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Potential Use of Wheat Straw, Grape Pomace, Olive Mill Wastewater and Cheese Whey in Mixed Formulations for Silage Production

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Abstract: Two experiments were conducted to investigate the chemical and fermentative characteristics of by-product-mixed silages consisting of wheat straw (WS), grape pomace (GP), olive mill wastewater (OMWW) and cheese whey (CW) at 7, 30 and 90 days. The silage formulations were based on a ratio of 60% solids (WS + GP) and 40% liquids (CW + OMWW), with the addition of water (W) where necessary to achieve 40% of liquids. In experiment 1, the effects of the inclusion of GP or CW in a mixture of WS and OMWW were studied according to two silage formulations: SIL-A, WS40% + OMWW5% + GP20% + W35%; SIL-B, WS60% + OMWW5% + CW35%. In experiment 2, the effects of two levels of CW and the inclusion of OMWW in mixed silages based on WS, GP, and CW were studied according to four silage formulations: SIL-C, WS40% + GP20% + CW20% + W20%; SIL-D, WS40% + GP20% + CW20% + OMWW5% + W15%; SIL-E, WS40% + GP20% + CW35% + W5%; SIL-F, WS40% + GP20% + CW35% + OMWW5%. In experiment 1, the silage formulation affected the chemical composition showing a greater ($p < 0.05$) content of DM in SIL-B; crude protein, ether extract and ADL contents were higher ($p < 0.05$) in SIL-A. In experiment 2, no differences ($p > 0.05$) in the chemical characteristics of the silages were found. In both of the experiments, the chemical composition and total phenol content did not change ($p > 0.05$) during the ensiling period. Fermentative characteristics were not affected ($p > 0.05$) by the by-product combination nor the ensiling period and proved to be adequate for good-quality silages. The Flieg's scores at D30 and D90 were greater than a 100 score in all the experimental silages, leading to the conclusion that WS, GP, OMWW and CW can be effective for producing silage.

Keywords: wheat straw; grape pomace; olive mill wastewater; cheese whey; silage; feeding use; sustainability; resource use efficiency

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1. Introduction

In recent decades, the awareness of environmental issues and climate change have exerted considerable pressures to reduce the environmental impact of agricultural and agro-industrial activities and to adopt sustainable processes, in line with the circular economy and bioeconomy. This new economic model represents one of the major local and global challenges that the agricultural sector is facing for sustainable development, linked to the use of natural resources and the considerable production of wastes that can cause environmental impact. On the other hand, the biomass with value-addition value, such as by-products, can represent an essential input for the bioeconomy, contributing to the development of more green processes for food, feed, bioproducts and bioenergy, with a general reduction of greenhouse gas emissions [1]. In this context, the use of agro-industrial by-products in animal feeding constitutes an important strategy for mitigating the impact related to livestock breeding, with particular reference to ruminants. A considerable number of studies focus on the use of by-products deriving from crops, fruit and vegetable processing in animal feeding [2–5].

In the Mediterranean area, several types of agro-industrial waste deserve a lot of attention since their disposal generates significant environmental and economic impacts, but they may have further productive purposes. The main agro-industrial by-products include straw, olive mill wastewater, grape pomace and cheese whey.

Straw is an agricultural by-product in dried form. It is used as roughage in ruminants' diet and as bedding for the shelter in livestock farming, but in several areas, most of the straw is burned in the fields, causing environmental pollution problems due to CO_2 emissions [6].

The Mediterranean area produces more than 30 million m^3 per year of OMWW that corresponds to 95–97% of global production [7,8]. It consists of olive-vegetation waters (the watery part of the drupe) and the water used during oil processing. The amount varies in relation to the olive oil production process (3.25–5.0 m^3 per ton of olive oil produced [7]. OMWW has a high pollution load since it is characterized by a high degree of organic pollution, with chemical oxygen demand (COD) values up to 220 $gO_2\ L^{-1}$ and a COD/biochemical oxygen demand (BOD5) ratio between 2.5 and 5. It also has a high content of polyphenols (up to 80 g L^{-1}) that, together with some organic acids, cause phytotoxic and antimicrobial activities [9]. In many countries, OMWW discharge is a significant environmental issue [10]. Usually, OMWW is sprinkled on soils and water bodies, and its uncontrolled disposal represents an environmental hazard [11].

Grape pomace (GP) is the primary waste resulting from the wine industry, after grape pressing, and constitutes, on average, 25% of the grape mass used for wine production. It mainly consists of the stalks, skins and grape pulp, and contains the seeds that remain after the grape crushing and pressing steps. GP contains many polyphenols [12]. Generally, GP is disposed in open areas and, if it is released without proper treatment, it causes environmental problems such as surface and groundwater pollution (phytotoxicity and toxicity in relation to soil and aquatic micro-organisms) [13].

Cheese whey is a liquid waste deriving from the dairy industry and constitutes over 80% of the milk used in cheese making [14]. It has a high pollution load; its BOD and COD values are 27–60 kg m^{-3} and 50–102 kg m^{-3}, respectively; the BOD5/COD ratio is commonly more than 0.5 [15].

It is worth considering that these agro-industrial wastes can be used in animal feed because they contain carbohydrates, proteins, minerals and lipids and are rich in bioactive components [16], especially polyphenols. The polyphenols are bioactive compounds, due to their antioxidant and scavenging activities, and play important roles in the inhibition or delay of lipid peroxidation and may have beneficial effects on animal health [17,18] and quality of the products [19–21].

The use of agricultural and agro-industrial by-products in animal feed can be advantageous for the livestock sector in terms of environmental sustainability and profitability [22]. This can effectively contribute to generating environmental benefits, mitigate climate change, increase the efficiency of resource use, and create value through the improvement of the production technology. The limits are represented by the seasonal availability of the agro-industrial by-products; ensiling is regarded as a suitable method for stabilizing the nutritional value of by-products to be efficiently incorporated into diets for livestock production, thus reducing feeding cost.

In several studies, OMWW and GP have been used in silage production for the feeding of animals such as lambs, pigs and broiler chickens [23–27].

The use of various wastes of the agro-food industry for mixed-silage production is increasing in some countries (Japan, Israel, China), mainly in farms of dairy cattle [28,29]. This system can be useful for the production of good quality silage using different kinds of wastes which can contain high moisture and unpalatable characteristics [30–33]. For the production of total mixed ration silage, the choice of inoculum is crucial for the fermentation quality, aerobic stability, in vitro gas production kinetics and digestibility. A recent study showed that in particular environmental conditions, such as high altitude and low ambient temperatures like Tibet, it is recommended to use a chemical additive (sodium diacetate) in comparison with lactic acid bacteria [34] due to its inhibitory effect on undesirable microorganisms, which thus promotes lactic acid fermentation [35].

In a recent study, innovative silages produced by mixing maize grains, olive mill wastewater, grape pomace and cheese whey, subjected to preliminary processes, were optimized [36]. In this study, we wanted to investigate the possibility of using wheat straw with grape pomace, olive mill wastewater and cheese whey wastes as raw ingredients for by-products-mixed silages.

Therefore, the aim of this study was to evaluate the chemical composition, phenolic content and fermentative parameters of by-product-based silages in six different mixtures of wheat straw, grape pomace, olive mill wastewater and cheese whey at different ensiling periods.

