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Quantitative Determination of Phenolic Acids and Flavonoids in Fresh Whole Crop Rice, Silage, and Hay at Different Harvest Periods

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Abstract: Whole crop rice (WCR) is used as an important feed for livestock ruminants. In this study, "Yeongwoo" variety WCR (cultivated on the Korean peninsula) was harvested at three different maturity stages (booting, heading, and milk) and their phenolic acid and flavonoid profiles in the lactic acid bacteria (LAB)-inoculated and noninoculated silage, hay, and fresh freeze-dried WCR extract were quantified. The alterations in the phenolic and flavonoid contents of the selected WCR during maturation in different samples were analyzed by the High-Performance Liquid Chromatography- Diode Array Detector (HPLC–DAD) technique. The six phenolics (caffeic acid, ferulic acid, p-coumaric acid, chlorogenic acid, dihydroxy benzoic acid, and prophyl gallate) and six flavonoids (rutin hydroxide, luteolin, kaempferol, vitexin, myricetin, and quercetin) were noted to have slight differences between the LAB-inoculated and noninoculated silage samples; however, the phenolics and flavonoids were higher in hay WCR at the milk stage compared to the silage and fresh freeze-dried samples. The results indicate that WCR harvest times have different phenolic compounds in the WCR silage, hay, and fresh samples. The phenolic and flavonoid compounds were higher ($p < 0.05$) with the increase in maturity (Stage 1–3). The stage of WCR maturity was positively related ($p < 0.05$) to the amount of phenolic acid and flavonoid contents (μg/g) in hay, silage, and fresh freeze-dried extract. We observed the WCR had high amounts of phenolic acid and flavonoid concentrations at milk stage (Stage 3) hay (quercetin, kaemferol, luteolin, ferulic acid, caffeic acid, and coumaric acid were 1.28, 1.29, 0.54, 1.54, 1.92, and 1.81 μg/g, respectively) compared with the booting and heading stages (Stages 1 and 2), with acceptable accuracy on a pilot scale. Based on these results, it could be concluded that LAB (*Lactobacillus plantarum*)-inoculated whole crop rice silage (WCRS) did not affect the phenolics and flavonoids of secondary plant metabolites in fermented silage. However, phenolics and flavonoids were of higher rations in WCR at the milk stage. Furthermore, this phenolic acid and flavonoid effect needs to be confirmed using large-scale in vivo analysis.

Keywords: whole crop rice; maturity; lactic acid bacteria; phenolics; flavonoids; HPLC–DAD

1. Introduction

Phenolics consist of a broad range of secondary metabolites that are subdivided into different classes, including phenolic acids (hydroxybenzoic and hydroxycinnamic acid derivatives), flavonoids (flavonol, flavanol, isoflavonoids, and anthocyanin), coumarins, lignans, and tannins

(hydrolyzable and condensed tannins) [1,2]. Phenolic compounds are an important group of secondary plant metabolites that have been shown to be responsible for many health benefits such as antimicrobial, antioxidant, anticancer, antidiabetic, and anti-inflammatory effects [3]. However, the phenolic compound contents and their pharmacological functions are vitally dependent on pre- and postharvest factors such as environmental and agronomic conditions, cultivar selection, stage of maturity, and extraction procedure [4]. Based on that, it is important to determine the optimal harvesting time for the highest amount of active ingredients (particularly phenolic acids and flavonoids) and maximum pharmacological activity [5,6]. Plant maturity is the stage of development in crops where there are close relationships between plant features and their nutritive values. Plant maturation analysis may be used as a low-cost tool to analyze the nutritive composition of forages that provide relationships between nutritive value and suitable harvest time [7]. Additionally, forage maturity analysis is an important concept used to evaluate the yield and quality of the crop. Recently, Kim et al. [8] studied the forage growth stage as one of the most important factors influencing nutritional composition. However, when a plant matures, the cytoplasmic compartment levels of the cells decrease and the quantities of proteins, lipids, soluble carbohydrates, and minerals are also reduced. In contrast, the dry matter (DM) content increases as the WCR crop matures. Therefore, the concentrations of phenolic acids and flavonoids in WCR may differ based on the different cultivar, environment, and stages of maturity [9].

