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Polyphenol Characterization and Antioxidant Capacity of Multi-Species Swards Grown in Ireland—Environmental Sustainability and Nutraceutical Potential

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Abstract: Ruminant production systems are major contributors to greenhouse gases emissions, with animal feeding practices being the main cause for methane and nitrous oxide's release. Although feeding animals forages has been proven to be more sustainable, traditional ryegrass monocultures still require a lot of input (e.g., fertilisers and pesticides). Multi-species swards, consisting of different swards, such as grasses, forage legumes and herbs, need less management and fertiliser, produce more dry matter, and also add a variety of phytochemicals into the animal diet. In particular, polyphenols have been associated with a positive impact on animal health and productivity. However, data on the phenolic composition of multi-species sward components is still scarce, and little is known about the change in concentration over the grazing season. The present study investigated the antioxidant activity of six forage species (perennial ryegrass, timothy, white clover, red clover, chicory and plantain) over the Irish grazing season, using FRAP, DPPH$^{\bullet}$ and ORAC assays. The forages were screened for individual phenolic compounds using Liquid-Chromatography-Triple-Quadruple-Mass-Spectrometry. Plantain exhibited the highest antioxidant capacity, being almost one and a half times higher than timothy and double that of chicory. Chlorogenic acid was the most abundant polyphenol in perennial ryegrass, timothy and plantain. Overall, formononetin and biochanin A levels were higher in red clover, white clover and in chicory, in comparison to other forages ($p < 0.05$). Variations in antioxidant capacity and polyphenol composition were more significant between species ($p < 0.01$) than between season within species ($p > 0.05$). This study suggests that multi-species swards, regardless of the grazing month, offer a potential sustainable alternative to monoculture swards with significant antioxidant activity and nutraceutical compounds.

Keywords: chicory; *Chicorium intybus*; multi-species; timothy; *Phleum pratense*; plantain; *Plantago lanceolata*; polyphenols; red clover; *Trifolium pratense*; LC-MS-QqQ

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1. Introduction

In accordance with the National Energy and Climate Plan for 2021–2030, Ireland has committed to cut down 30% of its 2005 emission levels by 2030, and to reach a net zero emission status by 2050 [1]. Animal feeding practices still represent a main contributor to greenhouse emissions, with methane and nitrous oxide accounting for 21.33% of total national emissions [2]. The global warming potential of methane and nitrous oxide is, respectively, 36 and 298 times higher than carbon dioxide [3]; for this reason, there is a strong need to find mitigating strategies to reduce emissions in animal production systems.

Feeding animals with forages is a prevalent practice in temperate regions around the world, such as Ireland, UK and New Zealand. Common forage ecosystems consist of intensively managed monoculture swards, such as perennial ryegrass and Italian ryegrass,

which lead to a high stocking rate but require high inputs, such as fertilisers and herbicides [4]. Thus, monoculture swards may not be suitable for green environmental strategies, as they increase the nitrogen in the soil, greenhouse emissions and water pollution.

The advantages of using multi-species swards (the combination of grasses, legume forages and herbs) have recently become recognised. Legume forages, such as white and red clover, have bacteria in their roots which can capture the nitrogen from the air, trap it in the roots and convert it into fertiliser for neighbouring plants [5]. Herbs, such as chicory and plantain, are both tolerant to drought and heat, in addition to producing more dry matter (DM) than perennial ryegrass–clover swards [6]. Multi-species swards improve soil function and increase biodiversity in the land. Furthermore, they also contain a variety of biogenic elements, such as sodium, calcium, zinc and potassium, and phytochemicals such as alkaloids, steroids and polyphenols, which have the potential to positively impact the health and productivity of the animal [7].

Phytochemical composition and concentration vary among forages, with polyphenols comprising the largest group and having been associated with many health properties. For example, Biochanin A, an isoflavone abundantly present in legume forages, was shown to improve the weight gain of grazing steers by promoting cellulolytic bacteria in the rumen [8]; meanwhile, quercetin, a flavonol commonly found in herbs, was linked to possibly reducing biomarkers associated with liver damage and somatic cell count in cows [9,10]. Formononetin and daidzein, both found in legume forages, increased the amount of equol in milk, which has been linked to a reduction in the development of osteoporosis, cardiovascular diseases and some types of cancer in humans [11]. In a recent review, a positive correlation between higher polyphenols dietary intake and the reduction in enteric emissions was also demonstrated [12]. Indeed, polyphenols have the capacity to inhibit the methanogenic pathways in the rumen or improve nitrogen utilisation by protecting proteins against proteolysis. Since cattle can consume up to 500 g of polyphenols per day when eating forages [13], there is a particular general interest in employing these compounds as natural additives to animal feed. Nevertheless, information on the phenolic profile of forages is still very scarce, and even less is known about the change in the phenolic composition and concentration over the grazing period.

In this study, the phenolic composition and concentration of multi-species swards components (i.e., perennial ryegrass (*Lolium perenne*), timothy (*Phleum pratense*), white clover (*Trifolium repens*), red clover (*Trifolium pratense*), chicory (*Chicorium intybus*) and plantain (*Plantago lanceolata*)) was investigated over five months during a typical Irish grazing season (April–August). The polyphenolic concentrations of each species were assessed using colorimetric assays and the Liquid Chromatography-Electrospray Ionisation-Triple-Quadruple-Mass Spectrometry (LC-ESI-QqQ-MS) technique in Multiple Reaction Monitoring (MRM) mode. The antioxidant capacity of the species was also studied over the investigated grazing period. Improved understanding of the phenolic composition of the forage species may help to optimise feeding strategies and enhance the understanding of the relationship between phytochemical supply, and animal health and productivity.

2. Materials and Methods

HPLC grade (≥99.9) and LCMS (≥99.9) grade methanol were purchased from Thermo Fisher Scientific (Dublin, Ireland); Acetic acid, aluminium chloride, DPPH•, ferric (III) chloride, Folin–Ciocalteu's reagent, phosphate buffer saline (pH 7.4), sodium carbonate, sodium nitrite, 2,4,6-Tris(2-pyridyl)s-triazine (TPTZ), Trolox, and fluorescein sodium salt were purchased from Sigma-Aldrich (Arklow, Ireland). Polyphenols standards were purchased from Stratech (Ely, UK).

