Original Article

Submerged fermentation improves bioactivity of mulberry fruits and leaves

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A R T I C L E   I N F O

A B S T R A C T

Objective: Mulberry (*Morus* spp.) fruits and leaves have been proven to possess nutraceutical properties. Due to its fast and easy growing characteristics, mulberry fruits (MF) and leaves (ML) potentially emerge as a great source of functional foods. This study aims to enhance bioactivities (antioxidant, anti-inflammation, and hypoglycemic activity) of MF and ML via submerged fermentation using bacteria (*Lactobacillus plantarum* TAR 4), yeast (Baker's yeast and red yeast) and fungi (*Tempeh* and *Tapai* starter).
Methods: In this study, 25% (mass to volume ratio) of MF and ML were fermented (48 h) with 1% (mass to volume ratio) of different microbial cultures, respectively. Effects of different fermentations on MF and ML were determined based on the changes of total phenolics (TPC), flavonoids (TFC), anthocyanins, total sugar, DPPH activity, ferric reducing antioxidant power (FRAP), albumin denaturation inhibition activity (ADI), anti-lipoxygenase activity and α-amylase inhibition activity (AI).
Results: Generally, ML had higher AI than MF. However, MF exhibited higher DPPH, FRAP and anti-lipoxygenase activity than ML. After all forms of fermentation, DPPH and AI activity of MF and ML were increased significantly ($P < 0.05$). However, the effects of fermentation on TPC, FRAP, ADI and anti-lipoxygenase activity of MF were in contrast with ML. TPC, FRAP and anti-lipoxygenase activity of ML were enhanced, but reduced in MF after fermentation. Although the effects exerted by different microorganisms in MF and ML fermentation were different, the bioactivities of MF and ML were generally improved after fermentation. Fermentation by *Tempeh* starter enhanced TPC (by 2-fold), FRAP (by 2.3-fold), AI (at 10% increment) and anti-lipoxygenase activity (by 5-fold) of ML, whereas *Tapai* fermentation effectively enhanced the DPPH (at 17% increment) and ADI (by 2-fold) activity of MF.
Conclusion: Findings of this study provide an insight into the future process design of MF and ML processing into novel functional foods.

1. Introduction

Mulberry (*Morus* spp.), a plant of the Moraceae family has attracted interest from the community recently due to its high economy, nutraceutical, and medicinal values. Mulberry tree is a plant that grows easily. Due to its good tolerance and adaptability to harsh environment, it is widely cultivated throughout the world, ranging from tropical to temperate regions in different soil conditions (Krishna et al., 2020; Wani et al., 2017). A mulberry tree grown from seed takes approximately 10 years to bear fruits and the fruits spend around 2–4 weeks to ripe. The ripe fruits vary in white, red, purple, and black color depending on the species. The high moisture (70.0%–87.4%) and slight acidic pH (3.20–5.33) of

the fruits contributed to the sweet–sour juiciness mouthfeel (Imran, Khan, Shah, Khan, & Khan, 2010; Liang et al., 2012).

Numerous studies have proven that mulberry fruit is a good source of phenolics and flavonoids, which contributed to the antioxidant, anti-diabetic, anti-obesity, anti-tumor, anti-neuroinflammatory and anti-aging potential (Ahmed, Ali, El-Kholie, El-Garawani, & Sherif, 2016; Huang, Ou, & Wang, 2013; Turan et al., 2017; Wang et al., 2013; Xu, Huang, Xu, He, & Wang, 2020). The major phytochemicals in mulberry fruits are quercetin, kaempferol, chlorogenic acid, cinnamic acid, *p*-hydroxybenzoic acid, protocatechuic acid and vanillic acid (Sánchez-Salcedo, Mena, García-Viguera, Martínez, & Hernández, 2015a; Xu, Huang, Xu, He, & Wang, 2020). In the study by Yu, Lim, Lee, Choi, and Kim (2021), ethanolic extract of mulberry fruits have proven to exhibit protective effects against oxidative stress and inflammatory responses in RAW 264.7 macrophages. Besides phenolics and flavonoids, other compounds such as loliolide, odis-

olane indole and its derivatives, which are the common pharmaceutical agents, were also detected in the extract. In addition, Xu et al. (2020) had also successfully identified 50 compounds, including the three new compounds in the methanolic extract of mulberry fruits. These compounds had been proven to significantly inhibit nitric oxide (NO) production induced by lipopolysaccharides (LPS), reduce expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), as well as block LPS-induced nuclear translocation of NF-κB. Complex interactions between the chemical constituents are postulated as the factors that contribute to the nutraceutical properties of mulberry fruits. Furthermore, Xu et al. (2020) also recommended that a reasonable consumption of mulberry fruits could prevent and reduce the prevalence of neuroinflammatory and oxidative related disorders. However, the chemical composition and nutritional value of mulberry fruits differ among cultivars. Each cultivar has its own nutrition strength (Liang et al., 2012). Thus, mulberry fruits are claimed to have great potential to be developed into novel nutritious raw ingredient for diverse health food applications.