Rice (*Oryza sativa* L. Japonica) is an important food crop in South East Asian countries. Particularly in Korea and South Asia, rice is a community and a socioeconomic entity. Over the centuries, the Koreans have cultivated rice in hot and humid summer conditions. After harvesting the rice crop, the straw is used to feed ruminant animals, and the grains are consumed as a staple food. In addition, high-yield rice varieties are cultivated and these varieties greatly improve the agricultural inputs per acre yield of rice in the Korean peninsula [10]. The whole crop rice forage, including leaves, panicles, and stems, is harvested at different maturity stages, such as heading, milky, yellow-ripen, grain, and grain maturity, which are conditioned into whole-crop rice silage, hay, and haylage development for feed for livestock animals. Yamada et al. [11] reported that whole crop rice silage (WCRS) prepared at the yellow-ripe stage contained approximately 6–7% crude protein (CP), which is similar to whole crop barley silage. In addition, WCRS usually contains a high amount of α-tocopherol (lipid-soluble vitamins) at the yellow-ripe stage compared with the milky stage of WCR [12]. Many studies have also shown that the stage of maturity of crops may affect chemical compositions and fermentation profiles, as well as the net energy content of forages [7,13]. The stage of maturation has a vital role in the nutritive value and ensiling process of the forage [14]. Arieli and Adin [15] reported that whole crop wheat harvested at the flowering stage for silage had improved dietary fiber, digestibility, and milk quality and yields than other maturity stages. On the other hand, Ashbell et al. [16] reported that the best harvest time for wheat silage was the grain stage in terms of the yield and nutrient composition. Based on the reports, the most suitable stage to harvest whole crop rice for ensiling and optimum phenolic and flavonoid profiles remains unclear [17].

The chemical composition and fermentability profiles are the key indications of the nutritive quality of the crop, which may be affected by the variety and stages of maturity, although no such research has been found on the effect of phenolic acid and flavonoid contents on the stages of maturity in relation to the different types of WCR silage, hay, and fresh extract. Therefore, the objective of this study is to determine the effects of different harvest times on silage, hay, and fresh freeze-dried WCR samples and to find the maximum phenolic and flavonoid profiles using analysis by the HPLC–DAD method.

2. Materials and Methods

2.1. Chemical Used

HPLC-grade solvents methanol, acetonitrile, ethanol, and water (99.9%) were purchased from Fisher Scientific Korea Ltd. (Seoul, Korea). Formic acid was procured from Lab-Honeywell, (Sigma-Aldrich, MI, USA). Acrodisc LC syringe filter 0.2 µm GHP membranes were from PALL,

Life Sciences, (Ann Arbor, MI, USA). The HPLC column, YMC-Triart C18 (150 × 4.6 mm I.D., S-3 µm, 12 nm), was from YMC Co. Ltd. (YMC, Kyoto, Japan), while other accessories such as glass inlet filters and solvent filters were purchased from Agilent Co. (Santa Clara, CA, USA). Phenolic acid standards (including caffeic acid, ferulic acid, p-coumaric acid, chlorogenic acid, di-hydroxy benzoic acid, and prophyl gallate) and flavonoid standards (rutin hydroxide, luteolin, kaempferol, vitexin, narcissoside, myricetin, and quercetin) were obtained from Sigma (St. Louis, MO, USA) and Fisher Scientific company (Seoul, Korea), respectively.

2.2. WCR Sample Collection

Whole crop rice (*O. sativa* L. Japonica) "Yeongwoo" breed is widely cultivated on the Korean Peninsula. The whole crop rice was grown on a commercial farm in Cheonan, Korea. The selected rice crop was sown under similar environmental conditions on the farm, and it was harvested at three stages of maturity, namely, booting (stage 1), heading (stage 2), and milk stages (stage 3). The rice crop was harvested physically using a scythe at approximately 10–15 cm above the soil and immediately covered in poly bags (20 × 30 cm dimensions, FoodSaver vacuum sealer bags, Seoul, Korea), without wilting, and transported to the laboratory for further analysis.

2.3. WCR Silage, Hay, and Fresh Freeze-Dried Sample Preparation

The WCR samples were divided into three parts: the first part was used to characterize the phenolic and flavonoid components in the LAB-inoculated and noninoculated WCR silage with a high moisture level. Then, we further analyzed the phenolic and flavonoid profiles in hay and fresh freeze-dried samples.

WCR Silage: After harvesting, the whole crop rice sample was wilted at room temperature for 36–48 h to reach moisture below 60–70%, and then samples were chopped to 2–3 cm length using a hand cutter. After cutting, WCR forage was inoculated with silage bacteria (SB; Top Silage Products, Jeollanam-do, Korea) containing *Lactobacillus plantarum* KCC-10, *Lactobacillus plantarum* KCC-19, and *Lactobacillus plantarum* K-49 at a bacterial population of 1×10^5 CFU/g of forage. Then, 100 g of finely chopped WCR was mixed with LAB inoculants (1×10^5 CFU/g) in plastic bags, and without-LAB was maintained as control (Phosphate-Buffered Saline alone). The LAB-inoculated and noninoculated WCR silage bags were sealed by vacuum sealer machine (FoodSaver, FM-5460-071, EMK Co. Ltd., Gangnam-gu, Seoul, Korea) and then stored at room temperature (25–30 °C). Afterward, the sealed bags were monitored for 6 h to ensure no damage to the silage bags. All experimental samples were carried out in triplicate. After 60 days, the silage bags were opened, and a portion of samples from each group and each stage of maturity was analyzed for their phenolic and flavonoid contents. Another portion of the WCR silage sample was analyzed for pH, microbial population, and organic acid profile, and then samples were dried at 60 °C for 72 h and powdered using a grinding mill (Kunhewuhua, Seoul, Korea) with a 1-mm pore size sieve for nutrient and chemical analyses [18].

