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Posted Date: 16 September 2024

doi: 10.20944/preprints202409.1251.v1

Keywords: Cheese fatty acids; polyphenols; sustainability; consumer health

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Improving the Sustainability of Caciocavallo Ragusano Cheese Production and Enhancing Its Role on Consumer Health and Liking

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Abstract: In this study, Caciocavallo Ragusano, a typical cheese produced in Sicily Island (Italy), was obtained from milk of dairy cows fed with and without enriched olive cake (ECO and CTR, respectively) in order to evaluate nutritional, microbiological, volatile, and sensory differences in cheeses. ECO cheese showed greater ($p < 0.05$) MUFA and PUFA and polyphenols content, and lower SFA content than CTR cheese. Microbiological analyses revealed the absence of Salmonella, *Listeria monocytogenes*, *Escherichia coli*, and *E. coli* O157, and no significant differences in the viable counts of the remaining microbial groups analyzed, between samples. Thermophilic lactococci were more prevalent in ECO cheese. The implementation of culture-independent method, such as PCR-DGGE analyses, revealed the presence of a more diverse microbial population in both cheeses. Regarding the volatile compounds profile, long-chain free fatty acids were more abundant in the ECO cheese resulting in a healthier free fatty acid profile. This study also showed that, especially for their appearance and taste, the consumers mostly appreciated the ECO cheese. Results showed that using enriched olive cake could enhance the sustainability and the quality of Ragusano cheese, improving not only the health of the consumers but also positively influencing tastes and acceptability.

Keywords: cheese; fatty acids; polyphenols; sustainability; consumer health

1. Introduction

The European cheese production made from bovine milk showed in the last years an increase of +5.8% from 2018 (9,070,916 tons) to 2023 (9,614,010 tons) [1], with a growth forecast in 2024, arousing a particular interest in the environmental impact that the cheese production industry may have worldwide. In a recent study on life cycle assessment of cheese production [2], emerged that raw milk production was the major environmental impact category. Currently, there are several research on the use of waste by-products as natural biosource of beneficial added-value compounds [3], which both decrease the environmental impact caused by food-waste and, at the same time, enhance food nutritional quality [4,5]. To achieve these goals, different strategies are focused on the integration of agro-industrial byproducts in animal nutrition, due to their considerable amounts of bioactive compounds [6]. There are several research on the effect of integration of byproducts into the diet of dairy cows [7], such as dried apple pomace [8], pelleted citrus pulp [9], the mix of tomato and apple pomace [10], and olive cake [11,12]. These research evidence how the use of by-products can give

 doi:10.20944/preprints202409.1251.v

added value to the final product: for example, Amato et al. [13] have recently observed a significant increase of MUFA and PUFA and a decrease in SFA in the milk of cows fed with olive cake enriched in polyphenols. Furthermore, the addition of olive polyphenols in the diet may modify the microbiological profile of cheese as reported by Calabrese et al. [5], which after olive cake supplementation in dairy cow's diet showed an improvement of the microbiological quality of cheese.

Caciocavallo Ragusano is a traditional cheese produced in Sicily, with a production in 2023 of 199 tons and an export amounted to 1,637,550 kg [1], made with a traditional manufacturing process (e.g. using traditional wood tools), where the fermentation is driven by indigenous LAB, which arises from raw milk, biofilm present on the inner surface of the vats, and environment [14]. In this context, the fed supplementation with olive cake could represent a promising strategy to improve both sustainability of the Ragusano cheesemaking and quality of the traditional cheese, more appreciable by consumers. Thus, this study aimed to evaluate the nutritional value, microbiological profiles, sensory features, and volatile aroma compounds of Caciocavallo Ragusano produced with milk obtained from cows fed with olive cake, enriched in polyphenols (ECO).

2. Materials and Methods

2.1. Animal Management and Treatment

The experimental protocol was approved by the Ethical Committee of the Department of Veterinary Science, University of Messina, Italy (code 041/2020). The research complied with guidelines of Good Clinical Practices (The European Agency for the Evaluation of Medicinal Products [15]) and the Italian and European regulations on animal welfare (Council of The European Union [16]).

The study was conducted on a commercial dairy farm in Ragusa (Sicily, Italy), located 500 meters above sea level, with 460 healthy multiparous Friesian dairy cows enrolled in the trial. The animals were randomly allocated into two homogeneous groups (n = 230/group) according to body condition score (BCS; 2.43 ± 0.26), days in milk (DIM; 113 ± 47 d), and milk yield (MY; 31.42 ± 3.28 kg/d).

The management of the cows and the nutritional characteristics of the concentrates and of enriched olive cake were reported by Amato et al. [13].

The experimental group (ECO) received a concentrate supplemented with 7% unconventional OC on a DM basis, and the control group (CTR) received a concentrate with no olive cake incorporation. The enriched OC, with high polyphenols content, was supplemented according to approved UE disciplinary "QS Sicilia" that aims to recover agro-industrial by-products. A flowchart of enriched OC production were reported by Attard et al. [11].

2.1. Cheese Making

After an adaptation period of 3 weeks, from the start of the feeding treatment (April 2023), all cows were mechanically milked twice, evening and morning, and the milk from the two groups was collected separately and transported in refrigerated tanks (4 ± 2 °C) to commercial cheesemaking located in Ragusa. Four hundred (400) liters of milk from each group were used to produce Caciocavallo Ragusano cheese, according to the traditional flowchart reported by Licitra et al. [17]. Samples were brined for 10 days, vacuum-packed, and transported to the Departments of Veterinary Sciences of the University of Messina, Italy, and to the Department of Agriculture, Food and Environment, University of Catania, Italy, for further analyses.

2.2. Chemical, Polyphenols, and Fatty Acid Analyses

The physico-chemical composition of milk samples (pH, SH°, fat, total protein and casein, lactose, and total solids) was determined using a pH meter (Hannah HI9023 pH meter, Hannah Instruments, Keysborough, VIC, Australia) and a Milkoscan FT3 (Foss Electric, Denmark) apparatus.

