Research article

Ergosterol and polyphenol contents as rapid indicators of orchardgrass silage safety

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ABSTRACT

The ergosterol (ERG) has been proposed as a potential indicator of fungal contamination, along with polyphenol content analysis to predict silage safety. Despite efforts in controlling fungal growth in silage, mycotoxin co-contamination represents a possible risk for animal and human health. Modern analytical techniques determine a multitude of fungal metabolites contaminating feed. Nonetheless, these methods require sometimes arduous sample pre-treatment, long separation times, and expensive standard compounds to identified contaminants. Thus, the goal of this study was to suggest a rapid analysis of ERG and polyphenol contents to assess silage hygienic quality in ten orchardgrass varieties ensiled without and with biological and chemical additives. The determination of ERG on samples was performed by high-performance liquid chromatography using UV detection and UV/Vis spectrophotometry to determine the polyphenol content. Statistically significant differences ($P < 0.05$) between varieties, years and silage additives were found. Bepro was the unique variety that did not present ERG in the first cut in 2012. ERG content increased in the first cut in 2013 using biological additives as well as ERG and polyphenol contents in the first cut in 2013 using chemical additives compared with untreated silage. In addition, biological and chemical additives used in this study did not satisfactorily reduce the content of ERG and polyphenols in silage grass. Consequently, our results provide fast information about the progressive fungal contamination of grass silage. To our knowledge, it is the first time that the presence of ERG and polyphenols is determined in ten different orchardgrass varieties treated without and with additives. In general, ERG and polyphenol contents showed to be good indicators of orchardgrass silage safety.

1. Introduction

Silage is the major component of ruminant feed rations worldwide which represents more than 50% of dairy cow feed in winter [1]. In particular, orchardgrass (*Dactylis glomerata* L.) is the fourth most important forage grass in the world [2], widely cultivated on a global scale for grazing, hay or silage production due to its high adaptability under different environmental conditions and

considerable tolerance to extreme heat [3,4].

The low availability of water in semi-arid and temperate climates produces a limitation in the growth and development of forage, reducing yield [5]. In view of current climate conditions, silage is the best method to preserve fresh forage [6]. However, silage is susceptible to infection by different fungi during storage periods negatively affecting the normal rate of growth and development of the animal [7,8]. In fact, *Aspergillus fumigatus* is one of the well-documented moulds in silage that produces mycotoxins whose spores can be inhaled causing aspergillosis disease [9] as well as Farmers Lung disease in humans with altered lung function [10].

The additives have been widely used to improve the quality and the fermentation process in order to avoid silage deterioration from possible pathogens [11]. In this sense, silage safety can be affected by the additives used during the fermentation period by enabling the production of more organic acids and greater reductions in pH, as well as by effectively inhibiting the activity of undesirable microorganisms such as moulds [11,12]. In spite of additives inhibit the activity of moulds and pathogens, it is necessary to evaluate the possible contamination through reliable biochemical indicators to ensure silage quality and animal health because additives do not always completely prevent this type of contamination.

Frequently, techniques employed for contamination detection are expensive, require long analysis times and, the contamination is detected in the final stages of infection [13]. Thus, an earlier contamination detection is necessary to prolong the life span of silages remaining their safety. In this manner, different researchers have been proposed the determination of ergosterol (ERG) content as a rapid, sensitive and cheap indicator of food safety [13–18]. ERG is the main sterol of fungal mycelium membranes of moulds and yeasts. In particular, this compound represents between 10 and 80% of sterols from fungal mycelium membranes (depending on the fungal growth phase) [18]. Moreover, ERG is a primary metabolite that shares a biosynthetic pathway with other harmful mycotoxins such as deoxynivalenol and nivalenol [19]. In this way, different researchers have observed that ERG is a more sensitive indicator than enzyme-linked immune sorbent assays-polyclonal antibody, polymerase chain reaction or direct plating to detect yeast and mould colonization in foods [13,17,20]. In fact, this compound has been better correlated with fungal biomass than traditional fungal colony-forming unit count [18,20–22] and that is why ERG has been proposed as an indicator of fungal contamination for various matrices such as solid plant substrates [13], foods and raw materials [23], grasses [24] and recently in grass silage [22]. Furthermore, recent studies proved that ERG is significantly correlated with mycotoxins in foods [25,26].

In addition, the determination of total phenolic contents as indicator of silage safety has been proposed because a deterioration of silage implies an increase of phenolic contents as response to the biological stress suffered by the plant cell against the fermentation period and mould infections [27,28]. An increase of phenolic compounds can produce a colour change in the food, that is attributed to enzymatic browning reactions caused by the oxidation of tannins or quinones that combined with free amino acids and proteins originates complexes of dark coloration [29,30]. The binding of proteins by these oxidation products influences the digestibility of proteins and therefore in its absorption affecting the health of the animal. In general, polyphenols can provide astringency to feed-stuffs, when the ensiled forage is consumed by ruminants due to the precipitation of salivary proteins [31]. In addition, the silage is affected by rancidity and other sensorial properties such as colour, flavour, and texture alteration by polyphenols decreasing its palatability. In this sense, tannins and polyphenols have been historically known as antinutritional factors for ruminant because they decrease the voluntary feed intake and nutrient digestibility [32,33].