Fresh WCR: Fresh WCR forage (2 kg) in each stage was cut into small pieces (2–3 cm), put into small gauze bags (10 × 15 cm dimensions, Asahikasei, Tokyo, Japan), and soaked in liquid nitrogen (−170 °C) for 5–10 min. Then, the samples were dried using a freeze dryer and kept at −80 °C for 4–5 days. Afterward, the samples were powdered in a grinding mill to pass through a 1-mm sieve, and the powdered material was carefully stored at room temperature for further chemical profile analysis.

WCR hay: The WCR samples were cleaned well and dried in a shadow condition for 5 days, and then samples were kept in an air-forced drying oven at 65 °C for 24 h. The dried WCR samples were ground well in a hammer mill (MF-220, CapsulCn International, Shanghai, China) to 1-mm particle size, kept in airtight plastic containers, and stored at 4 °C until further use. The details of the WCR samples and the different experimental set-ups used in this study are summarized in Table 1.

Table 1. Whole crop rice (WCR) samples and the different experimental set-ups used in this study.

S. No	Treatments	Samples Condition	WCR Harvest Period		Methods for Sample Preparation
1	Group 1	Fresh freeze-dry	Stage 1	Booting	Liquid N_2 freeze-dried at −80 °C
2		Fresh freeze-dry	Stage 2	Heading	
3		Fresh freeze-dry	Stage 3	Milk	
4	Group 2	LAB-inoculated WCRS	Stage 1	Booting	KCC-10-, KCC-19-, and K46-inoculated and incubated for 60 d at RT
5		LAB-inoculated WCRS	Stage 2	Heading	
6		LAB-inoculated WCRS	Stage 3	Milk	
7	Group 3	LAB-noninoculated WCRS	Stage 1	Booting	Without KCC-10, KCC-19, and K46 and incubated for 60 d at RT
8		LAB-noninoculated WCRS	Stage 2	Heading	
9		LAB-noninoculated WCRS	Stage 3	Milk	
10	Group 4	WCR Hay	Stage 1	Booting	Oven dried at 60 °C
11		WCR Hay	Stage 2	Heading	
12		WCR Hay	Stage 3	Milk	

Here, the WCR samples were analyzed with three replicates each.

Analysis of Organic Acids Profile in WCR Silage Samples

Here, we extracted and detected three major organic acids (lactic, acetic, and butyric acids) in microbial fermented WCR samples using high-performance liquid chromatography equipped with a UV/RID detector. HPLC separation was performed using an Aminex HPX-87 column (Bio-Rad, Life Science, Hercules, CA, USA) and the mobile phase was 0.005 M H_2SO_4 isocratic elution at a flow rate of 0.7 mL/min. The column temperature was maintained at 35 °C, and organic acids were detected at a wavelength of 210 nm. Sample injection volume was 10 mL injected through an auto-sampler system. The concentration of lactic, acetic, and butyric acids was calculated by retention time and area of standard and unknown spectra data. The total run time between injections was 25 min [18].

2.4. Extraction of Phenolic Acids and Flavonoids in WCR Samples

Extraction of flavonoids and phenolic acids from WCR silage, hay, and fresh samples was carried out by Kuppusamy et al.'s method [19]. Five grams of each WCR powder sample was mixed with 200 mL of ethanol–water (65:35, *v*/*v*) to extract the phenolic and flavonoid compounds. The mixture was kept in a hot water bath at 70 °C for 2 h with mild shaking; then, extracts were cooled down and centrifuged at 8000 rpm for 20 min. After that, the supernatant was collected and the solvent evaporated under a rotary vacuum evaporator. The extract was weighed and further diluted in deionized water. The crude samples were subjected to purification in an SPE-LC-18 cartridge column. Initially, the LC-18 cartridges were preactivated by 5 mL each of methanol and acidified deionized water (pH 2.5), and then 2 mL of crude extract was poured into LC-18 cartridges (535 m^2/g, BET method, 58 micron, 58 Å) to elute phenolic acid and flavonoid fractions using 10 mL of distilled water and 100% methanol, respectively. Each eluate was dried in a fume hood for 3 days, and then fractions were redissolved in 1 mL of acetonitrile solvents to quantify the phenolic profiles [20].

