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Silage of the By-Products of Mollar de Elche and Wonderful Pomegranate Varieties Preserves Nutritional Value and Antioxidant Activity of Ruminant Feed

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Abstract

The valorization of agro-industrial by-products for their use in animal feed leads to a reduction in inputs, creating the opportunity to optimize the sustainability of the agri-food chain, a priority of the SDG 2030 strategy; it also leads to a reduction in production costs. The objective of this study was to examine the changes that occur during the silage process of the pomegranate varieties Mollar de Elche (PDO) and Wonderful in terms of their nutritional and antioxidant characteristics for subsequent use in ruminant feed. Microsilos were created with the by-products of these two different pomegranate varieties. Two different microsilos for each variety were monitored on days 0 (raw material), 14, 35, 60, and 180. The variables studied included microbiology tracks, fermentation products, pH, dry matter (DM), macronutrient composition, organic acid and sugar contents, and antioxidant activity. The results show that, for both varieties, the silage process was successful; the stability of the fermentation process was determined by day 35, and its viability was ensured for a minimum period of 6 months. Furthermore, the nutritional characteristics of the raw material were preserved in the ensiled product. An evaluation of the total phenols and antioxidant capacity (ABTS and DPPH) showed that they remained stable throughout the monitoring period, despite the decrease in bioactive compounds (total phenols) at the end of the study period. It was concluded that silage is an effective preservation method for the by-products of Mollar de Elche and Wonderful pomegranate varieties, and its outcome presents valuable potential as a sustainable nutritional resource for ruminants.

Keywords: circular economy; sustainability; animal feed; fermentation; antioxidants

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1. Introduction

The food industry generates vast amounts of agricultural waste, posing significant environmental and sustainability challenges worldwide. In 2017 alone, ≈37 million tons of agricultural by-products (peels, stems, seeds, and pulp) were produced globally [1]. If not properly managed, these by-products can lead to environmental degradation and represent a lost opportunity for valorization. Pomegranate (*Punica granatum* L.) was first

domesticated in ancient Persia and later spread throughout the Mediterranean basin by Persians, Greeks, and Romans. Today, Turkey, Tunisia, Israel, and Spain rank among the leading producers in cultivated area, fruit yield, and export volume [2]. In Spain, pomegranates are primarily processed for juice, which generates large quantities of peel and seeds [3]. The peel alone accounts for ≈30–40% of the fruit and is especially rich in polyphenolic compounds, phenolic acids, tannins, flavonoids, and anthocyanins [3]. These constituents underlie the peel's high antioxidant capacity [4,5], a trait linked to reduced coronary heart disease risk and improved cellular longevity [6,7]. Because of this rich bioactive profile, pomegranate peel has attracted interest as an ingredient for both human and animal nutrition.

Numerous studies have evaluated its use as a ruminant feed supplement [8–11]. In vitro studies have shown that both the peel and seeds are readily fermented by rumen microorganisms, indicating good potential as ruminant feed [8]. For instance, Jami et al. [9] reported that supplementing dairy cow diets with up to 4% concentrated pomegranate residue extract improved dry matter intake, nutrient digestibility, and milk yield. Furthermore, the tannins in the peel may reduce ruminal protein degradation, thereby enhancing post-ruminal protein availability [9]. More recent research has focused on the use of pomegranate peel in silage. Sadhasivam et al. [10] found that incorporating pomegranate peel extract into maize silage suppressed fungal growth and mycotoxin production, thereby enhancing silage safety. Similarly, Ahmed et al. [11] demonstrated that co-ensiling pomegranate peel with molasses and berseem significantly improved fermentation quality and reduced methane emissions, likely due to the tannin-induced inhibition of methanogenic archaea. These findings are further supported by studies evaluating the ensiling capacity and quality of pomegranate by-product silages. Zhang et al. [12] reported that pomegranate peel could be successfully ensiled when mixed with high-moisture substrates such as apple pomace. The resulting silage exhibited favorable fermentation characteristics, including rapid acidification (pH < 4.5), elevated lactic and acetic acid production, and reduced ammonia-N levels, indicating efficient preservation and microbial stability [12]. Notably, the antioxidant properties of the peel were well retained during the ensiling process, which contributed to reduced nitrogen losses and methane emissions [12]. Kara et al. [13] also observed improved fermentation dynamics and digestibility when pomegranate pomace was ensiled with apple pomace, confirming its value as a co-ensiling material. Additionally, in vivo trials by Kazemi et al. [14] and Eliyahu et al. [15] demonstrated positive effects on feed intake, nutrient digestibility, growth performance, and meat quality in fat-tailed lambs and sheep when pomegranate by-product silage was incorporated into total mixed rations. These results confirm that pomegranate peel and pomace not only have a good ensiling capacity but also enhance the nutritional, microbial, and functional quality of silage.

Therefore, the aims of the present study were to evaluate the ensiling capacity (round bale, 300 kg) and conservation characteristics of the peel by-products of two pomegranate varieties, namely, "Mollar de Elche PDO" and "Wonderful", locally produced in the Valencian Region in Spain, and to assess their safety and suitability for ruminant feeding for 6 months.

2. Materials and Methods

2.1. Facilities, Experimental Design, and Sample Management

An experiment was carried out at a pilot plant located in the facilities of the "Granja Caprina", part of the Polytechnic School of Orihuela (EPSO) at Miguel Hernández University of Elche (UMH), Spain.