Considering the global production of silage and the disadvantages of analytical capabilities; spoilage moulds and mycotoxin contamination will continue to be an area of concern for regulatory agencies, the feed industry, and animal nutrition. Consequently, it is fundamental to find reliable biomarkers to reach a quick and sufficiently accurate diagnosis of silage quality, which allows for decision-making concerning the safe use of ensiled forage in animal feed.

It is hypothesized that the method based on the determination of ergosterol content can be used as an indicator of fungal metabolites contaminating to determine silage safety. Therefore, the aim of this study was to propose the analysis of ERG and phenolic compounds to assess silage hygienic quality in ten orchardgrass varieties. In addition, the effect of biological and chemical additives used in different orchardgrass varieties was evaluated as inhibitors of fungal contamination.
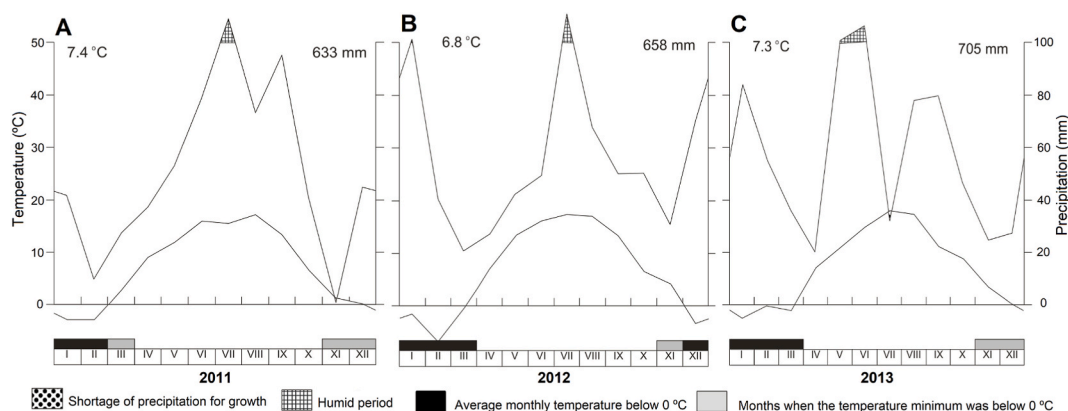

Fig. 1. Precipitation and temperatures in years 2011, 2012 and 2013 at Research Station.

2. Materials and methods

2.1. Plant material and growth conditions

This study was based on a previous investigation obtained by Alba-Mejía et al. [34], where the fermentation characteristics and nutritive values of the same orchardgrass silage used in this study were determined. The results obtained in the previous work suggest that the treatment of silage grass with biological inoculant improves the quality of the chemical composition and fermentation process.

In this study, ten varieties of orchardgrass from different European places were used. The plant material was composed of forage grass such as Greenly and Starly obtained from France, Otello from Italy, Sw-Luxor from Sweden, Amera, Dika and Bepro from Poland, Husar from Germany, and Dana and Vega from the Czech Republic. The plots were established during the second week of April 2011, and the laboratory assessment was conducted in 2012 and 2013 in the Czech Republic at the Research Station of Fodder Crops in Vatín (49°31′N, 15°58′E, 560 masl). The research site presents a precipitation and the average annual temperature of 632, 658 and 705 mm and 7.4, 6.8 and 7.3 °C in 2011, 2012, and 2013, respectively (Fig. 1).

The experimental design consisted of a plot with a soil type of Cambisol with a sandy-loam texture with a dimension of 1.5 m × 10 m (three replicates). For harvesting, a self-propelled mowing machine (HEGE 212 harvester, Wintersteiger, Ried im Innkreis, Austria) with a mowing width of 1.25 m was used. The harvested area of individual plots was 12.5 m^2, with a remaining stubble height of 7 cm where 20 kg/ha dose of seeds was applied during sowing. Fertilisation consisted of a 60 kg/ha N dose per year in urea form. The first and second growth were harvested at the heading phase (vegetation stage when inflorescence is emerging). The biomass was wilted on the plot for 14 h to reduce the water content after mowing. Afterwards, the forage samples (10 kg per treatment) were taken to the laboratory and chopped with a conventional forage harvester to a particle length of 40–60 mm.