The cheese samples were analysed for moisture content (AOAC, 2000; method 948.12), protein content (AOAC 2000; method 920.123), and fat content (AOAC 2000; method 933.05). All samples were analysed in triplicate. Fatty acid methyl esters (FAMEs) were obtained from milk and cheese fat

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trans-methylated and analysed through GC-FID, as described by Di Bella et al. [19]. Individual FAMEs were identified, based both on retention time and on the comparison with a standard mixture of 37 pure components (Supelco 37 Component FAME Mix, Merck Life Science, Milano, S.r.l, Italy). Single standards (marked with an asterisk) not included in the Mix were identified as a percentage of the area and the retention time of the individual standard. The determination of milk and cheese total polyphenols was carried out according to the Folin-Ciocalteau method as described by Singleton et al. [20] with some modifications [21]. To 300 µL of cheese extract, 1.5 mL of ultrapure water was pipetted, and reagents were added. First, 2.5 mL of Folin-Ciocalteu reagent, then 2 mL of Na_2CO_3. The tubes containing the solutions were placed in the dark for 90 min and the absorbance was measured at 725 nm against the blank solution. Gallic acid solutions were used as standards.

2.3. Microbiological Analyses

Milk samples were analyzed for the presence of *Enterobacteriaceae*, *Escherichia coli* and coliforms, total mesophilic bacteria, *Listeria monocytogenes,* and *Salmonella* as previously reported by Calabrese and coworkers [5]. In addition, Lactic Acid Bacteria (LAB) were enumerated on de Man, Rogosa, and Sharpe (MRS) agar, enterococci on kanamycin azide agar, both incubated anaerobically at 37 °C for 48 h, whereas Sabouraud Dextrose Agar (SDA), added with chloramphenicol, incubated at 25°C for 72 h, was used for the enumeration of yeasts.

Samples from the surface and inner mass (i.e., rind and core, respectively) of the cheese wheels (weight 500 g) were aseptically collected with a sterile knife from 0-day-ripened cheese wheels [22]. Twenty-five grams of both CTR and ECO cheeses were homogenized with 225 mL of sterile peptone water (Oxoid, Basingstoke, UK) in a Stomacher apparatus (Interscience) for 2 min at 260 rpm [23]. Serial tenfold dilutions were prepared and 100 µL of each dilution was inoculated in duplicate as previously reported by Calabrese et al. [5]. All the media were from Liofilchem (Roseto degli Abruzzi, Italy).

2.4. Total DNA Extraction and PCR-DGGE Analyses

The CTR and ECO milk and cheese samples were subjected to direct extraction of total bacterial DNA as previously reported. After DNA extraction, PCR amplification was performed according to the protocols reported, using the universal PCR primers U968-GC and L1401-r, targeting the V6 to V8 regions of eubacterial 16S rDNA [24]. After verification by electrophoresis on a 1.2% (w/v) agarose gel, 20 µL of the PCR products were used for DGGE analysis on the Dcode System apparatus (BioRad, Hercules, CA, USA). The run was carried out in 8% polyacrylamide (acrylamide/bis-acrylamide mix 37.5:1, w/v) gels with a 40.0 to 60.0% urea–formamide (w/v) gradient (100% denaturant was 7 M urea plus 40%, w/v, formamide) increasing in the direction of electrophoresis. Gels were subjected to a constant voltage of 85 V for 8 h at 60 °C for 16 h. The DNA bands were visualized by silver staining and were developed as previously described [25].

2.5. Volatile Aroma Compound Analysis

The volatile aroma compounds of milk, feeds, and cheese were analyzed by Head Space-Solid Phase Micro Extraction-Gas Chromatography-Mass Spectrometry (HS-SPME-GC-MS). 20 mL of milk, 2 g of feed + 15 mL of saturated NaCl, and 10 g of finely cut cheese + 10 mL of saturated NaCl solution were placed into a 40 mL glass vial and equilibrated for 20 minutes at 40 °C, respectively. A triphasic DVB/CARB/PDMS fiber was then exposed to the headspace for 30 minutes for the extraction of volatile compounds and successively transferred to the injection port of the GC at 260 °C and kept for 3 min. A Shimadzu GC 2010 Plus gas chromatographer coupled with a TQMS 8040 triple quadrupole mass spectrometer (Shimadzu, Milan, Italy) equipped with a VF-WAXms capillary column (60 m. 0.25 mm i.d.; coating thickness 0.25 µm) was used for the GC-MS analyses by using the following conditions: injector temperature, 260 °C; injection mode, splitless; oven temperature, 45 °C held for 5 minutes, then increased to 80 °C at a rate of 10 °C $minute^{-1}$, and to 240 °C at a rate of 2 °C $minute^{-1}$; carrier gas, helium used at a constant flow of 1 ml min^{-1}; transfer line temperature, 250 °C; acquisition range 40-400 m z^{-1}; scan speed, 1250 amu s^{-1}. The identification of compounds was

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made by mass spectral data, NIST'20 (NIST/EPA/NIH Mass Spectra Library, Wiley USA) FFNSC 3.0 database, Linear Retention Indices (LRI), literature data, and the injection of the available standards. The LRI was calculated according to Van den Dool and Kratz equation. The results were expressed as peak area percentages.