By-product was obtained from the local pomegranate juice industry. The manufacturing process followed the method described in the patent by Díaz et al. [16]. Briefly, silos

were formed using an Agronic MR 820 rotary baler (AGRONIC OY, Haapavesi, Finland), with a capacity of 0.64 m^3 and a weight of 300 kg per bale. To ensure proper compaction and firmness, each bale was wrapped with five layers of netting. Additionally, 13 layers of plastic film (Karatzis, Heraklion, Greece) were applied to maintain an airtight seal and prevent oxygen infiltration. The Mollar and Wonderful by-products consisted of pomegranate peels. To enhance compaction, 20% of chopped barley straw (2 cm approx.) was added, and, to promote anaerobic conditions within the silos, this raw material was chopped and homogenized using a FASTER MIX 1 V mixer wagon (Compar, Sant Pere de Torelló, Spain) (Figure 1). A silage inoculant, Magniva Platinum 3 (Lallemand, Blagnac, France), containing the lactic acid bacteria Lactobacillus hilgardii CNCM I-4785 and Lactobacillus buchneri NCIMB 40788 in a 1:1 ratio (7.50×10^{10} CFU/g), was added at a concentration of 2 g per ton of by-product.

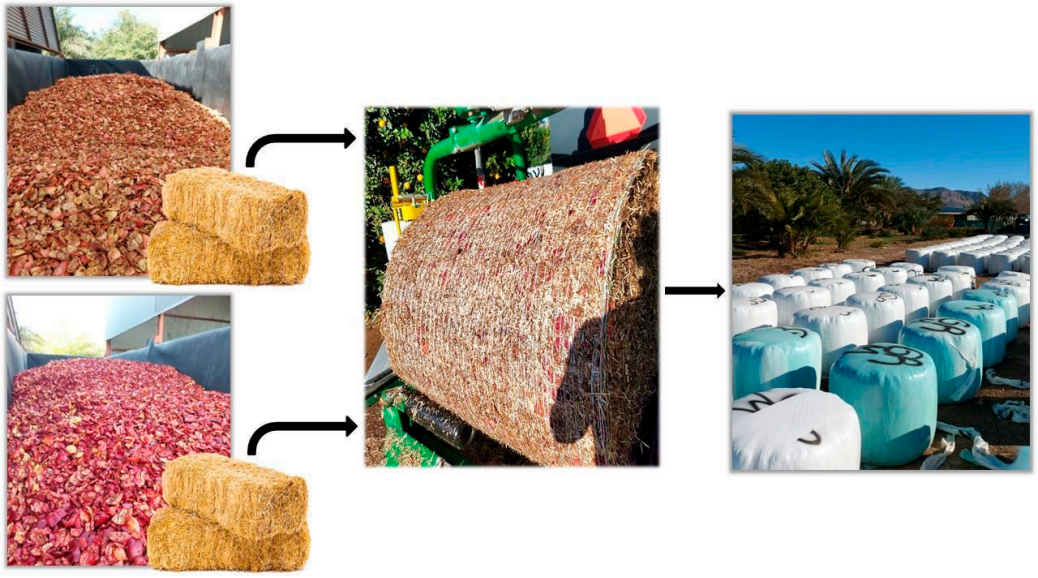

Figure 1. By-products studied (Mollar de Elche, upper side, and wonderful, bottom side, pomegranate varieties) and round bales manufactured (microsilos).

To monitor the changes over time in both pomegranate varieties studied (Mollar de Elche PDO: Mollar and Wonderful), samples were aseptically collected at five intervals: days 0 (the day of silo preparation), 14, 35, 60, and 180. On day 0, three samples were taken from three different sections of the entire batch of material intended for silaging. For every sampling day, two different bales were selected, and a 1 kg sample was extracted using a manual auger from three distinct zones of each bale: the middle, the upper section, and 20 cm from the base. These three sub-samples from each bale were then combined into a composite sample.

The samples were transported to the laboratory, where some analyses, such as microbiological assays and pH measurements, were performed immediately. The remaining samples were stored at −80 °C for subsequent analyses.

2.2. Microbiological Determination

For microbiology tracking, the samples were transported to the laboratory in aseptic plastic bags. Subsequently, 25 g of each sample was placed into aseptic plastic bags with a lateral strainer and homogenized with 225 mL of peptone water using a stomacher (BagMixer® 400, Interscience, Puicapel, Cantal, France). Microbiological cultures were prepared for the enumeration of enterobacteria and aerobic mesophilic bacteria, and these were directly incubated on EB and AC 3M™ Petrifilm plates (3M Microbiology, Minnesota,

USA) at 37 °C for 24 h, respectively. For lactic acid bacteria counts, the samples were diluted in MRS broth (Liofilchem, Roseto degli Abruzzi, Italy) and incubated on LAC 3M™ Petrifilm plates at 37 °C for 48 h in an anaerobic jar. Molds and yeasts were cultured on YM Petrifilm plates and incubated for 72 and 120 h, respectively. Butyric spore count was determined using the most probable number technique (MPN) and Bryant and Burkey broth (BBB, Merck, Darmstadt, Germany), following the methodology indicated in Arias et al. [17]. The results are expressed as log10 cfu/g of fresh sample, following the AENOR guidelines (Spanish Association for Standardization and Certification, 2015). All microbiological analyses were conducted at the Animal Science Laboratory, part of the Agro-Food Technology Department at EPSO-UMH.

2.3. Quantification of Fermentation Products

To study fermentative dynamics during the silage process, the key metabolites involved in this reaction were quantified using humid frozen samples. Sugars naturally present in the fruit (sucrose, fructose, and glucose), which act as substrates in silage fermentation, were analyzed. Additionally, short-chain organic acids (VFAs) (acetic, butyric, and propionic acids) and lactic acid and ethanol, considered the main fermentation products, were also analyzed. Extraction was performed using a 1:2 ratio of humid sample to distilled water. The mixture was homogenized using a high-speed disperser (T18 digital ULTRA-TURRAX®, IKA, Staufen, Germany) and then centrifuged. The resulting supernatant was filtered through 0.45 µm membrane filters. Quantification was performed following the method described by Feng-Xia et al. [18] using high-performance liquid chromatography (HPLC; Agilent 1200, Agilent, Santa Clara, CA, USA). The analysis employed a 30 cm × 7.8 mm DI C610H column (Supelcogel, Sigma-Aldrich Co., Taufkirchen, Germany), with 0.1% orthophosphoric acid as the mobile phase. The results are reported as g/kg of dry matter (DM).