2.2. Treatment of grass materials with biological and chemical additives

Representative forage samples (6 kg) were placed in experimental silos of polyvinyl chloride (PVC) and compacted to a pressure of 600 kg/m^3. The experimental silos (three repetitions per treatment) were sealed with a lid and stored at room temperature (28 °C) for 90 days. In order to evaluate the effectivity of additives in the orchardgrass silage, the following treatments were applied to the forage: a) silage without inoculants, used as a control; b) silage containing biological inoculants [*i.e.*, *Lactobacillus plantarum* (DSMZ 16568) in a concentration of 5×10^{10} colony-forming unit (CFU)/g, *Lactobacillus buchneri* (DSMZ 22501/CCM 1819, DSZM: German collection of microorganisms and cell cultures; CCM: Czech collection of microorganisms) in a concentration of 1.25×10^{10} CFU/g, and *Entero-coccus faecium* (DSMZ 22502/NCIMB, NCIMB: Scottish national collection of industrial and marine bacteria) in a concentration of 6.25 $\times 10^{10}$ CFU/g (manufacturer CHR. HANSEN A/S, Denmark)]; and c) silage containing chemical additives [*i.e.*, formic acid, propionic acid, benzoic acid, ammonium formate, E150d-sulfite ammonia, caramel and water (manufacturer BIOFERM CZ, spol. s r.o., Czech Republic)]. Biological inoculants were applied at a rate of 2 g/ton, and chemical additives were applied at 4 L/ton, according to the manufacturer's recommendations. At the end of the ensiling period (90 days), the silos were opened, and samples were taken for subsequent analysis. Dry matter was analysed after drying the biomass at 103 °C. Meanwhile, the pre-drying silage samples was performed in a specific drying oven at 60 °C for 48 h. Subsequently, the forage samples were ground in a mill and then filtered through a 1 mm sieve. The pulverized silage samples were stored under darkness at room temperature of 28 °C until their analysis.

2.3. Ergosterol determination

The extraction of ERG content was performed using a mixer (MS2 Minishaker IKA, USA) and a thermostat (Evaterm, Labicom, CZ). Then, the detection and quantification of ERG was carried out by HPLC system from Agilent (Agilent Technologies, USA) equipped with an ultraviolet detector (UV) using the method developed by Skládanka et al. [35].

2.4. Phenolic compound determination

Total concentration of phenolic compounds was determined by the Folin-Ciocalteu spectrophotometric method, described by Šimek et al. [36]. Besides the concentration of phenolic compounds was expressed as g gallic acid (GAE)/kg DM.

2.5. Statistical analyses

The statistical software STATISTICA version 12 (StatSoft, Inc.) was used to process the data. The Shapiro-Wilk test was applied to verify the normal distribution of the data. The results are expressed as the mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) by Fisher's exact test was used to discriminate on the least significant difference LSD ($P < 0.05$) to compare differences among orchardgrass varieties treated with biological and chemical additives as well as untreated orchardgrass of ERG and phenolic with a 95% confidence level.

3. Results

3.1. Analysis of ergosterol by HPLC

The ERG content in fresh forage of orchardgrass varieties increased from the first cut of 2012 [0.0–6.6 mg/kg dry matter (DM)] to the second cut of 2013 (22.8–39.5 mg/kg DM). In the first cut of 2012, ERG was detected only in Greenly, Starly and Amera varieties but as of the second cut of 2012 it was detected in all orchardgrass varieties. In 2013, Husar from the first cut and Greenly, Starly, Amera, Bepro and Vega varieties from the second cut showed high ERG content (>30 mg/kg DM) (see Table 1).

As can be observed in Table 2, ERG content indicated that the contamination by fungi depended on the silage variety ($P < 0.05$). In addition, an increase of ERG was observed over time being higher ($P < 0.05$) in 2013 than in 2012. In 2012, the ERG content ranged from 0.0 to 23.5 mg/kg DM in the first cut and 13.1–25.4 mg/kg DM in the second cut. In the same year (2012), the varieties Greenly, Amera and Dana (first cut) presented the highest ($P < 0.05$) ERG content, with Bepro (first cut) and Dika (second cut) showing the lowest ($P < 0.05$) ERG content. Interestingly, 'Bepro' variety did not present ERG (0.0 mg/kg DM) in the first cut in 2012. On the other hand, the ERG content in 2013 ranged from 34.1 to 54.6 mg/kg DM in the first cut and 62.7 and 83.2 mg/kg DM in the second cut. In the same year (2013), Amera variety showed the lowest ($P < 0.05$) ERG content in the first cut. Nevertheless, in the second cut of 2013, P-value (0.2377) after ANOVA analysis predicted that no significant differences among varieties but when Fisher's test was applied the significant differences appeared.

Regarding the ensiled varieties in relation to cuts in 2012, Sx Luxor, Otello, Husar, Dika, Bepro and Vega presented the lowest ($P < 0.05$) ERG content in the first cut compared to the second cut. In 2013, all varieties showed the highest ($P < 0.05$) ERG content in the second cut compared to the first cut, except for Sx-Luxor variety did not show any significant differences ($P > 0.05$) between both cuts. In terms of years, it is important to mention that in 2012 there was less mean annual precipitation and temperature compared to 2013. In this sense, the weather change condition, characterized by the co-occurrence of co-varying environmental variables, often affects plant epiphytic microbial community occurrence differently. In our study, the weather change condition seems to have altered the ERG content in all ten varieties of orchardgrass in both cuts and years, being the highest in 2013 compared to 2012.