2.5. Qualitative Descriptive Analysis

The Qualitative Descriptive Analysis (QDA) of Caciocavallo Ragusano cheese, was carried out according to Merlino et al. [26]. The panel was trained according to ISO 8586-1:1993;9 and a common vocabulary was developed in preliminary sessions for the explanation of the sensory descriptors and to familiarize themselves with scales and procedures. Each descriptor was extensively described and explained to avoid any doubt about the relevant meaning. Based on the frequency of citations, 21 descriptors were selected (yellow color, color uniformity, presence of fracture, presence of lighter colored spots, glossy, milk odor, butter odor, green odor, hay odor, olive cake odor, ripened cheese odor, rancid odor, salty taste, bitter taste, sour taste, pungent taste, juiciness, tenderness, friability, springiness, greasiness, adhesiveness). The judges evaluated the intensity of each descriptor by assigning a score between 1 (absence of the sensation) and 9 (extremely intense). Each judge evaluated the samples in four sessions. All evaluations were carried out from 10.00 to 12.00 A.M. in individual booths illuminated by white light. The order of presentation was randomized among judges and sessions. Water and unsalted crackers were provided to judges between samples. All data were acquired by a direct computerized registration system (FIZZ Byosistemes. ver. 2.00 M, Couternon, France). The results were expressed as the average for each sensory attribute.

2.6. Consumer Acceptability Test

Consumer acceptability was assessed by randomly selected consumers (n=80, 37 males and 43 females, 24–60 years) among the students and personnel of the University of Messina. Participation in the consumer survey was voluntary. Consumers evaluated the samples based on 4 attributes namely appearance, odor, taste, and texture. The scores were recorded based on a 9 points hedonic scale method where 9 = like extremely and 1 = dislike extremely.

2.7. Statistical Analysis

All statistical calculations were carried out using SPSS 13.0 for Windows software (SPSS Inc., Chicago, IL, USA). The initial multivariate matrix consisted of 6 cases (Caciocavallo cheese analyzed) and 54 variables. The data were divided into two groups according to the diet (CTR and ECO). Firstly, the significance of the differences between samples from different diets was assessed using the non-parametric Mann-Whitney U test. The data were then normalized to obtain the independence of the scaling factors of the different variables. After checking the adequacy of the initial data using the Kaiser-Meyer-Olkin (KMO) test and Bartlett's test, the data were subjected to Principal Component Analysis (PCA) to try to differentiate samples from different diets.

One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was applied to microbiological data using the Statistica software (version 10.0 for Windows, TIBCO Software, Palo Alto, CA, USA). Differences were considered statistically significant at $p < 0.05$.

Data from sensory and volatile constituent analyses were analysed using XLStat software, version 2019.1.2 (Addinsoft, Damremont, Paris, France). Two-way Analysis of Variance (ANOVA) and Duncan's multiple range test at a confidence level of 95% were applied to determine significant differences among samples.

3. Results and Discussion

3.1. Chemical Composition

The chemical composition of milk and Caciocavallo Ragusano cheese is shown in Table 1 and in Table 2, respectively. No differences in chemical composition of milk were detected between ECO and CTR. This result shows a different trend from that reported by Chiofalo et al. [27] and Castellani et al. [28], where milk protein of animals fed diets supplemented with olive cake was higher than in

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the relevant control groups. However, differences in chemical composition were observed between ECO and CTR cheese. In fact, the percentage of total lipids of cheese was influenced by the dietary treatment ($p < 0.05$). Cheeses made from milk in the CTR group had a higher fat content (27.49%) than cheese in the ECO group (25.03%). However, no significant differences ($p > 0.05$) were found in cheese protein between the groups.

Table 1. Chemical composition, total polyphenols, and fatty acid profile of milk.

Milk				
	Groups		**SEM**[1]	***p*-value**
Item	**CTR**	**ECO**		
PH	6.68	6.65	0.07	0.26
SH°	3.2	3.2	0.01	0.31
Moisture (%)	86.82	88.21	0.09	0.51
Total proteins (%)	3.36	3.30	0.02	0.27
Casein (%)	2.64	2.59	0.02	0.21
Total lipids (%)	3.76	3.59	0.08	0.06
Lactose (%)	4.54	4.49	0.03	0.33
Total polyphenols (mg/kg)	62.13	109.25	0.12	**0.04**
Fatty acids (g/100g FA)				
C4:0	2.47	2.51	0.13	0.70
C6:0	2.06	1.96	0.18	0.85
C8:0	0.97	1.02	0.05	0.26
C10:0	2.77	2.60	0.04	0.55
C11:0	0.06	0.13	0.45	**0.05**
C12:0	3.52	3.15	0.11	0.09
C13:0	0.09	0.13	0.07	**0.04**
C14:0	12.92	12.10	0.04	**0.05**
C15:0 iso*	0.13	0.14	0.07	0.19
C15:0 anteiso*	0.01	0.01	0.0001	1.00
C15:0	1.53	1.48	0.04	0.51
C16:0	28.14	27.02	0.02	0.06
C17:0	1.22	0.93	0.16	**0.05**
C18:0	13.50	12.17	0.06	**0.05**
C20:0	0.56	0.30	0.38	0.09
C21:0	0.07	0.05	0.19	0.06
C22:0	0.04	0.09	0.16	**0.05**
C23:0	0.08	0.09	0.09	**0.05**
C24:0	0.02	0.01	0.05	0.20
SFA	70.17	65.92	0.04	**0.05**
C14:1	0.91	0.95	0.06	0.50

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C15:1	0.12	0.11	0.17	0.66
C16:1	1.79	1.93	0.05	**0.05**
C16:1 trans*	0.01	0.01	0.000	1.00
C16:1 n-5	0.01	0.01	0.01	1.00
C17:1	0.27	0.30	0.11	0.27
C18:1 cis9	15.91	18.26	0.08	**0.05**
C18:1 trans9	0.68	0.78	0.09	**0.05**
C18:1 cis11*	3.08	3.64	0.11	0.07
C18:1 trans11*	0.25	0.31	0.17	0.13
C20:1 n-11	0.06	0.06	0.08	0.98
C22:1 n-9	0.01	0.01	0.36	0.94
C24:1 n-9	0.03	0.04	0.05	0.11
MUFA	23.14	26.42	0.07	**0.05**
C16:3 n-4*	0.04	0.07	0.30	0.06
C18:2 cis9 cis12	1.38	1.60	0.21	0.09
C18:2 trans9 trans12	0.67	0.66	0.09	0.98
C18:3 cis6 cis9 cis12	0.05	0.05	0.03	0.19
C18:3 cis9 cis12 cis15	0.71	1.06	0.24	**0.05**
C20:2 n-6	0.05	0.06	0.15	0.09
C20:3 n-6	0.04	0.03	0.16	0.45
C20:3 n-3	0.01	0.01	0.28	0.32
C20:4 n-6	0.04	0.05	0.19	0.09
C20:5 n-3	0.06	0.07	0.12	0.09
C22:2	0.10	0.14	0.24	0.06
C22:6 n-3	0.02	0.04	0.31	0.07
PUFA	3.15	3.85	0.13	**0.05**
n3	0.80	1.18	0.23	**0.05**
n6	2.22	2.45	0.14	0.51
n6/n3	2.82	2.10	0.25	0.27