2.4. Assessment of Physicochemical Properties

The physicochemical properties of the silage were evaluated on the same day as sampling. The pH was determined following the procedure described by Cherney et al. [19], using a 1:10 (w/v) ratio of fresh sample to double-distilled water. The mixture was stirred and then allowed to rest at room temperature for 1 h before pH measurement (GLP 21 pH meter, Crison, L'Hospitalet de Llobregat, Spain). The dry matter (DM) content was determined as g/kg according to AOAC Method 930.5 [20]; this was carried out by dehydrating a known amount of fresh sample, which was weighed and dried in a forced-air oven at 105 °C until a constant weight was achieved. Silage quality was assessed by calculating the Flieg score for each sample using the equation proposed by Kilic [21]:

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

Silage was categorized based on the Flieg scores as follows: <20 points indicated very low-quality silage; 21–40 points indicated low-quality silage; 41–60 points indicated medium-quality silage; 61–80 points indicated high-quality silage; and >81 points indicated very high-quality silage.

Further analyses were conducted on the samples dehydrated at 60 °C and ground to a 1 mm particle size. The following parameters were analyzed using AOAC methods: ash (g/kg DM, method 934.01), crude protein (CP, g/kg DM, method 988.05), ether extract (EE, g/kg DM, method 920.39), crude fiber (CF, g/kg DM, method 978.10), and total sugars (g/kg DM, method 974.06). The contents of neutral detergent fiber (NDF, g/kg DM), acid detergent fiber (ADF, g/kg DM), and acid detergent lignin (ADL, g/kg DM) were determined according to the method by Van Soest et al. [22]. Non-protein nitrogen

(NPN, g/kg DM) was quantified using the Cornell method for feed nitrogen fractionation described by Licitra et al. [23]. Starch content was determined using the polarimetric method described by Ewers [24].

2.5. Antioxidant Capacity

To evaluate the antioxidant potential, extraction was performed on microsilo frozen samples (−80 °C) using acetone (70% *v*/*v*, HPLC grade). The solution was thoroughly mixed using an agitator, prepared under a fume hood, and maintained at an appropriate temperature with ice during the extraction process to preserve the integrity of the compounds. This extraction was used for the analysis of the total phenol (TP) content using the Folin–Ciocalteu method described by Kim et al. [25], with the results expressed as mg of gallic acid equivalents (GAE) per gram of dry matter (mg GAE/g DM). It was also used to determine the reducing power using DPPH and ABTS, with the results expressed in mg Trolox eq/g DM. The DPPH analysis (reduction in the 1,1-diphenyl-2-picrylhydrazyl radical) was conducted following a protocol modified by Cheng et al. [26], developed initially by Brand-Williams et al. [27]. For the ABTS analysis (reduction in the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical), the method described by Leite et al. [28] was followed. The results of these reactions were measured via spectrophotometry using a UV–VIS spectrophotometer (Zuzi 4255/50, Auxilab, Beriain, Spain) at wavelengths of 750, 515, and 734 for TP, DPPH, and ABTS, respectively.

2.6. Statistical Analysis

All the determined variables were analyzed using a general linear model (Proc.GLM, SAS V 9.4, 2022), according to the following equation:

$$Y = \mu + Bi + Dk + (BixDk) + e$$

Here, Y is the dependent variable, μ is the intercept, Bi is the fixed effect of the ensiling day (i = 0, 14, 35, 60, and 180 days), Dk is the fixed effect of the pomegranate variety (k = Mollar or Wonderful), Bi x Dk is the interaction of both, and e is the residual error. To interpret the differences between the levels of the fixed effect, least-squares means were calculated, representing model-adjusted estimates. Mean comparisons between the levels of the fixed effect were performed by testing the null hypothesis: H0: LSMean (h) = LSMeann (j), where (h) and (j) represent the different levels of the fixed effect. The significance of these comparisons was evaluated using $Pr > t$. If $p < 0.05$, then the null hypothesis is rejected, indicating that the differences between the levels are statistically significant.

3. Results

3.1. Microbiology Tracking

The evolution of aerobic bacteria, lactic bacteria, enterobacteria, molds, yeasts, and butyric spores during the ensiling period is shown in Table 1. A significant effect of ensiling day ($p \leq 0.01$) was observed for all microbial groups. In contrast, the effect of variety was not significant for any of the studied populations. Significant variety × ensiling day interactions were observed for enterobacteria and molds ($p \leq 0.001$). Aerobic bacteria were abundant in the fresh material (day 0), with counts of 7.63 and 7.05 log cfu/g FM in Mollar and Wonderful, respectively. Their levels decreased over time, reaching lower and more stable counts by day 180, especially in Mollar (4.60 log cfu/g FM). The decay of the aerobic population was more intense in Mollar in the first 35d than in Wonderful, with similar values obtained at 60 d of ensiling. In the beginning, lactic acid bacteria (LAB) showed low counts in both varieties (5.87 in Mollar and 5.55 in Wonderful), but they increased significantly ($p < 0.01$) by day 14 (8.74 and 8.43, respectively), remaining relatively high

until day 180. Enterobacteria populations showed higher counts at day 0 in the Mollar variety ($p < 0.10$), although these bacteria declined sharply and were undetectable by day 35 in both varieties. The interaction of variety × ensiling day was highly significant ($p < 0.001$), as the decline was more pronounced in Mollar. Regarding molds, Wonderful showed higher counts at day 0, although with a significant and relevant decrease over ensiling days; nevertheless, it always showed higher values than Mollar. Mold levels gradually declined such that, by day 180, more than 50% of the initial mold population disappeared in both varieties. Yeasts increased from day 0 to day 14 and then began to decline. By day 180, yeast counts were higher in Mollar (5.26 log cfu/g FM) than in Wonderful (3.33 log cfu/g FM), with the overall time effect being significant ($p < 0.01$). Butyric spores were detected in fresh material, reaching a peak by day 14 in Mollar (2.55 log cfu/g FM) and by day 60 in Wonderful (2.55 log cfu/g FM), and then they notably declined in both varieties, with the lowest levels observed at day 180 (0.15 and 1.90 log cfu/g FM for Mollar and Wonderful, respectively). Only a significant effect of time ($p < 0.01$) was observed.

