https://doi.org/10.48048/tis.2024.7483

Metabolomics Profiles of Solid State and Submerge Fermentation of Corn Silk using Mixed Microbes

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Received: 8 September 2023, Revised: 28 September 2023, Accepted: 2 October 2023, Published: 5 February 2024

Abstract

Corn silk is a waste product when harvesting corn, but it contains many bioactive compounds. This research characterized and assessed the antioxidant bioactivity of compounds isolated from corn silk. Two different biological fermentation processes were compared: Solid-state fermentation (SSF) and submerged fermentation (SmF). Fresh corn silk (*Zea mays* L.) from both sweet corn and feed corn were fermented separately with various microorganisms (*Bacillus subtilis*, *Lactobacillus* sp. and *Saccharomyces cerevisiae*). Biological compounds were identified using LC-MS/MS. The extract was then tested for total phenolic content (TPC) and total flavonoid content (TFC). Next, the extract was tested for xanthine oxidase inhibition and antioxidant inhibition using the ABTS method. Potential contamination with the heavy metals As, Pb, and Hg was checked. The results show that fermented corn silk extract contains numerous bioactive compounds and amino acids. Among the different extract process variations, the highest TPC and TFC were 6.1 ± 0.7 µg GAE/g dry wt. and 68.3 ± 1.1 µg QE/g dry wt., respectively, and the highest xanthine oxidase inhibition was 76.5 ± 6.4 %. The above highest results were obtained using SmF of sweet corn for 60 days. In contrast, the highest antioxidant activity of 66.9 % was found using SSF of sweet corn for 20 days. Neither sweet corn nor feed corn were found to contain heavy metal ions. In summary, fermented corn silk extract samples contain numerous bioactive compounds, including both flavonoid and phenolic compounds, and the extract demonstrates antioxidant activity. Corn silk extracts from fermentation thus have potential for utilization in health products.

Keywords: Corn silk, Heterofermentative microbe, Heatmaps, LC-MS/MS, Xanthine oxidase inhibition

Introduction

Corn silk consists of silky yellow plant fibers that grow on top of the ear of the corn plant (*Zea mays* L.), and it is a part of the plant's female flower (stigma). Corn silk makes up approximately 2 % of the weight of a fresh corn ear [1]. Corn is the third-largest cash crop in the world. In most countries, corn can be grown year-round. As a result, corn silk is a major by-product of the corn processing industry, and it is traditionally discarded as eco-friendly agricultural waste or used as animal feed [2]. However, corn silk is rich in beneficial nutrients, including carbohydrates, proteins, vitamins, and minerals, besides containing resins, mucilage, and fibers [2]. Corn silk also contains a wide range of bioactive compounds in the form of volatile oils, steroids, and other natural antioxidants, such as polyphenols and flavonoids [3]. Several *in vivo* and clinical studies have reported that corn silk is safe for human consumption [4]. Given these benefits, corn silk is now being utilized in the development of value-added foods, such as beverages. Previous studies of 10 different corn silk genotypes found a significant number of bioactive components, including flavonoids and phenolics, and revealed antioxidant activity of corn silk polysaccharides [5]. However, those studies utilized a variety of techniques. Also, content of nutrients and bioactive compounds in corn silk can vary widely depending on soil characteristics, environmental conditions, and the specific cultivar tested.

Corn silk possesses potentially high medicinal value because of its diverse bioactive phytochemical compounds. Corn silk contains important phenolic compounds that may fight dementia. The bioactive compounds of corn silk have demonstrated antioxidant, antimutagenic, antiproliferative, anti-inflammatory, antihyperglycemic, antidiabetic, antibacterial, antifungal, antihyperlipidemic, antidepressant, antihypertensive, antihyperlipidemic, antiadipogenic, and anti-fatigue activities [1,6]. In addition, research

by Lertkaeo *et al.* [7] reported a synergistic effect when mixing corn silk extract with silver nanoparticles, a combination that can inhibit inflammation as well as the growth of *Escherichia coli* and *Pseudomonas aeruginosa* bacteria.

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms such as bacteria and fungi. In the course of this metabolic breakdown, they also release several compounds including primary metabolites and secondary metabolites or bioactive compounds. The development of techniques such as submerged fermentation (SmF) and solid-state fermentation (SSF) should be performed. SmF or liquid fermentation is best suited for microorganisms such as bacteria that require high moisture of substrates are utilized quite rapidly; hence need to be constantly supplemented with nutrients. This process has led to industrial-level production of bioactive compounds. Solid-State Fermentation (SSF) is best suited for fermentation techniques involving microorganisms, mainly fungi and moulids that require less moisture content. This technique impersonates the natural environment of most microorganisms. It is less susceptible to bacterial contamination and enables an increased enzyme efficiency for many enzymes. However, the selection of a substrate, microorganisms and process conditions influence the desired enzyme production [8]. SSF is considered to be a suitable tool for transforming agro-industrial wastes and by-products into value-added products, such as bioactive compounds, cosmetic, food and feed products. Typically, the dynamic changes within the fermentation are tracked over the incubation period. However, such research on fermented corn silk is limited.

The current study highlights the value of corn silk processed by submerged fermentation and solid-state fermentation with mixed microorganisms including *Saccharomyces cerevisiae*, *Bacillus subtilis*, *Bacillus* sp. and *Lactobacillus* sp. for various periods of time. There is a long history of studying beneficial microbes and metabolites produced through many different kinds of fermented foods, and high-performance liquid chromatography (HPLC) and mass spectrometry (MS) are commonly used to find metabolite profiles of fermented foods.

Materials and methods

Sample collection and preparation

The sweet corn silk (*Zea mays*) variety used in this study was “Organic2”, and it was provided by the National Corn and Sorghum Research Center, Nakhon Ratchasima, Thailand. The feed corn silk variety was “Pacific392”, and this was provided by a local farm in Phitsanulok, Thailand. After the mature corn was harvested, corn silk samples were collected and washed under running tap water. The corn silk was kept in a polyethylene bag with a vacuum seal and maintained below −20 °C until use.

When the corn silk was going to be used, it was dried in a hot air oven at 60 °C for approximately 12 h until the weight was constant after water evaporation. The dried corn silk was then kept in a polyethylene zip-lock bag and maintained in a desiccator cabinet until use.

Sample extraction

Water extraction

The water extract of dried corn silk was prepared by placing 150 g of dried corn silk and 500 mL of water in a conical flask, which was autoclaved at 121 °C for 15 min. The extract solution was kept in a sterile tube at −20 °C. This water extract was used to determine antioxidant content and activity.