2.5. Instrumentation HPLC–DAD Analysis

The purified fractions were quantified for phenolic acids and flavonoids using the HPLC–DAD (Agilent 1200, San Diego, CA, USA) technique. Chromatographic analysis and data were managed with Agilent Chem-station software (Agilent Scientific Instruments, San Diego, CA, USA). Chromatographic separation was carried out using a YMC C18 (5 µm, 150 × 4.6 mm i.d; YMC, Kyoto, Japan). The column temperature was operated at 35 °C. Sample injection volume was 10 µL, and the gradient elution was used. The mobile phase consisted of 1% (*v*/*v*) formic acid in HPLC water (Solvent A) and 100% (*v*/*v*) acetonitrile (Solvent B). The gradient elution condition was the

following: 0–25 min, 14–24% B; 25–35 min, 24–85% B; 35–45 min, 85% B; 45–48 min, 90–14% B (Table 2). Each sample was individually run and analyzed the presence of the following polyphenolic compounds: caffeic acid, ferulic acid, p-coumaric acid, chlorogenic acid, di-hydroxy benzoic acid, prophyl gallate, rutin hydroxide, luteolin, kaempferol, vitexin, and myricetin. The identification of the chromatographic peaks was performed by comparing the retention times and absorption spectra of the sample peaks with those of the standard compounds. For quantitative determination, the standard curve was made by known concentrations of standard compounds. Standard compounds were prepared in the concentration range of 10–100 µg/mL.

Table 2. Gradient elution for the HPLC–DAD mobile phase solvent system.

Time Step (min)	Elution Method	Solvent System	
		Solvent A (%) 1% Formic Acid in Water	Solvent B (%) 95% Acetonitrile
0–25	Gradient	75	25
25–35	Gradient	15	85
35–45	Gradient	15	85
45–48	Gradient	10	90
48–65	Gradient	60	40
65–75	Gradient	85	15

Quantification of Phenolic Acids and Flavonoids

Next, 10 µL of each concentration of purified WCR sample was injected into HPLC–DAD twice, and the calibration curves were prepared by plotting the concentration against the area of the peak. Each phenolic acid and flavonoid in the WCR samples was quantified using a formula previously reported by Inbaraj et al. [20].

2.6. Statistical Analysis

The data were expressed in the form of mean ± SD. One-way analysis of variance was used to compare the mean values of the phenolic and flavonoid contents in the different harvest stages of WCR using the LSD method and Student's *t*-test, at $p < 0.05$ statistical significance.

3. Results

The extract yield of phenolic acids and flavonoids from the WCR samples was observed for WCR LAB-inoculated silage Stages 1, 2, and 3 (1301.51, 1445.73, and 1456.25 mg/5 g), followed by WCR silage LAB-noninoculated silage Stage 1, 2, and 3 (1271.23, 1387.47, and 1376.58 mg/5 g), WCR hay (1415.16, 1487.15, and 1490.32 mg/5 g), and WCR fresh freeze-dry sample (987.33, 1012.47, and 1065.15 mg/5 g).

The quantified phenolic acids and flavonoids in the WCR silage materials are shown in Table 3. The phenolic acid and flavonoid contents in the LAB-inoculated and noninoculated WCR silage samples showed no remarkable differences between these two samples. However, myricetin was quantified at 1.14 µg/g in the milk stage and not identified in the heading and booting stages of the LAB-inoculated WCR silage sample. In addition, the concentrations of flavonoids and phenolic acids were slightly higher at the milk stage except for those of rutin and chlorogenic acid compounds, compared to other stages (Table 3). Luteolin was detected at the milk stage but not detected at the booting stage of the WCR sample. However, the quercetin, rutin, prophyl gallate, and caffeic acid contents were observed to have no significant changes at the milk stage of both the LAB-inoculated and noninoculated WCR extracts. Therefore, LAB inoculation did not alter the phenolic acid and flavonoid contents of fermented WCR silage. Chlorogenic acid, rutin, and luteolin at the milk stage were increased slightly in LAB-noninoculated WCR silage than in the LAB-inoculated WCR silage; they were 0.77, 1.15, and 0.58 µg/g, respectively, and they were higher at the milk and heading stages than at the booting stage of WCR silage samples ($p < 0.05$). The flavonoid compounds, particularly luteolin and myricetin, were identified at the milk stage of LAB-noninoculated WCR silage, which was

not detected at the booting and heading stages of the LAB-inoculated WCR sample. It may be due to microbial community utilization as a nutrient or degradation into monomers in LAB-inoculated WCR silage (Table 3).