* Individual standards not included in the Mix. [1] SEM (Standard Error of Mean).

Table 2. Chemical composition, total polyphenols, and fatty acid profile of Caciocavallo cheeses.

	Caciocavallo cheese			
	Groups		SEM[1]	*p*-value
Item	**CTR**	**ECO**		
PH	-	-	-	-
SH°	-	-	-	-
Moisture (%)	37.74	39.96	0.01	**0.04**
Total proteins (%)	30.24	31.57	0.02	0.27

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Casein (%)	-	-	-	-
Total lipids (%)	27.49	25.03	0.02	**0.05**
Lactose (%)	-	-	-	-
Total polyphenols (mg/kg)	110.91	232.85	0.18	**0.03**
Fatty acids (g/100g FA)				
C4:0	2.73	2.65	0.07	0.27
C6:0	2.37	2.58	0.02	**0.05**
C8:0	1.80	1.74	0.02	0.27
C10:0	3.58	3.65	0.01	0.51
C11:0	0.03	0.06	0.13	**0.04**
C12:0	3.70	3.58	0.01	**0.05**
C13:0	0.09	0.11	0.05	**0.04**
C14:0	11.11	10.37	0.02	**0.05**
C15:0 iso*	0.25	0.23	0.03	0.10
C15:0 anteiso*	0.01	0.01	0.000	1.00
C15:0	1.00	1.01	0.01	0.66
C16:0	27.93	27.85	0.003	0.83
C17:0	0.55	0.46	0.06	**0.05**
C18:0	15.03	14.45	0.01	**0.04**
C20:0	0.94	1.04	0.03	0.51
C21:0	0.04	0.03	0.08	0.09
C22:0	0.05	0.07	0.07	**0.03**
C23:0	0.04	0.05	0.09	**0.04**
C24:0	0.04	0.04	0.07	0.19
SFA	71.29	69.98	0.005	**0.05**
C14:1	0.97	0.98	0.01	0.38
C15:1	0.21	0.28	0.07	**0.05**
C16:1	1.35	1.71	0.07	**0.05**
C16:1 trans*	0.01	0.01	0.000	1.00
C16:1 n-5	0.01	0.01	0.000	1.00
C17:1	0.59	0.52	0.06	0.51
C18:1 cis9	16.43	18.12	0.03	**0.04**
C18:1 trans9	1.67	0.98	0.12	**0.05**
C18:1 cis11*	2.10	2.41	0.05	0.38
C18:1 trans11*	0.01	0.02	0.15	0.11
C20:1 n-11	0.10	0.10	0.02	0.19
C22:1 n-9	0.01	0.01	0.000	1.00
C24:1 n-9	0.02	0.03	0.11	0.09
MUFA	23.48	25.19	0.02	**0.03**

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C16:3 n-4*	0.01	0.01	0.000	1.00
C18:2 cis9 cis12	0.75	0.93	0.05	**0.05**
C18:2 trans9 trans12	0.40	0.38	0.02	0.37
C18:3 cis6 cis9 cis12	0.27	0.31	0.04	**0.05**
C18:3 cis9 cis12 cis15	0.41	0.43	0.04	0.83
C20:2 n-6	0.05	0.05	0.05	0.19
C20:3 n-6	0.06	0.04	0.07	0.07
C20:3 n-3	0.01	0.01	0.000	1.00
C20:4 n-6	0.02	0.03	0.16	0.09
C20:5 n-3	0.06	0.07	0.08	0.09
C22:2	0.26	0.22	0.05	0.27
C22:6 n-3	0.01	0.02	0.15	0.11
PUFA	2.30	2.50	0.02	**0.05**
n3	0.48	0.53	0.03	0.12
n6	1.55	1.74	0.03	**0.05**
n6/n3	3.22	3.30	0.03	0.83

*Individual standards not included in the Mix. [1]SEM (Standard Error of Mean).

The fatty acid composition of milk and cheese is reported in Table 1 and Table 2, respectively. An increase in monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) and a decrease in saturated fatty acid (SFA) were observed in the ECO samples. In fact, the diet showed an increase in C16:1, C18:1 cis9, C18:3 cis6 cis9 cis12 and a decrease in C12:0, C14:0, C17:0 and C18:0. Among MUFAs, C16:1, C18:1cis9 and C18:1 trans9 are statistically different between the two groups ($p < 0.05$), where only C18:1 trans9 is significantly higher in the CTR samples. In contrast, among the PUFAs, the only fatty acid that is statistically different between the two groups is C18:3 cis6 cis9 cis12, which is significantly higher in the ECO samples. As far as the fatty acid profile is concerned, the supplementation of olive cake reduced the short- and medium-chain fatty acids synthesized entirely "de novo" and partially by the mammary gland from rumen-produced acetate and beta-hydroxybutyrate, respectively [29]. Medium-chain fatty acids are responsible for increased concentrations of low-density lipoprotein cholesterol in the blood if they are not associated with the correct levels of linoleic acid [30]. The high C18:1 cis9 content observed in ECO cheeses is probably related to two reasons: the high oleic acid content of olive pomace, and probably the desaturation of stearic acid in the mammary gland by delta9-desaturase [31], encoded by the stearoyl-coenzyme A desaturase gene [32], as reported by Chiofalo et al. [27] and Vargàs-Bello-Pérez et al. [33] in dairy sheep, and by Terramoccia et al. [34] in buffaloes fed with an olive pomace supplementation; the other reason could be a reduced biohydrogenation rate of the oleic acid intermediate by Butyrivibrio [35]. Similar conclusions were reached by Vargàs-Bello-Pérez et al. [36] on milk samples from small ruminants fed with olive pomace, Symeou et al. [37] in studies on dairy sheep, and Castellani et al. [28], Neofytou et al. [38], and Amato et al. [13] in studies on dairy cattle fed with olive cake.