2. Materials and Methods

2.1. Study Design and Silage Preparation

Two experiments on a laboratory scale were conducted to investigate the chemical composition, phenolic content and fermentation parameters (pH, Flieg's score, lactic acid and volatile fatty acids) of raw by-product-mixed silages based on the use of straw with raw wine, oil and cheese by-products at different ensiling times. In particular, we used raw OMWW on the basis of previous results relating to a higher content of phenols in raw material compared to filtrate and retentate (3.90 vs. 2.65 and 3.69 mg GAE/g DM).

The by-products considered were wheat straw (*Triticum durum*; WS); grape pomace (GP), obtained from the vinification of *Vitis vinifera* L. (cv. Primitivo); cheese whey (CW), obtained from the dairy processing of bovine milk; and olive mill wastewater (OMWW), produced from milling olives (*Olea europea*, cv. Coratina). The by-products were taken from agro-industrial companies of the local area (southern Italy, Apulia region); they were stored in the refrigerator (2–4 °C) until use (12–24 h).

The materials were sampled and analyzed for their chemical composition and total phenol content. The different proportions of the ingredients were established in order to obtain the fresh matter ratio of 60% solids (WS + GP) and 40% liquids (CW + OMWW) [37]. In the mixtures where CW and OMWW amounted to less than 40%, water (W) was added to reach 40% liquids.

A set-up with two lab scale experiments was designed. Out of a total of six, different silage-mixtures were considered in experiment 1 and experiment 2, according to the proportions reported in Table 1.

Table 1. Experimental design and mass ratios of ingredients of the produced silages (% fresh matter).

Silage	WS	GP	CW	OMWW	W
Experiment 1					
SIL-A	40	20	0	5	35
SIL-B	60	0	35	5	0
Experiment 2					
SIL-C	40	20	20	0	20
SIL-D	40	20	20	5	15
SIL-E	40	20	35	0	5
SIL-F	40	20	35	5	0

Note: WS: wheat straw; GP: grape pomace; CW: cheese whey; OMWW: olive mill wastewater; W: water.

For silage preparation, WS was chopped into an average length of 2–3 cm and mixed thoroughly by hand to silage material with the other ingredients. A commercial freeze-dried starter culture of *Lactiplantibacillus plantarum* (14/DCSL CECT 4528; 250×10^9 cfu g^{-1};

Lactosil, CSL Zelo Buon Persico, Lodi, Italy) was used as a silage additive; it was homogeneously sprayed with a plastic sprayer to silage mixture and mixed by hand.

Samples were collected from each mixture of the by-products at the pre-ensiling time (D0) for the evaluation of the initial characteristics. Next, 1-kg samples were vacuum-packed into plastic HPDE bags, in triplicate, heat sealed and stored for 7, 30 and 90 days at room temperature. At every sampling day, three sub-samples were taken, mixed and pooled to constitute one sample. Subsequently, the sample was divided into three parts: one for the analyses on the fresh sample (dry matter, pH, fermentation characteristics), another was dehydrated (60 °C, 48 h) and another was frozen (−20 °C) for analyses of the chemical composition and total phenolic content.

2.2. Chemical Analysis

Ground samples of silages (1 mm) were analyzed for dry matter (DM, method 950.46), crude protein (method 990.03), ether extract (method 920.39) and ash (method 920.153), according to the standard methods of AOAC [38]. The neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined according to Van Soest et al. [39].

Determination of Total Phenolic Content

Total phenol contents of the extract from the individual by-product and the experimental silages were estimated according to the Folin-Ciocalteau's assay [40]. Briefly, phenolic compounds were extracted from the materials with hydro-alcoholic solution (methanol:water, 80%, *v*/*v*). The extracts were filtered through Whatman No. 1 paper. The filtrate was centrifuged at 2000× *g* for 10 min and supernatants were collected and evaporated under vacuum at ≤40 °C using a laboratory rotavapor (BÜCHI Labortechnik AG, Flawil, Switzerland). The fractions were dissolved in methanol and kept at −30 °C for analysis. For the phenols quantification, 100 µL of extract was dilute with 6 mL of distilled water, then was mixed with 0.5 mL of Folin–Ciocalteu's reagent (Sigma Aldrich, St. Louis, MO, USA). After 10 min, 1.5 mL of sodium carbonate solution (20%, *w*/*v*) was added. The mixture was vortexed and stood for 60 min at room temperature. The absorbance was measured at 760 nm spectrophotometrically (Cary 60 UV-Vis Spectrophotometer, Agilent, Santa Clara, CA, USA). The total phenolic concentration was expressed as milligram of gallic acid equivalents (GAE) per g of dry matter (DM).

2.3. Fermentation Characteristics

2.3.1. pH Determination

For the pH determination, 10 g of each sample of silages was added to 90 mL of distilled water and mixed for five minutes. Then, the pH value was measured with WTW InoLab pH Level 3 (Xylem Analytics Germany Sales GmbH & Co. KG, WTW, Weilheim in Oberbayern, Germany). For each experimental silage, two replicates were performed.

2.3.2. Flieg's Point Calculation

As a silage quality index, Flieg points were calculated with the following equation [41]:

$$\text{Flieg's score} = 220 + (2 \times \%\text{DM} - 15) - 40 \times \text{pH}$$

According to this index, the quality of silage is classified as very good (score: from 81 to 100); good (score 61 to 80); medium (score: 41 to 60); inferior (score: 21 to 40); very inferior (score less than 20).

2.3.3. Lactic and Volatile Fatty Acids

Lactic acid of the silages was determined spectrophotometrically according to Madrid et al. [42]. Briefly, a silage sample (20 g) was macerated in deionized water (100 mL) for 1 h, and filtered. The filtered liquid was diluted with deionized water (1:100) and 1 mL of this was used for the analysis. For the determination, 6 mL of concentrated H_2SO_4 in borosilicate tubes was added to 1 mL of standard (0–30 µg mL^{-1} lactic acid solutions) and/or to silage sample solutions, and mixed in a vortex mixer. The mixed solutions were incubated at 95–100 °C for 10 min in a steam water bath. The tubes were cooled to room temperature using a water bath. Subsequently, 100 mL 4% $CuSO_4 \cdot 5H_2O$ in deionized water reagent, followed by 200 mL 1.5% p-phenylphenol reagent in 95% of ethanol, were added and mixed well using a vortex mixer. The tubes were kept at room temperature ($\geq$20 °C) for at least 30 min. The absorbance was measured at 570 nm (Cary 60 UV-Vis Spectrophotometer, Agilent, Santa Clara, CA, USA).

Volatile fatty acid (VFA) concentration of the silages (acetic acid; propionic acid; isobutyric acid; and butyric acid) was determined according to the method of Lashkari et al. [43], slightly modified. Briefly, the silage samples were blended with deionized water for 15 min. The homogenate was filtered through filter paper (Whatman grade 1; Sigma Aldrich, Darmstadt, Germany); the filtrate (5 mL) was added with meta-phosphoric acid (25%, *wt*/*vol*; 1 mL). After shaking (10 min), volatile fatty acids were extracted with toluene, and quantified by flame ionization detection gas chromatograph (Agilent 7890A GC, Agilent Technology Italia Spa, Roma, Italia) and a capillary column (SACtm-5 column 300 cm × 0.25 mm, Supelco, Bellefonte, PA, USA). Free fatty acids were identified and quantified on the basis of standard elution times (Volatile Free Acid Mix Supelco, Bellefonte, PA, USA). The sum of total VFA (acetic, propionic, isobutyric and butyric acids), total acids (VFA plus lactic acid) and their percentages (total VFA/total acids) were calculated.