The phenolic acid and flavonoid levels of WCR hay were also investigated. There were slight differences in phenolic acid and flavonoid contents between the three stages of harvest periods; in particular, the maximum phenolic acids and flavonoids were observed at the milky stage compared to the booting and heading stages of WCR hay. The WCR milk stage was quantified with higher concentrations of chlorogenic acid, prophyl gallate, luteolin, kaemferol, and quercetin (1.71, 0.79, 0.54, 1.29, and 1.28 μg/g, respectively; Table 3). However, chlorogenic acid, prophyl gallate, luteolin, kaemferol, and quercetin were lower at the booting stage, followed by the heading stage of WCR hay. The heading stage of WCR hay phenolic acids showed a similar trend to the milk stage: in particular, caffeic acid, ferulic acid, p-coumaric acid, chlorogenic acid, and prophyl gallate were 1.85, 1.81, 1.23, 1.74, and 0.82 μg/g, respectively, and the phenolic acid content was not significantly changed compared to the milky and heading stages. Furthermore, the quercetin and kaempferol contents gradually increased in all stages after heading, but most of the metabolites were increased in (milk) Stage 3 alone.

Fresh freeze-dried WCR extract was analyzed for phenolic acid and flavonoid concentration at different harvesting periods, such as the booting, heading and milky stages. Among them, the milk stage of fresh WCR had the highest phenolic acid profile (ferulic acid, p-coumaric acid, chlorogenic acid, prophyl gallate, vitexin, myricetin, and quercetin were 2.49, 1.93, 1.97, 1.57, 0.98, 1.14, and 1.55, μg/g, respectively). However, similar metabolites were noted at lower values at the booting and heading stages. The luteolin range decreased with maturity. On the other hand, the contents of caffeic acid, rutin, and kaempferol were significantly higher in fresh freeze-dried WCR at the milk stage than that at the booting and heading stages ($p < 0.05$). Overall, the whole crop rice fresh freeze-dried extract had a higher phenolic acid profile at the milky stage compared to the booting and heading stages. In addition, there were no significant changes in the flavonoid content between the milky and heading stages ($p < 0.05$; Table 3). The heading stage of WCR hay had a slightly higher amount of phenolic acids and flavonoids among WCR silage and fresh samples at the heading stage; however, ferulic acid and p-coumaric acid were detected at higher levels at the milk stage of WCR silage, hay, and fresh freeze-dried extract. The stage of maturity may also have a significant impact on fermentation efficiency in terms of the organic acid profile, nutrient composition, and microbial diversity (Table 4) of the WCR silage/haylage samples. Hence, the optimum level of phenolic acids and flavonoids may depend on the stage of crop maturity, and it could be one of the important factors influencing feed quality for silage-making and/or feed development.

In addition, the pH, organic acid profiles, and microbial data of LAB-inoculated and noninoculated WCR silage samples are summarized in Table 4. The LAB-inoculated WCRS at the milk stage (Stage 3) showed a higher concentration of lactic acid and a lower amount of butyric acid than the noninoculated WCRS; it clearly indicates that the inoculated LAB utilize the WSC in WCRS efficiently and improve the WCRS fermentation quality. Lower ($p < 0.05$) concentration of organic acid profiles at the booting stage (Stage 1) is due to lower LAB survival and nutritional availability in the WCRS silage, which may not be suitable for feeding ruminants. Therefore, WCRS preparation should be required for an optimum harvesting period.

Table 3. Quantification of phenolic acids and flavonoids from WCR silage, hay, and fresh freeze-dried extract at different stages of maturity (μg/g).