After 90 days of ensiling, the ERG values of the tested orchardgrass cultivars were significantly affected ($P < 0.05$) by the additives and years. The results showed that silages group treated with biological additives improved in the first cut of 2012, decreasing the ERG content without any statistical differences ($P > 0.05$) in comparison with untreated silage. In contrast, silages treated with chemical additives showed an increase of ERG content in the first cut of 2012 compared to untreated silage, but no significant differences ($P > 0.05$) were observed. Nevertheless, in comparison with the control, silages treated with both types of additives (biological and chemical) presented higher ($P < 0.05$) ERG content in the first cut of 2013, while in the second cut of 2012 and 2013, they did not present statistically significant differences ($P > 0.05$) in ERG content among treated and non-treated silage (see Table 2). In relation to the cuts and years, the silage groups treated with both types of additives (biological and chemical) showed the lowest ($P < 0.05$) ERG content in the first cut compared to the second cut in 2012 and 2013, respectively. Moreover, ERG content was significantly higher ($P < 0.05$) in 2013 compared to 2012.

3.2. Analysis of phenolic compounds by Folin-Ciocalteu assay

Table 3 showed that in the fresh grass, the concentration of phenolic compounds increased in the first cut in both years (2012 and 2013), where the first cut from 2013 presented a high concentration of phenolic compounds ranging from 38.1 to 63.1 g gallic acid per kg of DM (g GAE/kg DM).

The total phenolic concentration showed differences ($P < 0.05$) among orchardgrass varieties and years (Table 4). The concentration of phenolic compounds increased in the first cut in both years (2012 and 2013), where the first cut from 2013 presented a high concentration of phenolic compounds ranging from 51.2 to 82.3 g gallic acid per kg of DM (g GAE/kg DM). Regarding varieties in 2012, Amera (first cut) and Starly (second cut) showed the highest ($P < 0.05$) phenolic concentration. In 2013, no significant differences ($P > 0.05$) were found among varieties, except for Greenly and Starly varieties (second cut) which showed the highest ($P < 0.05$) phenolic concentration. Regarding ensiled varieties in relation to cuts in 2012, Greenly, Starly, Amera, Dika, Dana and Vega

Table 1
Content of ergosterol (mg/kg dry matter) in samples of fresh forage.

Varieties	2012		2013	
	I Cut	II Cut	I Cut	II Cut
Greenly	6.6	23.8	29.9	39.5
Starly	5.6	5.3	28.1	32.5
Sw Luxor	0.0	14.7	26.7	29.4
Otello	0.0	5.6	20.1	28.5
Husar	0.0	8.1	30.9	22.8
Amera	5.7	16.2	15.4	30.1
Dika	0.0	13.0	22.0	23.9
Bepro	0.0	10.4	18.7	30.7
Dana	0.0	11.4	18.4	29.7
Vega	0.0	6.5	21.8	31.1

Table 2
Content of ergosterol (mg/kg dry matter ± standard error of the mean) in orchardgrass silage.

Varieties	2012		2013		P value
	I Cut	II Cut	I Cut	II Cut	
Greenly	19.7 ± 3.5 $^{a}_{c}$	18.5 ± 2.7 $^{bcd}_{c}$	49.9 ± 2.5 $^{a}_{b}$	77.8 ± 9.9 $^{ab}_{a}$	0.0258
Starly	11.5 ± 4.0 $^{b}_{c}$	19.9 ± 1.1 $^{abc}_{c}$	44.1 ± 2.2 $^{ab}_{b}$	67.3 ± 3.9 $^{bc}_{a}$	<0.0001
Sw Luxor	7.2 ± 2.4 $^{bc}_{c}$	22.9 ± 1.6 $^{ab}_{b}$	54.6 ± 5.7 $^{a}_{a}$	62.7 ± 4.1 $^{c}_{a}$	0.0055
Otello	8.1 ± 2.0 $^{bc}_{d}$	25.4 ± 1.6 $^{a}_{c}$	48.1 ± 3.0 $^{a}_{b}$	72.0 ± 7.1 $^{abc}_{a}$	<0.0001
Husar	3.4 ± 1.4 $^{cd}_{d}$	23.3 ± 1.8 $^{ab}_{b}$	53.9 ± 8.3 $^{a}_{b}$	83.2 ± 4.4 $^{a}_{a}$	<0.0001
Amera	23.5 ± 3.8 $^{a}_{c}$	21.5 ± 1.9 $^{ab}_{c}$	34.1 ± 3.6 $^{b}_{b}$	68.2 ± 3.9 $^{bc}_{a}$	<0.0001
Dika	4.7 ± 0.7 $^{bcd}_{d}$	13.1 ± 3.1 $^{d}_{c}$	44.0 ± 3.3 $^{ab}_{b}$	76.2 ± 3.2 $^{abc}_{a}$	<0.0001
Bepro	0.0 ± 0.0 $^{d}_{d}$	14.4 ± 2.2 $^{cd}_{c}$	53.7 ± 2.2 $^{a}_{b}$	75.0 ± 3.5 $^{abc}_{a}$	<0.0001
Dana	19.7 ± 2.3 $^{a}_{c}$	23.6 ± 1.9 $^{ab}_{c}$	44.8 ± 2.1 $^{ab}_{b}$	70.9 ± 4.6 $^{abc}_{a}$	<0.0001
Vega	6.9 ± 1.7 $^{bcd}_{d}$	18.2 ± 2.9 $^{bcd}_{c}$	50.2 ± 5.3 $^{a}_{b}$	69.9 ± 1.2 $^{abc}_{a}$	<0.0001
P value	<0.0001	0.0023	0.0421	0.2377	
Silage additives					
US	10.4 ± 1.5 $^{ab}_{d}$	19.3 ± 1.5 $^{a}_{c}$	42.7 ± 2.3 $^{b}_{b}$	70.3 ± 2.6 $^{a}_{a}$	<0.0001
BSA	6.9 ± 1.8 $^{b}_{d}$	18.5 ± 1.5 $^{a}_{c}$	49.7 ± 2.5 $^{b}_{b}$	70.9 ± 2.7 $^{a}_{a}$	<0.0001
CSA	14.1 ± 2.7 $^{a}_{d}$	22.4 ± 1.1 $^{a}_{c}$	50.8 ± 2.5 $^{b}_{b}$	75.7 ± 3.2 $^{a}_{a}$	<0.0001
P value	0.0495	0.1152	0.0476	0.3503	