2.4. Statistical Analyses

All statistical analyses were performed by SPSS 23.0 (SPSS software for Windows, release 23.0., Inc., Chicago, IL, USA). In experiment 1 and 2, the main effects of silage formulation and ensiling time, and their interaction were analyzed by a two-way analysis of variance and tested using Bonferroni's test at $p = 0.05$. Data of total VFA, total acids and percentage of VFA /total acids at D30 (experiment 1 and experiment 2) were analyzed by one-way analysis of variance.

3. Results and Discussion

3.1. Experiment 1—Chemical Composition

The chemical composition and phenol content of the individual by-products prior to ensiling, used in experiment 1 and experiment 2, are listed in Table 2. In particular, OMWW and GP are characterized by phenol content (37.5 and 11.40 mg GAE g^{-1} DM), and CW by protein content (11.30% DM).

Table 2. Chemical composition and phenols content of by-products used in mixed silages (mean ± SD).

	WS	GP	CW	OMWW
Dry matter (%)	92.94 ± 0.84	51.63 ± 4.64	6.65 ± 0.35	10.18 ± 0.59
Crude protein (% DM)	3.18 ± 0.08	9.75 ± 2.19	11.30 ± 3.01	-
Ether extract (% DM)	1.20 ± 0.01	4.55 ± 0.21	0.79 ± 0.02	9.58 ± 0.16
Ash (% DM)	6.11 ± 0.06	6.76 ±1.60	7.53 ± 1.40	6.13 ± 0.17
Crude fat (% DM)	44.44 ± 0.62	20.72 ± 0.57	-	-
NDF (% DM)	73.00 ± 0.74	50.48 ± 0.95	-	-
ADF (% DM)	48.13 ±0.15	42.15 ± 1.49	-	-
ADL (% DM)	7.08 ± 0.07	15.39 ± 0.37	-	-
Total phenols (mg GAE/100 g^{-1} DM)		11.40 ± 0.10		37.50 ± 2.10

Note: WS: wheat straw; GP: grape pomace; CW: cheese whey; OMWW: olive mill wastewater. NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; GAE: mg gallic acid equivalents per g of dry matter (DM).

The variations in the chemical characteristics of SIL-A and SIL-B are presented in Table 3. Before ensiling (D0), as expected, SIL-B had greater ($p < 0.05$) dry matter in comparison to SIL-A silage, which showed higher ($p < 0.05$) crude protein, ether extract and ADL contents. This was determined by the different amounts of the by-products used in the silage formulations, mainly straw at 60% or 40% and grape pomace at 20% or 0%, in SIL-A and SIL-B, respectively. This affected the composition of the two silages in terms of their individual chemical characteristics.

Table 3. Changes in chemical composition (% of dry matter) of the mixed by-products-based silages at different ensiling times.

Items	**Treatment**			**Effect**		
	SIL-A	**SIL-B**	**SEM**	**Treatment**	**Time**	**Interaction**
Dry matter (%)				*	ns	ns
0 d	47.62 ^{a}	59.10 ^{b}	0.185			
7 d	46.15 ^{a}	58.87 ^{b}	0.069			
30 d	47.55 ^{a}	58.75 ^{b}	0.225			
90 d	45.89 ^{a}	58.58 ^{b}	0.053			
Crude protein (%)				*	ns	ns
0 d	4.23 ^{a}	3.44 ^{b}	0.024			
7 d	3.93	3.28	0.029			
30 d	3.98	3.37	0.021			
90 d	4.04	3.77	0.029			
Ether extract (%)				*	ns	ns
0 d	1.87 ^{a}	1.51 ^{b}	0.017			
7 d	1.90 ^{a}	1.45 ^{b}	0.018			
30 d	1.88 ^{a}	1.45 ^{b}	0.013			
90 d	1.87 ^{a}	1.46 ^{b}	0.009			
Crude fiber (%)				ns	ns	ns
0 d	37.19	37.91	0.019			
7 d	37.27	38.32	0.056			
30 d	38.25	38.70	0.020			
90 d	37.28	37.78	0.020			
Ash (%)				ns	ns	ns
0 d	6.35	6.17	0.023			
7 d	6.46	6.21	0.020			
30 d	6.39	6.28	0.011			
90 d	6.28	6.30	0.014			
NDF (%)				ns	ns	ns
0 d	66.04	67.29	0.029			
7 d	67.98	69.30	0.043			
30 d	67.80	68.71	0.018			
90 d	66.21	68.84	0.011			
ADF (%)				ns	ns	ns
0 d	44.61	45.21	0.058			
7 d	46.78	47.14	0.010			
30 d	46.81	47.86	0.027			
90 d	45.58	47.49	0.027			
ADL (%)				*	ns	ns
0 d	8.87 ^{a}	6.42 ^{b}	0.027			
7 d	8.41 ^{a}	6.46 ^{b}	0.027			
30 d	8.27 ^{a}	7.20 ^{b}	0.037			
90 d	8.80 ^{a}	7.12 ^{b}	0.058			

Table 3. *Cont.*

Items	Treatment			Effect		
	SIL-A	SIL-B	SEM	Treatment	Time	Interaction
Total phenols, mg GAE g^{-1} DM				ns	ns	ns
0 d	0.63	0.57	0.030			
7 d	0.61	0.59	0.020			
30 d	0.63	0.60	0.030			
90 d	0.60	0.58	0.010			

* = $p < 0.05$; ns = not significant. [a,b] Means with different letters on the same row are significantly different ($p < 0.05$). Note: SIL-A: wheat straw 40% + olive mill wastewater 5% + grape pomace 20% + water 35%; SIL-B, wheat straw 60% + olive mill wastewater 5% + cheese whey 35%. NDF: neutral detergent fiber; ADF = acid detergent fiber; ADL = acid detergent lignin. GAE: mg gallic acid equivalents per g of dry matter (DM).

Similarly, at D30, the SIL-B was characterized by a higher ($p < 0.05$) dry matter content, while SIL-A had a higher ($p < 0.05$) content of ether extract and ADL, and a slightly higher content of crude protein (+0.5% DM; $p > 0.05$; Table 3). Overall, these differences between the two silages remained almost constant for the whole ensiling period, showing a slight reduction at D90. Ash, crude fiber and its fractions (NDF, ADF and ADL) were not influenced ($p > 0.05$) by the silage formulation; their values fluctuated slightly during the silage period without significant differences ($p > 0.05$; Table 3).

3.2. Fermentation Characteristics

The fermentation characteristics are reported in Figures 1 and 2, and Table 4. At day 0, SIL-B showed a higher pH value (5.78) than SIL-A (4.84). Thereafter, in both the silages, it decreased ($p < 0.01$) with ensiling time, reaching the same value at D7 (4.3), and 3.73 and 3.92, respectively, in SIL-B and SIL-A, at D30, which can be considered the end of lactic fermentation and the silages' maturation time. At D90, it increased slightly, reaching 4.3–4.4 (Figure 1). Thus, 30 days can represent the end of lactic fermentation and be considered as the silages' maturation time, in line with other studies [36,44].