		Phenolic Acids (μg/g)							Flavonoids (μg/g)				
Treatments/Sample Types	**Harvest Period**	**Caffeic Acid**	**Ferulic Acid**	**P-Coumaric Acid**	**Chlorogenic Acid**	**Di-hydroxy Benzoic Acid**	**Prophyl Gallate**	**Rutin Hydroxide**	**Luteolin**	**Kaempferol**	**Vitexin**	**Myricetin**	**Quercetin**
Group I Fresh freeze-dried	Stage 1	0.84 ± 0.01 ^{b}	2.11 ± 0.01 ^{c}	1.85 ± 0.03 ^{c}	1.92 ± 0.02 ^{b}	ND	1.57 ± 0.01 ^{a}	1.23 ± 0.06 ^{b}	0.72 ± 0.02 ^{a}	1.42 ± 0.04 ^{c}	0.21 ± 0.03 ^{c}	1.03 ± 0.03 ^{c}	1.50 ± 0.03 ^{c}
	Stage 2	0.88 ± 0.05 ^{a}	2.21 ± 0.06 ^{b}	1.89 ± 0.03 ^{b}	1.96 ± 0.04 ^{a}	ND	1.56 ± 0.03 ^{b}	1.25 ± 0.05 ^{a}	0.63 ± 0.03 ^{b}	1.76 ± 0.03 ^{a}	0.94 ± 0.01 ^{b}	1.12 ± 0.02 ^{b}	1.54 ± 0.03 ^{b}
	Stage 3	0.87 ± 0.02 ^{a}	2.49 ± 0.04 ^{a}	1.93 ± 0.05 ^{a}	1.97 ± 0.02 ^{a}	ND	1.57 ± 0.03 ^{a}	1.22 ± 0.05 ^{b}	ND	1.75 ± 0.03 ^{b}	0.98 ± 0.02 ^{a}	1.14 ± 0.04 ^{a}	1.55 ± 0.02 ^{a}
Group II LAB-inoculated silage	Stage 1	0.65 ± 0.04 ^{c}	1.94 ± 0.06 ^{c}	1.71 ± 0.06 ^{c}	0.64 ± 0.04 ^{b}	ND	0.93 ± 0.05 ^{c}	0.98 ± 0.03 ^{b}	0.46 ± 0.03 ^{a}	1.17 ± 0.03 ^{c}	ND	ND	1.03 ± 0.02 ^{c}
	Stage 2	0.73 ± 0.05 ^{b}	2.11 ± 0.05 ^{b}	1.75 ± 0.04 ^{b}	0.69 ± 0.03 ^{a}	ND	1.23 ± 0.04 ^{b}	1.01 ± 0.03 ^{a}	ND	1.34 ± 0.03 ^{b}	0.95 ± 0.03 ^{b}	ND	1.18 ± 0.02 ^{b}
	Stage 3	0.93 ± 0.04 ^{a}	2.47 ± 0.05 ^{a}	1.81 ± 0.04 ^{a}	0.68 ± 0.04 ^{a}	ND	1.57 ± 0.04 ^{a}	1.02 ± 0.02 ^{a}	ND	1.36 ± 0.04 ^{a}	0.97 ± 0.03 ^{a}	1.14 ± 0.04 ^{a}	1.29 ± 0.01 ^{a}
Group III LAB-noninoculated silage	Stage 1	0.71 ± 0.03 ^{c}	2.13 ± 0.04 ^{c}	1.69 ± 0.03 ^{c}	0.72 ± 0.02 ^{c}	ND	1.21 ± 0.03 ^{c}	1.03 ± 0.05 ^{c}	0.53 ± 0.03 ^{b}	1.28 ± 0.03 ^{b}	0.81 ± 0.03 ^{c}	ND	1.04 ± 0.02 ^{c}
	Stage 2	0.86 ± 0.02 ^{b}	2.40 ± 0.03 ^{b}	1.74 ± 0.03 ^{b}	0.77 ± 0.02 ^{a}	ND	1.46 ± 0.03 ^{b}	1.15 ± 0.04 ^{a}	0.58 ± 0.03 ^{a}	1.38 ± 0.03 ^{a}	0.96 ± 0.03 ^{b}	1.01 ± 0.02 ^{b}	1.21 ± 0.02 ^{b}
	Stage 3	0.91 ± 0.02 ^{a}	2.55 ± 0.04 ^{a}	1.83 ± 0.04 ^{a}	0.75 ± 0.03 ^{b}	0.36 ± 0.04 ^{a}	1.49 ± 0.02 ^{a}	1.10 ± 0.04 ^{b}	ND	1.38 ± 0.04 ^{a}	0.98 ± 0.03 ^{a}	1.15 ± 0.02 ^{a}	1.28 ± 0.03 ^{a}
Group IV Hay	Stage 1	1.85 ± 0.03 ^{b}	1.81 ± 0.03 ^{a}	1.23 ± 0.02 ^{b}	1.74 ± 0.03 ^{a}	2.01 ± 0.04 ^{c}	0.82 ± 0.02 ^{a}	0.86 ± 0.04 ^{b}	0.48 ± 0.04 ^{b}	1.23 ± 0.03 ^{c}	0.55 ± 0.04 ^{c}	0.92 ± 0.03 ^{b}	1.19 ± 0.03 ^{c}
	Stage 2	1.87 ± 0.04 ^{a}	1.80 ± 0.03 ^{a}	1.43 ± 0.03 ^{a}	1.70 ± 0.02 ^{b}	2.58 ± 0.03 ^{a}	0.54 ± 0.02 ^{c}	0.88 ± 0.04 ^{a}	0.53 ± 0.03 ^{a}	1.25 ± 0.04 ^{b}	0.75 ± 0.03 ^{a}	0.95 ± 0.03 ^{a}	1.24 ± 0.03 ^{b}
	Stage 3	1.84 ± 0.04 ^{b}	1.92 ± 0.02 ^{b}	1.51 ± 0.04 ^{c}	1.81 ± 0.03 ^{b}	2.62 ± 0.04 ^{b}	0.89 ± 0.02 ^{b}	0.91 ± 0.03 ^{c}	0.54 ± 0.04 ^{a}	1.29 ± 0.04 ^{a}	0.92 ± 0.03 ^{b}	0.98 ± 0.03 ^{c}	1.28 ± 0.04 ^{a}

(Stage 1: booting stage; Stage 2: heading stage; Stage 3: milk stage; ND: not detected. ^{a,b,c} different superscript letters represent data that are statistically significant between the stages of each group ($p > 0.05$).