US: untreated silage; BSA: biological silage additives; CSA: chemical silage additives. ^{abcd}Superscript letters indicate significant differences among varieties or among treated silages with additives in the same column (P < 0.05) determined by Fisher's test. ^{abcd}Subscript letters indicate significant differences among cuts and years in the same row (P < 0.05) determined by Fisher's test.

Table 3
Concentration of phenolic compounds (g gallic acid/kg dry matter) in samples of fresh forage.

Varieties	2012		2013	
	I Cut	II Cut	I Cut	II Cut
Greenly	33.9	19.6	40.4	26.4
Starly	33.6	27.5	38.1	25.2
Sw Luxor	26.3	19.0	53.0	27.1
Otello	22.4	14.7	59.6	21.2
Husar	26.1	22.3	63.1	23.7
Amera	39.3	19.1	58.0	21.9
Dika	23.3	20.7	54.1	21.6
Bepro	23.9	19.0	51.6	23.8
Dana	23.8	21.1	61.5	23.6
Vega	26.5	22.8	50.4	23.0

Table 4
Concentration of phenolic compounds (g gallic acid/kg dry matter ± standard error of the mean) in orchardgrass silage.

Varieties	2012		2013		P value
	I Cut	II Cut	I Cut	II Cut	
Greenly	41.0 ± 1.6 $^{b}_{a}$	26.7 ± 0.7 $^{b}_{c}$	51.2 ± 3.9 $^{d}_{a}$	34.0 ± 1.1 $^{ab}_{bc}$	<0.0001
Starly	36.6 ± 2.7 $^{bc}_{a}$	29.4 ± 0.7 $^{a}_{c}$	52.0 ± 3.3 $^{cd}_{a}$	33.3 ± 0.4 $^{ab}_{bc}$	0.0003
Sw Luxor	29.0 ± 1.6 $^{de}_{b}$	23.0 ± 0.5 $^{d}_{c}$	69.0 ± 6.9 $^{ab}_{a}$	29.6 ± 0.9 $^{b}_{b}$	0.0002
Otello	26.3 ± 0.4 $^{e}_{b}$	22.8 ± 0.9 $^{d}_{b}$	76.8 ± 4.5 $^{ab}_{a}$	25.2 ± 0.8 $^{d}_{b}$	<0.0001
Husar	29.3 ± 2.6 $^{de}_{b}$	26.4 ± 1.3 $^{bc}_{b}$	75.7 ± 3.3 $^{ab}_{a}$	28.8 ± 0.3 $^{b}_{b}$	<0.0001
Amera	49.0 ± 1.2 $^{a}_{b}$	24.1 ± 0.5 $^{dc}_{c}$	76.2 ± 10.7 $^{ab}_{a}$	26.4 ± 0.6 $^{cd}_{c}$	<0.0001
Dika	29.7 ± 1.5 $^{de}_{b}$	23.0 ± 1.3 $^{d}_{c}$	77.0 ± 1.4 $^{ab}_{a}$	28.8 ± 0.9 $^{b}_{b}$	<0.0001
Bepro	27.5 ± 4.5 $^{de}_{b}$	23.2 ± 1.1 $^{d}_{b}$	66.4 ± 3.7 $^{bc}_{a}$	28.9 ± 1.2 $^{b}_{b}$	<0.0001
Dana	31.6 ± 0.7 $^{cde}_{b}$	23.2 ± 0.7 $^{d}_{c}$	82.3 ± 2.1 $^{a}_{a}$	28.1 ± 0.5 $^{bc}_{b}$	<0.0001
Vega	32.6 ± 0.5 $^{cd}_{b}$	25.9 ± 0.3 $^{bc}_{c}$	76.1 ± 1.7 $^{ab}_{a}$	28.4 ± 0.8 $^{b}_{b}$	<0.0001
P value	<0.0001	<0.0001	0.0017	<0.0001	
Silage Additives					
US	32.7 ± 2.0 $^{a}_{b}$	24.0 ± 0.8 $^{a}_{c}$	63.9 ± 3.6 $^{b}_{a}$	30.1 ± 0.9 $^{a}_{bc}$	<0.0001
BSA	31.3 ± 2.6 $^{a}_{b}$	24.7 ± 0.8 $^{a}_{c}$	69.4 ± 3.6 $^{ab}_{a}$	27.8 ± 0.9 $^{a}_{bc}$	<0.0001
CSA	35.7 ± 2.5 $^{a}_{b}$	25.5 ± 0.7 $^{a}_{c}$	77.5 ± 3.8 $^{a}_{a}$	29.5 ± 0.9 $^{a}_{bc}$	<0.0001
P value	0.4241	0.4086	0.0439	0.1849	

