallard reactions, especially in the rich-starch materials.

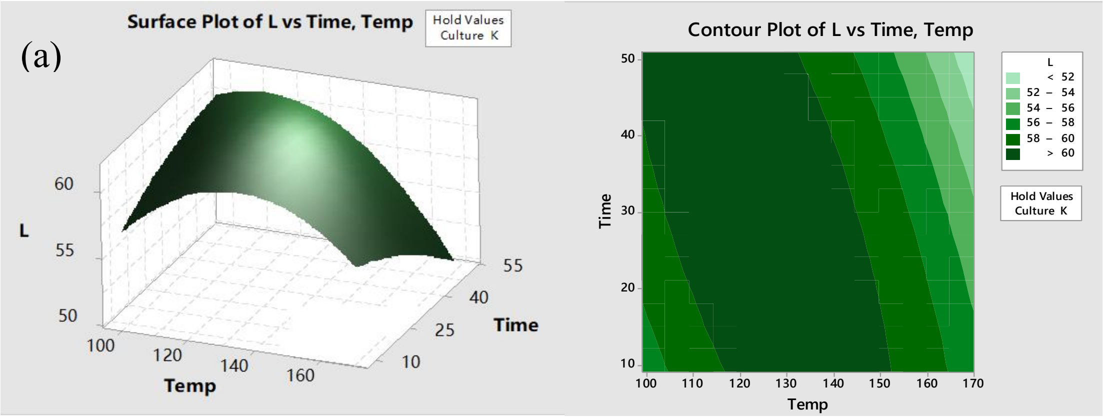
As for the effect of culture as seen in Figure 4.6c, the highest FRAP antioxidant value was found in roasted fermented avocado seeds inoculated with *L. plantarum*, compared to kefir and naturally fermented samples. This may be associated with the enzymatic hydrolysis by LAB during fermentation and roasting processing *(Hur, et al. 2014)*, as well as the inhibition effect of *L. plantarum* on the activity of oxidative enzymes, which may reduce the oxidation of antioxidants components *(Kachouri, et al. 2015)*.

Differences existed in the antioxidant assays of roasted fermented avocado seeds between CUPRAC and FRAP assays. This may be due to the different sensitivities for specific antioxidants *(Bean, et al. 2009)*. It was reported that the FRAP assay had higher sensitivity to ascorbic acid in rabbit tissues than the CUPRAC assay *(Bean, et al. 2009)*. *Çelik, et al. (2010)* found that the Trolox equivalent antioxidant capacity (TEAC) of different antioxidant compounds (quercetin, ferulic acid, and catechin) in green tea were significantly different using the CUPRAC assay, in comparison to the FRAP assay. The authors further explained that the significantly different results between CUPRAC assay and FRAP assay may be due to the redox reaction that occurred at different pH values that CUPRAC, FRAP and Folin-Ciocalteu assays were carried out at pH 7, pH 3.6, and pH 10 respectively. Bean, et al. (2009) further reported that pH value less than 7 would contribute to inhibition of reducing capacity. Furthermore, lipophilic antioxidants were not detected in FRAP assay but can be detected by CUPRAC assay, which can also account for differences between these two assays *(Çelik, et al. 2010)*.

In summary, results of chemical analysis in this study indicated that the TPC of roasted fermented avocado seeds significantly increased with increasing roasting time. The increase in TPC in roasted avocado seeds may be due to thermal processing that enhanced the release of bound polyphenols into free forms that resulted in the formation of higher antioxidant activity products *(Hur, et al. 2014)*. Moreover, the new antioxidants generated through Maillard reactions may also increase antioxidant capacity of samples, especially in the rich-starch materials *(Açar, et al. 2009)*. With FRAP assay, antioxidant values significantly increased with increasing roasting time from 120°C, reaching a maximum at 170°C. The initial decrease in FRAP values at lower roasting temperature may be due to the reduction of antioxidant polyphenols, and the later increase may be related to liberation of bound antioxidant polyphenols with higher roasting temperature *(Ioannone, et al. 2015)*. In terms of culture, roasted avocado seeds fermented by *L. plantarum* resulted in significantly higher TPC and antioxidant capacity compared to kefir and naturally fermented samples. This may be associated with the enzymatic hydrolysis by LAB during fermentation and roasting processing, as well as the inhibition effect of L. plantarum on the activity of oxidative enzymes, which may reduce the oxidation of antioxidants components.