Biological extraction by fermentation

Submerged fermentation (SmF): Both varieties of fresh corn silk, handled separately, were cut into a tiny segment of 0.2 - 0.3 cm and 300 g of these were then placed in 900 mL of water in a glass jar with a tight-fitting plastic lid. To this was added 300 g of sucrose, 0.2 g of the mixed microorganism powder (10^{10} cell/g: *Bacillus subtilis*, *Lactobacillus* sp., and *Saccharomyces cerevisiae*) provided by the Department of Agriculture, Thailand. The jar was incubated at 30 °C for 60 days under static condition. At the 30 days and 60 days marks, the fermented sample was suctioned, and its pH value was adjusted to 7.0 with 6 N NaOH. The sample was then filtered with a 0.2 µm sterile filter (Acrodisc) prior to storage at −20 °C.

Solid-state fermentation (SSF): Both varieties of fresh corn silk, handled separately, were cut into a tiny segment of 0.2 - 0.3 cm and 300 g of these were then placed in only 100 mL water in a glass jar with a tight-fitting plastic lid along with 30 g of sucrose and 0.2 g of the mixed microorganism powder (10^{10} cell/g: *Bacillus subtilis*, *Lactobacillus* sp., and *Saccharomyces cerevisiae*) were added. The jar was incubated at 30 °C for 20 days. At the 7 and 20 days marks, the fermented sample was suctioned, and its pH value was adjusted to 7.0 with 6 N NaOH. The sample was then filtered with a 0.2 µm sterile filter prior to storage at −20 °C.

Metabolomics by LC-ESI-QTOF-MS/MS analysis

Liquid Chromatography-Mass spectrometer system used was a modular Agilent 6540 Q-TOF-MS spectrometer (Agilent Technologies) coupled with an Agilent 1260 Infinity Series High performance liquid chromatography (HPLC) system. The chromatographic separation was performed on an Agilent Luna C_{18} column (4.6×150 mm^2, 5 µm) (Phenomenex). The flow rate of the mobile phase was 0.5 mL/min at 35 °C. The mobile phase A was purified water type I (Millipore) and B was acetonitrile. Both phases contained 0.1 % (v/v) formic acid. The gradient elution mode started with 5 % solvent B to 95 % B linear gradient within 30 min and held on at this ratio for 10 min. The injection volume was 5 µl. The operating parameters with electrospray ionization for ESI-MS detection were as follows: Drying gas (N_2) flow rate 10 L/min at 350 °C; nebulizer pressure 30 psi; capillary 3,500 V; skimmer 65 V; octupole RFV 750 V; and fragmentary voltage 250 V in negative mode and 100 V in positive mode. The mass range was set at m/z 100 - 1,000 Da with a 250 ms/spectrum. The chemicals used were of analytical grade (Sigma-Aldrich). Purified water was purified by a Milli-Q purification system (Millipore). Samples were diluted and filtered through a 0.2 µm Nylon membrane syringe filter (Acrodisc) before auto injecting to the system [9].

All acquisition and analysis of the data were controlled by MassHunter Data Acquisition Software version B.05.01 and MassHunter Qualitative Analysis Software B06.01, respectively (Agilent Technologies). Analysis of each sample was performed both in positive and negative ionization modes. The m/z and fragmentation patterns of each compound were identified using metabolites databases such as the METLIN PCD/PCDL database and public database as the Human Metabolome Database (http://www.hmdb.ca).

Bioactive compound analysis

Determination of total flavonoid compounds (TFC)

TFC analysis of the fermented extract was carried out using the protocol of Zannou *et al.* [10], somewhat modified. Into each well of a 96-well microplate (flat-bottom, sterile) was placed 87 µL of the fermented sample along with 26 µL of 5 % w/v $NaNO_2$. The mixture was then left in the dark for 5 min. Then 43 µl of 10 % w/v $AlCl_3$ was added. The mixture was left at ambient temperature for 6 min before adding 43 µL of 1 M sodium hydroxide and then left at ambient temperature for another 10 min. The mixture was measured at 510 nm by spectrophotometer (BioTek). The TFC was calculated from a calibration curve using quercetin as the standard ($Y = 0.0099x$; $R^2 = 0.9870$). The results were estimated as µg quercetin equivalents (QE)/mL.

Determination of total phenolic compounds (TPC)

The TPC of the fermented extract was determined using the Folin-Ciocalteu method adopted from Grobelna *et al.* [11]. Into each well of a 96-well microplate (flat-bottom, sterile) was placed 50 µL of the fermented extract along with 50 µL of 10 % Folin-Ciocalteu's phenol reagent. The mixture was left in the dark for 8 min before adding 100 µL of 7.5 % (w/v) sodium carbonate and then left in the dark again for another 30 min. The absorbance of the reactant was measured at 765 nm by microplate reader spectrophotometer (BioTek). The TPC in each sample was calculated from a calibration curve ($Y = 4.56x$; $R^2 = 0.9779$), using gallic acid as a standard. The results were reported as µg gallic acid equivalents (GAE)/ml.

Bioactivity capacity

ABTS radical cation scavenging activity assay

Antioxidant activity was measured using the ABTS method. The method used was modified from Grobelna *et al.* [12]. ABTS, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical agent was prepared by weighing 0.0063 g ABTS into 10 mL of distilled water. Next was added 0.0166 mg potassium persulphate, and the mixture was left in the dark at room temperature for 12 - 16 h. Into each well of a 96-well microplate (flat-bottom, sterile) was placed 180 µl of ABTS solution followed by 20 µl of fermented extract sample. The mixture was left in the dark for 15 min. The absorbance was then measured at a wavelength of 734 nm using a microplate reader (BioTek). The antioxidant activity in each sample was calculated from a calibration curve ($Y = -0.443x + 1.1096$; $R^2 = 0.9753$). The results were expressed as mmol Trolox equivalent (TE). The percentage of antioxidant activity inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{(\text{Asb. of ABTS} - \text{Asb. of sample})}{(\text{Asb. of ABTS} - \text{Asb. of ref.})} \times 100$$

The value IC_{50} of Antioxidant activity was calculated by the concentration of the fermented extract to inhibit 50 % of free radicals.

Xanthine oxidase inhibitory activity

XOD inhibitory activity of the fermented extract was determined using the modified method of Wang *et al.* [13]. Into each well of a 96-well microplate (flat-bottom, sterile) was placed 60 µl of phosphate-buffered saline solution (Sigma-Aldrich) and 30 µl of 0.06 mM xanthine solution (Sigma-Aldrich). Next was added 30 µl of the fermented extract. Separately, another microplate was prepared in the same way expect that 0.04 mM Allopurinol (Sigma-Aldrich) replaced the fermented extract. Allopurinol is a common gout medicine against which the fermented extract is compared here as a positive control. To each well of both microplates was added 30 µl of 0.2 U/mL xanthine oxidase, and the solution was mixed gently. The reaction was performed at 37 °C for 2 min and stopped by adding 30 µl of 0.5 M HCl. The reactant absorbance was measured at a wavelength of 290 nm using a microplate reader (BioTek). The percentage of xanthine oxidase inhibition was calculated as follows:

% of xanthine oxidase inhibition = [(A–B) – (C–D)/(A–B)]×100

A = Normal xanthine oxidase activity: Xanthine mixed with xanthine oxidase

B = The control of "A" is only xanthine.