US: untreated silage; BSA: biological silage additives; CSA: chemical silage additives. ^{abcde}Superscript letters indicate significant differences among varieties or among treated silages with additives in the same column (P < 0.05) determined by Fisher's test. ^{abc}Subscript letters indicate significant differences among cuts and years in the same row (P < 0.05) determined by Fisher's test.

showed the highest (P < 0.05) phenolic concentration in the first cut compared to the second cut. In 2013, all varieties presented the highest (P < 0.05) phenolic concentration in the first cut compared to the second cut. In terms of years, it is crucial to note that in 2013 there was higher mean annual precipitation and temperature compared to 2012, therefore, the incidence of rain could have affected the concentrations of polyphenols in silage. In this sense, the results from 2012 to 2013 showed that the first cut had a higher (P < 0.05) phenolic concentration compared to the second cut. Nevertheless, the first cut from 2013 presented the highest (P < 0.05) phenolic concentration compared to the second cut of the same year and with both cuts from 2012.

Regarding silage grass, the concentration of phenolic compounds did not show statistically significant differences (P > 0.05) among treated and non-treated silage, except in the first cut of 2013 where the concentration of phenolic compounds was higher (P < 0.05) in the silage group treated with chemical silage additives than control silages. However, there were no differences in phenolic compound concentrations among the silage groups (treated with biological and chemical additives) in both cuts of 2012 and 2013. In relation to the cuts and years, the silage treated with both types of additives (biological and chemical) showed the highest (P < 0.05) phenolic concentration in the first cut from 2012 to 2013 compared to the second cut in both years. Nevertheless, no significant differences (P > 0.05) in the concentration of phenolic compounds were found in the second cut from 2013 compared to both cuts from 2012. In addition, the first cut from 2013 presented the highest (P < 0.05) phenol concentration in this study.

3.3. Silage additives

As can be observed in Tables 2 and 4, ERG and polyphenol contents were different depending on the type of additive employed and varied over time in this study. ERG and polyphenol contents increased using chemical additives but without significant differences compared to control silage. However, the silage treated with biological and chemical additives showed a higher ERG content (P < 0.05) in the first cut of 2013 compared to control silage. Besides silage treated with chemical additives presented an increase of the polyphenol concentration in the first cut of 2013 (P < 0.05) compared to control silage.

4. Discussion

Actually, during silage-making practices, it is difficult to avoid mycotoxin contamination of forage crops [37]. Hence, ensiled forages may contain a mixture of mycotoxins originating from pre-harvest contamination and/or from postharvest contamination with toxigenic moulds that are common in silage [7]. This fact was demonstrated in our study, where evidently the grass silage showed signs of moulds contamination. As a result, the contamination by moulds increased during the ensiling period. Thus, a large number of silage additives are used worldwide during ensiling process to inhibit the growth of undesirable microorganisms, preventing spoilage of the feeds and minimizing nutrient losses and prolong aerobic stability [12]. Biological additives (homo- and hetero-fermentative strains) have been used to inhibit the growth of spoilage moulds and improve the lifespan of silage at feedout while chemical silage additives have been employed as antifungal and antibacterial [11]. However, the positive effects of silage additives were not observed in this study in the orchardgrass silage within the same cut for each variety, moreover, they did not reduce the content of harmful compounds. It has been shown that the nature and intensity of the effect of silage additives may differ across plant species [38], suggesting that either biological or chemical additives did not reduce the mould presence and oxidative damage in orchardgrass silage; as a consequence, there were no differences in the ERG and phenolic compound contents.