For over 5000 years, mulberry leaves are widely served as the food source for silkworm in the silk industry and ruminant feed due to its high protein and fiber content. Mulberry leaves were found to contain approximately 27%−37% (dry weight) of protein and 11%−17% (dry weight) of crude fiber, which are significantly higher than most green leafy vegetables (Yu et al., 2018). Besides that, mulberry leaves are also widely used in traditional Chinese medicine as folk remedy to treat fever, cold, high blood pressure, diabetes mellitus, liver, and kidney damage, as well as other chronic diseases (Huang et al., 2013; Thaipitakwong, Numhom, & Aramwit, 2018; Yu et al., 2018). Mulberry leaves are proven rich in various phytochemicals. The most abundant phenolic acid in mulberry leaf is chlorogenic acid, while isoquercitrin is the major flavonol glycoside (Sánchez-Salcedo, Mena, García-Viguera, Hernández, & Martínez, 2015; Yu et al., 2018). 1-Deoxynojirimycin (DNJ) is the main polyhydroxyalkaloid in mulberry leaves that is well-known for its hypoglycemic property. DNJ has a structure similar to glucose which effectively inhibits intestinal α-glucosidase activity, therefore control postprandial blood glucose level in human (Gao et al., 2016). DNJ content varies among mulberry leaf varieties. The China varieties were reported to contain 0.08 to 1.12 mg/g dry weight of DNJ whereas the Thailand varieties ranged from 0.3 to 1.7 mg/g dry weight (Vichasilp et al., 2012; Yu et al., 2018). In addition, DNJ also exhibits lipid-lowering activity, inhibits growth of colon cancerous cells and promotes growth of beneficial gut microbes (Hu et al., 2019; Kojima et al., 2010; Shuang et al., 2017). Furthermore, γ-aminobutyric acid detected in the mulberry leaves has also been proven to present anti-hypertensive and anti-fatigue properties (Chen et al., 2016; Yang, Jhou, & Tseng, 2012). With the excellent nutraceutical benefits of mulberry fruits and leaves, both are foreseen to be the potential health-promoting ingredients for functional foods development.

In the 21st century, consumer demand for foods no longer just for their satiety. Consumers are seeking for convenient, good tasting, healthy, nutritious, and value-added foods. To fulfill the present trend and demand for foods, there is a need to develop more economical and green processing technologies to improve nutritional quality of food materials. Fermentation has been long applied in food processing to diversify food variety, prolong shelf life, as well as enhance nutritional and organoleptic properties of foods, in a cost-effective manner. In recent years, effect of fermentation on various fruits such as grape, blueberry, apple, mango, and red dragon fruit have also been widely investigated. Fermented fruits were proven to exhibit higher bioactivities such as antioxidant, anti-inflammation, hypoglycemic, etc. (Ayed, M'hir, & Hamdi, 2020; Choo et al., 2018). Nevertheless, limited study was

conducted to determine the effects of fermentation on the bioactive constituents and bioactive properties of mulberry fruits and leaves. To date, research on fermentation of mulberry fruits and leaves was mostly limited to the fruit juice fermentation and solid-state fermentation of the leaves (Chaudhary, Sharma, & Saharan, 2019; Wang, Sun, Li, Yu, Liu, Huang, & Zhan, 2015; Zheng et al., 2014). Effect of fermentation on the recovery of bioactive constituents and bioactivities of mulberry fruits and leaves have yet to be explored. Therefore, this study was designed to evaluate the effect of submerged fermentation using bacteria (Lactobacillus plantarum TAR 4), yeast (Baker's yeast and red yeast) and fungi (Tempeh and Tapai starter) on the recovery bioactive compounds, and hence the bioactivities of mulberry fruits (MF) and mulberry leaves (ML). Effect of fermentation using different microorganisms on the antioxidant, anti-inflammation and hypoglycemic activities of MF and ML were determined. This study provides an insight into the setup of fermentation technology for future functional foods development using mulberry fruits and leaves.

2. Materials and methods

2.1. Materials

Dried mulberry fruits (Morus sp.) (origin: Xinjiang, China) used in this experiment were purchased from a local store, whereas the fresh mulberry leaves were collected from the mulberry trees planted in the garden of Tunku Abdul Rahman University College, Kuala Lumpur, Malaysia. The fresh leaves harvested were washed, then dried in a vacuum oven under pressure 5 kPa at 40 °C for 12 h. The dried leaves and fruits were ground into coarse powder by using a knife mill. Both dried mulberry fruits and leaves were kept refrigerated at 4 °C until further usage.