4.2.4 The response surface plots and contour plots

The response surface plots are displayed in a three-dimension view of roasting time and roasting temperature on the different responses of roasted avocado seed samples that were naturally fermented and fermented by *L. plantarum*, kefir, while the contour plots present a two-dimension view for each response. Only the significant interactions will be discussed. Based on the two-way ANOVA model (Table 4.5), only interactions for values of L*, b *, and CUPRAC assay were significant.



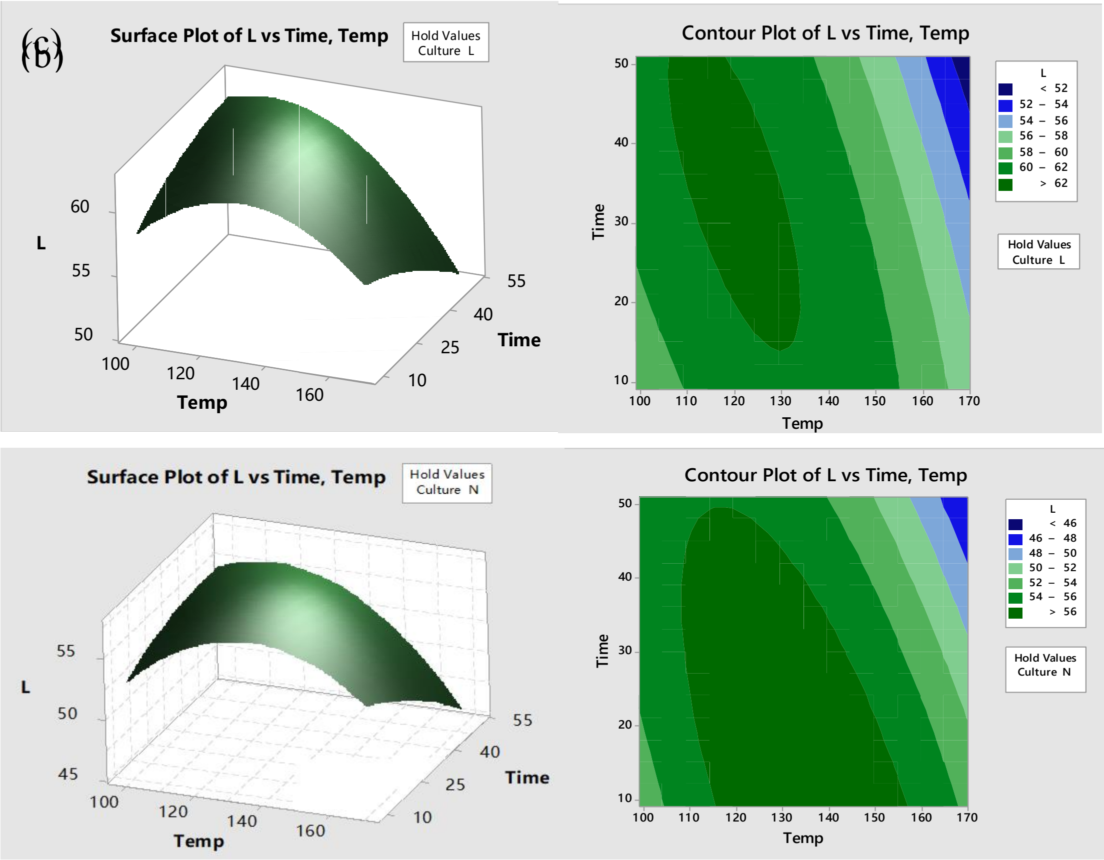
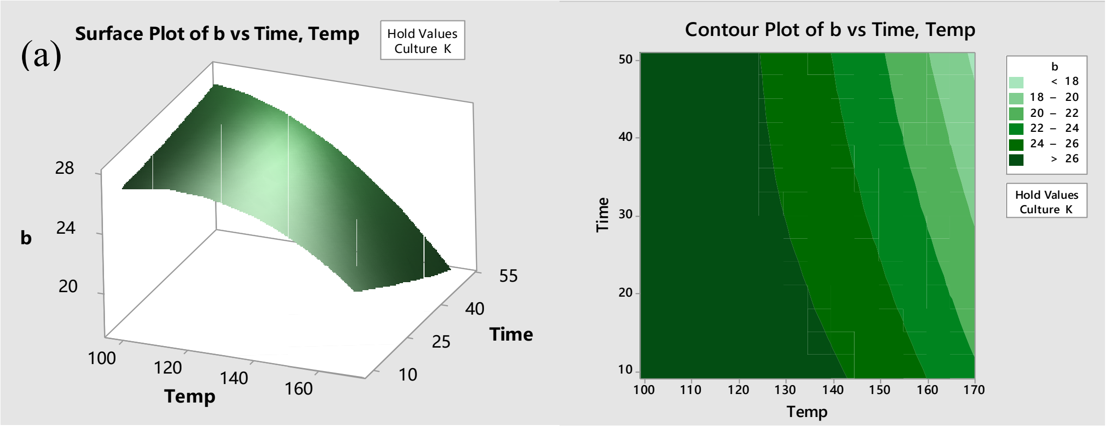
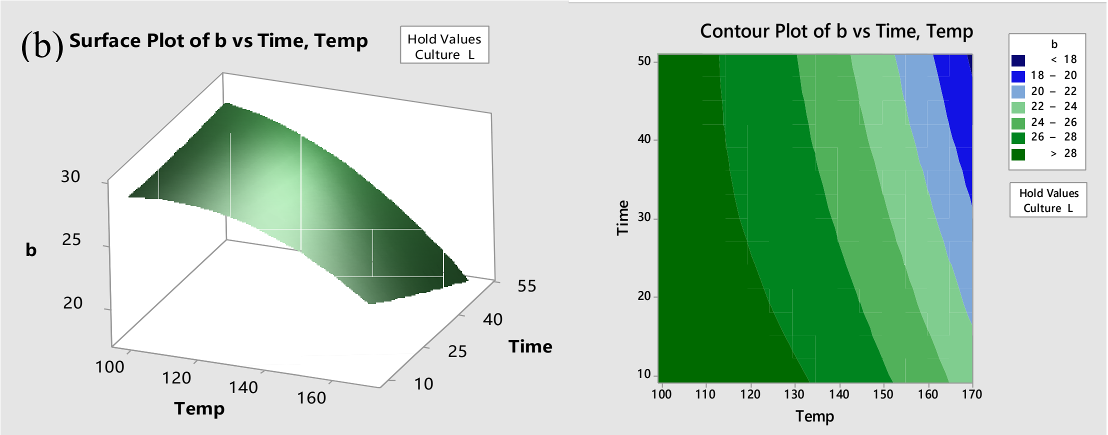