2.1. Plant Material

Multi-species swards of perennial ryegrass, timothy, white clover, red clover, chicory and plantain were cultivated at University College Dublin, Lyons Research Farm (Co. Kildare, Ireland). Four experimental paddocks were established, with each paddock

comprising 2-ha of swards. Initially, the site received 40 kg N ha^{-1}, 25 kg P ha^{-1} and 80 kg K ha^{-1}, followed by 92 kg N ha^{-1}, 18 kg P ha^{-1}, and 115 kg K ha^{-1}. Swards were harvested repeatedly between April 2020 and August 2020 on a monthly basis. Shortly after collection, the swards were separated according to species, washed using running tap water to remove any soil and dirt, and frozen for 24 h; this was followed by a lyophilization step. The dried forages were milled using a kitchen blender and stored away from light until further analysis.

2.2. Polyphenol Extraction

The extraction procedure was based upon a modified version of a protocol optimised by Gupta [14]. Milled forages (0.4 g) were combined with methanol (30 mL, 50%) in a flask capped with Parafilm at 40 °C for 120 min and placed in an orbital incubator shaker (Innova 42, Mason Technology, Dublin, Ireland) at 100 rpm under dark conditions. The flask content was then transferred into Nalgene tubes and centrifuged (10 min, 12,000× *g*, 4 °C) (Sigma 2K15, Mason Technology, Dublin, Ireland). The supernatant was retained, whereas the pellet was washed twice with methanol (5 mL, 50%). The pooled supernatant was filtered (Grade 1 filter paper, 11µm pore, Whatman International Limited, Maidstone, Kent, UK) and reduced to 10 mL by evaporation (Syncore Polyvap, Mason Technology, Dublin, Ireland). The samples were frozen at −20 °C for lyophilisation. Once freeze dried, the extracts were transferred to Eppendorf tubes and stored at −20 °C until further analysis. Extractions were carried out in triplicates.

2.3. Total Polyphenol Content and Total Flavonoid Content

The total polyphenol content (TPC) of the forages was determined using the Folin–Ciocalteu's method, as described in Jaiswal et al. [15]. Briefly, 100 µL aliquot of sample was placed under alkaline conditions with 2 mL of 2% sodium carbonate. After 2 min, 100 µL of 50% Folin–Ciocalteu reagent was added, and the mixture was left for 30 min at room temperature. Absorbance was measured at 720 nm using a spectrophotometer (Varioskan LUX, Thermo Scientific, Waltham, MA, USA). Gallic acid standard (0–500 µg/mL) was used to prepare the standard curve and calculate the TPC of the forages, which was expressed as Gallic Acid Equivalent of dried weight (mg GAE/g).

The total flavonoid content (TFC) of the forages was determined using the aluminium chloride assay, as described in Jaiswal et al. [15]. A 250 µL aliquot of sample was placed under alkaline conditions with 5% sodium nitrate. After 6 min, 150 µL of 10% aluminium chloride and 0.5 mL of 1 M sodium hydroxide were added to the sample. The mixture was left for 30 min at room temperature, before measuring the absorbance at 510 nm. Catechin standard (0–200 µg/mL) was used to prepare the standard curve and calculate the TFC of the forages, which was expressed as a Catechin Equivalent of dried weight (mg CE/g).

2.4. Characterisation of Polyphenols Using LC-ESI-QqQ-MS

Mass spectrometry was employed to characterise and quantify the individual polyphenols present in the forage samples. The instrument was composed of an Agilent Technologies 1290 Infinity series HPLC, coupled with an Agilent Technologies 6470 series electrospray ionization triple quadruple with electrospray ionization. A modification of Vlaisavljević's method [16] was performed. A 5 µL aliquot of sample was injected and the separation was carried out using a Poroshell 120 (3.0 mm × 100 mm × 2.7 µm) (Agilent Technologies, Cork, Ireland) held at 50 °C. The mobile phase solvent A consisted of 0.1% formic acid in water and the mobile phase solvent B consisted of 0.1% formic acid in methanol. Elution was performed at a flow rate of 0.5 mL/min using the following gradient: starting with 40% B, reaching 70% B in 6 min and holding until 10 min, with a post-time of 3 min.

The detection of eluted polyphenols was performed using Multiple Reaction Monitoring (MRM), in the following ion source: negative ion polarity, gas flow 13 l/min, nebulizer 40 psi, sheath gas 350 °C, and drying gas 9 l/min. LC-ESI-QqQ-MS parameters for stan-

dard compounds are presented in Table 1. Calibration curves were constructed from peak areas of different standard concentrations (0.01 to 3 µg/mL), using the equation for linear regression obtained from the calibration curves (R^2 = 0.99).

Table 1. LC-ESI-QqQ-MS data for the standard compounds.

Targets	Molecular Formula	Retention Time (min)	Fragmentor Voltage (V)	Collision Energy (V)	Precursor Ion (m/z)	Product Ion (m/z)
Chlorogenic acid	$C_{16}H_{18}O_9$	1.090	165	10	353.2	191.0
Naringin	$C_{27}H_{32}O_{14}$	2.137	225	33	579.4	271.2
Daidzein	$C_{15}H_{10}O_4$	3.700	145	31	253.0	208.0
Quercetin	$C_{15}H_{10}O_7$	4.094	130	15	301.1	151.1
Kaempferol	$C_{15}H_{10}O_6$	4.640	130	0	285.0	285.0
Luteolin	$C_{15}H_{10}O_6$	4.640	135	25	285.2	133.0
Formononetin	$C_{16}H_{12}O_4$	6.947	112	10	267.0	252.0
Biochanin A	$C_{16}H_{12}O_5$	8.636	135	17	283.0	268.0

2.5. Antioxidant Studies

2.5.1. Ferric Reducing Antioxidant Power (FRAP)

The FRAP method was used to determine the antioxidant capacity of forages, following the methods of Benzie and Strain [17], and Shannon [18]. The FRAP reagent (300 mM sodium acetate buffer pH 3.6, 20 mM ferric chloride solution and 10 mM 2,4,6-Tris(2-pyridyl)s-triazine (TPTZ) in 40 mM HCl; at a ratio of 10:1:1, $v/v/v$) was prepared and incubated at 37 °C for 5 min. In a 96-well plate, 100 µL of the FRAP reagent was added to 50 µL of the sample. After incubating the plate at 25 °C for 10 min, the absorbance was measured at 593 nm. Dried forage extract samples were diluted to a 0.25 mg/mL concentration. Trolox standard (0–140 µM) was used to prepare the standard curve and calculate the antioxidant capacity of forages, which was expressed in Trolox Equivalent of dried extract (µM TE/g).

2.5.2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH•)

The DPPH• method was used to estimate the free-radical scavenging activity of forages using the method of Jaiswal et al. [15]. The DPPH• radical solution was prepared in methanol (165 µM). Using six wells of the 96 well plate, 100 µL of each sample was pipetted. A 100 µL aliquot of H_2O was added to the first three wells (control test) and 100 µL aliquot of DPPH• was added to the other three wells (test). The plate was incubated in the dark at 30 °C for 30 min and the absorbance was measured at 517 nm. Dried forage extract samples were diluted to a 0.25 mg/mL concentration. L-ascorbic acid standard (0–25 mM) was used to prepare the standard curve and calculate the scavenging capacity of forages, which was expressed as % DPPH• inhibition.