Lactic acid bacteria (LAB) used in this experiment was an isolated strain Lactobacillus plantarum TAR4 (NCBI GenBank database under the accession numbers MH012173). The LAB was grown in MRS broth (Oxoid, England) and the live cells were harvested after 48 h through centrifugation at 6373 × g for 5 min. Commercial food grade Baker's yeast (Saccharomyces cerevisiae), red yeast (Monascus purpureus), Tempeh (Rhizopus oligosporus) and Tapai starter were purchased from the local market. Tapai starter (a.k.a ragi) was a traditional starter inoculum that was widely available in local market for Tapai production. It was a mixed culture comprised of diverse range of bacteria, yeast, and fungal species, include Amylomyces rouxii, Endomyces fibuligera, Chlamydomocular oryzae, Candida pelliculosa, Saccharomyces cerevisiae, Hansenula anomala, Bacillus sp. and Acetobacter sp. (Law, Abu Bakar, Mat Hashim, & Abdul Hamid, 2011).

Chemicals used include Folin-Ciocalteu reagent, sodium carbonate, gallic acid, quercetin, sodium hydroxide, methanol, sulfuric acid, ferrous sulfate, sodium acetate, ferric chloride, ascorbic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, albumin, etc. All chemicals used were analytical grade with the brand name of Sigma (U.S.), Merck (Germany) and Chemsoln (Malaysia).

2.2. Fermentation

All the glassware and utilities were sterilized in boiling water for 5 min before substrate preparation. About 25% (mass to volume) of mulberry leaves (ML) and fruits (MF) were prepared by completely soaking the ML and MF in distilled water. Next, about 1% (mass to volume) of microbial culture (LAB, Baker's yeast, red yeast, Tempeh and Tapai starter) was inoculated separately into ML and MF, respectively. Fermentation was carried out in the

screw-capped Scott bottle. LAB fermentation was conducted at 37 °C in an incubator, whereas fermentation by Baker's yeast, red yeast, *Tapai* and *Tempeh* starter were carried out at ambient temperature (25 °C). Both ML and MF were fermented for 48 h. Experimental control was carried out by incubating the ML and MF without any inoculation under the same condition.

2.3. Extraction

About 100 mL of distilled water was added into the fermented ML and MF, followed by a 1 h extraction by using an ultrasonicator (40 kHz and 500 W) at 50 °C. The ML and MF juices were obtained via pressing using 2-layers cheese cloth.

2.4. Chemical content determination

Total phenolics (TPC) and flavonoids (TFC) content of ML and MF juices were determined according to methods described by Wang et al. (2015). Gallic acid and quercetin were used as the calibration standard for TPC and TFC, respectively. Total anthocyanins content was determined through pH differential method described by Elisia, Hu, Popovich, and Kitts (2007). Total sugar content was determined according to Dubois method (Masuko et al., 2005).

2.5. Bioactivity characterization

DPPH scavenging activity of ML and MF juices was determined according to method Wang et al. (2015) with slight modifications. Briefly, 100 μL of sample was mixed with 3.9 mL of 0.6 mmol/L DPPH in methanol, then stand in dark at room temperature for 30 min. Absorbance of the mixture was measured at wavelength 517 nm. Methanol was used as the control. DPPH free radical scavenging activity was calculated using formula:

$$(A_{control} - A_{sample})/A_{control} \times 100\%$$

where $A_{control}$ and A_{sample} indicate absorbance of control and sample, respectively.

Ferric reducing antioxidant power (FRAP) of ML and MF juices was determined according to method Fernandes et al. (2016). Anti-inflammatory activity was evaluated based on albumin denaturation inhibition (ADI) and anti-lipoxygenase activities according

to procedures Rashid and Shafi (2018), whereas hypoglycemic activity was determined based on α-amylase inhibition (AI) activity (Abdul & Kasim, 2017).

2.6. Statistical analysis

All experiments were carried out in triplicates and the results were expressed as mean ± standard deviation. The statistical difference between data was determined via one-way ANOVA and LSD test. The results were considered significant when $P < 0.05$. Besides, correlation between the studied factors was determined via Pearson correlation analysis. All statistical analysis was conducted by using SPSS software version 20 (IBM, New York).