C = Xanthine oxidase activity inhibited using fermented extract or Allopurinol: xanthine and xanthine oxidase mixed with either fermented extract or Allopurinol

D = The control of "C" is xanthine mixed with either fermented extract or Allopurinol

One unit of xanthine oxidase is the amount of enzyme that converts xanthine to 1 mmol uric acid per minute at 37 °C.

Detection of heavy metal ions

Fermented corn silk extract was tested for 3 heavy metal ions: Arsenic (As), Lead (Pb), and Mercury (Hg). These tests were performed by Central Laboratory (Thailand) Co. Ltd. in Bangkok with their in-house method TE-CH-134 based on AOAC (2019) [14] using ICP-MS.

Data analysis and statistics

All experiments were conducted in triplicate (n = 3). The statistical analysis was performed using GraphPad Prism9 Software. The data are shown as mean ± standard deviation. One-way analysis of variance (ANOVA) and Tukey tests were used to evaluate the data. Values of *p* below 0.05 were considered statistically significant.

The data of LC-ESI-QTOF-MS/MS analysis presented as mean ± standard deviation. The metabolomic datasets were generated using the R software (version 4.2.1.) with the *gg*plot2 package. The heatmap was generated to visualize the conversion in metabolites. A total metabolite profiles derived from LC–MS/MS analyses in positive and negative ESI mode was identified. The color green (ESI^+) and blue (ESI^-) indicate a decreasing trend thus limited at ± 10, while yellow corresponds to a value of zero that identified definitely the substances.

Results and discussion

Metabolomics

Phytochemical screening for the presence of metabolites in fermented corn silk was characterized by LC-ESI-QTOF-MS/MS in both negative and positive ion modes. These compounds were identified on the basis of the accurate mass measurement (mass error), which was obtained by comparing the observed mass of their protonated $(M+H)^+$, deprotonated $(M-H)^-$, or other adduct ions to the theoretical exact mass in databases. The results demonstrated in **Figures 1** and **2** were tentatively identified only 26 compounds.

The results show different chemical profiles in the various fermentation processes. The metabolites found in the various fermentations of this study (**Table 1**). Also found in both the unfermented sweet corn and feed corn controls, but not in the fermentations, were numerous organic acids including malic acid, iso-citric acid, and aconitic acid. Some of the amino acids found, for example phenylalanine, leucine, valine, and their derivatives, were present in all the experimental groups but with varying chemical structures. SSF has been observed that prolonged incubation periods may lead to alterations in the compound, particularly affecting fatty acid, phenolic compounds, and flavonoid compounds. These changes can result in chemical structural modifications or reduced quantities, rendering the determination of chromatogram peaks

unfeasible. Conversely, the SmF resulted in the production of various compounds in the category of organic acids. The main flavonoid in this study was apigenin-6-C-glucoside that was detected in the initial stages of all the fermentations. And phenolic compounds; Ethyl 3,4-dihydroxybenzoate were found in samples of feed corn silk that were fermented with SFF for 7 days and with SmF for 30 days. It was also found in fermented sweet corn silk with SmF for 60 days. **Figure 3** shows heatmaps that represent the correlation coefficients (r) of the metabolite profiles derived from LC-MS/MS analyses. It provided elucidates the precision in identifying each in 26 types of compounds that were determined by High-Performance Liquid. Chromatography (HPLC) analysis, in comparison with the reference metabolites database. Typically, the correlation coefficients (r) of the metabolite profiles derived from LC–MS/MS analyses should fall within the range of −10 to +10. In the context of this study, they have revealed that the values are within the specified range, thus rendering the analyzed compounds reliable.