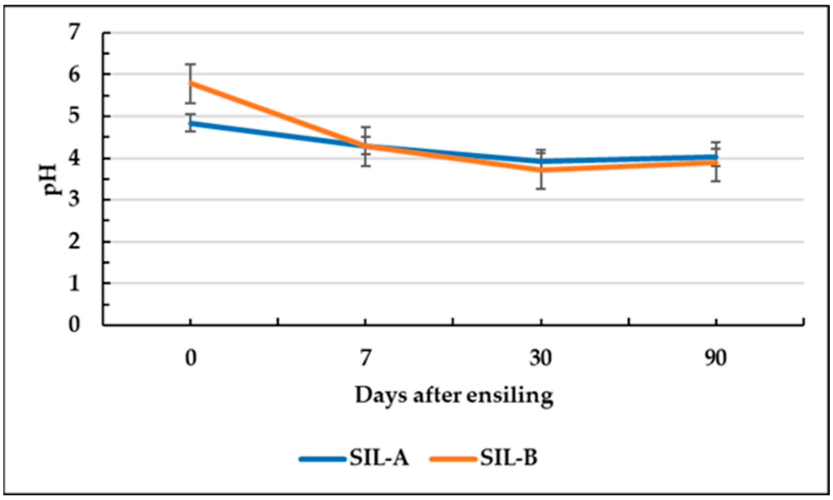


Figure 1. Changes in pH of the mixed by-products-based silages during fermentation (experiment 1). Note: SIL-A: wheat straw 40% + olive mill wastewater 5% + grape pomace 20% + water 35%; SIL-B: wheat straw 60% + olive mill wastewater 5% + cheese whey 35%.

The pH value is a pivotal parameter for the proper ensiling process and low pH values could be attributed to the fermentation of water-soluble carbohydrates, by lactic acid bacteria, into organic acids [45,46]. In this study, for both the experimental silages, the rapid drop in the pH observed in the first ensiling step and the final pH values near 4.2, which is generally considered as the maximum threshold for well-persevered silages, may be considered indexes of good fermentation quality, according to other authors [47,48]. The reduction of pH during the ensiling process is of crucial importance in preventing the growth of undesirable microbes such as clostridia, Enterobacteriaceae, listeria and molds [45].

The Flieg's score, based on the pH and dry matter content, is commonly used as an index to classify the quality of silage. In this study, the Flieg's score recorded at D30 was greater than 100 in SIL-A and SIL-B and, although it showed a decrease at D90, remained above 100 (Figure 2), indicating that both silage formulations were of a very high quality and stability [41,49].

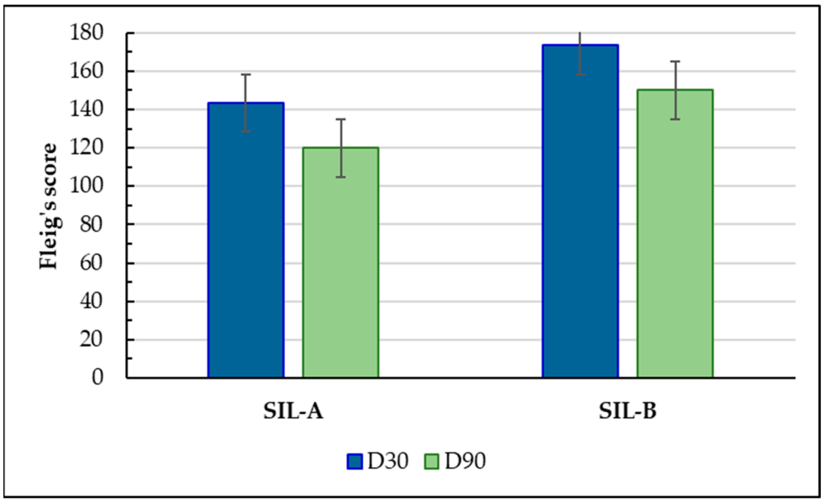


Figure 2. Flieg's score of the mixed by-products-based silages at 30 and 90 days after ensiling (experiment 1). Note: SIL-A: wheat straw 40% + olive mill wastewater 5% + grape pomace 20% + water 35%; SIL-B: wheat straw 60% + olive mill wastewater 5% + cheese whey 35%.

The concentrations of lactic acid and volatile fatty acids and their changes during fermentation are reported in Table 4.

Table 4. Profile of lactic acid and volatile fatty acids (VFA; g/kg of dry matter) of mixed silages at different ensiling times (experiment 1).

	Treatment			Effect		
	SIL-A	**SIL-B**	**SEM**	**Treatment**	**Time**	**Interaction**
Lactic acid				ns	**	ns
0 d	nd	nd				
7 d	11.03 ^{a}	12.29 ^{a}	0.021			
30 d	19.84 ^{b}	20.06 ^{b}	0.018			
90 d	20.21 ^{b}	19.93 ^{b}	0.027			
Acetic acid				**	**	*
0 d	nd	nd				
7 d	0.91 ^{1a}	0.18 ^{2a}	0.003			
30 d	0.78 ^{1b}	0.23 ^{2}	0.001			
90 d	0.75 ^{1b}	0.26 ^{2b}	0.002			
Propionic acid				ns	ns	ns
0 d	nd	nd				
7 d	nd	nd				
30 d	nd	nd				
90 d	nd	nd				

Table 4. *Cont.*

	Treatment			Effect		
	SIL-A	**SIL-B**	**SEM**	**Treatment**	**Time**	**Interaction**
Isobutyric acid				**	ns	*
0 d	nd	nd				
7 d	0.20	nd	0.002			
30 d	0.22 ^{1}	0.02 ^{2}	0.001			
90 d	0.27^{1}	0.05 ^{2}	0.001			
Butyric acid				*	**	**
0 d	nd	nd				
7 d	0.18 ^{1a}	0.08 ^{2a}	0.002			
30 d	0.47 ^{b}	0.51 ^{b}	0.009			
90 d	0.57 ^{b}	0.53 ^{b}	0.008			
At D30:						
Total VFA	1.47	0.76	0.164	**		
Total acids	21.31	20.84	0.292	ns		
% total VFA/total acids	7.03	3.63	0.764	**		

* = $p < 0.05$; ** = $p < 0.01$; ns = not significant; nd = not detected. ^{a,b} Means with different letters on the same column are significant different. ^{1,2} Means with different numbers on the same row are significant different. Note: SIL-A: wheat straw 40% + olive mill wastewater 5% + grape pomace 20% + water 35%; SIL-B, wheat straw 60% + olive mill wastewater 5% + cheese whey 35%.VFA: sum of acetic, propionic, isobutyric and butyric acids. Total acids: VFA plus lactic acid.