Olive cake supplementation results in increased total polyphenols in ECO samples of milk and cheese (Table 1 and Table 2), confirming that olive polyphenols are absorbed in the gastrointestinal tract [39]. However, it is worth noting that low concentrations of polyphenols have also been measured in CTR samples; in this regard, previous studies have shown that some polyphenols such as hydroxytyrosol and tyrosol are generated endogenously in small amounts as by-products of tyramine and dopamine metabolism [40]. However, the higher amount of polyphenols in ECO cheese and milk reflects the significant amounts of polyphenols present in the OC [41]. The intake of these

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bioactive compounds is considered beneficial to human health as they enhance the natural defence system by reducing the formation of free radicals and harmful oxidative events in metabolism [42].

3.2. Principal Component Analysis

PCA was applied to the normalized data using the variables found to be significantly different in the analysis to discriminate between the two diets. The suitability of the data for factor analysis was checked. The Kaiser-Meyer-Olkin sampling adequacy measure gave a value of 0.658 and Bartlett's sphericity test showed an approximate chi-square value of 710.157, so the correlation matrix was factored and fit for PCA.

According to the Kaiser-Guttman criterion, three principal components were extracted with eigenvalues greater than one (18.247, 2.019, and 1.314), which together explain 93.828% of the total variance (79.336%, 8.777%, and 5.714% respectively) for Caciocavallo cheese, whereas, three principal components were extracted with eigenvalues greater than one (18.177, 2.627 and 1.029), which together explain 92.036% of the total variance (78.683%, 7.676% and 5.677% respectively) for milk. Since there were no variables with low saturation in each factor and the commonality was always above 0.740, the extracted components were able to reproduce all the variables well. Analysis of the correlation matrix showed that the highest positive correlations were observed for C14:0 lipids (0.982), MUFA-C22:0 (0.976), MUFA-C18:1 cis9 and PUFA-C15:1 (0.960), whereas the highest negative correlations were observed for MUFA-C14:0 (-0.988), MUFA-C18:0 (-0.956) and C22:0 lipids (-0.949). Figure 1 shows the 2D scatterplots on the plane defined by PC1 and PC2 for the Caciocavallo cheese and milk samples.

 doi:10.20944/preprints202409.1251.v

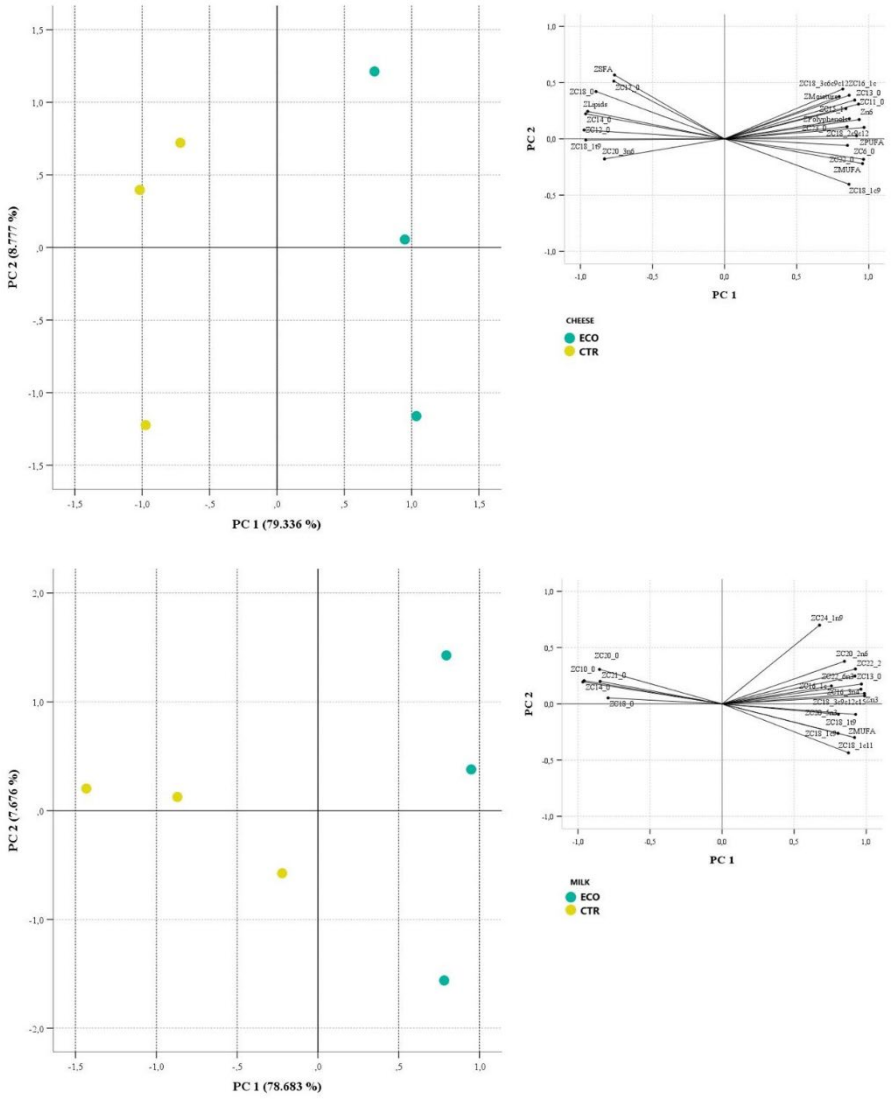

Figure 1. Two 2D Scatterplots for cheese and milk samples categorized by diet. Insert: loading plot for PC1 and PC2.