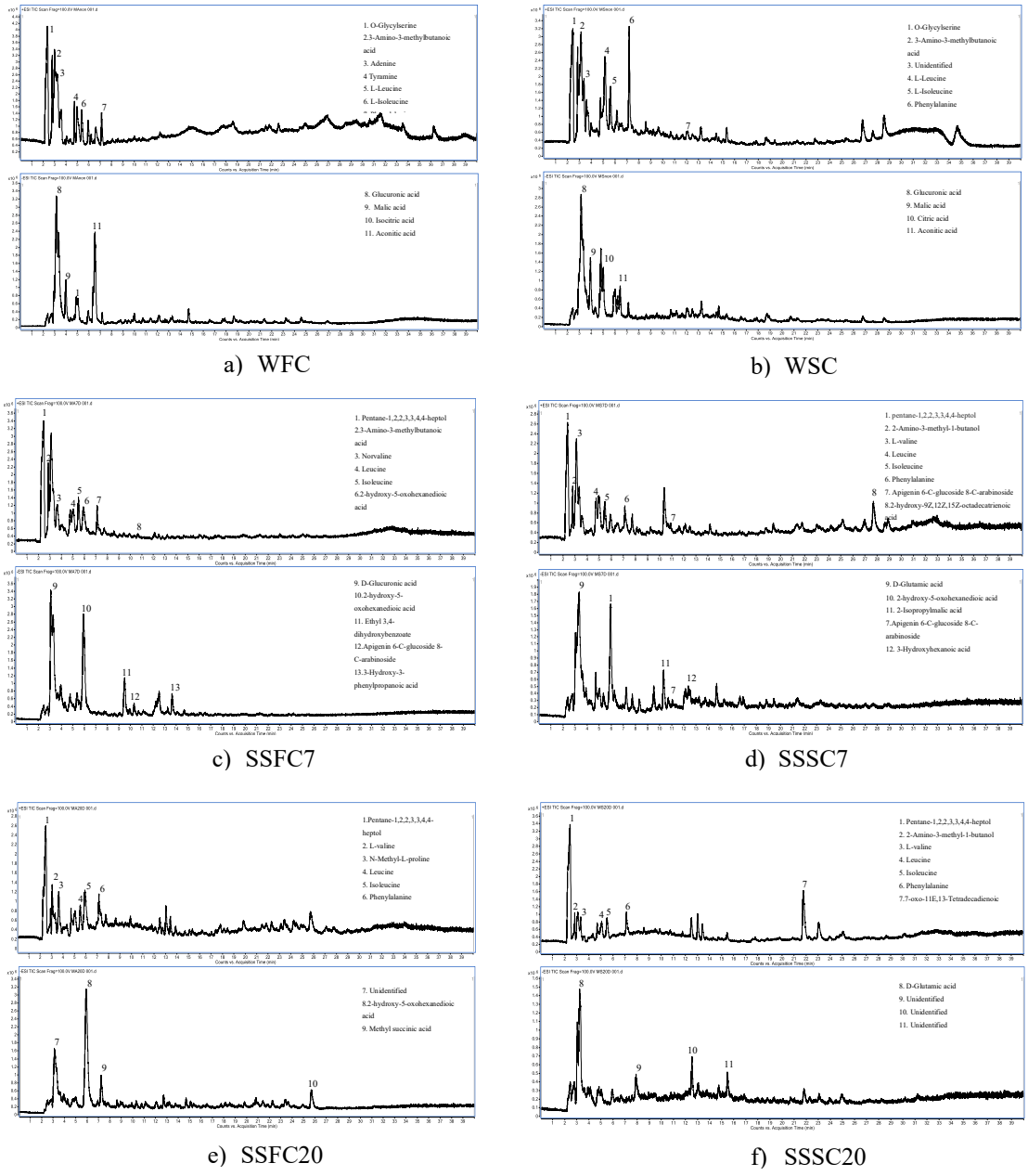
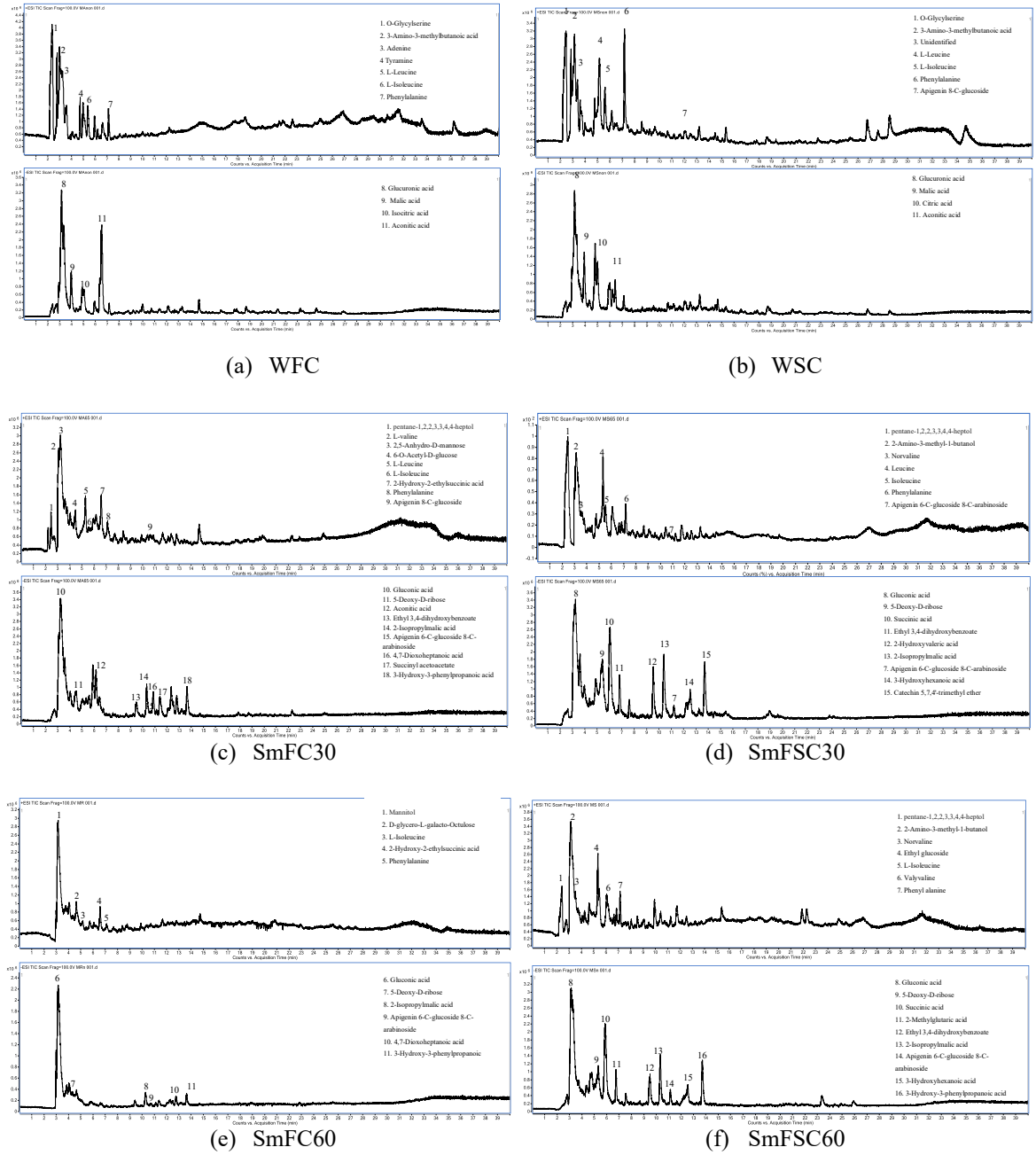


Figure 1 LC-MS/MS metabolite profiles of corn silk extracts from; (a) water extraction of feed corn, (b) water extraction of sweet corn, (c) solid-state fermentation (SSF) of feed corn for 7 days, (d) SSF of sweet corn for 7 days, (e) SSF of feed corn for 20 days, and (f) SSF of sweet corn for 20 days (in each extract profile: Above is ESP+ and below is ESP−.).