Table 1. Effect of ensiling on the microbial population in the by-products of two pomegranate varieties.

Variety	Days of Ensiling					SEM	F $_{p\text{-value}}$		
	0	14	35	60	180		v	d	x
				Aerobic bacteria (log cfu/g FM)					
Mollar	7.63	5.84	5.33	6.94	4.60	0.29	F = 2.2	F = 18.7	F = 3.1
Wonderful	7.05	5.81	6.31	6.84	5.67		p = 0.171	p = < 0.0001	p = 0.068
				Lactic bacteria (log cfu/g FM)					
Mollar	5.87	8.74	7.97	7.73	7.06	0.53	F = 0.3	F = 8.5	F = 0.04
Wonderful	5.55	8.43	7.96	7.70	6.85		p = ns	p = 0.003	p = ns
				Enterobacteria (log cfu/g FM)					
Mollar	6.40	1.09	0.00	0.00	0.00	0.25	F = 4.6	F = 217.4	F = 16.38
Wonderful	5.00	3.82	0.00	0.00	0.00		p = ns	p = 0.0001	p = 0.0002
				Molds (log cfu/g FM)					
Mollar	3.60	3.03	2.34	3.48	1.15	0.55	F = 16.1	F = 7.6	F = 0.33
Wonderful	5.72	4.26	3.72	4.39	2.50		p = 0.002	p = 0.0045	p = ns
				Yeast (log cfu/g FM)					
Mollar	6.05	7.31	6.81	4.58	5.26	0.54	F = 0.20	F = 8.9	F = 2.83
Wonderful	6.70	7.24	6.0	6.0	3.33		p = ns	p = 0.0024	p = ns
				Butyric spores (log cfu/g FM)					
Mollar	1.79	3.08	2.55	2.72	0.15	0.32	F = 0.07	F = 8.18	F = 5.81
Wonderful	1.82	2.31	1.95	2.55	1.90		p = ns	p = 0.005	p = 0.017

v: variety; d: ensiling day; x: day x variety interaction. ns: $p > 0.05$.

3.2. Fermentation Products

The sucrose, glucose, fructose, lactic acid, acetic acid, and ethanol concentrations in the Mollar and Wonderful pomegranate varieties during the ensiling process are presented in Table 2. A significant effect of ensiling day ($p < 0.01$) was observed for all of them, with no significant effect of variety for any variable. A significant variety × ensiling day interaction was observed only for sucrose ($p < 0.05$). The sucrose content decreased sharply from day 0 to day 14 in both varieties, from 19.2 to 8.72 g/kg DM in Mollar and from 15.1 to 8.62 g/kg DM in Wonderful, and it remained relatively stable until day 60. By day 180, the values dropped to 0.28 in Mollar and 0.00 g/kg DM in Wonderful. Glucose concentrations declined steadily over time. The initial values were 59.9 g/kg DM in Mollar and 55.23 g/kg DM in Wonderful, reaching 4.53 and 6.80 g/kg DM by day 180, respectively. Fructose followed a similar trend to glucose, decreasing from 95.7 to 17.8 g/kg DM in Mollar and from 86.1 to 13.4 g/kg DM in Wonderful over the 180 days. Ethanol increased markedly from day 0 to day 14 in both varieties, reaching peak values at day 14 (54.6 in Mollar and 59.5 g/kg DM in Wonderful), and then it gradually declined until day 180 (27.9 and 43.4 g/kg DM,

respectively). Lactic acid increased notably after day 0 in both varieties. In Mollar, it rose from 1.47 to 28.0 g/kg DM by day 14 and reached 38.7 g/kg DM by day 180. In Wonderful, the values increased from 1.26 to 14.1 g/kg DM by day 14 and reached 49.7 g/kg DM by day 180. Acetic acid showed a progressive increase over time. In Mollar, concentrations increased from 1.06 at day 0 to a peak of 18.5 g/kg DM at day 60, and then they decreased to 11.9 g/kg DM by day 180. Wonderful followed a similar trend, increasing from 0.85 to 17.86 g/kg DM by day 180. Butyric and propionic acids were not detected in either of the varieties.

Table 2. Effect of ensiling on sugar content and fermentative components in the by-products of the two pomegranate varieties, Mollar de Elche and Wonderful.

Variety	Days of Ensiling					SEM	F *p*-value		
	0	14	35	60	180		v	d	x
				Sucrose (g/kg DM)					
Mollar	19.24	8.72	8.55	9.79	0.28	1.27	F = 2.60	F = 44.7	F = 0.98
Wonderful	15.10	8.62	8.55	7.83	0.00		*p* = ns	*p* = <0.001	*p* = ns
				Glucose (g/kg DM)					
Mollar	59.86	12.20	7.27	5.63	4.53	6.38	F = 0.09	F = 23.66	F = 0.18
Wonderful	55.23	11.46	11.26	10.48	6.80		*p* = ns	*p* = <0.001	*p* = ns
				Fructose (g/kg DM)					
Mollar	95.67	29.26	19.08	18.97	17.78	9.45	F = 0.11	F = 22.49	F = 0.16
Wonderful	86.14	32.87	21.67	16.90	13.43		*p* = ns	*p* = <0.001	*p* = ns
				Lactic acid (g/kg DM)					
Mollar	1.47	28.00	33.81	27.53	38.70	3.07	F = 2.07	F = 50.92	F = 4.96
Wonderful	1.26	14.12	23.89	26.5	49.74		*p* = ns	*p* = <0.001	*p* = 0.018
				Acetic acid (g/kg DM)					
Mollar	1.06	5.13	10.6	18.5	11.92	2.67	F = 2.68	F = 10.23	F = 2.64
Wonderful	0.85	1.63	5.03	8.00	17.86		*p* = ns	*p* = 0.0015	*p* = ns
				Ethanol (g/kg DM)					
Mollar	1.78	54.59	46.43	38.84	27.95	5.19	F = 8.35	F = 36.12	F = 1.39
Wonderful	1.53	59.47	52.60	60.01	43.44		*p* = 0.016	*p* = <0.001	*p* = ns

v: variety; d: ensiling day; x: day x variety interaction. ns: $p > 0.05$.