Two groups can be clearly distinguished for cheese and two groups for milk: the ECO samples are separated from the CTR samples on the first component, which explains 79.336% and 78.683% of the total variances; the ECO samples are positioned at positive values of PC1 and are characterized by higher values of MUFA, C18:2 cis9 cis12, C22:0 and polyphenols, while the CTR samples are positioned at negative values of PC1 and have higher values of C12:0, C14:0 and lipids.

3.3. Microbiological Analysis

Microbiological analyses revealed the compliance of milk quality with the legal requirements (Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs (n.d.)) for the production of raw milk cheeses. Indeed, the Salmonella spp. and L. monocytogenes milk pathogens were absent, whereas the count of S. aureus was below the detection limits (Table 3).

Table 3. Microbial counts (expressed as mean log10 cfu/mL ± standard deviation) of milk samples.

Microbial groups	CTR	ECO	*p*-values
Enterococcus spp.	3.04±0.13	3.15±0.15	0.34
Enterobacteriaceae	2.70±0.35	2.43±0.23	0.24

 doi:10.20944/preprints202409.1251.v

S. aureus	0.93±1.60	0.95±0.1.64	0.11
Total mesophilic bacteria	4.54±0.25	4.42±0.26	0.13
Yeasts&moulds	2.57±0.20	2.42±0.10	0.84
Lactococcus spp.	3.11±0.02	3.20±0.02	0.41
Thermophilic lactococci	3.77±0.04	5.09±0.75	**0.01**
Lactic acid bacteria	4.55±0.18	4.45±0.08	0.41
E. coli	1.47±1.29	0.67±1.15	**0.01**
Total coliforms	1.80±1.56	1.33±1.15	0.41
Salmonella spp.	Absent	Absent	---
L. monocytogenes	Absent	Absent	---

Although many factors can influence the microbial composition of raw milk [43,44] such as milking conditions, temperature, duration of milk storage and transport as well as the diet of the cows [45–47] no statistical differences ($p > 0.05$) were observed for the analysed microbial groups, except for E. coli, and thermophilic lactococci densities (Table 3). Indeed, compared to CTR milk, in ECO samples the number of E. coli was lower, while the thermophilic lactococci were more abundant. Overall, both LAB and the total mesophilic bacteria were the most abundant counted groups, with a density above 4.00 log cfu/mL in both CTR and ECO milk, followed by lactococci and enterococci. Concerning Enterobacteriaceae, molds, and yeasts, the viable count was below 3.00 log cfu/mL and comparable for both milk samples, whereas the lowest count was observed for total coliforms, especially in ECO milk.

Regarding cheese samples, a general increase in almost all microbial groups investigated was observed. The levels of the viable count of the CTR and ECO cheese samples are shown in Table 4.

Table 4. Microbial counts (expressed as mean log10 cfu/g ± standard deviation) of cheese samples.

Microbial groups	CTR	ECO	P-values
Enterococcus spp.	5.04±0.19	5.15±0.15	0.65
Enterobacteriaceae	2.54±0.34	2.72±0.17	0.33
Coagulase+ staphylococci	4.48±0.01	5.29±0.30	0.13
Total mesophilic bacteria	6.38±0.11	6.23±0.11	0.62
Yeasts&moulds	6.57±0.26	6.42±0.17	0.84
Lactococcus spp.	6.91±0.02	6.68±1.42	0.35
Thermophilic lactococci	2.77±0.04	5.29±0.75	**0.01**
Lactic acid bacteria	7.34±0.48	7.03±0.28	0.41
E. coli O157	Absent	Absent	---
E. coli	Absent	Absent	---
Salmonella spp.	Absent	Absent	---
L. monocytogenes	Absent	Absent	---

Overall, all undesired species such as Salmonella spp., L. monocytogenes, E. coli, and E. coli O157, generally associated with poor hygiene of dairy production, were never detected in the analyzed samples, reinforcing the safety of dairy environment [14]. Moreover, the viable counts of the remaining analyzed microbial groups did not significantly differ ($p > 0.05$) among all samples, except for the thermophilic lactococci whose density was significantly higher ($p < 0.05$) in ECO cheeses (Table 4). Although, the cell density of thermophilic LAB usually increases after cheese manufacture, and it is well known that the microbial dynamics leading from raw cows' milk to pasta filata cheese include all typical manufacturing steps [48], the observed increase in thermophilic lactococci levels in milk and later in cheeses here detected can probably be explained by the resistance of this group to polyphenol content of the olive cake supplementation. A previous work revealed the high resistance of lactococci to different classes of polyphenols, intentionally inoculated into raw ewe's milk, showing their ability to perform a rapid acidification [49]. It is also noteworthy that

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Lactococcus genus, significantly contribute to the texture and flavor characteristics of fermented products, by enhancing the rheological properties of fermented milk products [50] through the production of extracellular polysaccharides, In this study, the lactococci counts were between 6.91 and 6.68 log cfu/g and presumptive LAB count ranged from 7.34 to 7.03 log cfu/g, in the CTR sample and the ECO cheese, respectively. Notably, the pro-technological groups, including LAB and both mesophilic and thermophilic cocci dominated the microbial community, which together with enterococci were not affected by the thermal treatment applied during stretching, whose consistent increase could be due to later milk contact with the wooden vat [51]. It is well known that these bacteria play a crucial role during cheese fermentation, mainly in the development of sensorial characteristics and, generally, in the consistent contribution to the typicality of traditional cheeses [52]. Although the stretching operation, typical of Caciocavallo Ragusano cheese making, is known to exert a sanitizing effect [53], the thermal shock applied with stretching did not determine the reduction of the presumptive coagulase-negative staphylococci group in cheeses, where their levels were quite consistent (4.48 in the CTR and 5.29 log cfu/g in the ECO samples). Concerning the enterococci group, the reached value was about 5.00 log cfu/g for both samples, while the Enterobacteriaceae count was comprised between 2.54 and 2.27 log cfu/g in the CTR and ECO cheese, respectively. Finally, no marked differences were observed between the cheeses for both total mesophilic bacteria and eumycetic population, which compared to milk have increased in the same way.