3. Results and discussion

3.1. Effect of fermentation using different microorganisms on chemical changes in ML and MF

Fig. 1 showed the effect of fermentation using different microorganisms on the changes of total phenolics (TPC), flavonoids (TFC), sugar and anthocyanins content in ML and MF. Results indicated that MF juice contained higher phenolics and sugar than ML juice. However, their total flavonoids and anthocyanins content were not significant difference ($P > 0.05$). ML juice was found to contain (0.62 ± 0.02) mg GAE g^{-1} of phenolics, (50.85 ± 0.75) mg QE g^{-1} of flavonoids, (0.13 ± 0.02) mg/g of anthocyanins and (89.33 ± 11.22) mg/g of sugar, whereas MF juice contains (3.21 ± 0.01) mg GAE g^{-1} of phenolics, (53.85 ± 3.44) mg QE g^{-1} of flavonoids, (0.12 ± 0.02) mg/g of anthocyanins and (553.23 ± 52.08) mg/g of sugar. A higher total sugar content in MF juice as compared to ML juice was expected, because simple sugars in fruits were always higher than leaf. According to Wang et al. (2015), MF contained 75–118 g/L of reducing sugars, at which 34–66 g/L was glucose and 38–73 g/L was fructose. In the study by Espada-Bellido et al. (2017), about 1.3 mg/g of phenolics and 0.2 mg/g of anthocyanins was successfully extracted from MF via ultrasound-assisted technique. Gao et al. (2020) successfully extracted 8.352 mg/g of phenolics from ML via microwave-assisted deep eutectic solvent extraction technique. Chemical composition of an extract was greatly affected by the selected extraction method.
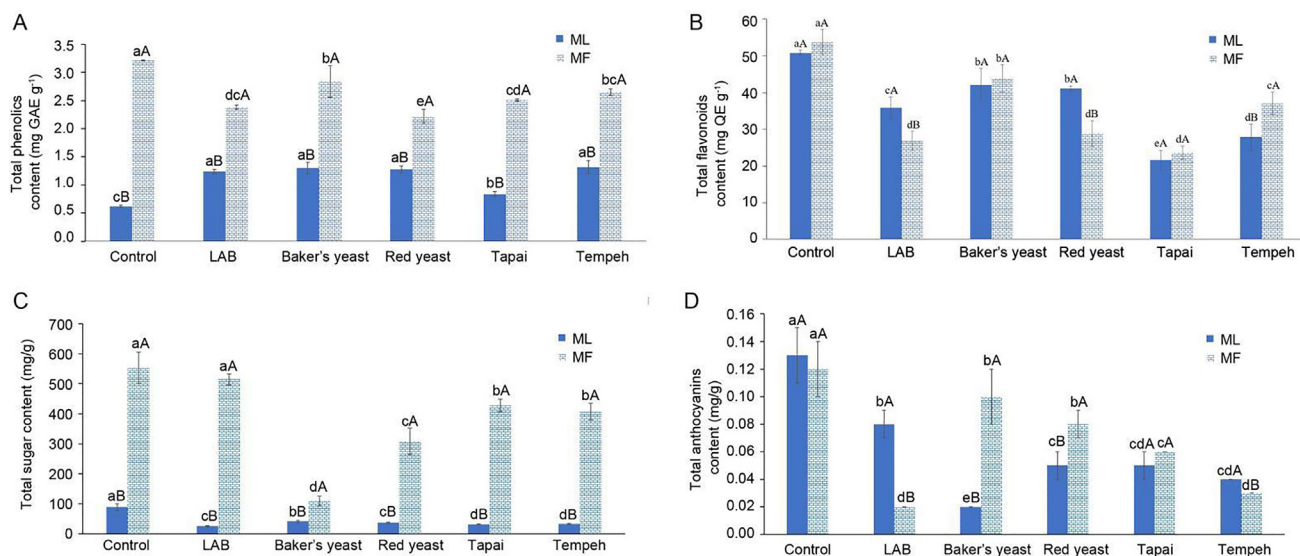

Fig. 1. Effect of fermentation using different types of microorganisms (LAB, Baker's yeast, red yeast, *Tapai* and *Tempeh*) on the changes of total phenolics (TPC) (A), total flavonoids (TFC) (B), total anthocyanins (C), and total sugar of mulberry leaves (ML) and fruits (MF) (D) (mean ± SD, n = 3).

Efficiency of an extraction method was influenced by the penetration of solvent, rate of mass transfer and solubility of the targeted compounds in the extraction solvent (Espada-Bellido et al., 2017). Therefore, lower TPC in ML juice compared with MF juice might be due to the limitation of the extraction method employed. The extraction efficiency might be higher on the fruits which had a softer texture than the waxy leaves.