For both the silages, a rapid increase in lactic acid concentration occurred from the day of ensiling (D0) to D7 after ensiling to reach approximately 20 g kg^{-1} DM at D30 ($p < 0.01$). At this time, no difference ($p > 0.05$) was found between SIL-A and SIL-B for lactic acid concentration. Subsequently, it remained substantially unchanged until D90. In both the experimental silages, the percentage of lactic acid content with respect to the total acids (88.41% and 96.35%, SIL-A and SIL-B, respectively) showed that lactic acid represented the principal compound derived presumably from good fermentative processes leading to high quality silages. In fact, as reported by Ward and Ondzarda [47], in a homo-fermentative ensiling process, lactic acid should be the principal end-product of fermentation, and the level of its concentration is considered to be a good indicator of silage quality [50]. Lactic acid (pKa of 3.86) is the fermentation compound that most contributes to the decrease in the pH of silage, because it is about 10 to 12 times stronger than any of the other major organic acids such as acetic (pKa of 4.75) and propionic acid (pKa of 4.87) [48,51]. A low pH derived from high lactic acid concentration is considered important for silage preservation [52]. Moreover, great lactic acid content typically corresponds to low dry matter loss [47]. In ruminants under normal feeding conditions, lactic acid ingested from silage is converted to propionic acid by rumen microbes such as *Selenomonas ruminantium*, *Megasphaera elsdenii* or Propionibacteria [48,53]. The propionic acid is absorbed by the rumen and is transformed into glucose by the liver. However, very high concentrations of lactic acid, as well as of total acids, in silage have negative effects, depressing feed intake and potentially contributing to subacute acidosis [53].

The concentration of acetic acid was significantly ($p < 0.01$) influenced by the silage formulations and ensiling time, and their interaction ($p < 0.05$). As a whole, SIL-A showed higher levels of acetic acid, reaching a concentration of 0.78 g kg^{-1} DM compared to 0.23 g kg^{-1} DM of SIL-B ($p < 0.01$); these values remained almost constant at D90. The concentration of acetic acid in SIL-A was lower compared with the results obtained by Belém et al. [54]. This is related to the effects of different quantities of grape pomace added to *Calotropis procera* silage, where acetic acid concentration increased when grape pomace was increased up to 22%, associated with enterobacteria and heterofermentative lactic acid bacteria activity [54]. Acetic acid is normally the acid with the second-highest concentration in silage (1 to 3% of DM) [48]. Its moderate concentration in silage, as for both the silage formulations in this study, is deemed useful for inhibiting yeast and to improve its stability in air. In ruminants, acetic acid from silage can be adsorbed by the rumen and used for energy or for milk or body fat synthesis [48].

Propionic acid was not detected in SIL-A nor in SIL-B. This could be attributed to appropriate moisture levels in the silages (DM range: 46–59%; Table 3), according to Kung et al. [48]. The latter reported that propionic acid may be undetectable in silage with a DM greater than 35%, as in this study, whereas it is commonly present in very wet silage (<25% DM).

Isobutyric acid was affected by silage formulation ($p < 0.01$) and significant was the interaction between treatment and time ($p < 0.05$; Table 4). Isobutyric acid concentrations were very low in both the silages, although it was more prevalent in SIL-A, where it ranged from 0.20 at D7 to 0.27 g kg^{-1} DM at D90, than in SIL-B (0.02 and 0.05 g kg^{-1} DM, at D30 and D90, respectively). The concentration of butyric acid was influenced ($p < 0.05$) by the silage formulation, time of ensiling and their interaction ($p < 0.01$) (Table 4). Butyric acid increased with the ensiling period (D7–D90; $p < 0.05$), showing, however, low concentrations in the two experimental silages. At D30, its concentration was 0.47 and 0.51 g kg^{-1} of DM, respectively, in SIL-A and SIL-B ($p > 0.05$), and increased slightly at D90 ($p > 0.05$). These values indicated that the two silage formulations had good fermentation quality, below the maximum acceptable concentration of 2.0 g/kg DM for a good quality silage [55]. Butyric acid formation during fermentation is, in fact, undesirable; the acid is produced due to an increase in *Clostridium* spp. if silage has a high humidity or low water-soluble carbohydrates and the pH is too high. In these conditions, Clostridia will increase their numbers and actively convert organic substances and lactic acid into butyric acid, acetic acid, NH_3, CO_2 and H_2, leading to the catabolism of amino acids and amides due to the coupled oxidation of two amino acids (Stickand reactions) [56]. It has been reported that the fermentation of glucose and lactic acid into butyric acid, CO_2 and hydrogen causes 51.1% of material losses [57]. In ruminants, butyric acid from silage is metabolized to ketone bodies [58], but butyric acid, ammonia and amines have been linked with reduced ad libitum feed intake [59].

The total VFA, comprising acetic, propionic, isobutyric and butyric acids, and its proportion to total acids, was lower ($p < 0.01$) in SIL-B compared to SIL-A. However, for both the silages the percentages of VFA were low overall (3.63 and 7.03%, SIL-B and SIL-A; Table 4), far below the threshold value indicated by Santoso et al. [46] (20%). This indicates a good fermentation efficiency, as the production of VFA has been related to an inefficient or secondary fermentation of lactic acid [46].

3.3. Experiment 2—Chemical Composition

The chemical composition of the experimental silages was not influenced ($p > 0.05$) by the formulation of by-products nor by the ensiling time. Therefore, it was referred to with the values observed at the ensiling process maturation time (D30; Table 5); the data at the different ensiling periods were presented in graphic form (Figure 3), and the discussion is limited to the highly significant findings.

Concerning the chemical composition, DM is considered an important index for nutrition retention after ensiling, and the protein content represents one of the most important attributes of its nutritive value [60]. Although several authors have reported that DM was not influenced by storage [61,62], normally the ensiling process determines losses of DM, reducing the nutritional value of the product as animal feed. These variations have been related to the initial DM content of the ingredients at the time of ensiling and to the conditions of its process [63]. In this study, DM and the chemical composition of all the experimental silages (SIL-C, SIL-D, SIL-E and SIL-F) at D0 remained almost unchanged over ensiling time (Figure 3)—probably facilitated by the efficiency of laboratory vacuum ensiling—indicating good retention of nutritional compounds. According to Borreani et al. [63], the maintenance of DM content could be attributed to the homofermentative process of glucose by LAB that produced only lactate, inducing no DM loss. On the contrary, LAB that ferment glucose heterofermentatively produce 1 mol of carbon dioxide per mol of glucose, leading to DM loss.

Table 5. Chemical composition (% of dry matter) of mixed silages at D30 of maturation (experiment 2).