The augmentative tendency of the ERG content found in the silage samples of orchardgrass from 2012 to 2013 was related to the higher rainy conditions in the second year, contributing to the occurrence and development of fungi. In fact, Kalač [24] reported that ERG content increases at high relative humidity and low temperatures. In addition, the high ERG content is related to the delayed harvest date [39] and the high frequency of rains [24] at the time of harvest, which explains why the ERG content was higher in the second cuts of each year than in the first cuts. In 2012, the measured precipitation and temperature values were lower by 47 mm and 0.5 °C, respectively, than those in 2013 (see Fig. 1). These parameters contributed to the higher incidence of fungi in the cuts of 2013 than those of 2012, resulting in a high ERG content in silage. This fact shows that ERG content can vary according to the environment (e.g., location and year). For this reason, ERG monitoring should be introduced to assess hygienic silage safety in livestock farms.

This study showed that the ERG content in silage increased in 2013 compared with 2012; thus, our findings are in good agreement with Skládanka et al. [40], who evidenced that the ensilage process does not decrease the content of moulds and ERG and hence represents a high risk of mycotoxin production in the forage. Consequently, a high fungal infestation by mould explains the significant correlations of ERG content [41].

The ERG content found in our study in the first cut of 2012 in the silage treated with biological additives was consistent with a previous study presented by Skládanka et al. [40], where ERG degradation was not statistical visible by inoculation with bacterial additives. Despite of the use of chemical silage additives based on organic acids and salts has been widely recommended in silage crops to limit the growth of fungi [42] this treatment had not a positive effect in our study. This may be due to the chemical additives employed in this study were not inhibit yeasts, thereby creating conditions for acetic bacteria to cause aerobic spoilage and a growth of undesirable microorganisms such as moulds. The high ERG content indicated that the chemical additives were not prevented the proliferation of fungi in our orchardgrass silages. Hence, it can be suggested that the effect of silage additives in the prevention of the growth of moulds and their metabolites is not always efficient. The reason for this may be because the disadvantage of some additives enhances the spoilage of silage upon exposure to air because they produce insufficient quantities of the antifungal acetic and propionic acids that inhibit the growth of spoilage yeasts and moulds. In this sense, the effect of additives may differ across forage plant species with high moisture content, besides the high levels of moulds contamination at harvest.

The ERG content in forage grasses can vary from 20 to 400 mg/kg DM depending on the grass species as well as the variety within

the species [24]. In addition, Kalač [24] reported that the lowest ERG content production correspond to the highest quality of silage. To date, a safe limit for the ERG content in silages has not been established because the limits depend on the mould species that are contaminating the silage, the mycotoxins that are being produced and the ensiled forage species. For example, 110 mg ERG/kg DM in *Festulolium* forage was considered low, due to this grass was resistant to mildew infestation, in comparison to 139.6 mg ERG/kg DM in *Arrhenatherum elatius* forage that was contaminated with a high content of zearalenone [43]. Furthermore, Skládanka et al. [44] suggested that the ERG amounts (ranging from 3.8 to 190.8 mg/kg DM) in different grasses (*Lolium perenne, Festulolium pabulare* and *Festulolium braunii*) harvested on different dates (summer and winter) showed a high content of mycotoxins [*e.g.*, deoxynivalenol (DON) and zearalenone (ZEN)] in the summer and in the beginning of winter period. These results could indicate that the ERG amounts recorded in this study (ranging from 42.7 to 75.7 mg/kg DM in 2013) without and with additives cannot be considered safe for silage. Moreover, previous studies claim that the results of analyses of the ERG content in cereals demonstrate a significant correlation with mycotoxin concentrations (such as zearalenone, deoxynivalenol and nivalenol) found in grain crops [20,45]. For example, Stuper-Szablewska and Perkowski [20] showed a statistically significant correlation between deoxynivalenol/ERG and nivalenol/ERG in cereal grains. As a result, high fungal infestation in mouldy parts may explain the high ERG content obtained in silage sub-samples [24]. In addition, Hossain [46] reported that the quality of maize is acceptable if the ERG content is less than 3 mg/kg while the content of 8 mg/kg was considered to be of inferior quality. Moreover, it is well known that in general, the levels of ERG in mouldy grain are in the range of 8–15 mg/kg [47]. Thus, there is a high possibility of fungal invasion and mycotoxin contamination if the ERG content exceeds more than 8 mg/kg. Because there are no specific regulations on mycotoxins in silage (*e.g.,* grass silage, where a guidance value is only available for maize-based products), currently recommended levels for animal feed could also be considered guidelines for silage [48]. In this sense, it is recommended that the presence of DON and ZEN in silage not exceed 12 mg/kg and 3 mg/kg, respectively [49]. Thus, our findings suggest that determining the ERG content as a biochemical indicator is relevant to assess silage safety but it does not allow the establishment of safe limits for ruminants.