Figure 4.7 The response surface plots and contour plots showing the effects of roasting time and temperature on L* value of three different roasted fermented avocado seeds. (a): kefir samples; (b): *L. plantarum* samples; (c): naturally fermented samples

L* value

The effects of roasting time and roasting temperature on L* value can be seen in Figure 4.7. There was a highly significant (P <0.001) interaction between roasting temperature and time on L* value (Table 4.5). The L* value increased in all roasted fermented avocado seeds at the lower range of temperature and roasting time for roasted kefir samples as seen in Figure 4.7a (from 110°C-150°C and 10-40 min), *L. plantarum* samples as seen in Figure 4.7b (from115-135°C and 15- 50 min), and naturally fermented samples as seen in Figure 4.7c (115-155°C and 10-48 mins). After that, an increase in roasting temperature to more than 150°C and an increase in time from 10 to 50 min decreased L* value, indicating darkening occurred in all three roasted fermented samples. Similarly, L* value in roasted cocoa bean *(Zzaman & Yang, 2014)*, roasted hazelnuts *(Ozdemi, et al., 2000)* and roasted corn kernels *(Chung, et al. 2014)* increased at the start of roasting and then declined at a higher temperature and longer time of roasting. The denaturation of soluble proteins by heat treatment *(Ozdemi, et al., 2000)* and lower moisture content may be factors that contributed to the initial lightening of roasted samples *(Chung, et al. 2014).* As seen in Figure 4.7 a, b, and c, the area with the lowest L* values were located on the upper right-hand side of the contour plot (165-170°C and 45-50min). This was supported by findings reported by *Chai, et al. (2019)* that showed increasing roasting temperature from 110 to 160 °C and time from 15 to 45 min significantly reduced the L* value of roasted fermented rambutan seeds. Similarly, *Bagheri, et al. (2018)* indicated that the peanut kernels became darker (L* value significantly decreased) with increasing roasting time (from 10 to 30 min) and infrared power (from 250 to 450 W), which favoured the non-enzymatic browning reaction and generated Maillard reaction products (brown nitrogenous polymers and melanoidins).





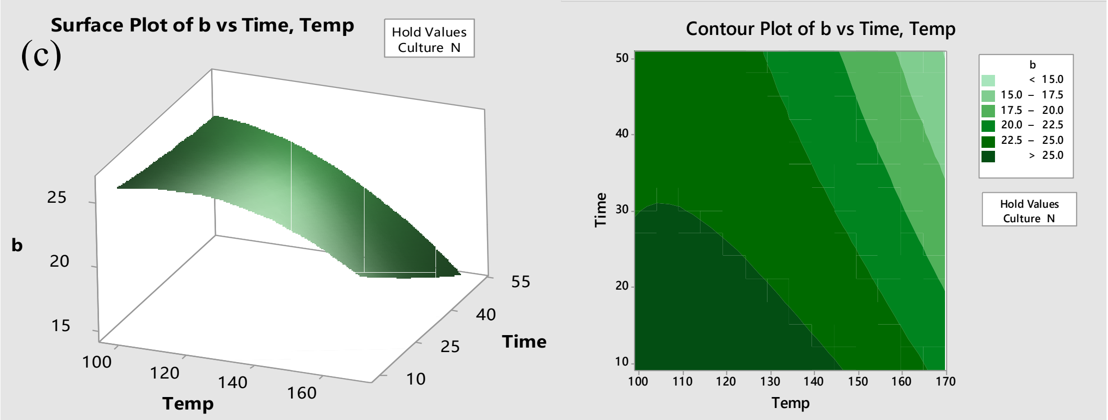


Figure 4.8 The response surface plots and contour plots showing the effects of roasting time and temperature on b* value of three different roasted fermented avocado seeds. (a): kefir samples; (b): *L. plantarum* samples; (c): naturally fermented samples

b* value

The changes in b* value during roasting are shown in Figure 4.8. There was a significant interaction between roasting temperature and time on b* value (Table 4.5). The b* value of the roasted avocado seed samples with the range of 17.7 to 29.7 depending on the different fermentation conditions (*L. plantarum*, kefir and naturally fermented samples). Additionally, the contour plot Figure 4.8 showed that the highest b* value was obtained in the range of 100-140°C and 10-50 min for roasted kefir samples (Figure 4.8a), 100-130°C and 10-50 min for roasted *L. plantarum* samples (Figure 4.8b), and 110-145°C and 10-30 min for naturally fermented samples (Figure 4.8c). After that, there was a significant decrease in the b * value with increase in roasting temperature and roasting time for all three fermented avocado seeds. As seen in Figure 4.8 a, b, and c, the areas with the lowest b* values were located on the upper right-hand side of the contour plot (165-170°C and 35-50 min). A similar decrease in b* value was observed with roasting of corn kernels *(Chung, et al. 2013)*, fermented cocoa beans *(García-Alamilla, et al. 2017)*, and Arabica coffee beans *(Somporn, et al. 2011)*, indicating the at increasing roasting temperature and time. The negative effect on b* value with higher temperature and longer time was probably due to the destruction and degradation of colour pigments in roasted products *(Somporn et al. 2011).*