2.5.3. Oxygen Radical Antioxidant Capacity (ORAC)

The ORAC method was used to determine the oxygen radical scavenging capacity following Ou's method [19]. A 75 µM Phosphate Buffered Saline (PBS) solution at pH 7.4 was prepared using mono and dibasic potassium phosphate. Forage extract samples, fluorescein sodium salt solution (4×10^{-3} mM) and APPH solution (153 mM) were prepared using the PBS. Prior to the assay, the fluorescein sodium salt solution was diluted 50:50, obtaining a concentration of 4×10^{-6} mM. A 25 µL aliquot of either sample, blank or standard, was added to each well, followed by 150 µL fluorescein sodium salt. The plate was incubated for 30 min in the spectrophotometer, which was preheated to 37 °C. Briefly, the plate was removed before adding (in a very dim light) 25 µL of the 2,2′-Azobis (2-amidinopropane) (APPH) solution. The plate was incubated again at 37 °C for 90 min and measurements were carried out. Dried forage extract samples were diluted to a 0.25 mg/mL concentration. Trolox standard (0–100 µM) was used to prepare the standard curve and calculate the ORAC value of forages, which was expressed in Trolox Equivalent of dried extract (µM TE/g).

2.6. Statistical Analysis

All the data were reported as means ± standard deviations of triplicate determinations. A statistical analysis was performed with SPSS Statistic Software (vers 28.0.0), using one-way analysis of variance (ANOVA). Differences at $p < 0.05$ were considered statistically different. LC-ESI-QqQ-MS data were analysed using Agilent Mass Hunter Workstation Software-Qualitative Analysis (vers 10) and Agilent QQQ Quantitative Analysis (vers 8).

3. Results

3.1. Total Polyphenol and Flavonoid Content

Forages are a rich source of polyphenols, but information on their concentration and composition is still scarce. The TPC and TFC of the multi-species sward components were studied over five months during the Irish grazing season (April–August). TPC is a standard assay used to determine the polyphenols content, as well as any other reducing compounds present in samples (i.e., vitamins, minerals). However, TFC is a more robust method for measuring polyphenols, as it specifically targets flavonoids (the largest subgroup of polyphenols found in plants). The TPC and TFC results are reported in Table 2.

Table 2. Total Phenolic Content (TPC) (mg GAE/g) and Total Flavonoid Content (TFC) (mg CE/g) of the multi-species swards components (perennial ryegrass, timothy, red clover, white clover, chicory, plantain) over the grazing season. Values are represented as the mean of three replicates ± standard deviation (in italics). Significant difference ($p < 0.05$) between species is indicated with letter superscript.

	Perennial Ryegrass				**Timothy**		
April	TPC	26.93 ^{a}	*±3.16*	**April**	TPC	52.37 ^{c}	*±4.32*
	TFC	14.57 ^{a}	*±1.25*		TFC	35.72 ^{b}	*±2.01*
May	TPC	29.43 ^{a}	*±1.72*	**May**	TPC	37.71 ^{c}	*±3.18*
	TFC	13.8 ^{a}	*±2.20*		TFC	23.11 ^{b}	*±4.86*
June	TPC	25.15 ^{a}	*±3.43*	**June**	TPC	95.45 ^{c}	*±4.26*
	TFC	12.97 ^{a}	*±3.82*		TFC	67.12 ^{b}	*±2.57*
July	TPC	23.01 ^{a}	*±2.93*	**July**	TPC	75.41 ^{c}	*±3.08*
	TFC	10.70 ^{a}	*±0.78*		TFC	60.29 ^{b}	*±2.98*
August	TPC	19.95 ^{a}	*±1.74*	**August**	TPC	76.95 ^{c}	*±4.33*
	TFC	11.73 ^{a}	*±0.93*		TFC	64.62 ^{b}	*±3.68*
	White Clover				**Red Clover**		
April	TPC	38.04 ^{a}	*±5.26*	**April**	TPC	40.57 ^{b}	*±4.92*
	TFC	17.06 ^{a}	*±1.07*		TFC	12.62 ^{a}	*±4.15*
May	TPC	20.16 ^{a}	*±3.85*	**May**	TPC	41.70 ^{b}	*±2.81*
	TFC	6.27 ^{a}	*±4.06*		TFC	13.06 ^{a}	*±2.88*
June	TPC	33.17 ^{a}	*±5.58*	**June**	TPC	47.49 ^{b}	*±2.97*
	TFC	12.33 ^{a}	*±3.30*		TFC	21.84 ^{a}	*±5.79*
July	TPC	39.63 ^{a}	*±4.13*	**July**	TPC	38.60 ^{b}	*±3.38*
	TFC	16.01 ^{a}	*±0.83*		TFC	15.52 ^{a}	*±5.27*
August	TPC	31.70 ^{a}	*±3.99*	**August**	TPC	43.49 ^{b}	*±2.54*
	TFC	12.64 ^{a}	*±1.63*		TFC	13.68 ^{a}	*±2.60*
	Chicory				**Plantain**		
April	TPC	71.01 ^{c}	*±5.89*	**April**	TPC	118.58 ^{d}	*±1.43*
	TFC	58.95 ^{b}	*±4.64*		TFC	81.01 ^{c}	*±7.37*
May	TPC	74.94 ^{c}	*±3.14*	**May**	TPC	137.11 ^{d}	*±9.40*
	TFC	53.67 ^{b}	*±2.52*		TFC	102.42 ^{c}	*±3.72*
June	TPC	67.77 ^{c}	*±6.47*	**June**	TPC	138.69 ^{d}	*±8.11*
	TFC	55.94 ^{b}	*±6.82*		TFC	101.12 ^{c}	*±3.36*
July	TPC	52.57 ^{c}	*±4.24*	**July**	TPC	112.27 ^{d}	*±8.50*
	TFC	40.55 ^{b}	*±5.27*		TFC	87.77 ^{c}	*±5.60*
August	TPC	47.50 ^{c}	*±4.40*	**August**	TPC	120.29 ^{d}	*±7.20*
	TFC	35.56 ^{b}	*±0.93*		TFC	96.24 ^{c}	*±5.83*

The TPC and TFC values were significantly different among species ($p < 0.01$), while there was no significant difference within species over the grazing season ($p > 0.05$). High TPC and TFC were found in plantain (138.69 mg GAE/g and 101.12 mg CE/g), timothy

(95.45 mg GAE/g and 67.12 mg CE/g) and chicory (74.94 mg GAE/g and 53.67 mg CE/g), whereas lower levels were found in red clover (38.60 mg GAE/g and 15.52 mg CE/g), perennial ryegrass (19.95 mg GAE/g and 11.73 mg CE/g) and white clover (20.16 mg GAE/g and 6.27 mg CE/g). Similar TPC and TFC values were reported for plantain [20], chicory [21,22] and both of the clover species investigated in this study [16,23]. There is little reported on timothy, whereas perennial ryegrass has been shown to have similar [24] and lower TPC [25] to the values reported here.