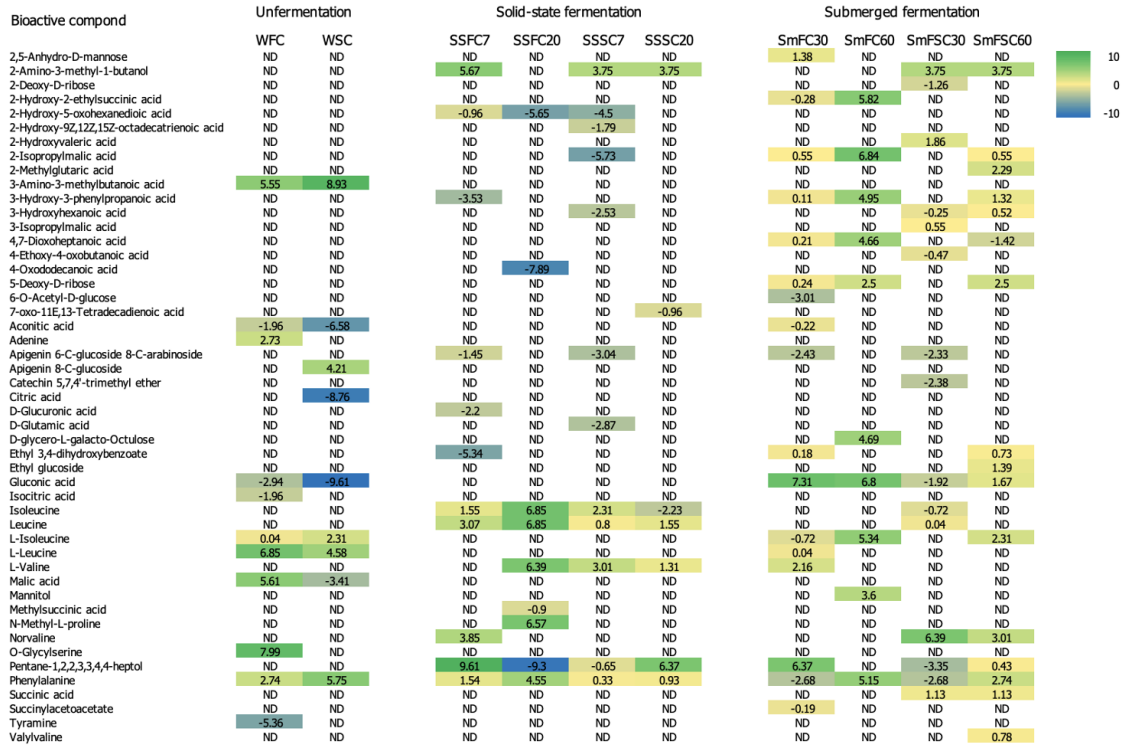


(a) WFC (b) WSC

(c) SmFC30 (d) SmFSC30

(e) SmFC60 (f) SmFSC60

Figure 2 LC-MS/MS metabolite profiles of corn silk extracts from; (a) water extraction of feed corn, (b) water extraction of sweet corn, (c) submerged fermentation (SmF) of feed corn for 30 days, (d) SmF of sweet corn for 30 days, (e) SmF of feed corn for 60 days, and (f) SmF of sweet corn for 60 days. (in each extract profile: Above is ESP^+ and below is ESP^-.).



ND = not detected

Figure 3 These heatmaps represent the correlation coefficients (r) of the metabolite profiles derived from LC–MS/MS analyses: WFC (water extraction of feed corn silk), WSC (water extraction of sweet corn silk), SSFC7 (SSF of feed corn silk for 7 days), SSSC7 (SSF of sweet corn silk for 7 days), SSFC20 (SSF of feed corn silk for 20 days), SSSC20 (SSF of sweet corn silk for 20 days), SmFC30 (SmF of feed corn silk for 30 days), SmFSC30 (SmF of sweet corn silk for 30 days), SmFC60 (SmF of feed corn silk for 60 days), and SmFSC60 (SmF of sweet corn silk for 60 days).

Table 1 Metabolomic analysis of extracts from unfermented and fermented corn silk using LC-MS/MS.

Unfermented		**Solid state fermentation**				**Submerged fermentation**			
Sweet corn silk	**Feed corn silk**	**Sweet corn silk**		**Feed corn silk**		**Sweet corn silk**		**Feed corn silk**	
		7 days	**20 days**	**7 days**	**20 days**	**30 days**	**60 days**	**30 days**	**60 days**
Amino acids and N-compounds									
O-Glycyl serine	3-Amino-3-methylbutanoic acid	2-Amino-3-methyl-1-butanol	2-Amino-3-methyl-1-butanol	2-Amino-3-methyl-1-butanol	L-Valine	2-Amino-3-methyl-1-butanol	2-Amino-3-methyl-1-butanol	L-Valine	L-Isoleucine
3-Amino-3-methylbutanoic acid	adenine	L-Valine	L-Valine	Norvaline	N-Methyl-L-proline	Norvaline	Norvaline	L-Leucine	Phenylalanine
L-Leucine	Tyramine	Leucine	Leucine	Leucine	Leucine	Leucine	L-Isoleucine	L-Isoleucine	
L-Isoleucine	L-Leucine	Isoleucine	Isoleucine	Isoleucine	Isoleucine	Isoleucine	Valylvaline	Phenylalanine	
Phenylalanine	L-Isoleucine	Phenylalanine	Phenylalanine	Phenylalanine	Phenylalanine	Phenylalanine	Phenylalanine		
	Phenylalanine	D-Glutamic acid							
Organic acids									

Unfermented		Solid state fermentation				Submerged fermentation			
Sweet corn silk	Feed corn silk	Sweet corn silk		Feed corn silk		Sweet corn silk		Feed corn silk	
		7 days	20 days	7 days	20 days	30 days	60 days	30 days	60 days
Gluconic acid	Gluconic acid	2-Isopropyl malic acid		D-Glucuronic acid	Methylsuccinic acid	Gluconic acid	Gluconic acid	2-Hydroxy-2-ethylsuccinic acid	Gluconic acid
Malic acid	Malic acid			3-Hydroxy-3-phenylpropanoic acid		Succinic acid	succinic acid	Gluconic acid	2-Hydroxy-2-ethylsuccinic acid
Citric acid	Isocitric acid					3-Isopropylmalic acid	2-Methylglutaric acid	Aconitic acid	2-Isopropylmalic acid
Aconitic acid	Aconitic acid						2-Isopropylmalic acid	2-Isopropylmalic acid	4,7-Dioxoheptanoic acid
							4,7-Dioxoheptanoic acid	4,7-Dioxoheptanoic acid	3-Hydroxy-3-phenylpropanoic acid
							3-Hydroxy-3-phenylpropanoic acid	3-Hydroxy-3-phenylpropanoic acid	
Fatty acids									
		2-hydroxy-9Z,12Z,15Z-octadecatrienoic acid	7-oxo-11E,13-Tetradecadienoic acid	2-hydroxy-5-oxohexanedioic acid	2-hydroxy-5-oxohexanedioic acid	4-Ethoxy-4-oxobutanoic acid	3-Hydroxyhexanoic acid		
		3-Hydroxyhexanoic acid			4-Oxododecanoic acid	2-Hydroxyvaleric acid			
		2-hydroxy-5-oxohexanedioic acid				3-Hydroxyhexanoic acid			
Flavonoids									
Apigenin 8-C-glucoside		Apigenin 6-C-glucoside 8-C-arabinoside		Apigenin 6-C-glucoside 8-C-arabinoside		Catechin 5,7,4'-trimethyl ether	Ethyl glucoside	Apigenin 6-C-glucoside 8-C-arabinoside	
						Apigenin 6-C-glucoside 8-C-arabinoside			
Phenolics									
				Ethyl 3,4-dihydroxybenzoate			Ethyl 3,4-dihydroxybenzoate	Ethyl 3,4-dihydroxybenzoate	
Miscellaneous									
unidentified		pentane-1,2,2,3,3,4,4-heptol	pentane-1,2,2,3,3,4,4-heptol	pentane-1,2,2,3,3,4,4-heptol	pentane-1,2,2,3,3,4,4-heptol	pentane-1,2,2,3,3,4,4-heptol	pentane-1,2,2,3,3,4,4-heptol	pentane-1,2,2,3,3,4,4-heptol	Mannitol