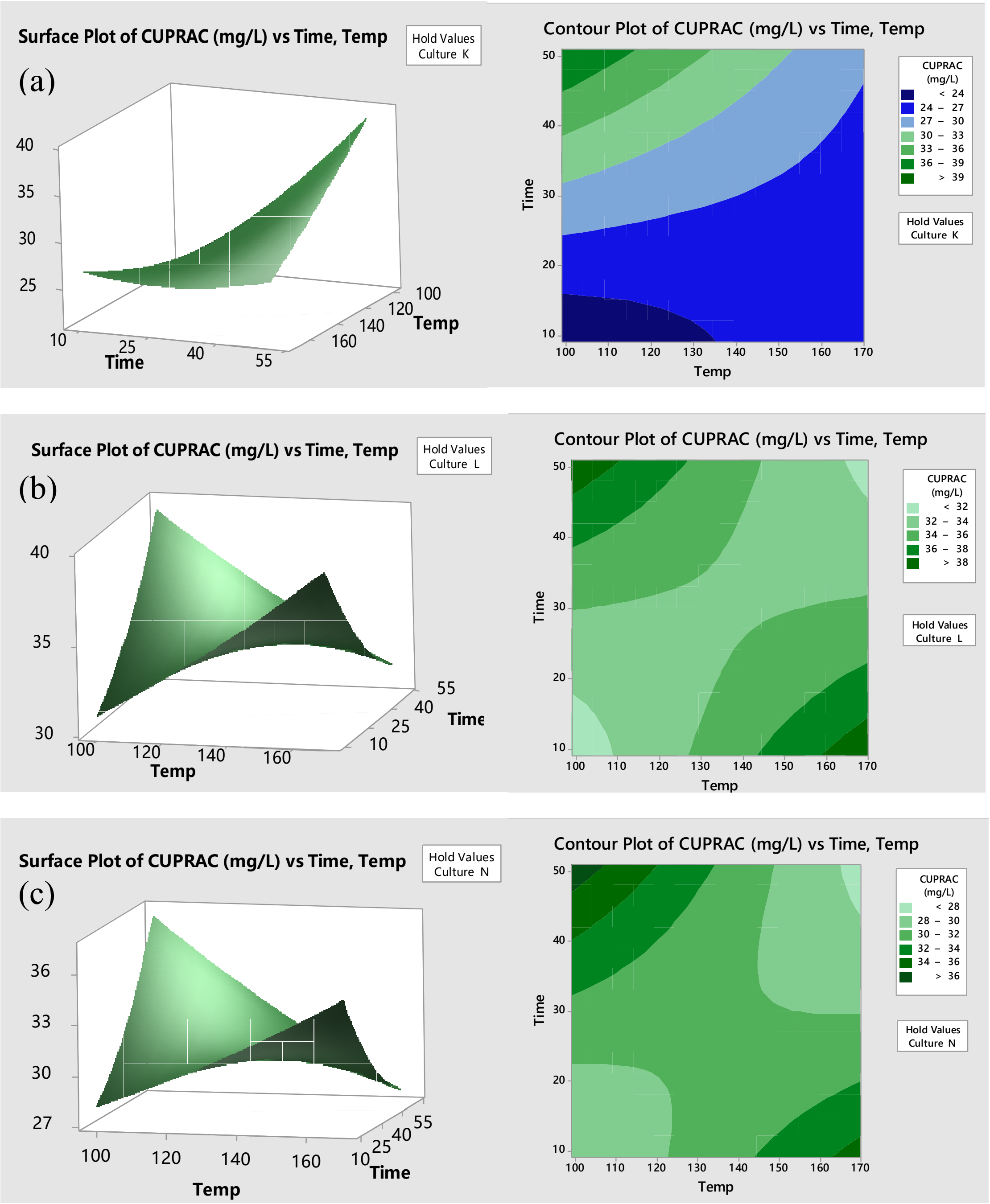


Figure 4.9 The response surface plots and contour plots showing the effects of roasting time and temperature on CUPRAC assay of three different roasted fermented avocado seeds. (a): kefir samples; (b): *L. plantarum* samples; (c): naturally fermented samples