3.2. Characterisation of Polyphenols Using LC-ESI-QqQ-MS

Characterisation of the phenolic compounds of the multi-species swards components was performed using high selective and specific LC-ESI-QqQ-MS in negative MRM acquisition mode. MRM mode is an accurate technique that monitors ions of the compounds of interest and provides more precise quantification at a lower detection limit. In this research work, one phenolic acid (chlorogenic acid), two flavonol (kaempferol, quercetin), one flavone (luteolin), one flavanone (naringenin) and three isoflavones (biochanin A, daidzein and formononetin) were studied. The eight polyphenols investigated were based on common presence among forages and their potential impact on enhancing animal health and animal products' nutritional value. Polyphenols were accurately detected by comparing the retention time and unique transition from parent ion to product ion between commercial standards and samples. The results of the LC-ESI-QqQ-MS are shown in Table 3.

Table 3. Quantification of selected polyphenols in multi-species sward components over April, May, June, July and August. Results are expressed in mg g^{-1} of dry weight (BcA = Biochanin A; CgA = Chlorogenic acid; Dz = Daidzein; Fmnt = Formononetin; Kae = Kaempferol; Lu = Luteolin; Nar = Naringenin; Que = Quercetin). Values are represented as mean ± standard deviation (in italics).

	Perennial Ryegrass							Timothy						
April	**BcA**	0.01	*±0.01*	**Kae**	0.05	*±0.02*	**April**	**BcA**	0.01	*±0.01*	**Kae**	0.07	*±0.04*
	CgA	6.62	*±0.41*	**Lu**	0.03	*±0.03*		**CgA**	14.61	*±1.21*	**Lu**	0.04	*±0.03*
	Dz	0.05	*±0.06*	**Nar**	0.05	*±0.07*		**Dz**	0.04	*±0.04*	**Nar**	0.04	*±0.05*
	Fmnt	0.05	*±0.02*	**Que**	0.03	*±0.03*		**Fmnt**	0.04	*±0.02*	**Que**	0.01	*±0.01*
May	**BcA**	0.05	*±0.04*	**Kae**	0.02	*±0.01*	**May**	**BcA**	0	*±0.01*	**Kae**	0.02	*±0.01*
	CgA	5.64	*±0.87*	**Lu**	0.01	*±0.01*		**CgA**	13.83	*±0.57*	**Lu**	0.04	*±0.01*
	Dz	0.03	*±0.03*	**Nar**	0	*±0.01*		**Dz**	0.02	*±0.01*	**Nar**	0.02	*±0.01*
	Fmnt	0.04	*±0.01*	**Que**	0.01	*±0.01*		**Fmnt**	0.06	*±0.01*	**Que**	0.01	*±0.01*
June	**BcA**	0.02	*±0.02*	**Kae**	0.02	*±0.01*	**June**	**BcA**	0.03	*±0.03*	**Kae**	0.02	*±0.02*
	CgA	4.51	*±0.48*	**Lu**	0.01	*±0.00*		**CgA**	16.61	*±0.29*	**Lu**	0.01	*±0.02*
	Dz	0.02	*±0.02*	**Nar**	0.01	*±0.01*		**Dz**	0.01	*±0.02*	**Nar**	0.01	*±0.00*
	Fmnt	0.06	*±0.03*	**Que**	0.01	*±0.00*		**Fmnt**	0.04	*±0.04*	**Que**	0.01	*±0.02*
July	**BcA**	0.01	*±0.01*	**Kae**	0.01	*±0.01*	**July**	**BcA**	0.06	*±0.05*	**Kae**	0.02	*±0.01*
	CgA	4.63	*±0.57*	**Lu**	0.03	*±0.03*		**CgA**	17.07	*±1.35*	**Lu**	0.03	*±0.01*
	Dz	0.01	*±0.02*	**Nar**	0.01	*±0.01*		**Dz**	0.01	*±0.01*	**Nar**	0.01	*±0.00*
	Fmnt	0.01	*±0.01*	**Que**	0.01	*±0.00*		**Fmnt**	0.15	*±0.03*	**Que**	0	*±0.00*
August	**BcA**	0.01	*±0.01*	**Kae**	0.01	*±0.01*	**August**	**BcA**	0.09	*±0.07*	**Kae**	0.02	*±0.01*
	CgA	1.94	*±0.33*	**Lu**	0.01	*±0.01*		**CgA**	20.69	*±0.56*	**Lu**	0.05	*±0.04*
	Dz	0.01	*±0.01*	**Nar**	0.01	*±0.01*		**Dz**	0.01	*±0.00*	**Nar**	0.03	*±0.03*
	Fmnt	0.03	*±0.01*	**Que**	0.01	*±0.02*		**Fmnt**	0.25	*±0.03*	**Que**	0.01	*±0.00*