3.4. PCR-DGGE Analyses

The DGGE profiles of the milk and cheese samples are shown in Figure 2.

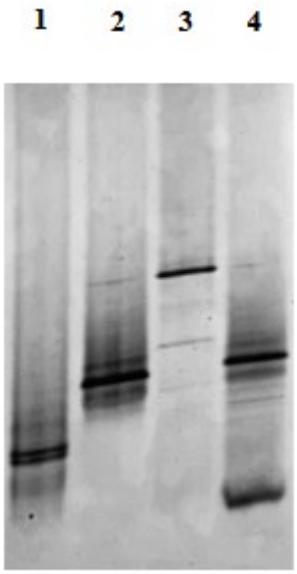

Figure 2. PCR-DGGE profiles of CTR milk (line 1), CTR cheese (line 2), ECO milk (line 3), and ECO cheese (line 4).

Overall, the analyses showed differences in terms of the number of bands or relative abundance and intensity between the two types of samples and within the same group. In particular, the DGGE profiles of the CTR and ECO milk samples (lines 1 and 3, respectively) consisted of a few bands located at different heights within the gel, showing substantial differences between the two samples. In particular, one to two marked bands were observed in each sample, while both profiles were characterized by a greater number of weak bands, occasionally found in the corresponding cheese samples. Similarly, the DGGE profiles of the cheese (lines 2 and 4) shared some bands, of varying intensity, one of which was also with the ECO milk sample, where it was more prominent. Furthermore, the ECO cheese showed several weaker bands, not revealed in the CTR sample, presumably attributable to other species involved in the cheesemaking. These results confirm the

 doi:10.20944/preprints202409.1251.v

importance to coupled different techniques (cultur-dependent and independent) to study more in-depth microbial population and its dynamics during the cheese production [54].

3.4. Volatile Constituents

3.5.1. Feeds and Olive Cake

In Table 5 the volatile percentage composition in single compounds and classes of substances for the feeds, CTR and ECO cheese, and olive cake are reported.

Table 5. Volatile composition (peak area % ± SD) of control and ECO feeds and olive cake.

Compounds	LRI	Control	ECO	Olive cake
Acids				
Acetic acid	1455	tr^{a}	tr^{a}	0.36±0.02^{b}
Propionic acid	1540	tr^{a}	tr^{a}	0.23±0.01^{b}
2-Methyl-propanoic acid	1566	0.62±0.04^{b}	0.55±0.04^{b}	0.28±0.02^{a}
Butanoic acid	1629	tr^{a}	tr^{a}	0.94±0.06^{b}
2-Methyl-butanoic acid	1666	3.14±0.12^{c}	2.64±0.22^{b}	0.85±0.05^{a}
Hexanoic acid	1844	1.62±0.18^{a}	5.02±0.41^{b}	5.58±0.41^{b}
Heptanoic acid	1950	tr^{a}	0.77±0.06^{b}	0.85±0.06^{b}
Octanoic acid	2060	0.26±0.03^{a}	0.94±0.07^{b}	1.06±0.08^{b}
Nonanoic acid	2165	tr^{a}	0.71±0.03^{b}	0.74±0.04^{b}
Tetradecanoic acid	2694	0.90±0.07^{a}	0.87±0.07^{a}	0.39±0.02^{b}
Pentadecanoic acid	2799	0.26±0.03^{b}	0.26±0.03^{b}	tr^{a}
Hexadecanoic acid	2906	3.23±0.16^{a}	3.63±0.18^{b}	3.89±0.31^{b}
All		**10.02±0.63^{a}**	**15.39±1.11^{b}**	**15.18±1.08^{c}**
Aldehydes				
Pentanal	985	0.85±0.05^{a}	1.23±0.07^{b}	0.66±0.04^{a}
Hexanal	1085	9.79±0.57^{c}	8.45±0.42^{b}	4.42±0.22^{a}
Heptanal	1188	1.21±0.08^{a}	2.44±0.17^{b}	2.03±0.14^{b}
Octanal	1292	3.54±0.14^{a}	5.76±0.23^{b}	6.86±0.27^{c}
(*Z*)-2-Heptenal	1328	0.58±0.03^{a}	0.50±0.02^{a}	1.39±0.07^{b}
Nonanal	1395	3.71±0.19^{a}	10.31±0.61^{b}	24.04±1.44^{c}
(*E*)-2-Octenal	1431	0.96±0.06^{a}	0.92±0.05^{a}	0.80±0.04^{a}
Decanal	1499	0.37±0.01^{a}	0.42±0.02^{a}	1.25±0.05^{b}
Benzaldehyde	1527	0.24±0.02^{a}	0.55±0.04^{a}	2.01±0.14^{b}
(*E*)-2-Nonenal	1535	0.39±0.02^{b}	0.26±0.02^{b}	tr^{a}
(*E*)-2-Decenal	1645	0.29±0.01^{a}	0.93±0.05^{b}	0.80±0.04^{b}
(*Z*)-8-Undecenal	1750	tr^{a}	0.30±0.02^{b}	tr^{a}
All		**21.95±1.18^{a}**	**32.07±1.72^{b}**	**44.27±2.45^{c}**
Alcohols				
Ethanol	937	tr^{a}	tr^{a}	0.23±0.01^{b}
5-Ethyl-2-heptanol	1121	tr^{a}	tr^{a}	0.25±0.02^{b}
1-Butanol	1143	0.32±0.02^{b}	0.29±0.01^{b}	tr^{a}
2-Methyl-1-butanol	1158	0.26±0.01^{b}	tr^{a}	tr^{a}
3-Methyl-1-butanol	1205	0.39±0.02^{b}	0.27±0.01^{a}	0.27±0.02^{a}
1-Pentanol	1247	6.27±0.34^{c}	4.75±0.19^{b}	tr^{a}
Heptan-2-ol	1315	0.35±0.02^{b}	tr^{a}	tr^{a}
3-Methyl-1-pentanol	1351	25.90±1.18^{c}	13.40±0.40^{b}	2.06±0.06^{a}
1-Octen-3-ol	1444	5.27±0.26^{c}	4.04±0.18^{b}	2.08±0.04^{a}
1-Heptanol	1450	7.57±0.45^{c}	5.77±0.28^{b}	0.29±0.01^{a}
6-Methyl-5-hepten-2-ol	1457	0.38±0.04^{b}	0.34±0.03^{b}	tr^{a}
2,4-Dimethyl-cyclohexanol	1478	0.31±0.02^{b}	tr^{a}	tr^{a}
2-Ethyl-1-hexanol	1484	0.26±0.01^{b}	tr^{a}	0.28±0.02^{b}