3.3. Physicochemical Parameters and Nutritional Composition

The evolution of pH and Flieg score during the ensiling process is shown in Figure 2. pH decreased during ensiling for both varieties, with significant effects detected for variety ($p < 0.01$), ensiling day ($p < 0.01$), and their interaction ($p < 0.01$). In Mollar, a marked decrease was observed in the first 14 days, from 4.75 to 3.92, being lighter during the rest of the conservation period; in Wonderful, a decrease from 4.61 to 3.89 was observed, although the greatest decrease was observed on day 60. The Flieg score improved slightly with time. The initial values were 104 for Mollar and 108 for Wonderful, reaching 123 and 119 by day 180, respectively. However, no significant ($p > 0.05$) effects were observed for variety, ensiling day, or their interaction.

The evolution of the DM, NDF, ADF, ADL, EE, CP, NPN, starch, and total sugar contents in the pomegranate varieties Mollar and Wonderful during the silage conservation time is shown in Table 3. The DM, CP, and total sugar contents were affected ($p < 0.05$) by the conservation time, and the variety and its interaction with conservation time were not significant for any variable. DM decreased notably by day 14 in both pomegranate varieties: from 412 to 325 g/kg DM in Mollar and from 469 to 325 g/kg DM in Wonderful. From that point onward, DM remained stable, with values of 355 g/kg DM in Mollar and 363 g/kg DM in Wonderful by day 180. NDF and ADF showed no significant changes over time or between varieties. ADL exhibited slight variation over time, with a general non-significant decreasing trend until day 180 in both varieties; Mollar values declined from 78.3 to 50.3 g/kg DM, while Wonderful values decreased from 64.1 to 51.5 g/kg DM.

The EE results were under 1 g/kg DM and remained relatively stable across the entire conservation period for both varieties. CP increased progressively in both varieties. Mollar rose from 34.5 to 43.5 g/kg DM, while Wonderful increased from 33.0 to 45.0 g/kg DM between days 0 and 180. NPN remained relatively unchanged throughout the study in both varieties, with values ranging from 5.50 to 7.00 g/kg DM. The starch content fluctuated non-significantly during the conservation time, with similar values at the end of the study (32.5 and 27.0 g/kg DM for the Mollar and Wonderful varieties, respectively). The total sugar content decreased sharply after day 0 in both varieties, from 123 to 22.0 g/kg DM by day 180 in Mollar and from 115 to 20.0 g/kg DM in Wonderful.

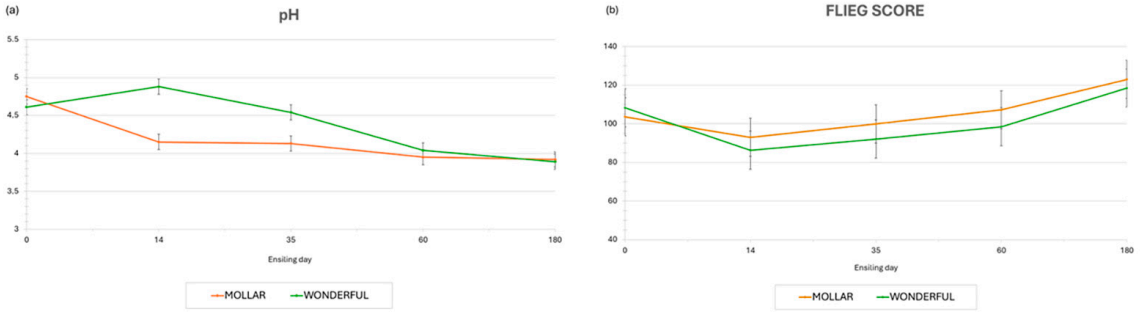

Figure 2. Effect of ensiling on pH (**a**) and Flieg score (**b**) of the by-products of two pomegranate varieties (estimated means and standard error bars).

Table 3. Effect of ensiling on the nutritional composition (estimated means) of the by-products of two pomegranate varieties, Mollar de Elche and Wonderful.

Variety	Days of Ensiling					SEM	F $_{p\text{-value}}$		
	0	14	35	60	180		v	d	x
				Dry Matter (g/kg FM)					
Mollar	412	325	326	350	355	15.7	F = 0.91 p = ns	F = 14.07 p = <0.001	F = 1.51 p = ns
Wonderful	469	325	318	312	363				
				Neutral Detergent Fiber (g/kg DM)					
Mollar	491	479	482	485	490	23.9	F = 0.00 p = ns	F = 1.75 p = ns	F = 1.57 p = ns
Wonderful	448	435	512	493	539				
				Acid Detergent Fiber (g/kg DM)					
Mollar	332	333	361	337	308	16.3	F = 0.18 p = ns	F = 2.50 p = ns	F = 1.00 p = ns
Wonderful	314	317	374	340	348				
				Ether Extract (g/kg DM)					
Mollar	0.75	0.70	0.90	0.90	0.90	0.72	F = 4.74 p = 0.059	F = 0.91 p = ns	F = 0.95 p = ns
Wonderful	0.75	0.85	0.85	0.90	1.05				
				Crude Protein (g/kg DM)					
Mollar	34.5	40.5	41.0	42.0	43.5	1.67	F = 0.04 p = ns	F = 8.59 p = 0.0014	F = 0.27 p = ns
Wonderful	33.0	42.0	41.0	41.0	45.0				
				Non-Protein Nitrogen (g/kg DM)					
Mollar	6.00	6.50	5.50	6.00	6.00	1.03	F = 0.03 p = ns	F = 0.32 p = ns	F = 0.05 p = ns
Wonderful	6.00	7.00	6.00	6.00	5.50				
				Starch (g/kg DM)					
Mollar	27.0	52.0	53.5	39.5	32.5	9.47	F = 3.89 p = ns	F = 0.67 p = ns	F = 1.01 p = ns
Wonderful	33.0	32.5	23.5	32.0	27.0				
				Total Sugar (g/kg DM)					
Mollar	123	30.0	19.0	13.0	22.0	5.21	F = 0.77 p = ns	F = 90.49 p = <0.001	F = 1.45 p = ns
Wonderful	115	46.0	20.5	20.0	19.5				