Table 3. *Cont.*

White Clover							**Red Clover**						
April	**BcA**	0.02	±0	**Kae**	0.03	±0.01	**April**	**BcA**	1.6	±0.11	**Kae**	0.05	±0.04
	CgA	1.05	±0.01	**Lu**	0.06	±0.02		**CgA**	0.11	±0.05	**Lu**	0.03	±0.02
	Dz	0.02	±0.01	**Nar**	0.02	±0.03		**Dz**	1.07	±0.15	**Nar**	0.01	±0.02
	Fmnt	0.57	±0.07	**Que**	0.01	±0.01		**Fmnt**	4.29	±0.69	**Que**	0.01	±0.00
May	**BcA**	0.02	±0.01	**Kae**	0.02	*±0.01*	**May**	**BcA**	2.22	*±0.55*	**Kae**	0.03	*±0.02*
	CgA	0.21	±0.05	**Lu**	0.02	*±0.01*		**CgA**	0.11	*±0.03*	**Lu**	0.05	*±0.00*
	Dz	0.01	±0	**Nar**	0.01	*±0.01*		**Dz**	0.43	*±0.02*	**Nar**	0.02	*±0.02*
	Fmnt	0.1	±0.02	**Que**	0.01	*±0.01*		**Fmnt**	5.64	*±0.68*	**Que**	0	*±0.01*
June	**BcA**	0.02	±0.02	**Kae**	0.03	*±0.01*	**June**	**BcA**	2.44	*±0.55*	**Kae**	0.03	*±0.03*
	CgA	0.19	±0.07	**Lu**	0.03	*±0.01*		**CgA**	0.06	*±0.01*	**Lu**	0.07	*±0.03*
	Dz	0.01	±0.02	**Nar**	0.01	*±0.01*		**Dz**	0.9	*±0.03*	**Nar**	0.01	*±0.01*
	Fmnt	0.19	±0.05	**Que**	0.01	*±0.01*		**Fmnt**	5.65	*±0.76*	**Que**	0.01	*±0.01*
July	**BcA**	4.04	±0.84	**Kae**	0.06	*±0.03*	**July**	**BcA**	2.57	*±0.53*	**Kae**	0.05	*±0.06*
	CgA	0.17	±0.03	**Lu**	0.05	*±0.05*		**CgA**	0.04	*±0.00*	**Lu**	0.03	*±0.00*
	Dz	0.04	±0.03	**Nar**	0.01	*±0.01*		**Dz**	0.44	*±0.05*	**Nar**	0.01	*±0.00*
	Fmnt	7.25	±1.11	**Que**	0.01	*±0.00*		**Fmnt**	5.86	*±0.76*	**Que**	0.01	*±0.00*
August	**BcA**	0.04	±0.02	**Kae**	0.02	*±0.02*	**August**	**BcA**	3.03	*±0.46*	**Kae**	0.02	*±0.01*
	CgA	0.08	±0.04	**Lu**	0.04	*±0.01*		**CgA**	0.07	*±0.06*	**Lu**	0.04	*±0.01*
	Dz	0.01	±0	**Nar**	0.01	*±0.01*		**Dz**	0.61	*±0.03*	**Nar**	0.01	*±0.01*
	Fmnt	1.71	±0.29	**Que**	0.04	*±0.06*		**Fmnt**	8.23	*±0.81*	**Que**	0.01	*±0.01*
Chicory							**Plantain**						
April	**BcA**	0.07	*±0.01*	**Kae**	0.84	*±0.13*	**April**	**BcA**	0.06	*±0.01*	**Kae**	0.04	*±0.02*
	CgA	0.77	*±0.05*	**Lu**	0.9	*±0.23*		**CgA**	7.36	*±0.69*	**Lu**	0.02	*±0.02*
	Dz	0.35	*±0.59*	**Nar**	0.02	*±0.02*		**Dz**	0.02	*±0.01*	**Nar**	0.01	*±0.01*
	Fmnt	0.3	*±0.01*	**Que**	0.01	*±0.01*		**Fmnt**	0.35	*±0.09*	**Que**	0.01	*±0.01*
May	**BcA**	0.06	*±0.01*	**Kae**	1.23	*±0.25*	**May**	**BcA**	0.05	*±0.02*	**Kae**	0.03	*±0.02*
	CgA	1.73	*±0.30*	**Lu**	1.29	*±0.17*		**CgA**	9.58	*±0.30*	**Lu**	0.03	*±0.02*
	Dz	0.01	*±0.01*	**Nar**	0.01	*±0.01*		**Dz**	0.01	*±0.01*	**Nar**	0	*±0.01*
	Fmnt	4.36	*±0.40*	**Que**	0.04	*±0.05*		**Fmnt**	0.11	*±0.02*	**Que**	0.01	*±0.01*
June	**BcA**	0.57	*±0.10*	**Kae**	0.89	*±0.00*	**June**	**BcA**	0.06	*±0.04*	**Kae**	0.02	*±0.02*
	CgA	1.55	*±0.20*	**Lu**	0.87	*±0.00*		**CgA**	8.08	*±0.58*	**Lu**	0.05	*±0.03*
	Dz	0.01	*±0.01*	**Nar**	0.02	*±0.01*		**Dz**	0.01	*±0.01*	**Nar**	0.02	*±0.01*
	Fmnt	1.38	*±0.35*	**Que**	0.01	*±0.00*		**Fmnt**	0.14	*±0.02*	**Que**	0.01	*±0.01*
July	**BcA**	0.38	*±0.14*	**Kae**	1.27	*±0.26*	**July**	**BcA**	0.21	*±0.03*	**Kae**	0.15	*±0.12*
	CgA	1.16	*±0.27*	**Lu**	1.25	*±0.27*		**CgA**	7.43	*±0.26*	**Lu**	0.39	*±0.27*
	Dz	0.02	*±0.01*	**Nar**	0.01	*±0.01*		**Dz**	0.01	*±0.01*	**Nar**	0.01	*±0.01*
	Fmnt	1.51	*±0.44*	**Que**	0.01	*±0.00*		**Fmnt**	0.74	*±0.24*	**Que**	0	*±0.00*
August	**BcA**	3.25	*±0.00*	**Kae**	0.03	*±0.00*	**August**	**BcA**	0.45	*±0.18*	**Kae**	0.13	*±0.05*
	CgA	0.83	*±0.06*	**Lu**	0.06	*±0.03*		**CgA**	5.49	*±0.89*	**Lu**	0.1	*±0.06*
	Dz	0.28	*±0.00*	**Nar**	0.01	*±0.00*		**Dz**	0.01	*±0.02*	**Nar**	0	*±0.01*
	Fmnt	5.68	*±0.00*	**Que**	0.01	*±0.00*		**Fmnt**	0.86	*±0.30*	**Que**	0.01	*±0.01*

This study found chlorogenic acid to be the predominant polyphenol among the grass forages (i.e., perennial ryegrass, timothy) ($p < 0.05$), with concentrations accounting for 10–37% of the overall TPC, and varying within species and throughout the grazing season. In perennial ryegrass, chlorogenic acid continuously decreased from 6.62 mg/g in April to 1.94 mg/g in August, while in timothy it increased from 14.61 mg/g to 20.69 mg/g. Chlorogenic acid was also found in the herb forages (1–7% of the overall TPC), and concentrations were at their highest in the month of May (9.58 mg/g in plantain and 1.73 mg/g in chicory).

Formononetin and biochanin A were found in red clover (10–19% and 4–7% of the overall TPC, respectively), white clover (0–18% and 0–10%) and chicory (0–12% and 0–9%) ($p < 0.05$). Concentrations of the two flavonoids in red clover almost doubled during the grazing season (4.29 mg/g of formononetin and 1.60 mg/g of biochanin A in April, 8.23 mg/g and 3.03 mg/g in August), possibly due to the maturation of the plant. Indeed, biochanin A and formononetin are the predominant isoflavones in all the plant parts of the red clover (e.g., leaves, stems, flowers) [26]; as the plant grows, an increase is expected in the overall concentration of these compounds. Daidzein, a derivative of biochanin A, was also detected in red clover ($p < 0.05$) and its concentration remained constant throughout the season.