After fermentation, all chemical components (TPC, TFC, anthocyanins and sugar) of MF and ML juices were reduced significantly ($P < 0.05$), except TPC of ML juice. TPC of ML juice was increased significantly ($P < 0.05$) after fermentation. TPC of ML juice (0.62 ± 0.02 mg GAE g^{-1}) was doubled (1.2–1.3 mg GAE g^{-1}) after fermentation by red yeast, Baker's yeast, LAB and *Tempeh* starter. The results were in accordance with study by Wang, Luo, Wu, Liu, and Wu (2018). According to the study, TPC of guava leaves increased after fermentation by *Monascus anka* and *Saccharomyces cerevisiae*. Besides, study by Degrain, Manhivi, Remize, Garcia, and Sivakumar (2020) also revealed that total phenolic acids of African nightshade leaves was increased by 1.3–1.4 times after lactic acid bacteria fermentation. Furthermore, Xiao et al. (2016) reported that *Rhizopus oligosporus* fermentation significantly enhanced total phenolics and isoflavone aglycone content of soybean. During fermentation, several carbohydrate-hydrolyzing enzymes, such as cellulase, estarase, xylanase and β-glucosidase are secreted by the microorganisms. These enzymes catalyze the hydrolysis of covalent bonds between the phenolics and lignocellulose in the plant cell wall matrix, hence release them into the extract. Therefore, both free and bound form of phenolics in the extract will be enhanced after fermentation (Thai, Camp, Smagghe, & Raes, 2014). According to Guo et al. (2020), cellulase and β-glycosidase activities were increased significantly during mulberry leaves fermentation. The hydrolytic action of these enzymes promotes cell wall break down, and subsequently dissociates the phenolics. However, the levels of enzymes secreted by the microorganisms during fermentation differ with the fermentation stage. At the early stage of fermentation, the microorganisms secreted enzymes such as amylase which dissociate nutrients from the substrate for its growth. Amylase activity was also proven highly correlated with the rate of liberation of soluble phenolics during fermentation (Bei, Chen, Lu, Wu, & Wu, 2018). However, high sugar level in MF juice may cease the amylase secretion by the microorganisms because the fermentation medium had supply adequate carbon source to support microorganism growth. Furthermore, fast growth rate of microorganisms with the high supply of carbon source might cause over-fermentation of MF, thus causing TPC and TFC reduction (Wang, Luo, Wu, & Wu, 2018). Nevertheless, further study needs to be conducted to determine the carbohydrate hydrolase activity and microbial growth rate in the extract to verify this hypothesis. Among the fermented MF, MF fermented with red yeast and LAB experienced the most drastic TPC and TFC reduction, which were approximately 30% and 50%, respectively. Although fermentation always associated with increased TPC and TFC yield, the recovery of TPC and TFC might also be reduced during fermentation if the soluble TPC and TFC bind with other soluble constituents in fermentation medium due to prolong fermentation, shifting of metabolic pathway of fermenting microorganisms to metabolize phenolics and degradation/hydrolysis of soluble TPC and TFC by the microbial enzymes (Adebo & Medina-Meza, 2020).

Sugar is the main carbon source for microorganisms during fermentation. Significant reduction of total sugar in MF and ML postulates microbial growth in the medium during fermentation. Among the samples, LAB fermentation in MF did not cause significant reduction of total sugar as compared with the unfermented MF. In contrary, LAB fermentation in ML caused substantial total sugar reduction. Among the fermented samples, LAB-fermented ML contained the lowest total sugar content [(25.53 ± 1.71)

mg/g]. In study by Chaudhary et al. (2019), *L. plantarum* was proven to grow well in mulberry fruit juice. The unchanged total sugar content in MF after 48 h fermentation might be due to the action of amylase enzyme on carbohydrate breakdown in MF during fermentation (Plaza-Vinuesa, Hernandez-Hernandez, Moreno, Rivas, & Muñoz, 2019). Sugars released from the carbohydrate breakdown had countered sugar consumption by *L. plantarum* during fermentation. In a Pearson correlation analysis, total sugar content in fermented ML was found negatively correlated with TPC ($r = -0.695$ at $P < 0.01$), but positively correlated with TFC ($r = 0.695$ at $P < 0.01$) and anthocyanin ($r = 0.731$ at $P < 0.01$). This finding proposes that microbial growth in ML facilitates the release of phenolics, but concurrently promotes the degradation of flavonoids and anthocyanins during fermentation. Among the fermented samples, Baker's yeast fermented MF contained the lowest total sugar content [(110.10 ± 16.09) mg/g]. Total sugar in MF was reduced by about 5-fold after Baker's yeast fermentation. Results of Pearson correlation analysis revealed that there was no significant correlation between total sugar content in the fermented MF with its TPC, TFC and anthocyanins yield. This finding unveiled that microbial growth in MF may not be the only leading factors that caused the changes of phenolics, flavonoids and anthocyanins in MF during fermentation.

According to Fig. 1D, anthocyanins content in MF and ML juice (0.12–0.13 mg/g) had no significant difference ($P > 0.05$). However, LAB and *Tempeh* starter fermentation on MF and Baker's yeast fermentation on ML had drastically reduced the anthocyanins content by about 80% to 0.02–0.03 mg/g. According to Zheng et al. (2014), release of hydrogen peroxide as the metabolite of LAB fermentation is the leading cause of anthocyanins degradation. Besides, pH change and action of microbial hydrolytic enzymes during fermentation are among the factors accelerate anthocyanins degradation (Kokkinomagoulos, Nikolaou, Kourkoutas, & Kandylis, 2020; Mushollaeni & Tantalu, 2020). In addition, anthocyanins will also react with the metabolites released during yeast fermentation, resulting in the formation of pyranoanthocyanins. The red–orange color pyranoanthocyanins are less sensitive to pH, hence less likely to be quantified through pH differential method (Ruta & Farcasanu, 2019). Moreover, anthocyanins in the fermentation medium may also be adsorbed on the yeast cell walls, thus reduces the anthocyanins content in the extract (Echeverrigaray, Scariot, Menegotto, & Delamare, 2020; Hornedo-Ortega et al., 2017).