Regarding silage safety, a high concentration of phenolic compounds gives us a hint of mould presence, as many plant tissues accumulate phenolic compounds on their cell walls by interactions with fungal pathogens. Consequently, their presence up to a certain threshold is actually considered to be positive due to their antioxidant activity and ability to chelate metals, inhibit lipoxygenase and scavenge free radicals [50,51]. Nevertheless, the *in vivo* antioxidant effects are rather complex. In fact, polyphenols can exert direct antioxidant activity as a consequence of their absorption along the gastrointestinal tract and because of their deposition in the tissues [31]. Considering that dietary polyphenols are poorly absorbed in the intestine [52], in particular in ruminant species, their effects could be mediated by chelating pro-oxidant metals at the intestine level with a reduction of lipid peroxide production [31]. Thus, in order to prevent an adverse effect in the animal feed caused by phenolic concentration in silage, the determination of phenolic content has been suggested too as an indicator of silage safety.

In this sense, total phenolic concentration in orchardgrass silage models ranged from 24.0 to 77.5 g GAE/kg DM, which proved a safety issue with the silage due to minimum phenolic compound [condensed tannins (CTs)] concentration needed to make forages bloat-safe has been proposed to be 5 g/kg [53]. Consequently, forage crops with higher CT concentrations than >55 g/kg DM reduce the voluntary feed intake due to astringency in the diet and digestibility of nutrients and besides depress rates of body and wool growth in ruminants [32,33,54]. Moreover, it is have been widely known that hydrolysable tannins (HTs) have a smaller molecular weight (500–3000 Da) than CTs (1900–28,000 Da), and have a weaker affinity for proteins and thus, are more easily absorbed from the intestine increasing potential toxicity to the animals [55].

In terms of ensiled grasses, Sw Luxor, Otello, Husar, Dika, Bepro and Vega varieties from the first and second cuts from 2012 showed acceptable results regarding ERG content, thus they can be considered suitable for ensiling because they seem to be more resistant to fungal infestation. While in 2013 all varieties from both cuts presented a high content of ERG which indicates a high level of fungal contamination and the simultaneous occurrence of mycotoxins and as a result, poor-quality silage [56]. On the other hand, Greenly, Starly, Amera, Dika, Dana and Vega varieties from the first and second cuts from 2012 and all varieties from the second cut from 2013 presented tolerable and low levels of phenolic compounds, in this sense, our findings are consistent with previous studies reported by Mazza et al. [32] and Nascimento et al. [33]. However, in 2013 all varieties from the first cut showed a high level of polyphenols, which may be due to the high oxidative stress caused by the fungal infestation. Given the above, ensiled varieties with a high phenolic concentration can generate an inverse effect in animals, decreasing voluntary feed intake due to astringency in the diet and digestibility of the nutrients from the microbial activity [33]. Furthermore, the high levels of ERG and phenols recorded in this study are also related to the rainfall recorded in 2013, at the time of harvest, as described above which indicates that the environment drastically influences plant physiology and subsequent forage quality. In this sense, to secure future productivity of livestock the development of new forage varieties should take account of the environmental effects on grass production and quality parameters [57].

5. Conclusions

The use of many of the sophisticated analytical methods for the hygienic assessment of agricultural commodities contaminated with moulds and mycotoxin is restricted, due to the problems associated with arduous laboratory work, exposure to high-risk toxins and the use of expensive standard compounds. This study provided rapid results about the silage quality of ten orchardgrass varieties by determining the ERG and phenolic contents as indicators of fungal contamination. In fact, these analyses allowed to observe the effectivity of the use of chemical and biological additives on silage. Results indicated that biological and chemical additives used in this study were not effective to counteract the increase of ERG and phenolic contents, because not significant statistical differences (P > 0.05) were observed among treated silages with additives compared to untreated silage in all orchardgrass varieties. Consequently, these results prove a safety issue for the silage quality considering the ensiled varieties could not be suitable for animal feed.

This study identified several specific and potential limitations that will need to be overcome to improve the production of silage. The following priorities should be considered for the benefit of silage safety since the studies on ergosterol in the world literature are quite limited regarding silage: (1) carry out a complete study of fresh and ensiled forage on ergosterol-forming moulds and mycotoxin-producing moulds to appreciate and associate their close relationship in terms of silage spoilage to prevent the adverse effects of mycotoxins before it is used in livestock feed and (2) create specific lines of research regarding the study of ergosterol in silage to determine the effectivity of this method and suggest its possible use and standardization on dairy farms due to current studies are mostly on the presence and occurrence of ergosterol in grains and its derivatives, foods (*e.g.,* tomatoes, apple juice, figs, nuts), air and soil.

In general, this work presents an alternative to determine the safety of silages using ERG and polyphenol contents as indicators of fungal contamination in silage. However, further studies are needed in this field to establish the hygienic quality of silage from the perspective of animal health.

Declarations

Author contribution statement

Jhonny E. Alba-Mejía: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data; Wrote the paper.

Vlastimil Dohnal, Veronika Mlejnková, Jiří Skládanka: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Gloria Domínguez-Rodríguez, Tomáš Středa, Miroslav Klíma: Analysed and interpreted the data; Drafted and revised the paper.

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Data availability statement

Data associated with this study has been deposited at https://doi.org/10.17632/v8cy8jf664.2.

Declaration of interest's statement

The authors declare no competing interests.

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