CUPRAC values

Changes of the CUPRAC assay in this study are shown in Figure 4.9. There was a significant interaction ($p < 0.05$) between roasting temperature and time (Table 4.5). affected the result of the CUPRAC assay. As seen in Figure 4.9, the highest CUPRAC assay values were obtained in the upper left-hand corner for all three fermented groups ranging from 100-110°C and 45-50 min (Figure 4.9 a, b and c). In fact, in the areas located in the lower right-hand corner of the contour plots for the *L. plantarum* (Figure 4.9b) and naturally fermented samples (Figure 4.9c), high CUPRAC values were obtained in the range of 160-170°C and 10-15 min. This was probably since the fact high temperature-short time (HTST) roasting and low temperature-long time (LTLT) may preserve more polyphenols content than other conditions during roasting as these conditions reduce the loss of polyphenol *(Ioannone, et al. 2015; Jan, et al. 2019)*. Similarly, *Jan, et al. (2019)* stated that roasted kalonji seeds had higher antioxidant activity (DPPH assay) under HTST conditions (over 180°C and 3 min) that generated more melanoidin pigments compared to normal roasting. Moreover, Lin, et al. (2016) reported that total antioxidant capacity (TEAC) significantly increased with the increasing time from 5-20 min at 150°C. The increase in antioxidant capacity with longer roasting time was likely related to the liberation of bound antioxidant components or the formation of antioxidant Maillard reaction products (brown nitrogenous polymers and melanoidins) *(Lin, et al. 2016)*, especially in the rich-starch materials *(Açar, et al. 2009)*. *Araújo, et al. (2018)* reported that avocado seeds contained approximately 30% of starch.

5. Conclusions and recommendations

This study set out to determine the influence of fermentation and roasting of avocado seeds on colour, TPC and antioxidant activities. In the first part of the study, avocado seed samples were subjected kefir, *L. plantarum*, and natural fermentation over 7 days. Results showed that the increase in fermentation time significantly ($p < 0.001$) increased LAB, AAB and yeasts counts, reaching a peak at day 5 over 7 days of fermentation. Although statistically significant difference existed between the day 5 and day 7 counts of LAB and yeasts, the increase was no more than 1 log cfu after 5 days indicating that growth had reached its stationary phase. Hence, further determination of TPC and antioxidant capacity of fermented avocado seeds in this study were only carried out on day 5 samples according to the microbial results obtained. This study has shown that TPC and antioxidant capacity of both inoculated and naturally fermented avocado seeds significantly decreased after fermentation compared to unfermented (day 0) samples. However, TPC and antioxidant capacity of *L. plantarum* fermented samples were significantly the highest compared to kefir and naturally fermented avocado seeds.

The second purpose of this study was to determine how roasting of fermented avocado samples influenced colour, TPC and antioxidant capacity. The fermented avocado seeds roasted at varying roasting time and temperature influenced colour, TPC, and antioxidant activity of powders. L*and b* values of roasted avocado seeds significantly ($P <0.001$) declined with increase in roasting temperature and time in my results, Roasting significantly increased the TPC with increasing roasting time. In terms of antioxidant activity, only the value of FRAP assay significantly increased with increasing roasting temperature and time. Interestingly, roasted avocado seeds fermented by *L. plantarum* resulted in significantly higher TPC and antioxidant capacity compared to kefir and naturally fermented samples. The findings of this study suggested that a combination of fermentation and roasting of avocado seeds yielded a powder rich in bioactive compounds that could potentially be used as a functional food additive and nutraceutical.

These findings contributed in several ways that increased our understanding on how fermentation and roasting can be used to convert food by-products into functional ingredients and provided a basis for further research into identifying bioactive components that are responsible for the increased bioactivity. It would also be interesting to further isolate and identify natural LAB from avocado seeds and further investigate the use of these microorganisms for fermentation.