3.2. Effect of fermentation using different microorganisms on bioactivity changes of ML and MF

Fig. 2 indicated the effect of fermentation using different microorganisms on free radical scavenging activity (DPPH) and ferric reducing antioxidant power (FRAP) of ML and MF juices. The results showed that MF juice exhibited higher DPPH activity and FRAP than ML juice. DPPH activity of both ML and MF juices was increased significantly ($P < 0.05$) after fermentation by all types of microorganisms. DPPH activity of MF juice was increased from about 45% to 51%−53% after fermentation by Baker's yeast and *Tapai* starter. For ML, LAB fermentation was the best to enhance DPPH activity. DPPH activity of ML juice was increased by about 45% from (25.43 ± 0.22)% to (36.90 ± 0.76)% after fermentation. However, the DPPH activity was not correlated with its TPC, TFC and anthocyanin content. This finding postulated that there might be other antioxidant compounds, other than phenolics, flavonoids and anthocyanins were present in the fermented ML and MF which contributed to DPPH activity. This hypothesis was supported by the previous report by Guo et al. (2020) and Kwaw et al. (2018). In their study, DPPH activity of mulberry fruits and leaves was increased after fermentation, but the activity had a very weak correlation with its TPC, TFC and anthocyanin. According to Hur, Lee,
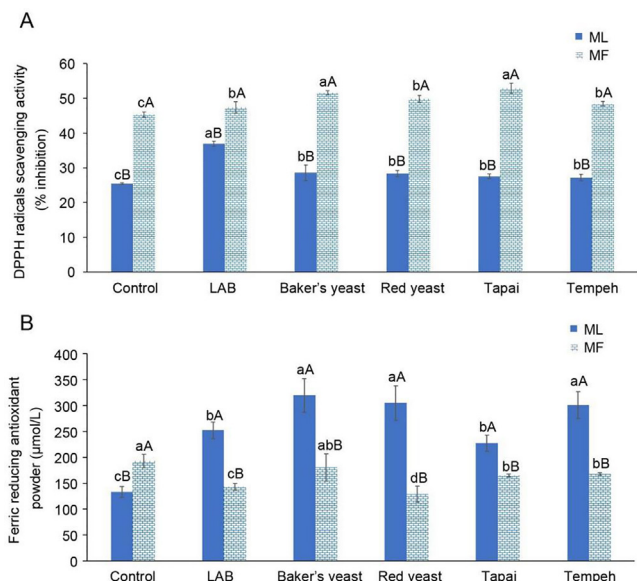
A

B

Fig. 2. Effect of fermentation using different types of microorganisms (LAB, Baker's yeast, red yeast, *Tapai* and *Tempeh*) on the changes of (A) DPPH scavenging activity and (B) ferric reducing antioxidant power (FRAP) of mulberry leaves (ML) and fruits (MF) (mean ± SD, n = 3).

Kim, Choi, and Kim (2014) and Iqbal et al. (2012), compounds such as ascorbic acid, peptide, amino acids, and fiber were also contribute to DPPH free radical scavenging activity. Antioxidant compounds were not limited to phenolic compounds, flavonoids, or anthocyanins only. Furthermore, Adesulu-Dahunsi, Jeyaram, Sanni, & Banwo, 2018; Wang et al., 2017 also revealed that exopolysaccharides secreted by *L. plantarum* during fermentation also exhibited free radicals scavenging and ferrous ion chelating activities in a dose-dependent manner. However, an opposite trend was observed in FRAP of MF juice. Fermentation caused reduction of FRAP in MF juice. FRAP of ML juice (133.47 ± 10.54 μmol/ml) was increased by about 2.4-folds after Baker's yeast, *Tempeh* starter and red yeast fermentation. However, the same fermentation caused 6.5% (Baker's yeast), 13% (*Tempeh* starter) and 33% (red yeast) of FRAP reduction, respectively in MF juice. The increase of TPC released during fermentation due to the action of carbohydrate and cell wall degrading enzymes were believed to be the cause of the increment of FRAP in ML (Guo et al., 2020). This hypothesis was supported by the results of Pearson correlation analysis, in which the FRAP of ML was found correlated with its TPC (r = 0.900 at P < 0.01). Furthermore, Yu et al. (2018) also proved that chlorogenic acid isolated from the mulberry leaves exhibited a strong positive correlation with FRAP. Although study by Abd Razak, Abd Rashid, Jamaluddin, Sharifudin, and Long (2015) and Kwaw et al. (2018) found that *Rhizopus oligosporus* and *Monascus purpureus* fermentation on rice bran and lactic acid bacteria fermentation on mulberry fruit juice enhanced FRAP of both products, results of this study were not in line with their studies. Results of Pearson correlation analysis in this study unveils that FRAP of MF juice was correlated with its TPC (r = 0.724 at P < 0.01) and TFC (r = 0.653 at P < 0.01), while fermentation was proven to cause a significant reduction in TPC, TFC and anthocyanins of MF. Thus, FRAP reduction after fermentation in MF was within expectation.