Kaempferol and luteolin were found in chicory ($p < 0.05$) and detected at a range of 0.84–1.27 mg/g and 0.90–1.29 mg/g, respectively, throughout the grazing season (accounting for 1–3% of the overall TPC). Quercetin was not found among any of the six species studied ($p < 0.05$). Quercetin is one of the strongest antioxidants present in nature, but it is usually found in plants in glycoside forms (e.g., glucorhamnoside rutin). Several studies have reported the presence of glycoside quercetin in red clover, in chicory [27] and plantain [28], while few have reported the aglycone forms. Hence, the absence of quercetin in this study could be due to structural reasons.

3.3. Antioxidant Studies

Diets high in antioxidants have been proven to be advantageous to livestock, as they decrease the incidence of mastitis and lower the development of off-flavour compounds in milk. Cattle can be supplemented with selenium and vitamin E, but this method can be expensive and could impact the consumer desire to consume organic products. Forages rich in antioxidants could represent a more appealing option to farmers, as they offer a natural and economic option, while also possessing environmental benefits. The antioxidant capacity of the multi-species sward components was investigated with three assays (FRAP, DPPH$\bullet^{\bullet}$ and ORAC), and the results are reported in Table 4.

FRAP is an electro-transfer assay which measures the ability of the antioxidant to transfer electrons onto a reducing agent. In this case, the antioxidant agent donates electrons to the Fe^{3+} ion, reducing it into Fe^{2+} [29]. DPPH$\bullet^{\bullet}$ and ORAC are free radical scavenging assays that also measure the antioxidant capacity. While DPPH$\bullet^{\bullet}$ measures the ability of an antioxidant to scavenge a free radical by a donating electron, ORAC measures it by a donating hydrogen atom [29].

Plantain showed the highest FRAP, DPPH$\bullet^{\bullet}$ and ORAC values throughout the study period (482.49 µM TroloxE/g, 80.94% and 2478.93 µM TroloxE/g, respectively), almost one and a half more than timothy (352.00 µM TroloxE/g, 68.07% and 1665.46 µM TroloxE/g), and twice as much as chicory (286.71 µM TroloxE/g, 58.98% and 1594.22 µM TroloxE/g). The antioxidant capacity was not found to be significantly different over the grazing season ($p > 0.05$). Timothy exhibited a high antioxidant capacity, in addition to being the species with the highest concentration of chlorogenic acid. A strong correlation ($p < 0.05$) between the antioxidant capacity and chlorogenic acid concentrations was also found in this study. The Antioxidant capacity of forages can be explained by the presence of polyphenols, and also by non-polyphenols phytochemicals. For example, plantain and chicory are rich in other reactive oxygen species-scavenging compounds, such as iridoids (e.g., aucubin, catalpol, acteoside) and sesquiterpene lactones [30]; it is plausible that these compounds contribute to the high antioxidant capacity of these forage species.

Table 4. FRAP (μM TroloxE/g), DPPH•• (%) and ORAC (μM TroloxE/g) values of the mix swards components (perennial ryegrass, timothy, red clover, white clover, chicory, plantain) over the grazing season. Values are represented as mean of three replicates ± standard deviation (in italics). Significant difference ($p < 0.05$) between species is indicated with letter superscript.

	Perennial Ryegrass				**Timothy**		
April	FRAP	90.04 ^{a}	*±3.01*	**April**	FRAP	216.75 ^{b}	*±7.92*
	DPPH••	20.93 ^{a}	*±3.19*		DPPH••	42.96 ^{c}	*±7.51*
	ORAC	1053.55 ^{a}	*±111.18*		ORAC	1027.61 ^{c}	*±106.38*
May	FRAP	80.26 ^{a}	*±5.38*	**May**	FRAP	154.15 ^{b}	*±8.03*
	DPPH••	19.27 ^{a}	*±3.54*		DPPH••	43.90 ^{c}	*±2.75*
	ORAC	1038.14 ^{a}	*±109.19*		ORAC	1243.69 ^{c}	*±132.59*
June	FRAP	102.77 ^{a}	*±3.92*	**June**	FRAP	352.00 ^{b}	*±6.97*
	DPPH••	23.01 ^{a}	*±5.98*		DPPH••	68.07 ^{c}	*±2.81*
	ORAC	960.15 ^{a}	*±100.64*		ORAC	1586.34 ^{c}	*±68.96*
July	FRAP	79.04 ^{a}	*±6.76*	**July**	FRAP	307.18 ^{b}	*±8.98*
	DPPH••	25.87 ^{a}	*±3.31*		DPPH••	62.48 ^{c}	*±6.50*
	ORAC	769.36 ^{a}	*±46.27*		ORAC	1510.63 ^{c}	*±73.57*
August	FRAP	67.36 ^{a}	*±7.96*	**August**	FRAP	326.84 ^{b}	*±4.62*
	DPPH••	23.50 ^{a}	*±4.97*		DPPH••	60.05 ^{c}	*±1.93*
	ORAC	755.08 ^{a}	*±156.06*		ORAC	1665.46 ^{c}	*±94.46*
	White Clover				**Red Clover**		
April	FRAP	108.57 ^{a}	*±6.43*	**April**	FRAP	97.25 ^{a}	*±8.43*
	DPPH••	23.30 ^{ab}	*±5.54*		DPPH••	23.74 ^{b}	*±4.57*
	ORAC	1251.90 ^{b}	*±108.17*		ORAC	1082.85 ^{b}	*±81.19*
May	FRAP	50.58 ^{a}	*±4.33*	**May**	FRAP	89.54 ^{a}	*±7.22*
	DPPH••	24.40 ^{ab}	*±5.52*		DPPH••	29.22 ^{b}	*±3.76*
	ORAC	1009.17 ^{b}	*±85.25*		ORAC	1176.31 ^{b}	*±102.53*
June	FRAP	97.95 ^{a}	*±6.20*	**June**	FRAP	136.05 ^{a}	*±5.35*
	DPPH••	27.21 ^{ab}	*±3.85*		DPPH••	37.58 ^{b}	*±2.87*
	ORAC	1185.22 ^{b}	*±65.08*		ORAC	1220.49 ^{b}	*±71.11*
July	FRAP	94.53 ^{a}	*±6.20*	**July**	FRAP	90.91 ^{a}	*±7.62*
	DPPH••	30.19 ^{ab}	*±4.34*		DPPH••	30.39 ^{b}	*±4.51*
	ORAC	1319.97 ^{b}	*±82.71*		ORAC	1051.56 ^{b}	*±93.29*
August	FRAP	69.07 ^{a}	*±3.26*	**August**	FRAP	92.02 ^{a}	*±7.28*
	DPPH••	18.44 ^{ab}	*±2.71*		DPPH••	23.36 ^{b}	*±6.86*
	ORAC	1219.08 ^{b}	*±107.26*		ORAC	988.17 ^{b}	*±53.15*
	Chicory				**Plantain**		
April	FRAP	270.24 ^{b}	*±10.53*	**April**	FRAP	368.70 ^{c}	*±9.40*
	DPPH••	54.96 ^{c}	*±8.00*		DPPH••	71.59 ^{d}	*±5.22*
	ORAC	1594.22 ^{c}	*±132.29*		ORAC	2200.39 ^{d}	*±123.70*
May	FRAP	286.71 ^{b}	*±12.19*	**May**	FRAP	448.14 ^{c}	*17.78*
	DPPH••	56.22 ^{c}	*±4.92*		DPPH••	79.94 ^{d}	*±5.22*
	ORAC	1289.67 ^{c}	*±112.20*		ORAC	2478.93 ^{d}	*±125.47*
June	FRAP	244.75 ^{b}	*±8.35*	**June**	FRAP	482.49 ^{c}	*±6.75*
	DPPH••	58.98 ^{c}	*±6.78*		DPPH••	80.94 ^{d}	*±3.50*
	ORAC	1224.31 ^{c}	*±71.76*		ORAC	2230.17 ^{d}	*±73.90*
July	FRAP	194.44 ^{b}	*±4.14*	**July**	FRAP	457.90 ^{c}	*±7.12*
	DPPH••	50.83 ^{c}	*±6.47*		DPPH••	78.21 ^{d}	*±3.80*
	ORAC	1306.99 ^{c}	*±69.78*		ORAC	2032.51 ^{d}	*±132.40*
August	FRAP	211.46 ^{b}	*±8.90*	**August**	FRAP	450.94 ^{c}	*±14.06*
	DPPH••	35.74 ^{c}	*±2.16*		DPPH••	79.82 ^{d}	*±4.00*
	ORAC	1301.14 ^{c}	*±13.97*		ORAC	2394.90 ^{d}	*±109.01*