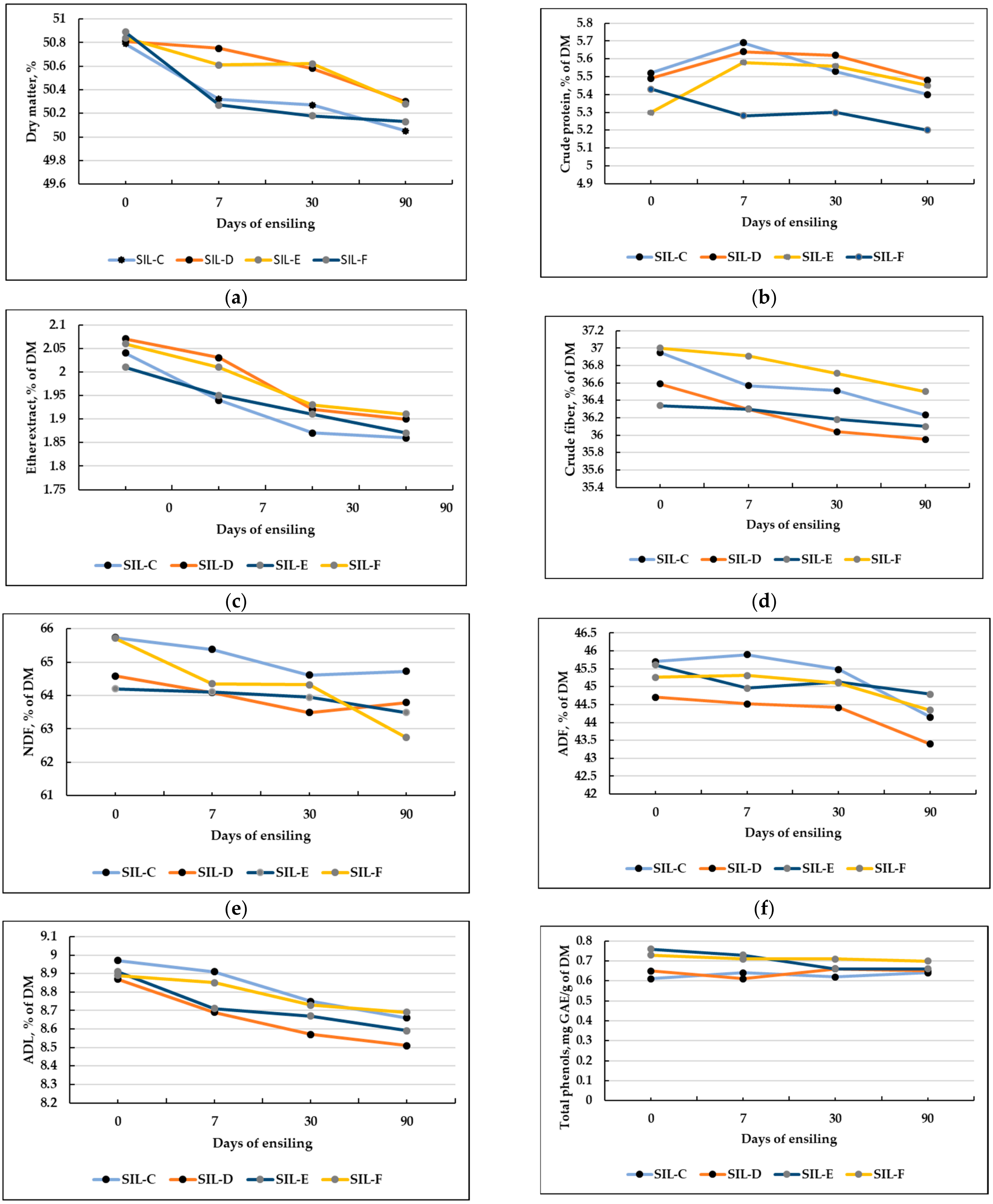
	Treatment					**Effect**
	SIL-C	**SIL-D**	**SIL-E**	**SIL-F**	**SEM**	**Treatment**
Dry matter	50.32	50.75	50.61	50.27	0.085	ns
Crude protein	5.53	5.62	5.58	5.20	0.049	ns
Ether extract	1.87	2.04	2.01	1.93	0.013	ns
Crude fiber	36.51	35.95	36.34	36.91	0.016	ns
Ash	5.93	5.66	5.64	5.69	0.012	ns
NDF	65.74	64.09	64.11	64.35	0.021	ns
ADF	45.48	44.52	45.13	45.26	0.025	ns
ADL	8.75	8.21	8.67	8.85	0.025	ns
Total phenols, mg GAE g^{-1} DM	0.62	0.66	0.66	0.71	0.025	ns

ns: not significant. Note: SIL-C: wheat straw 40% + grape pomace 20% + cheese whey 20% + water 20%; SIL-D: wheat straw 40% + grape pomace 20% + cheese whey 20% + olive mill wastewater 5% + water 15%; SIL-E: wheat straw 40% + grape pomace 20% + cheese whey 35% + water 5%; SIL-F: wheat straw 40% + grape pomace 20% + cheese whey 35% + olive mill wastewater 5%. NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin. GAE: mg gallic acid equivalents per g of dry matter (DM).

Moreover, the protein content of the experimental silages did not show substantial variations during the ensiling period. A possible explanation for the retention of the protein content over time could be related, in particular, to the inclusion of GP in all the silage formulations. GP could have inhibited proteolysis, due to deactivation of proteolytic enzymes and/or through the formation of protein–polyphenol complexes [64], according to other studies on ensiled forage [65,66]. Ke et al. [67] have reported that the use of grape pomace when ensiling alfalfa could improve the utilization of industrial waste as feed but could also effectively inhibit proteolysis and improve N utilization by ruminants. Fermentation quality, nutritive value and in vitro digestibility of mixed silages containing crop straws and tall fescue was improved by the inclusion of alfalfa [68], confirming the positive association between a poor ingredient (straw) and another plant [69]. No important losses in nutritional quality and dry matter were observed in silages produced with broccoli and artichoke by-products [44].

Phenolic compounds have strong antioxidant activity that affects the antioxidant potential of a feed [70,71]. Among the experimental silage formulations, the total phenol content at D30 was the highest in SIL-F and lower in SIL-C (0.72 vs. 0.62 mg GAE g^{-1} DM; Table 5); however, the difference was not significant.

The total phenol content was the highest in SIL-F and lower in SIL-C (0.72 vs. 0.62 mg GAE g^{-1} DM; Table 5); however, the difference was not significant ($p > 0.05$). In the four silage formulations, the total phenol content did not decrease during the whole ensiling period studied (D0 to D90; $p > 0.05$; Figure 3), showing a good ensiling process in preserving phenolic content over time as well. A decrease in phenol content during the ensiling process was reported in other studies [72–74]. According to Esparza et al. [75], the stability of phenolic compounds depends on its chemical nature as well as on the overall composition of the matrix because the different compounds present enhance or mitigate the degradation processes.



(**a**) (**b**)

(**c**) (**d**)

(**e**) (**f**)

(**g**) (**h**)

Figure 3. Variations of chemical composition of the mixed by-products-based silages during fermentation (experiment 2). (**a**) Dry matter (DM), %; (**b**) Crude protein, %DM; (**c**) Ether extract, % DM; (**d**) Crude fiber, % DM; (**e**) NDF (neutral detergent fiber), % DM; (**f**) ADF (acid detergent fiber), % DM; (**g**) ADL (acid detergent lignin), % DM: (**h**) Total phenols content (mg GAE (gallic acid equivalents)

per g of DM. Note: SIL-C: wheat straw 40% + grape pomace 20% + cheese whey 20% + water 20%; SIL-D: wheat straw 40% + grape pomace 20% + cheese whey 20% + olive mill wastewater 5% + water 15%; SIL-E: wheat straw 40% + grape pomace 20% + cheese whey 35% + water 5%; SIL-F: wheat straw 40% + grape pomace 20% + cheese whey 35% + olive mill wastewater 5%.

3.4. Fermentation Characteristics

The fermentation characteristics of the silages in experiment 2 are reported in Figures 4 and 5, and Table 6.