Unfermented		Solid state fermentation				Submerged fermentation			
Sweet corn silk	Feed corn silk	Sweet corn silk		Feed corn silk		Sweet corn silk		Feed corn silk	
		7 days	20 days	7 days	20 days	30 days	60 days	30 days	60 days
			Unidentified		Unidentified	2-Deoxy-D-ribose	5-Deoxy-D-ribose	5-Deoxy-D-ribose	D-glycero-L-galacto-Octulose
			Unidentified					2,5-Anhydro-D-mannose	5-Deoxy-D-ribose
			Unidentified					Succinyl Acetoacetate	
								6-O-Acetyl-D-glucose	

Table 2 Total flavonoid and phenolic contents in extracts from unfermented and fermented corn silk samples and bioactivity of crude extracts.

Fermentation	Corn silk	Time (day)	Total flavonoid content (µg QE/g dry wt.)	Total phenolic content (µg GAE/g dry wt.)
Unfermented (Water extraction)	Sweet corn		52.1 ± 4.4^{ab}	3.6 ± 0.2^{a}
	Feed corn		52.2 ± 2.9^{a}	2.6 ± 0.3^{b}
Solid State Fermentation	Sweet corn	7	50.5 ± 5.7^{ab}	2.3 ± 0.5
		20	54.9 ± 2.4^{ab}	3.2 ± 0.3^{a}
	Feed corn	7	65.7 ± 2.9^{b}	2.7 ± 0.3^{bc}
		20	61.9 ± 1.2^{b}	3.4 ± 0.2^{c}
Unfermented (Water extraction)	Sweet corn		52.1 ± 4.4^{c}	3.6 ± 0.2^{a}
	Feed corn		52.2 ± 2.9^{c}	2.6 ± 0.3^{b}
Submerged fermentation	Sweet corn	30	60.7 ± 2.7^{cd}	3.6 ± 0.0^{a}
		60	68.4 ± 1.1^{d}	6.1 ± 0.7^{a}
	Feed corn	30	61.8 ± 2.2^{cd}	2.3 ± 0.0^{b}
		60	67.89 ± 9.5^{d}	0.68 ± 0.0^{b}

QE = quercetin equivalents, GAE = gallic acid equivalent, TE = Trolox equivalent
Values in the same column with a common letter indicate no statistical difference among conditions ($p \geq 0.05$).

Bioactive compound content

Table 2 shows that the total flavonoid content (TFC) of the unfermented sweet and feed corn extracts were 52.1 ± 4.4 µg QE/g dry wt. and 52.2 ± 2.9 µg QE/g dry wt., respectively. The total phenolic content (TPC) of the unfermented sweet and feed controls was 3.6 ± 0.2 µg QE/g dry wt. and 2.6 ± 0.3 µg QE/g dry wt., respectively. **Table 2** shows that the TFC of the unfermented sweet and feed corn silk extracts was not significantly different when $p > 0.05$. On the other hand, a significant difference when $p \leq 0.05$ was observed in the total phenolic content of the unfermented sweet and feed corn silk extracts.

The TFC results of the SSF, revealing that the highest level of flavonoids, specifically 65.7 ± 2.9 µg QE/g dry wt., was obtained after fermenting feed corn for 7 days. When comparing different corn types under SSF, it was observed that on Day 7 of fermentation, the quercetin equivalent content in sweet corn (50.5 ± 5.7 µg QE/g dry wt.) was significantly lower than that of feed corn (65.7 ± 2.9 µg QE/g dry wt.).

The length of the fermentation period had no statistically significant difference on the quercetin equivalent content of sweet corn fermented for various periods. However, the fermentation period did cause a significant increase in the quercetin equivalent content of feed corn, starting from 52.2 ± 2.9 µg QE/g dry wt. (unfermented), increasing to 65.7 µg QE/g dry wt. on Day 7 when $p < 0.01$, but then decreasing to 61.9 ± 1.2 µg QE/g dry wt. on Day 20 of fermentation (**Table 2**).

The SmF total flavonoid content results, show that the highest flavonoid content came from sweet corn fermented for 60 days at 67.9 ± 9.5 µg QE/g dry wt. The type of corn had no significant effect on the quercetin equivalents in the 2 types of corn fermented for the same periods. The fermentation period did not cause significant differences in quercetin equivalents in either sweet or feed corn when comparing Day 30 and Day 60 of fermentation. However, the fermentation period did cause a significant change when $p < 0.05$ after 60 days of fermentation in feed corn (67.89 ± 9.52 µg QE/g dry wt.) and sweet corn (68.4 ± 1.1 µg QE/g dry wt.) compared to unfermented feed corn (52.2 ± 2.9 µg QE/g dry wt.) and unfermented sweet corn (52.1 ± 4.4 µg QE/g dry wt.) (**Table 2**).

The TPC results of the SSF, revealing that the highest level of phenolic content was 3.4 ± 0.2 µg GAE/g dry wt. in feed corn fermented for 20 days. The type of corn did not cause a significant difference in phenolic content between sweet corn silk and feed corn silk fermented for the same amount of time. However, the fermentation period caused a significant difference when $p < 0.05$ in the case of fermented sweet corn silk extract, with the gallic acid equivalent rising from 2.3 ± 0.5 µg GAE/g dry wt. on Day 7 to 3.2 ± 0.3 µg GAE/g dry wt. on Day 20. The fermentation period had no significant effect in the case of feed corn (**Table 2**).

Looking at the TPC results of SmF, the highest level of phenolic content was 6.1 ± 0.7 µg GAE/g dry wt. in sweet corn fermented for 60 days. Neither the type of corn nor the period of fermentation affected the phenolic content significantly in SmF (**Table 2**).

In summary, the results show that the highest TPC and highest TFC were both found in sweet corn silk fermented for 60 days through SmF. Thus, this experiment suggests that if a fermentation product with a high concentration of phenols and flavonoids is desired, the SmF process should be employed using sweet corn silk fermented for 60 days.