A correlation between the total phenolic content (TPC), total flavonoid content (TFC) and the antioxidant capacities (FRAP, DPPH•• and ORAC) is shown in Figure 1. The results show a positive linear correlation between the phenolic component of each species and the antioxidant capacities.

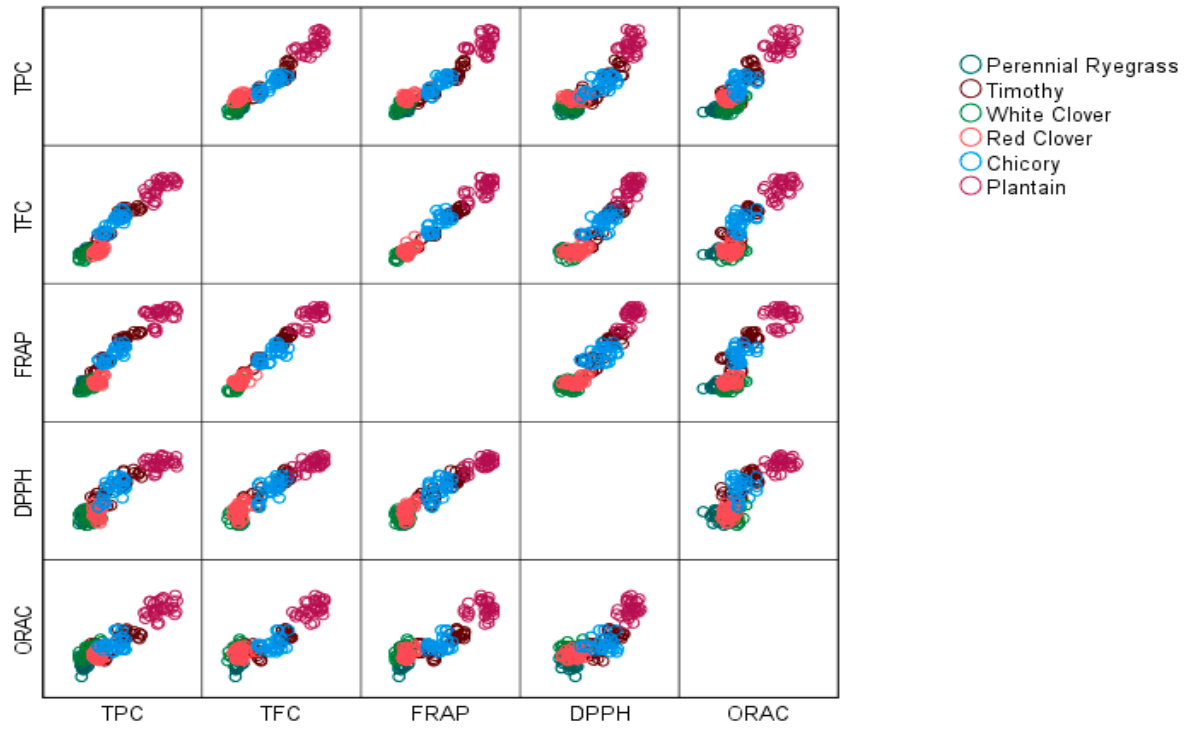

Figure 1. Matrix correlation between total phenolic/flavonoid content and the antioxidant capacity.

The correlation between phenolic content and antioxidant was at its strongest with FRAP (TPC, R = 0.968; TFC R = 0.984), followed by DPPH•• (TPC, R = 0.923; TFC R = 0.953), and ORAC (TPC, R = 0.921; TFC R = 0.899). These results show that the phenolic compounds, such as phenolic acids and flavonoids, are some of the main factors contributing to the antioxidant capacity of the forages.