v: variety; d: ensiling day; x: day x variety interaction. ns: $p > 0.05$.

3.4. Antioxidant Capacity

The total phenol content (mg GAE eq/g DM) and the antioxidant activity by DPPH and ABTS (mg Trolox eq/g DM) are presented in Figure 3a, Figure 3b, and Figure 3c, respectively. The total phenols remained relatively high ($p > 0.05$) until day 35 in Wonderful and day 60 in Mollar. In Mollar, the total phenols increased from 123 to 159 mg GAE/g DM by day 60, while in Wonderful, they rose from 160 to 173 mg GAE/g DM over the same time period; however, they decreased to 95 and 78 mg GAE/g DM by day 180 in Mollar and Wonderful, respectively. No significant effect of variety was detected. Interaction was significant at day 35 ($p = 0.0479$). Time significantly affected the phenolic content ($p < 0.05$), as there was a final decrease at 180 days compared to the initial content, indicating a temporal influence on phenol degradation. Antioxidant activity, determined via a DPPH assay, exhibited non-significant variety and variety x time effects, whereas ensiling day had a highly significant effect ($p < 0.001$), indicating a temporal response of both varieties to ensiling day. DPPH was slightly higher ($p > 0.05$) in the raw material of Mollar (day 0) than in that of Wonderful (59 vs. 53 mg Trolox eq/g DM, respectively), increasing ($p < 0.001$) for both pomegranate varieties until day 60, reaching a peak at day 35 (77 vs. 79 mg Trolox eq/g DM for Mollar and Wonderful, respectively), and then declining ($p < 0.001$) at day 180. ABTS showed a different evolution between the two varieties. In Mollar, values peaked at day 60 (205 mg TE/g DM), while in Wonderful, the highest level was recorded at day 35 (228 mg TE/g DM). Despite the apparent changes, no statistically significant effects were observed for variety, ensiling time, or their overall interaction, except for on day 35 ($p = 0.0182$).

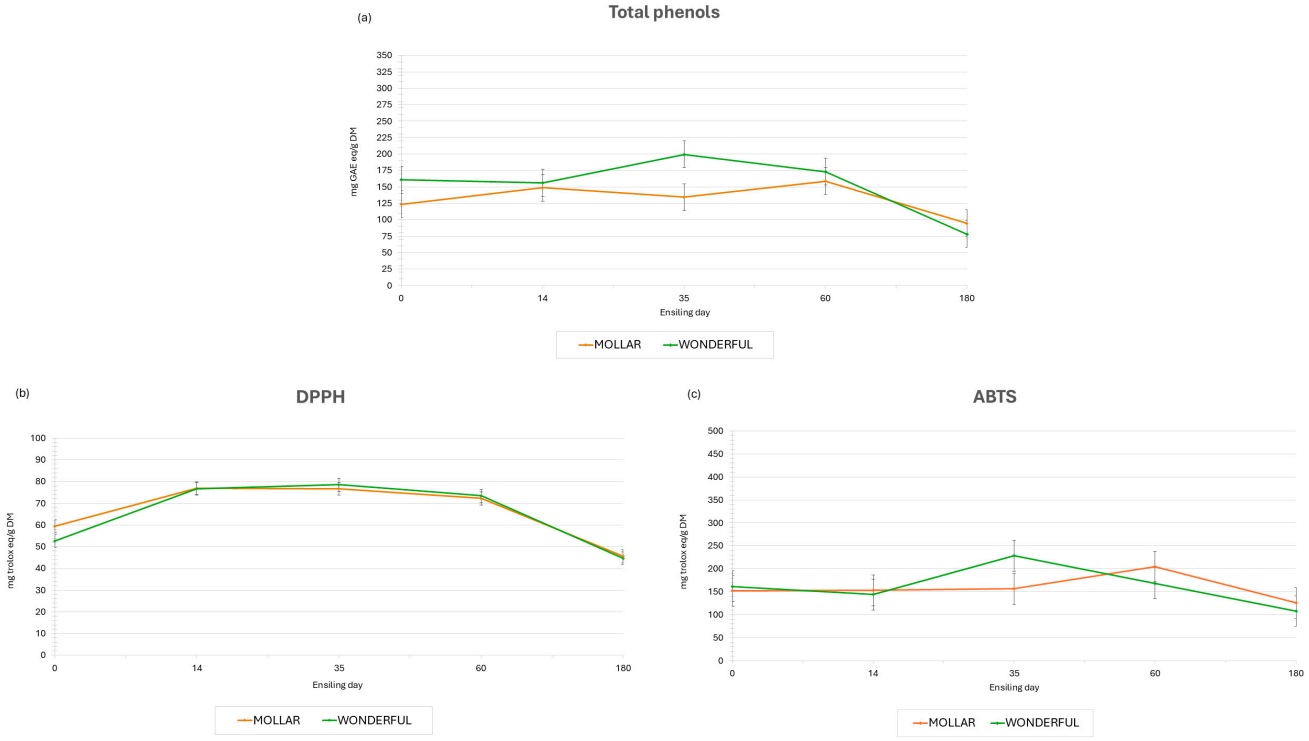

Figure 3. Effect of ensiling on total phenols (**a**), DPPH (**b**), and ABTS (**c**) in the by-products of two pomegranate varieties (estimated means and standard error bars).