Antioxidant capacity

Figure 4 shows the ABTS inhibition results for SSF and SmF, including the results for the unfermented water extract controls, which appear identically in both SSF and SmF **FigureS 4(a) - 4(b)** for easy comparison against other results. However, the unfermented water extract controls, was no statistically significant difference in the ABTS inhibition percentage between unfermented sweet corn silk and unfermented feed corn silk.

Turning to the results of the SSF extracts in **Figure 5(a)**, the highest ABTS inhibition result of 66.9 % was found in sweet corn silk fermented for 7 days, and this was significantly different compared to the unfermented extract. Neither the type of corn silk nor the period of fermentation had a statistically significant effect on the ABTS inhibition percentage in SSF.

In the SmF ABTS inhibition results, the highest ABTS inhibition result of 61.6 ± 4.8 % was found in sweet corn silk fermented for 60 days. The type of corn silk had no statistically significant effect on the ABTS inhibition. However, the period of SmF had some effect. The ABTS inhibition in fermented sweet corn silk extract was 44.1 ± 2.9 % on Day 30, and this increased significantly when $p < 0.05$ to reach 61.6 ± 4.8 % on Day 60 of fermentation. In the case of feed corn silk in SmF, the fermentation period had no statistically significant effect on ABTS inhibition **Figure 4(b)**.

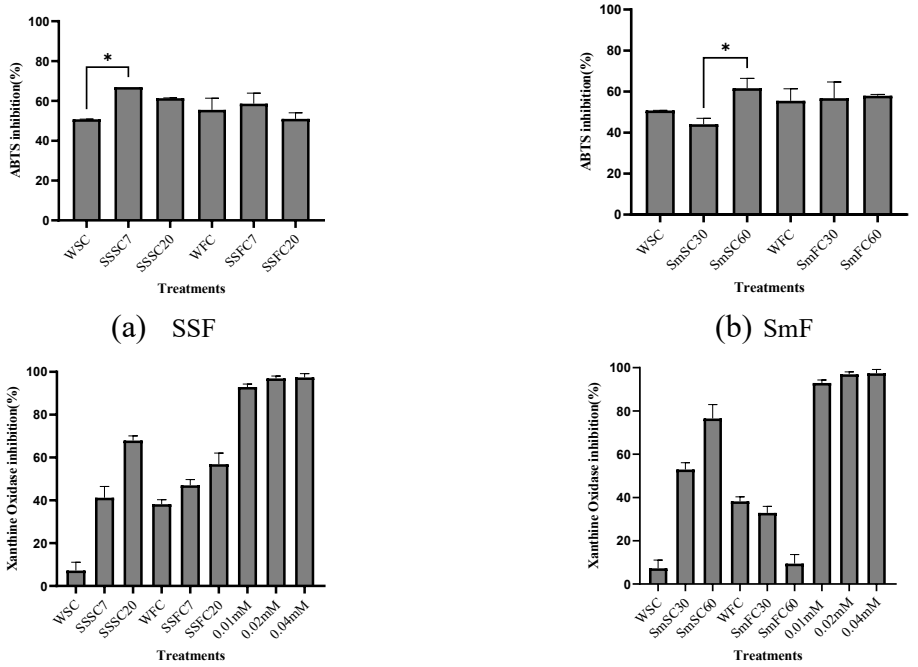
Xanthine oxidase inhibition

Figure 4 shows the xanthine oxidase (XO) inhibition results for SSF and SmF, including the results for the unfermented water extract controls, which, similar to before, appear in both SSF and SmF were compared. The unfermented water extra controls, the percentage of xanthine oxidase inhibition in feed corn silk extract (38.2 ± 2.1 %) was significantly when $p < 0.01$ higher than in sweet corn silk extract (7.2 ± 3.8 %).

The XO inhibition results for SSF extracts, shown the highest percent of result was 67.9 ± 2.2 % in sweet corn silk on Day 20, and it was significantly different when compared to the Allopurinol positive control **Figure 4(c)**. In SSF, the type of corn silk had no statistically significant effect on the XO inhibition. However, both fermented corn silks had a statistically significant when $p < 0.0001$ increase in the percentage of XO inhibition as the fermentation period increased. However, the XO inhibition results for SmF extracts, shown the highest percent of xanthine oxidase inhibition was 76.5 ± 6.5 % in sweet corn silk fermented for 60 days, and it was significantly different when compared to the different concentrations of

Allopurinol **Figure 4(d)**. Either the type of corn silk or the fermentation period had a statistically significant when $p < 0.0001$ effect on the XO inhibition percentage in SmF.

Considering the result for both ABTS inhibition and XO inhibition, this study determined that sweet corn silk fermented through SmF for 60 days had both the highest percent of ABTS inhibition and the highest percent of XO inhibition. This experiment suggests that if a fermentation product with both high XO inhibition and high ABTS inhibition is desired, the SmF process should be employed using sweet corn silk fermented for 60 days.



(a) SSF (b) SmF

(c) SSF (d) SmF

ns: SSSC7 & SSFC7, SSSC20 & SSFC20, SSFC7 & SSFC20, WFC & SSFC30, and concentration of Allopurinol

: SSSC20 & 0.01, 0.02, 0.04 mM Allopurinol *: Other pair

Figure 4 ABTS inhibition under (a) solid-state fermentation (SSF) and (b) submerged fermentation (SmF), along with xanthine oxidase inhibition under (c) SSF and (d) SmF, where WSC = water extraction of sweet corn (unfermented), WFC = water extraction of feed corn (unfermented) CSE, SSSC7 = SSF of sweet corn for 20 days, SSFC7 = SSF of feed corn for 20 days, SSSC20 = SSF of sweet corn for 20 days, SSFC20 = SSF of feed corn for 20 days, SmSC30 = SmF of sweet corn for 30 days, SmFC30 = SmF of feed corn for 30 days, SmSC60 = SmF of sweet corn for 60 days, and SmFC60 = SmF of feed corn for 60 days. 0.01, 0.02, and 0.04 mM refer to the concentration of Allopurinol, which is used as a reference point here. Differences were analyzed by one-way ANOVA. Data is shown as the mean ± SD. $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Biosafety of fermented corn silk

After conducting tests on both variants of corn silk slurry, it was determined that there was no measurable trace of contamination by any of 3 heavy metals: Arsenic, lead, or mercury. As such, it can be concluded that, regarding these 3 heavy metals, both forms of corn silk are safe for utilization as raw materials in fermentation processes.