4. Discussion

Variations in the polyphenol composition of plants are dictated by the botanical classification. For instance, species from the *Leguminosae* family (e.g., red clover and white clover) tend to be a rich source of isoflavonoids [31], whereas perennial herbs from the *Plantaginacea* (e.g., plantain) and *Asteraceae* (e.g., chicory) families have high levels of phenolic acids, flavones and flavonols [27,28]. The *Pomacea* family, which includes grass species (e.g., perennial ryegrass), is a rich source of hydroxycinnamic acids, particularly chlorogenic acid and its isomers [32]. Conversely, changes in phenolic compound concentrations over time are dependent on the season, phenological stage, stress response of the plant and weather conditions. In particular, defoliation from repeated grazing can impact the polyphenol content of the plant [33]. Thus, predicting patterns of polyphenols' biosynthesis and accumulation can be challenging [34]. This study showed that the sum of chlorogenic acid from perennial ryegrass, timothy, chicory and plantain remained consistent every month through the grazing season (7.5 mg/g); therefore, it would be possible for multi-species swards to somehow supply animals with a constant rate of dietary chlorogenic acid. Chlorogenic acid, and its several ester forms, have been linked to improved animal performance through their role as substrates for polyphenol oxidase (PPO). Activated PPO oxidises ***o***-diphenols into ***o***-quinones, which are reactive metabolites able to bind to proteins and protect them against proteolysis. The presence of ***o***-quinone has been shown to ensure more protein assimilation during animal digestion, leading to an improved animal performance [35]. *In vitro* studies with chlorogenic acid have demonstrated promising results for reducing the risk of mastitis, caused by *Staphylococcus aureus* [36]. Mastitis is an inflammation of the cow's mammary gland that is usually caused by a bacterial infection; it causes significant economic losses through veterinary costs, decreased production and discarded

milk. Gong [36] reported that 30 µg/mL of chlorogenic acid was enough to inhibit *S. aureus* growth. As cattle tend to consume between 16 and 18 kg of DM a day [37], the utilisation of high chlorogenic acid could support mastitis reduction. More animal studies are necessary in order to consolidate the association between forage-derived chlorogenic acid, and a positive impact on animal performance.

Concentrations of formononetin and biochanin A varied throughout the grazing season in white clover (7.25 mg/g of formononetin and 4.04 mg/g of biochanin A in July, 1.71 mg/g of formononetin in August), as well as in chicory (4.36 mg/g formononetin in May, 5.68 mg/g of formononetin and 3.25 mg/g biochanin A in August). The non-linear change in the concentration of isoflavones could be due to a change in the phenological stage (i.e., appearance and disappearance of flowering parts) or change in leaf to stem ratio of the plant. Indeed, this study did not take into account the compositional differences between parts of the plants, therefore, variations within species could be expected. Furthermore, field sampling was carried out randomly every month, resulting in various parts of the forages being collected at different growth stages. Nevertheless, combining red clover, white clover and chicory still resulted in an increase in the overall isoflavones' concentration through time (combined red clover, white clover and chicory had 1.72 mg/g of formononetin and 0.56 mg/g of biochanin A in April, and 5.2 mg/g and 2.1 mg/g in August), demonstrating that multi-species swards can provide an increasing supply of these compounds throughout the grazing season. The animals' intake of isoflavones through their diet, particularly formononetin, can impact the quality of the milk; this is because these compounds are precursors to equol. Equol is an oestrogen receptor modulator with indications of a possible positive impact on human bone health, blood pressure, cardiovascular conditions and oestrogen-related cancer types (e.g., breast, prostate) [38]. While only one-third of the human population is able to produce equol in the gut, ruminants are able to naturally convert formononetin into daidzein, and subsequently into equol. A Finnish study established that animal diets that are rich in legume-derived formononetin, can be associated with a higher concentration of equol in milk [39]. While the specific concentrations required to achieve these human-health benefits have not yet been established, forages with high isoflavones can present opportunities for enhancing the nutraceutical properties of milk and the subsequent benefits to the consumer.

Although kaempferol and luteolin were present at a lower concentration than the other polyphenols analysed in this study, ruminants tend to consume great proportions of chicory due to its palatability and rapid digestion. Thus, it could be possible that enough of these two flavonoids are ingested to impart biological activity. Further *in vivo* studies are required to establish an association between the concentration of flavonoids and specific animal health benefits. Kaempferol and luteolin are considered important in the ruminal microbial fermentation, as they can reduce methanogenic activity in the rumen [40,41]. It has been argued that flavonoids, in general, can act against methanogenic bacteria by inhibiting cytoplasmatic membrane function, bacterial cell wall synthesis, or nucleic acid synthesis; flavonoids, therefore, decrease methane production [42]. Sinz [41] reported the ability of luteolin to reduce ammonia formation during ruminal fermentation, thus improving nitrogen utilisation. Ammonia is a by-product of the dietary protein breakdown during animal digestion. Dietary protein structures are metabolised in the rumen by the microbial population and degraded into peptides, amino acids, and, ultimately, ammonia. Ammonia is expelled through urines, and, once it is in the soil, it gets converted into nitrous oxide, which is a powerful green-house gas. Luteolin has been shown to have the ability to bind proteins in ***o***-quinone-protein complexes; therefore, it potentially reduces protein degradation and ammonia formation [43]. It is worth noting that the studies presented above were carried out *in vitro*, therefore, they did not necessarily consider other variables that might be present in the rumen. Nonetheless, such findings are of interest and could have far reaching environmental impacts. Certainly, further *in vivo* animal studies are required to confirm associations between flavonoids and reduced gas emissions.

Overall, grass species were found to have chlorogenic acid, clover species were found to have formononetin and biochanin A, and herbs were found to contain chlorogenic acid, kaempferol and luteolin. Complementing grasses with legumes and herbs creates a synergetic nutritional effect, as it combines different phenolic compounds, as well as other properties peculiar to individual species. For instance, red clover is rich in PPO, which reduces proteolysis and lipolysis in the rumen. While reducing proteolysis is considered beneficial because it improves protein utilisation, reducing lipolysis increases the fat concentration in milk. Herbs contain high mineral content, which is indispensable for optimal animal performance and productivity. Grasses tend to have a high neutral detergent fibre (NDF) value [44], which is an indicator of the content of dietary soluble carbohydrates that can be converted into acetate and, further, into fatty acids. Diets rich in NDF are important for the fat component in milk, as they aid fat synthesis and avoid fat depression [45].

Furthermore, from an Irish climate-adaptability point of view, perennial ryegrass, plantain and timothy grow well during springtime, while white clover, red clover plantain and chicory grow well during summertime [46]. Hence, farmers establishing multi-species swards can find forages rich in nutrients that can potentially improve animal productivity and product quality, as well as have diversified pastures available all year around.

5. Conclusions

Multi-species swards are a sustainable alternative to ryegrass monoculture swards due to their capacity to improve soil functionality, increase biodiversity and reduce fertilization requirement. Additionally, multi-species swards include various phenolic compounds that have been associated with antioxidant and anti-inflammatory properties, which are identified as precursors for nutraceutical compounds in milk and linked to animal emission reduction. Variations in the phenolic concentration and antioxidant capacity were found to be more significant between species than between seasons within species. Farmers should consider implementing multi-species swards, as they can provide micronutrients linked to animal health and productivity, in addition to positively impacting the environment.

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