4. Discussion

Ensiling is a commonly applied preservation technique for terrestrial forages intended for livestock feed [29]. More precisely, ensiling is an anaerobic, lactic acid bacteria (LAB)-driven fermentation process that safely stores forage by decreasing the pH with

organic acids, thereby stabilizing the feed for ruminant diets [30–32]. Good-quality silage is achieved when the fermentation process is successfully completed [33]. The silages of the Mollar de Elche and Wonderful pomegranate varieties showed adequate microbial activity for good-quality silage, as the number of aerobic bacteria decreased sharply and remained low at days 35, 60, and 180. This pattern is consistent with the conversion of fresh, oxygenated substrate in anaerobic fermentation, which suppresses aerobic flora [34], giving space to LAB. LAB became the dominant microbiota during fermentation, and their count increased as ensiling proceeded, reflecting the active fermentation of sugars to acids [34]. The pH value decreased to 4.15 and 4.54 for Mollar and Wonderful on day 14, respectively, and it continued to decrease throughout the conservation process, maintaining an environment unsuitable for spoilage micro-organisms. Enterobacteria are gram-negative facultative anaerobes commonly found on fresh plants, and their count declined strongly with ensiling time, as the acidity produced by LAB and the low pH likely suppressed them [35]. As in Monllor et al. [36], enterobacteria counts decreased faster in Mollar, as the pH dropped faster, probably due to its higher sugar content, achieving low levels from day 14 (early silage) and undetectable levels from day 35. In both varieties, mold populations were low and fell quickly to negligible levels once anaerobic fermentation began, as they require oxygen. Similar findings were reported by Dolci et al. [34]. Yeasts also declined markedly over time, with minimal levels in both varieties. This result agrees with previous observations that yeast populations drop dramatically in properly fermented silage [34] because of the sugar unavailability and increase in acetic acid concentrations in the silage, known for antifungal and bactericidal activities [37]. Butyric spore counts remained very low in all silages until late storage (180 days). This outcome is desirable, as butyric clostridia are associated with spoilage and can produce butyric acid and ammonia, which degrade silage quality [38]. The overall pattern of rapid loss of undesirable aerobes/enterobacteria, dominance of LAB, and suppression of molds/yeasts maintained for both Mollar de Elche and Wonderful are expected for a well-preserved silage [34].

Fermentation products are a core measure of silage success. They determine its stability, safety, and palatability for ruminant animal feed [37] and, therefore, affect feed intake, productivity, and the quality of their derivatives [39]. The fermentation process is influenced by the predominant micro-organisms in the silage, the fermentable substrates present, and the nature of the fermentation that occurs during the whole ensiling process [40]. The Mollar variety had a higher sugar content, which resulted in higher peaks of lactic and acetic acid concentrations and therefore a more accelerated fermentation process than the Wonderful variety (Table 2). The concentration of lactic acid remained higher than that of the other organic acids detected, such as acetic acid, throughout the whole conservation period (180 days). The lactic/acetic acid ratio, as an indicator of the type of fermentation during the ensiling process and its quality, was higher than 3:1, which was described as ideal by Kung et al. [37]; this value was first observed on day 14 in both varieties, and it remained above this level until day 180. The ethanol concentrations in this study are much higher than those reported by Galvez-Lopez et al. [41] for white grape pomace silage. This could be explained by the higher level of sugars available in the pomegranate peel than in the white grape pomace by-product. Yeasts, such as *Saccharomyces cerevisiae* and related species, are the main ethanol producers, as they ferment water-soluble carbohydrates to ethanol and CO_2 [37]. In our findings, yeast counts remained above 5 log cfu/g FM, even on day 180. This supports our hypothesis, as the yeast levels observed in the present study were notably higher than those reported in other by-products such as those of white grape pomace (1 log cfu/g FM on day 60) [41] and prickly pear "pastazzo" silage (<1 log cfu/g FM on day 46) [42]. Other high water-soluble carbohydrate crops, such as beets, fruit pulps, melon, and alfalfa wilted with high sugar, feed yeast, and high moisture (low

dry matter), can prolong fermentation and help yeasts persist [37]. Additionally, it has been reported that some heterofermentative LAB also produces ethanol as a by-product. However, common silage inoculants, such as *Lactobacillus buchneri,* can help convert lactic acid to acetic acid and 1,2-propanediol (with little ethanol), inhibiting yeast growth and ethanol production and helping to delay the increase in pH after aerobic exposure [43]. In our study, despite the addition of *Lactobacillus buchneri*, ethanol and yeast levels remained high. This could be explained by the high concentration of water-soluble carbohydrates (WSC), which likely stimulated early yeast proliferation—particularly before the lactic acid bacteria (LAB) population could become dominant. According to McDonald et al. [40], *L. buchneri* typically becomes active later in the fermentation process, and therefore may not effectively suppress early yeast activity. Moreover, the effectiveness of microbial inoculants depends on factors such as the initial microbial load and the compatibility of the LAB strain with the specific substrate. In some cases, indigenous yeasts or other native microorganisms in the silage may be more competitive or diverse, limiting the impact of the added inoculant (Filya et al. [44]). A higher dose or concentration of *L. buchneri*, combined with an increase in the dry matter (DM) content of the raw material, could help enhance the activity and effectiveness of the inoculant. High amounts of ethanol are also associated with high losses of DM and energy, and, when fed in large quantities, they can cause off flavors in milk. Although cases of ethanol poisoning have been reported in ruminants [45], this is unlikely to occur with most commonly fed silages, even those with high concentrations of ethanol [37]. Mitigating strategies include ensiling with a higher DM content via the inclusion of raw materials such as straw or bran (reducing yeast growth), rapid sealing, and using additives (e.g., *L. buchneri* or formic acid) to decrease the moisture content and therefore yeast proliferation [40].