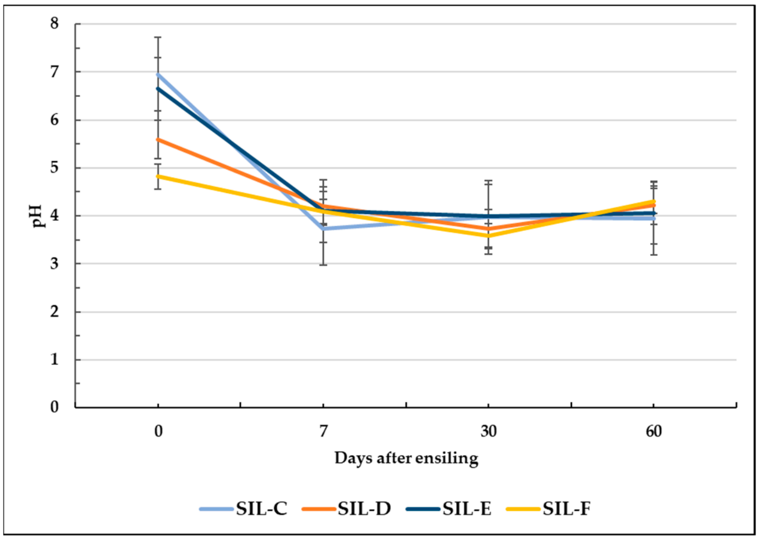


Figure 4. Changes in pH during fermentation of the by-products-based silages during fermentation (experiment 2). Note: SIL-C: wheat straw 40% + grape pomace 20% + cheese whey 20% + water 20%; SIL-D: wheat straw 40% + grape pomace 20% + cheese whey 20% + olive mill wastewater 5% + water 15%; SIL-E: wheat straw 40% + grape pomace 20% + cheese whey 35% + water 5%; SIL-F: wheat straw 40% + grape pomace 20% + cheese whey 35% + olive mill wastewater 5%.

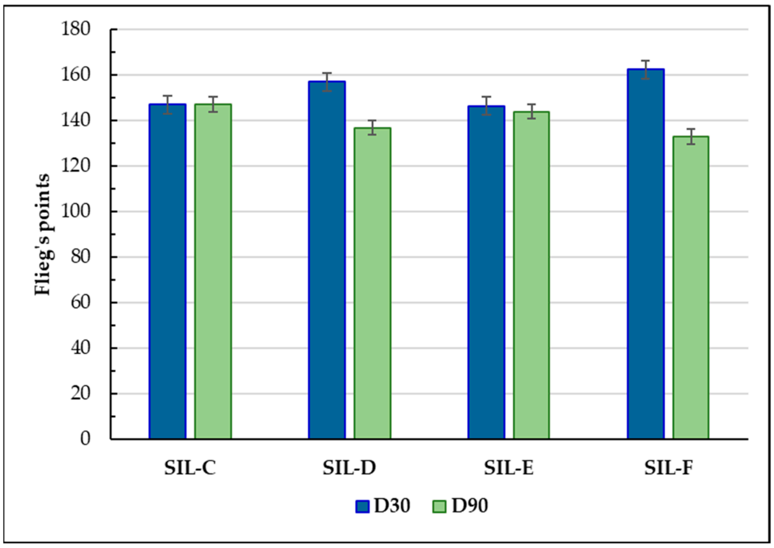


Figure 5. Flieg's score of mixed silages at 30 and 90 days after ensiling (experiment 2). Note: SIL-C: SIL-C: wheat straw 40% + grape pomace 20% + cheese whey 20% + water 20%; SIL-D: wheat straw 40% + grape pomace 20% + cheese whey 20% + olive mill wastewater 5% + water 15%; SIL-E: wheat straw 40% + grape pomace 20% + cheese whey 35% + water 5%; SIL-F: wheat straw 40% + grape pomace 20% + cheese whey 35% + olive mill wastewater 5%.

Table 6. Changes in lactic acid and volatile fatty acids (VFA; g/kg of dry matter) contents of mixed silages during the ensiling period (experiment 2).

	Treatment					**Effect**		
	SIL-C	**SIL-D**	**SIL-E**	**SIL-F**	**SEM**	**Treatment**	**Time**	**Interaction**
Lactic acid						*	**	*
0 d	nd	nd	nd	nd				
7 d	15.09 ^{a}	16.88 ^{a}	15.11 ^{a}	17.33 ^{a}	0.013			
30 d	28.21 ^{1b}	29.88 ^{1,2b}	30.27 ^{2b}	30.91 ^{2b}	0.017			
90 d	27.77 ^{1b}	29.14 ^{b}	29.93 ^{b}	30.86 ^{2b}	0.011			
Acetic acid						*	**	**
0 d	0.74 ^{1a}	0.53 ^{a}	0.37 ^{2a}	0.33 ^{2a}	0.001			
7 d	2.68 ^{1b}	2.97 ^{2b}	2.62 ^{1b}	2.88 ^{2b}	0.002			
30 d	5.34 ^{1c}	4.49 ^{1c}	6.82 ^{2c}	7.89 ^{2c}	0.002			
90 d	5.43 ^{1c}	4.50 ^{1c}	7.03 ^{2c}	7.92 ^{2c}	0.003			
Propionic acid						ns	ns	ns
0 d	nd	nd	nd	nd				
7 d	nd	0.02	0.08	0.01	0.001			
30 d	nd	0.02	0.08	0.04	0.001			
90 d	0.02	0.02	0.06	0.01	0.001			
Isobutyric acid						*	**	*
0 d	0.08 ^{a}	0.02 ^{a}	nd	0.03 ^{a}	0.001			
7 d	0.18 ^{1b}	0.20 ^{1b}	0.12 ^{1,2}	0.10 ^{2a}	0.001			
30 d	0.12 ^{1b}	0.20 ^{1b}	0.16 ^{1}	0.60 ^{2b}	0.002			
90 d	0.15 ^{1b}	0.22 ^{1b}	0.17 ^{1}	0.63 ^{2b}	0.001			
Butyric acid						*	*	ns
0 d	nd	nd	nd	nd				
7 d	1.53 ^{1,2a}	1.35 ^{1}	1.85 ^{2a}	2.01 ^{2a}	0.002			
30 d	1.86 ^{1b}	1.35 ^{2}	2.09 ^{1a}	2.22 ^{1b}	0.001			
90 d	1.91 ^{1b}	1.37 ^{2}	2.22 ^{1b}	2.29 ^{1b}	0.001			
At D30:								
Total VFA	7.31 ^{A}	6.11 ^{B}	9.15 ^{C}	10.77 ^{D}	0.535	**		
Total acids	35.54 ^{A}	35.94 ^{AB}	39.07 ^{Ca}	41.64 ^{Cb}	0.780	**		
% total VFA/total acids	24.09 ^{A}	16.58 ^{B}	23.43 ^{AC}	25.63 ^{A}	0.508	**		

* = $p < 0.05$; ** = $p < 0.01$; nd: not detected; ns: not significant. Means with different letters on the same column are significant different: ^{A,B,C,D} $p < 0.01$; ^{a,b,c} $p < 0.05$. ^{1,2} Means with different numbers on the same row are significant different. Note: SIL-C: wheat straw 40% + grape pomace 20% + cheese whey 20% + water 20%; SIL-D: wheat straw 40% + grape pomace 20% + cheese whey 20% + olive mill wastewater 5% + water 15%; SIL-E: wheat straw 40% + grape pomace 20% + cheese whey 35% + water 5%; SIL-F: wheat straw 40% + grape pomace 20% + cheese whey 35% + olive mill wastewater 5%. VFA: sum of acetic, propionic, isobutyric and butyric acids. Total acids: VFA plus lactic acid.