Moreover, fermentation had successfully turn ML became a better source FRAP than MF. FRAP of all fermented ML juices was significantly (P < 0.05) higher than MF juices. Generally, FRAP was always positively correlated with TPC and TFC due to their strong reducing power, thereby forming stable compounds. However, the correlation between FRAP and TPC/TFC was not always com-

patible under all circumstances. Not all phenolics and flavonoids exhibit equally strong reducing power (Kwaw et al., 2018; Wang et al., 2015; Yu et al., 2018). In study by Yu et al. (2018), FRAP of ML was greatly affected by its rutin and isoquercitrin content, whereas quercetin-malonyl-glucoside and kaempferol-malonyl-glucoside were contributed to FRAP weakly. Sample varieties and species, types of microorganisms used, fermentation conditions and extraction methods were the factors that cause different post-fermentation changes in chemical profile and antioxidant activity (Hur et al., 2014).

Fig. 3 demonstrated the effect of fermentation using different microorganisms on α-amylase inhibition activity of ML and MF juices. ML juice contained higher α-amylase inhibition activity than MF juice. α-amylase inhibition activity of both ML and MF was increased significantly (P < 0.05) after fermentation except red yeast-fermented ML. Red yeast fermentation did not cause significantly change (P > 0.05) in α-amylase inhibition activity of ML. Inhibiting carbohydrate-hydrolyzing enzymes such as α-amylase and α-glucosidase activity has been long adopted as one of the effective therapeutic approaches to monitor type 2 diabetes. Enzyme inhibition led to the delay in carbohydrate digestion and absorption, hence retard the substantial rise of postprandial blood glucose level (Adisakwattana, Ruengsamran, Kampa, & Sompong, 2012). Many studies have proven the remarkable effectiveness of fermentation in enhancing hypoglycemic property of foods. Shori (2020) proved that α-amylase inhibition activity of herbal fortified yogurt was five times higher than the plain yogurt, even after two weeks of storage. The fermented wheat sourdough bread had higher α-amylase inhibition activity than the common yeast bread (Diowksz, Malik, Jaśniewska, & Leszczyńska, 2020). Apart from this, Guo et al. (2020) found that hypoglycemic potential of mulberry fruits had been enhanced after *Monascus anka* fermentation. The α-glucosidase inhibition activity was increased by three-folds after fermentation. Results of Pearson correlation analysis reveals that α-amylase inhibition activity of MF was negatively correlated its TPC (r = −0.499 at P < 0.05), TFC (r = −0.551 at P < 0.05) and anthocyanins content (r = −0.639 at P < 0.01). This finding suggested that α-amylase inhibition activity of MF was not due to the phenolics, flavonoids and anthocyanins contained in it. High TPC, TFC and anthocyanins content in MF might slightly diminish its α-amylase inhibition activity. According to Jeong, Lee, Cho, Jeong, and Jeong (2014) and Tian, Tang, and Zhao (2016), anti-diabetic potential of mulberry leaves is contributed by its 1-deoxynojirimycin, fagomine, flavonoid and polysaccharide content. TPC and TFC were not always positively correlated with α-amylase inhibition activity because of the compound species and their interactive effects (Xiong et al., 2020). The configuration and polarity of phenolic compounds may also affect the ability of the compounds to bind to the active site of α-amylase enzyme (Li et al., 2018). Moreover, negative correlation between TPC and TFC in
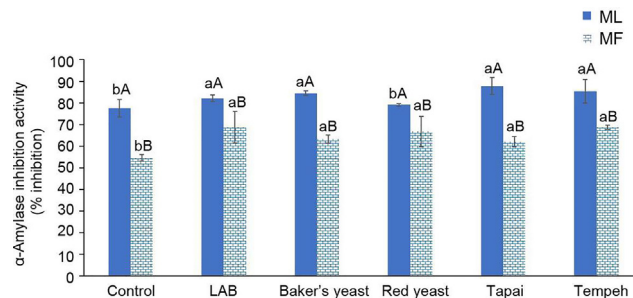

Fig. 3. Effect of fermentation using different types of microorganisms (LAB, Baker's yeast, red yeast, *Tapai* and *Tempeh*) on changes of α-amylase inhibition activity of mulberry leaves (ML) and fruits (MF) (mean ± SD, n = 3).

the plant extracts with their inhibitory effect toward α-amylase enzyme was also reported by Hyun, Ra, Han, and Kim (2018) and Loh and Hadira (2011).

Fig. 4 elicited the effect of fermentation using different microorganisms on albumin denaturation inhibition and anti-lipoxygenase activity of ML and MF juices.