The results of this study indicate that fermented corn silks have nutritive values. The fermented corn silk extracts in this study contain numerous biological components, including amino acids such as valine, leucine, and phenylalanine, which is consistent with previous studies. Gut bacteria (*Bacillus-Lactobacillus* groups) have developed proteolytic systems, presumably to compensate for their reduced or even absent capabilities to synthesize amino acids. These proteolytic systems include extracellular proteases that degrade proteins into oligopeptides, and amino acids. Generally, they contain a higher proportion of BCAA

(Branched-Chain Amino Acids) relative to other amino acids [15]. And Corn silk contains up to 9.65 % protein, the Karpiuk *et al.* [16] found 11 free amino acids and 16 bound amino acids after hydrolysis of aqueous solutions of the roots, leaves, and silk of *Z. mays*. Cysteine was the bound amino acid that appeared in greatest quantity in Karpiuk's samples. The different quantities of amino acids in different studies may be a result of variations in the extraction methods or corn varieties. The current study showed that the fermentation process induces changes in the types and amounts of amino acids in corn silk. This is caused by metabolic activities of microorganisms. Some microorganisms, including *Bacillus subtilis,* and *Lactobacillus* sp., and *Saccharomyces cerevisiae* can utilize amino acids as a nitrogen source, leading to the breakdown of amino acids into simpler compounds like ammonia and organic acids, which serve as nutrients for microorganisms. Consequently, specific amino acids may be consumed or depleted during fermentation. Conversely, some microorganisms involved in fermentation can synthesize and release amino acids, because they possess specialized enzymes or pathways for amino acid synthesis [17,18]. As a result, the levels of certain amino acids may increase during fermentation. There are also some microorganisms that can enzymatically convert or modify amino acids. For instance, specific bacteria or yeast strains can decarboxylate amino acids, leading to the production of biogenic amines and altering the flavor, aroma, and safety characteristics of fermented products [19,20]. The presence of essential amino acids such as valine, leucine, and their derivatives in the fermented corn silk extract has potential health benefits, such as reducing purine nucleotide cycle activity, decreasing uric acid production, and reducing the incidence of gout. Additionally, phenylalanine is an essential amino acid that plays an important role in protein synthesis and various other biological processes. For example, phenylalanine is a precursor in the shikimate pathway to synthesis of flavonoids [10 ,22 ,23].

The fermented corn silk extract also contains various organic acids, fatty acids, flavonoid compounds, and phenolic compounds. Flavonoids such as arginine-glucoside, catechin, and ethyl glucoside can also be identified in the extract. In addition, Aires and Carvalho [23] extracted corn silk by ultrasound method. They found apigenin and pelargonidin. Santin *et al.* [24] and Bhuvaneshwari *et al.* [25] found phlobatannin, alkaloid, sterol, and terpenoids in corn silk extract by using water and ethanol for extraction. The resulting differences may be due to the influence of the extraction method, extraction substances, or extraction conditions, including the variety, the planting environment, the age, and the maturity of the corn used [1].

In this study it was found that some fermentation treatments increased the phenolic and flavonoid content of the extracts. The fermentation process involves microorganisms releasing enzymes such as glucosidase, amylase, cellulase, hemicellulose, chitinase, inulinase, phytase, xylanase, tannase, esterase, invertase, or lipase, some of which can hydrolyze glucosides and break down plant cell walls, leading to the release of phenolic and flavonoid compounds [26]. However, Yucharoen *et al.* [27] reported that the extract of corn silk using ethanol displayed highest levels of total phenolic and flavonoid contents, at 28.27 ± 0.86 mg gallic acid equivalent/g extract and 4.71 ± 0.79 mg quercetin equivalent/g extract, respectively. They showed different results from this study, which showed high flavonoid content, but low phenolic content. During fermentation, microorganisms such as yeast and bacteria (*Bacillus subtilis*, *Lactobacillus plantarum*) play a crucial role in metabolizing different compounds present in the starting material. This includes the transformation of phenolic compounds into flavonoids [28].

The antioxidant activity tests of fermented corn silk extract showed a high percentage of antioxidant inhibition in the range of 40 - 60 %. In addition, Zhang *et al.* [29] used ethanol to extract corn silk and found that the ability of corn silk extract to scavenge DPPH and ABTS was 84.38 and 89.11 %, respectively. The different results may be due to the use of different verification methods. As for the $ABTS^+$ scavenging assay, this assay is based on the inhibition of the radical cation $ABTS^+$ absorbance. This radical is soluble in water and organic solvents, therefore the assay is applicable to both lipophilic and hydrophilic compounds. However, this assay has been criticized, as the ABTS radical is not representative of biomolecules and not even found in any biological or food system [30]. In this study, the highest SFF and SmF ABTS inhibition results were no different because they contain ethyl 3,4-dihydroxybenzoate that is excellent antioxidants due to a 3′-4′ dihydroxy group in their B ring [31]. In the xanthine oxidase (XO) results, fermented corn silk had the highest XO inhibition. Xanthine oxidase is an enzyme in purine catabolism. Activation of xanthine oxidase results in increased uric acid levels, whereas inhibition of xanthine oxidase activity can block excessive synthesis of uric acid in the body [32]. Therefore, extracts obtained from fermented corn silk could potentially reduce the severity of diseases caused by the accumulation of uric acid, such as gout.

Overall, this study revealed the superiority of SmF (Submerged Fermentation) over SSF (Solid-State Fermentation) in corn silk fermentation. Specifically, the fermentation of corn silk using SmF for a period of 60 days yielded interesting substances that can be utilized in various ways. Additionally, the quantities of TFC, TPC, Antioxidant inhibition, and XO inhibition in this study's extracts were high. According to

previous studies, the wild-type strains of *Bacillus* spp. produced more biomass and lipopeptides while producing less primary metabolite synthesis in SSF than in SmF [33]. The drying method in SSF may reduce the level of TPC, TFC, and XO inhibition as the extract is easily oxidized and sensitive to heat treatments [33]. In addition, Kumar *et al.* [34] summarize that SSF offers several benefits over SmF for production of metabolites such as high volumetric productivity, lower energy requirements, less effluent generation and simplicity to work with. The conclusion of Kumar and colleagues may contradict the results of this study, possibly due to differences in the duration and the proportions of different ingredients used in the fermentation process.

Since no heavy metal contamination was discovered in either of the corn silk cultivars, corn silk extract can be created and used safely in a variety of industries.

Conclusions

The phytochemical profiling established in this work serves as a useful method for evaluating the biological compounds and phytonutrient for efficacy and safety. An aliquot of fermented corn silk contained a certain number of bioactive compounds and amino acids, including quantitative flavonoid and phenolic compounds which demonstrate antioxidant potential. Another possibility is that the active compounds could be selected as a marker for qualitative control. Fermented corn silk extracts show promise for utilization in healthy products.

Acknowledgements

This work was supported by Naresuan University (NU), Thailand and the National Science, Research and Innovation Fund (NSRF), Thailand (Grant No. R2565B064).

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