The ideal pH range (4.2–3.6), as described in [46], was reached on day 14 in Mollar (4.02) and on day 35 in Wonderful (4.04), and the ideal range was maintained for all 6 months. These values are lower than those found by Monllor et al. [36] when ensiling broccoli, artichoke, and artichoke plant stubble by-products, and they are even lower than those found by Galvez-Lopez et al. [41] when ensiling white grape pomace. Moreover, the Flieg score was above 80 for both varieties throughout the entire ensiling process, exceeding that observed by other authors when ensiling other agro-industrial by-products [36,41,47,48], indicating quality silage suitable for conservation for at least 6 months.

Ensiling is used to preserve fresh forage or agro-industrial by-products through controlled fermentation, and it must ensure a minimum loss of DM over time. The loss of DM and sugars during ensiling is due to their use by plant enzymes, aerobic microbes for respiration, and yeasts, producing CO_2 and heat [37]. In this study, the initial DM content was in the range of 250–300 g/kg, as recommended by McDonald et al. [40]. However, the losses of DM were higher than the values set by the same authors (2–5%), probably due to the higher moisture content in pomegranate peel. The apparent gain in CP was mostly relative. True protein was degraded to soluble N (peptides, NH_3), but because other DM components disappeared faster, the CP/DM ratio increased. The polyphenol content in pomegranate peel slows proteolysis, explaining the modest increase in NPN [49]. The increase in NDF in Wonderful by day 30 and later by day 180 was likely relative, and it was not absolute due to the fermentation loss of non-fiber components; NDF accounted for a larger proportion of the remaining DM, as described by Muck et al. [50]. The stability of the NDF content in Mollar may be due to its slow degradation and more consistent fermentation. The final drop in ADF, especially in the Mollar variety, may have resulted from the slow microbial breakdown of cellulose during storage; some silage microbes, such as LAB, may produce low levels of cellulases. Moreover, pomegranate tannins could slow fiber degradation but also preserve structure, so the balance varies by variety [40,51]. The

initial increase in ADL up to day 35 was probably relative, as other fractions disappeared. However, its final decline by day 180 may indicate the chemical breakdown of lignin bonds under acidic, anaerobic conditions during long storage, or it may have been caused by the interference of phenolic compounds, as reported by Van Soest et al. [52]. According to our findings, the CP content in both the raw materials and silages was around 3–4%, while the ADL content was approximately 8–9%, which may negatively affect intake and the digestibility of protein and fiber [52]. Phenolic compounds are initially preserved or even slightly released due to the breakdown of cellular structures during early fermentation. However, prolonged ensiling leads to the degradation of polyphenols via oxidation or microbial metabolism [5,53]. The superior performance of Wonderful in phenolic retention may be attributed to the fresh material having an inherently richer polyphenolic profile (day 0). The increase in DPPH activity until day 35 suggests the release of antioxidant compounds as plant cell walls are broken down. However, by day 180, DPPH activity decreased considerably. This trend aligns with the phenolic content patterns and reinforces the idea that early ensiling may enhance antioxidant extractability, but that extended fermentation leads to the degradation of these compounds [54,55]. The ABTS assay, which also measures radical scavenging abilities but is more sensitive to hydrophilic and lipophilic compounds, showed a significant increase in antioxidant activity between days 35 and 60 for Wonderful and Mollar. Wonderful exhibited the highest and earliest peak in ABTS values but also a more rapid decline by day 180 when compared to Mollar. These findings suggest that the earlier release of reducing compounds in Wonderful, as evidenced by the initial increase in antioxidant activity, rendered these compounds more susceptible to prolonged ensiling conditions, including pH reduction and microbial load. This pattern supports the findings of Eliyahu et al. [15] and Ahmed et al. [11], who noted that ensiling can both enhance and degrade antioxidant properties depending on the duration and plant matrix.

5. Conclusions

The ensilage strategies implemented in this study effectively preserved both Mollar *de Elche* and Wonderful pomegranate by-products over a 180-day period, ensuring microbial safety while maintaining key nutritional and functional qualities. Both varieties underwent successful lactic acid bacteria (LAB)-driven fermentation, which resulted in favorable Flieg scores and suppressed spoilage organisms. These factors facilitated the retention of most nutritional compounds. Both varieties showed ethanol production, likely due to yeast fermentation, which warrants monitoring to prevent potential quality losses. The total phenol content and antioxidant activity (ABTS and DPPH assays) were also maintained after 180 days. Overall, the peels of both Mollar *de Elche* and Wonderful pomegranates present a viable and nutritious feed resource when properly ensiled and managed, offering the ruminant livestock sector a sustainable alternative that supports both economic viability and environmental stewardship.

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Abbreviations

ABTS	2,2′-azino-bis [3-ethylbenzothiazoline-6-sulfonate)
ADF	Acid Detergent Fiber
ADL	Acid Detergent Lignin
AENOR	Spanish Association for Standardization and Certification
AOAC	Association of Analytical Communities
CP	Crude Protein
DM	Dry Matter
DPPH	1,1-diphenyl-2-picrylhydrazyl
EE	Ether Extract
FM	Fresh Matter
GAE	Gallic Acid Equivalents
GLM	General Linear Model
LAB	Lactic Acid Bacteria
PDDO	Protected Designation of Origin
NDF	Neutral Detergent Fiber
NPN	Non-Protein Nitrogen
SDGs	Sustainable Development Goals
SEM	Standard Error of the Mean
TP	Total Phenolic
VFAs	Short-Chain Organic Acids

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