Table 4. pH, organic acids, and microbial count analyses of LAB-inoculated and non-inoculated WCR silage samples.

Treatments	Harvest Period	Types of Samples	pH	LA (%)	AA (%)	BA (%)	LAB ($\times 10^7$ cfu/g)
Group 3 and Group 4	Stage 1	LAB-noninoculated	4.05	3.11 ± 0.03	0.33 ± 0.01	0.97 ± 0.12	4.23
		LAB-inoculated	3.65	4.65 ± 0.06	0.39 ± 0.01	0.55 ± 0.45	6.25
	Stage 2	LAB-noninoculated	4.12	2.79 ± 0.02	0.51 ± 0.04	1.12 ± 0.03	4.12
		LAB-inoculated	3.51	4.88 ± 0.01	0.39 ± 0.03	0.06 ± 0.02	6.73
	Stage 3	LAB-noninoculated	4.13	2.42 ± 0.02	0.31 ± 0.03	0.73 ± 0.02	4.15
		LAB-inoculated	3.55	5.16 ± 0.02	0.33 ± 0.01	0.11 ± 0.05	6.93

LA: lactic acid; AA: acetic acid; BA: butyric acid; LAB: lactic acid bacteria (mixture of KCC-10, KCC-19, and K46); cfu/g: colony-forming unit per gram.

4. Discussion

Phenolic compounds are a large cluster of secondary plant metabolite families. To date, more than 8000 molecules have been identified and documented in chemical databases. Among them, the phenolic group is most important because of their potent biological properties. Particularly, hydroxycinnamic acids are chlorogenic acid, caffeic acid, ferulic acid, benzoic acid, kaempferol, catechin, myricetin, and p-coumaric acid, which are widely distributed in plant cell walls and other parts of plants such as root, grain, flower, and stem [21]. In this study, we found that different harvesting periods may affect the phenolic acid and flavonoid profiles in WCR silage, hay, and fresh samples. However, there are increased phenolic acid and flavonoid compounds in milk-stage ($p < 0.05$) hay than the heading and booting stages in silage WCR. In addition, the fresh freeze-dried WCR sample had significantly higher amounts of ferulic acid, p-coumaric acid, kaemferol, and myricetin (249, 1.93, 1.75, and 0.98 µg/g, respectively). It clearly shows that fresh WCR at the milk stage is also a potential source of feed development due to the higher concentration of phenolic acids and flavonoids. Thus, the quantified secondary metabolites play a key role in quality crop production for food development, and it is also useful for choosing a good harvesting time to produce quality silage. Previously, Xie et al. [17] reported that whole crop wheat nutritive quality and silage fermentation potential depend on the maturation stage of the crop. Additionally, they found that dry matter (DM) content and DM yield of wheat improved with maturity. The highest value was reached at the dough stage, which may influence the increasing ratio of grain to biomass in wheat forage. Therefore, the stages of maturity and processing methods may also be responsible for most of the variations, including the phenolic acid and flavonoid profiles of the crop. Islam et al. [22] reported that the stage of maturity can be used as an important tool to predict the content of metabolizable energy and metabolizable protein of WCR silage. The ideal harvest time is important to obtain the essential nutrient composition and avoid the loss of essential plant metabolites in the WCR crop. In addition, the harvesting time of between 35 and 40 days after flowering is good for WCR silage preparation, and elsewhere, other harvest times may affect the nutritional quality in WCR silage. Therefore, WCR silage or hay development requires harvest at a specific time to improve the quality and nutrient composition of the WCR.

LAB silage additives can increase the organic acid content in the fermented silage, and LAB utilized simple water-soluble carbohydrates and produced lactic and acetic acids in preserved forage crops that were incubated in vitro [23]. However, there has been no clear evidence about the phenolic profile at stages of maturity of WCR silage, hay, and fresh extract. In this study, we report the phenolic acid and flavonoid contents of Yeongwoo–WCR silage, hay, and fresh freeze-dried extract may be influenced by the stage of maturity; however, no significant changes were observed in phenolic compounds in LAB-inoculated WCR silage compared with the LAB-noninoculated silage. Collar et al. [24] reported that in the small grain, during early growth up to the flowering stage, the acid detergent fiber (ADF) and neutral detergent fiber (NDF) contents increased as the crop matured. Additionally, the grain had no significant changes or only slightly lower fiber levels at the dough and boot stages due to the increase in nonstructural carbohydrate (e.g., starch) storage during grain development. Recently,