Before ensiling (D0), the pH in the different formulations of silages ranged from 4.82 (SIL-F) to 6.95 (SIL-C). Afterward, in all the silages, the pH decreased as the ensiling progressed, reaching 3.60 to 4.00 ($p > 0.05$) at maturation time (D30) and showing a slight increase (range: 3.95–4.30) at D90 (Figure 4).

Overall, it should be considered that, at the completion of the maturation period (D30), all the silages based on the different combinations of the by-products in the study (experiment 1 and experiment 2) showed pH values below 4.2. This is regarded as an indicator of good fermentation quality in silage [48], which can prevent the proliferation of enterobacteria. In addition, at D90, the pH can be considered suitable for all the silage formulations, according to Kim et al. [76]. The latter reported that, in animal feeding, a pH of 4 to 5 of the fermented feed ingredients is suitable because a pH below 4 decreases voluntary feed intake, whereas a pH above 5 may favor microbial spoilage.

At the maturation time (D30), the Flieg's score of the four experimental silages ranged from 146 to 162. At D90, the Flieg's score showed a slight decrease, but always remained above the score of 100 (Figure 5), indicating that all the experimental by-product-based silages were high in quality [41]. Considering the Fleig's score of both the experiments, we can confirm the suitability of ensiling by mixing GP, OMWW, CW and straw in terms of nutritional composition and conservation over time.

As observed in experiment 1, in experiment 2, lactic acid was the dominant fermentation product in all the experimental silages produced during the ensiling period (Table 6). Its concentration was affected by the silage composition ($p < 0.01$), time of ensiling and their interaction ($p < 0.05$). From D0, when it was not detected, the lactic acid content increased rapidly ($p < 0.01$), reaching the maximum level at the end of fermentation (D30) and then remaining stable until D90. At D30, the silages produced with the inclusion of the higher amount of CW (35%; SIL-E and SIL-F) had a higher content of lactic acid, proving significant ($p < 0.05$) in comparison with SIL-C (CW at 20%). High lactic acid concentration in silage is considered beneficial for ruminants because it can be metabolized into propionic acid by *Megasphaera elsdenii* and used as a precursor for gluconeogenesis [77].

Acetic acid content was influenced by the by-product combination ($p < 0.01$), time of ensiling ($p < 0.05$) and their interaction ($p < 0.01$). It showed the highest ($p < 0.05$) values in SIL-E and SIL-F compared with SIL-C and SIL-D. These differences could be attributed to the additional supply of LAB from cheese whey (+15%) in SIL-E and to the bacteria given through the commercial inoculum (*Lactiplantibacillus plantarum*) and/or to an improvement of some environmental factors on which lactic and acetic acid strains could have been depended in SIL-F. It has been reported that acetic acid together with other VFA, by inhibiting the growth of yeasts and mold, improve the aerobic stability of ensiled forage [78,79]. A moderate concentration of acetic acid in ruminants' feed may have positive effects as it can be used to produce energy or fat for body reserves or milk production [80].

Considering both the main fermentation products: lactic and acetic acids, a ratio of 3:1 has been indicated as the best for a good silage quality [48]. In this study, this ratio was respected in both the experiments (experiment 1 and experiment 2), from day 7 to day 90.

At the end of silage maturation (D30), propionic acid was found in a negligible amount in all the silages, reaching the maximum concentration of 0.08 g kg^{-1} DM in SIL-E (Table 6). Low propionic acid content might be considered an indicator of well fermented silages. In fact, although Propionibacteria produce this acid from glucose and lactic acid, they are very intolerant of a low pH, and high levels of propionic acid (>0.3 to 0.5%) have been associated with poorly fermented silages and/or the presence of some strains of Clostridia [53].

Isobutyric and butyric acid contents were affected by silage composition and time of ensiling ($p < 0.05$); the ra was significant for the isobutyric acid ($p < 0.05$). For both the acids, the contents were the lowest ($p < 0.05$) in SIL-D (1.35 g kg^{-1} DM) in comparison with the other silages and remained stable at D90 (Table 6). However, their content was low and very close to the acceptable value of 2.0 g kg^{-1} DM, as reported by Moselhy et al. [81], in SIL-C, SIL-E and SIL-F too. A low butyric acid content has been related to high lactic bacteria populations that reduce enterobacteria, clostridia and yeast populations [45].

The concentration of total VFA and the proportion of VFA to total acids were the lowest ($p < 0.01$) in SIL-D, indicating greater fermentation efficiency compared to the other silages. However, the values found in these silages (SIL-C, SIL-E and SIL-F) were close to 20% (range: 20.6% to 25.8%), which, according to Santoso et al. [46], is the ideal maximum value. In a study of rice crop residue-based silage, the proportions of VFA to total acids ranged from 31.9% to 37.2%.

4. Conclusions

The results of this study showed that the chemical composition of the silages based on different proportions of WS, GP, CW and OMWW (experiment 1 and experiment 2) was not influenced by the ensiling time, remaining almost stable. At D30, in the silage formulations based on WS and OMWW (experiment 1), the inclusion of CW (SIL-B) resulted in greater DM in comparison with the inclusion of GP (SIL-A), which, in contrast, showed greater crude protein, ether extract and ADL contents. The fermentation characteristics of both the silages showed good patterns in terms of pH and lactic acid concentration from the 7th to 30th days after ensiling. Total FVA and its proportion to total acids were higher in SIL-A compared with SIL-B, but all the values were low overall. The Flieg's score was always greater than 100 in both the silages. In experiment 2, which considered the silages produced mixing WS, GP and CW, at two levels, with or without the addition of OMWW, at D30, the pH ranged from 3.6 to 4.0 ($p > 0.05$). The inclusion of the higher amount of CW (35%; SIL-E and SIL-F) affected the highest content of lactic and acetic acids, whereas the addition of CW at 20% plus OMWW (SIL-D) resulted in the lowest content of butyric acid, total VFA and its proportion to total acids. In all four experimental silages, the Flieg's score was greater than 100. According to the values of the fermentative components, the stabilization of the by-product-mixed silages, in the different formulations, was reached on D30, where the final pH value, and lactic and VFA concentrations were within the optimal threshold for silage. The Flieg's score, as a quality index, was always greater than 100, indicating that the experimental silages were of a very good quality that remained until D90. We can state that ensiling mixed by-products (straw, GP, OMWW and CW) is a suitable, practical technique that seems promising since it enables the production of feed that is stable over time in nutritional composition and a source of antioxidant compounds for the animals. Further research is needed to evaluate their effects on animal welfare and product quality. Moreover, the evaluation of feeding costs and environmental benefits, as a complementary strategy to improve the sustainability of livestock systems, is worthy of further consideration.

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