ML and MF demonstrated the same albumin denaturation inhibition activity before fermentation. The albumin denaturation inhibition activity of ML was decreased significantly ($P < 0.05$) after fermentation by all types of microorganisms. However, the activity was increased in MF. The albumin denaturation inhibition activity of MF juice was almost doubled after fermentation. Contrarily, MF juice exhibited higher anti-lipoxygenase activity than ML juice. Fermentation significantly increased ($P < 0.05$) the anti-lipoxygenase activity of ML juice. This finding proposes that both ML and MF exhibited anti-inflammation activity, but work via different pathways. Protein denaturation causes the production of autoantigens that trigger inflammation in condition such as rheumatic arthritis, cancer, and diabetes (Dharmadeva, Galgamuwa, Prasadinie, & Kumarasinghe, 2019). Lipoxygenase is one of the enzymes that plays important role in inflammation, particularly in the biochemical processes of leukotrienes. Leukotrienes are the main regulator of allergic response and inflammation (Sari, Elya, & Katrin, 2017). Either inhibiting protein denaturation or the activity of lipoxygenase enzyme could halt inflammation. Results of Pearson correlation analysis reveals that anti-lipoxygenase activity of MF was positively correlated with FRAP ($r = 0.718$ at $P < 0.01$), whereas albumin denaturation inhibition activity was correlated with DPPH activity ($r = 0.878$ at $P < 0.01$). These results disclosed that anti-inflammation activity of MF was closely related to its antioxidant activity. According to Arulselvan et al. (2016), antioxidant plays crucial role in inhibiting inflammation because overproduction of reaction oxygen species will cause oxidative damage to body cells, eventually induce inflammation. Yu et al. (2021) and Xu et al. (2020) suggested that indole, loliolide, odisolane, quercetin, kaempferol, eriodictyol and artonin are the compounds that contribute to anti-inflammation activity in mulberry fruits. However, there was no significant positive correlation found between anti-inflammation and antioxidant activity of ML. Besides phenolic

compounds, other component such as polysaccharide of mulberry leaves has also been proven to exhibit anti-inflammation activity (He et al., 2018). However, Zhang et al. (2016) discovered that there was a synergistic interaction between flavonoid and mulberry leaf polysaccharide on the antioxidant activity. Mulberry leaf polysaccharide alone has weak antioxidative effect. Thus, it was strongly believed that anti-inflammation activity of ML was likely contributed by non-antioxidant compounds.

4. Conclusion

Both mulberry fruit and leaf juices demonstrated remarkable antioxidant, anti-inflammation, and anti-diabetic activities. Mulberry fruits exhibited higher DPPH free radical scavenging activity, ferric reducing antioxidant powder and anti-lipoxygenase activity than the leaves. However, mulberry leaves displayed higher α-amylase inhibition activity than the fruits. Although different microorganisms (lactic acid bacteria, red yeast, Baker's yeast, *Tapai* starter and *Tempeh* starter) were found to exert different effects on the mulberry fruits and leaves, their antioxidant, hypoglycemic and anti-inflammation activities had been improved substantially after fermentation. In overall, fermentation by *Tempeh* starter was proven as the best technique to enhance total phenolics, ferric reducing antioxidant power, α-amylase inhibition activity and anti-lipoxygenase activity of mulberry leaves, whereas *Tapai* fermentation effectively enhanced the DPPH scavenging activity and albumin denaturation inhibition activity of mulberry fruits. Fermentation is recommended as an economic green approach to improve nutritional value of both mulberry fruits and leaves.

Future research to determine the chemicals profile in the *Tempeh* starter-fermented mulberry leaves and *Tapai* starter-fermented mulberry fruits is recommended. Besides, the strains in the *Tempeh* and *Tapai* starters should also be identified so that the biochemical reactions that happened during fermentation, which eventually contribute to the increase of antioxidant, anti-inflammation, and hypoglycemic activities of mulberry fruits and leaves can be elucidated.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
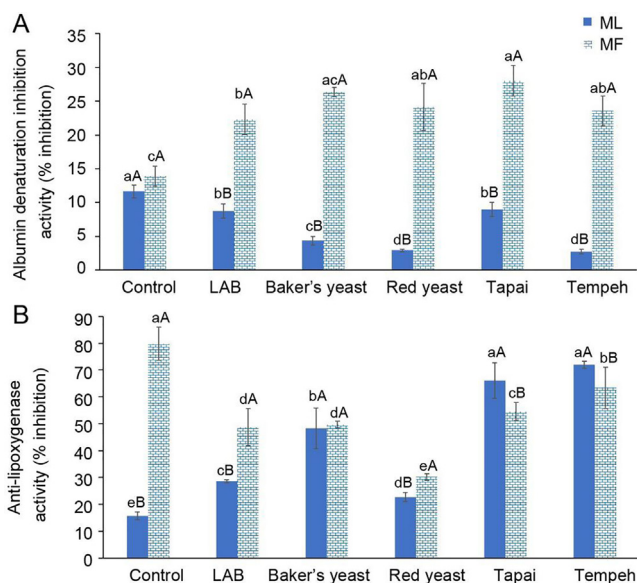
Fig. 4. Effect of fermentation using different types of microorganisms (LAB, Baker's yeast, red yeast, *Tapai* and *Tempeh*) on the changes of (A) albumin denaturation inhibition activity and (B) anti-lipoxygenase activity of mulberry leaves (ML) and fruits (MF) (mean ± SD, $n = 3$).

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