Kim et al. [8] reported that the fiber range exhibits the same trend and cannot be relied on to increase with increasing maturity. On the other hand, the crop yield increased with the stage of maturity and a similar nutritional pattern was noted in the plant heading stage. Additionally, WCR fermentability was not good with the increase in maturity. This difference in the stages of maturity changes the composition of botanical fractions significantly and has a direct impact on chemical constituents [7]. Moreover, we found that the organic acid contents and pH differed in LAB-inoculated WCR silage and may also influence the stages of maturity. In general, the milk stage has more water-soluble carbohydrates and less hardened stem than the grain matured stage; therefore, organic acid production was higher in forage varieties than grain varieties that were easily degraded by the LAB in WCR silage. Recently, Miyagusuku-Cruzado et al. [25] reported the LAB strains from the Ohio State University–Parker Endowed Chair (OSU–PECh), including *Enterococcus mundtii*, *Lactobacillus plantarum*, and *Pediococcus pentosaceus*, were able to synthesize phenolic acid decarboxylase (PAD) to decarboxylation p-coumaric, caffeic, and ferulic acids. *E. mundtii* OSU-PECh-39B, *P. pentosaceus* OSU-PECh-27B, and *L. plantarum* OSU-PECh-BB were capable of decarboxylating all of the p-coumaric acid, caffeic acid, and ferulic acid. However, they did not completely decarboxylate ferulic acid, as they did with caffeic acid and p-coumaric acid. Therefore, the LAB strain can synthesize PAD and degrade the phenolic acids and flavonoids in the preserved silages. Similarly, Ripari et al. [26] reported a novel LAB strain that promoted the bioconversion of hydroxycinnamic acids (HCAs); mainly, the LAB strains decarboxylated ferulic acid into 4-vinyl guaiacol in sourdough bread and functional beverages attained from microbial fermentation. In addition, De Las Rivas et al. [27] studied the decarboxylation pattern for LAB, where the strains were able to break down p-coumaric acid and caffeic acid more completely than the ferulic acid that was only partially degraded in food and beverage products. In contrast, we found that the WCR silage inoculated with the selected KCC-10, KCC-19, and K49 strains were observed to have slight changes in the phenolic acid and flavonoid concentrations of the control and treated WCR silage samples.

Furthermore, Oszmiański et al. [28] determined the highest amount of polyphenolics and some terpenoids in mature cranberry, whereas it was decreased at the semi-mature stage. Additionally, the amounts of bioactive compounds were altered at the mature stage and the semi-mature stage of ripening, and they were not uniform concentrations. Similarly, N'Dri et al. [29] studied the important role of ripening stages in Gnagnan berries that increased the content of caffeic acid, caffeoylquinic acids, flavonol glycosides, and naringen in red berries, while the content of some phenolic acids, such as p-coumaric acid and feruloylquinic acids, was not significantly changed among the three ripening stages. Lachowicz et al. [30] analyzed the sugar, pectin, ash, and protein contents of two different genotypes of clones of fruit cultivars. In the Polish and Canadian cultivars' fruits, the full ripening stage showed the highest amount of phytochemicals and antioxidant compounds. Similarly, Assefa et al. [31] reported that the composition and content of antioxidant and metabolite activities varied significantly, depending on the cultivar, color, and stage of maturity. In particular, the lettuce phenolic acid components of kaempferol derivatives were more dominant than tartaric acid derivatives. Moreover, Amira et al. [32] clearly demonstrated that the concentration of phenolics and the antioxidant ability of fruits were altered by maturation stages. The harvest periods have different levels of some phenolic acids, total flavonoids, and antioxidant capacities during the ripening period, and most of them are present at the end of the ripening stage. Accordingly, our study explores that the milky stage of maturity in WCR samples might have increased the phenolic profile, which could be a useful indicator to harvest WCR at the stages of maturity. Therefore, this study may provide (milk stage) the most suitable time to harvest WCR silage, hay, and fresh WCR for animal feed development and food-related applications.

5. Conclusions

In conclusion, we studied the effect of the harvesting period on the phenolic acids and flavonoids in WCR silage, hay, and fresh freeze-dried extract. The whole crop rice was harvested at the three

different harvest stages (booting, heading, and milk stages); among them, the milk stage (Stage 3) in hay, fresh WCR, and silage with/without LAB-inoculated had the highest phenolic acids and flavonoids and the lowest contents of phenolics were quantified at the WCR booting stage (Stage 1). Although WCR hay at the heading stage had the best phenolic acid and flavonoid contents for hay production, overall phenolic content was obviously inferior to that at the milk stage (Stage 3) in all WCR samples, including silage, hay, and fresh freeze extract. The best harvesting time for WCR may possibly predict the quality of phenolic acid and flavonoid contents of WCR silage, hay, and fresh extract, as well as the quality of feed production for livestock ruminants. In order to maximize the WCR feed quality by phenolic profile, WCR should be harvested at the milky stage (Stage 3) after heading the rice crop. However, the accuracy of phenolic estimation needs to be validated with a large-scale experiment. Additionally, further experiments using WCR silage, hay, and fresh extract feeding should be investigated using lactating dairy cows for milk quality, yield, and cattle muscle quality.

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