6. References

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7. Appendix 1（Regression Equations）

A response equation(Y=f (A, B) for each response variable in each fermentation condition ((1) kefir, (2) *L. plantarum* and (3) naturally fermented) was obtained from the two- way ANOVA results from Table 4.5, in terms of two independent process variables A (roasting temperature) and B (roasting time).

7.1 The regression equations of L* value

(1) K L = -5.95 + 0.960 Temp + 0.510 Time - 0.003455 Temp*Temp - 0.00161 Time*Time - 0.003322 Temp*Time

(2) L L = -3.06 + 0.944 Temp + 0.509 Time - 0.003455 Temp*Temp - 0.00161 Time*Time - 0.003322 Temp*Time

(3) N L = -10.89 + 0.972 Temp + 0.478 Time - 0.003455 Temp*Temp - 0.00161 Time*Time - 0.003322 Temp*Time

7.2 The regression equations of a* value

(1) K a = 7.7 + 0.222 Temp - 0.064 Time - 0.001078 Temp*Temp - 0.00028 Time*Time + 0.00031 Temp*Time

(2) L a = 5.6 + 0.233 Temp - 0.059 Time - 0.001078 Temp*Temp - 0.00028 Time*Time + 0.00031 Temp*Time

(3) N a = 0.1 + 0.267 Temp - 0.017 Time - 0.001078 Temp*Temp - 0.00028 Time*Time + 0.00031 Temp*Time

7.3 The regression equations of b* value

(1) K b = 3.33 + 0.4009 Temp + 0.1860 Time - 0.001659 Temp*Temp + 0.000365 Time*Time - 0.001881 Temp*Time

(2) L b = 6.94 + 0.3843 Temp + 0.1706 Time - 0.001659 Temp*Temp + 0.000365 Time*Time - 0.001881 Temp*Time

(3) N b = 2.23 + 0.4073 Temp + 0.1313 Time - 0.001659 Temp*Temp + 0.000365 Time*Time - 0.001881 Temp*Time

7.4 The regression equations of phenolic content

(1) K F-C (mg/L) = 1.22 + 0.0313 Temp + 0.184 Time - 0.000005 Temp*Temp + 0.000350 Time*Time - 0.001025 Temp*Time

(2) L F-C (mg/L) = 4.44 + 0.0352 Temp + 0.129 Time - 0.000005 Temp*Temp + 0.000350 Time*Time - 0.001025 Temp*Time

(3) N F-C (mg/L) = 1.01 + 0.0428 Temp + 0.155 Time - 0.000005 Temp*Temp + 0.000350 Time*Time - 0.001025 Temp*Time

7.5 The regression equations of CUPRAC assay

(1) K CUPRAC (mg/L) = 12.7 + 0.035 Temp + 0.802 Time + 0.00032 Temp*Temp + 0.00273 Time*Time - 0.00557 Temp*Time

(2) L CUPRAC (mg/L) = 18.9 + 0.085 Temp + 0.592 Time + 0.00032 Temp*Temp + 0.00273 Time*Time - 0.00557 Temp*Time

(3) N CUPRAC (mg/L) = 18.5 + 0.059 Temp + 0.607 Time + 0.00032 Temp*Temp + 0.00273 Time*Time - 0.00557 Temp*Time

7.6 The regression equations of FRAP assay

(1) K FRAP (mg/L) = 9.66 - 0.1197 Temp + 0.1079 Time + 0.000526 Temp*Temp - 0.000826 Time*Time - 0.000073 Temp*Time

(2) L FRAP (mg/L) = 11.30 - 0.1146 Temp + 0.0789 Time + 0.000526 Temp*Temp - 0.000826 Time*Time - 0.000073 Temp*Time

(3) N FRAP (mg/L) = 11.82 - 0.1267 Temp + 0.0796 Time + 0.000526 Temp*Temp - 0.000826 Time*Time - 0.000073 Temp*Time