 doi:10.20944/preprints202409.1251.v

1-Octanol	1553	7.21±0.10^{c}	6.78±0.06^{b}	0.98±0.01^{a}
(Z)-2-Octen-1-ol	1612	tr^{a}	0.23±0.01^{b}	0.26±0.01^{b}
1-Nonanol	1656	0.87±0.04^{b}	1.17±0.06^{c}	tr^{a}
Guaiacol	1859	tr^{a}	tr^{a}	0.29±0.02^{b}
Benzyl alcohol	1875	tr^{a}	0.47±0.04^{b}	0.89±0.07^{b}
Phenethyl alcohol	1909	tr^{a}	0.58±0.05^{b}	1.10±0.09^{c}
Creosol	1954	tr^{a}	tr^{a}	3.30±0.26^{b}
4-Ethyl-phenol	2176	tr^{a}	0.64±0.05^{b}	6.04±0.48^{c}
All		**55.37±2.51^{c}**	**38.74±1.37^{b}**	**18.33±1.12^{a}**
Ketones				
Heptan-2-one	1185	1.21±0.09^{b}	0.90±0.07^{a}	0.78±0.05^{a}
2-Octanone	1287	0.47±0.03^{a}	0.43±0.02^{a}	0.72±0.06^{b}
1-Octen-3-one	1304	tr^{a}	tr^{a}	0.25±0.02^{b}
6-Methyl-5-hepten-2-one	1336	2.27±0.18^{b}	1.67±0.11^{a}	2.12±0.09^{b}
4-Ethyl-cyclohexanone	1344	0.51±0.03^{b}	tr^{a}	tr^{a}
2-Nonanone	1389	0.60±0.04^{a}	0.66±0.03^{a}	1.79±0.13^{b}
Oct-3-en-2-one	1409	1.96±0.14^{a}	3.07±0.31^{b}	3.40±0.45^{b}
6-Methoxy-2-hexanone	1421	0.27±0.03^{b}	tr^{a}	tr^{a}
2-Decanone	1492	0.29±0.04^{a}	0.45±0.05^{b}	0.48±0.05^{b}
3,5-Octadien-2-one	1520	tr^{a}	0.52±0.03^{b}	0.31±0.01^{b}
(*E*,*E*)-3,5-Octadien-2-one	1572	0.48±0.04^{b}	0.41±0.03^{b}	tr^{a}
6-Methyl-3,5-heptadiene-2-one	1594	tr^{a}	0.40±0.02^{b}	1.02±0.05^{c}
Acetophenone	1652	tr^{a}	tr^{a}	0.35±0.02^{b}
Nerylacetone	1851	tr^{a}	tr^{a}	0.29±0.01^{b}
All		**8.06±0.62^{a}**	**8.51±0.67^{a}**	**11.51±0.94^{b}**
Esters				
Methyl acetate	833	tr^{a}	tr^{a}	0.68±0.04^{b}
Ethyl acetate	893	tr^{a}	tr^{a}	0.55±0.03^{b}
Methyl propionate	912	tr^{a}	tr^{a}	0.38±0.03^{b}
Ethyl propanoate	961	tr^{a}	tr^{a}	0.29±0.02^{b}
Methyl butyrate	991	tr^{a}	tr^{a}	0.25±0.02^{b}
Ethyl butanoate	1038	tr^{a}	tr^{a}	0.41±0.03^{b}
Methyl nonanoate	1489	tr^{a}	tr^{a}	0.71±0.05^{b}
Pentyl hexanoate	1511	0.24±0.02^{a}	0.50±0.04^{b}	0.51±0.04^{b}
Hexyl hexanoate	1607	tr^{a}	0.66±0.05^{b}	Tr^{a}
All		**0.24±0.02^{a}**	**1.17±0.09^{b}**	**3.78±0.26^{c}**
Lactones				
γ-Octalactone	1916	0.36±0.02^{b}	0.76±0.05^{c}	tr^{a}
γ-Nonalactone	2028	1.09±0.07^{a}	1.43±0.12^{b}	6.04±0.47^{c}
All		**1.45±0.09^{a}**	**2.19±0.17^{b}**	**6.04±0.47^{c}**
Oxides				
cis-Linalool oxide	1439	0.95±0.08^{c}	0.58±0.03^{b}	tr^{a}
trans-Linalool oxide (furanoid)	1467	0.44±0.02^{c}	0.31±0.01^{b}	tr^{a}
All		**1.39±0.10^{c}**	**0.89±0.04^{b}**	**tr^{a}**
Furans				
2-Pentyl-furan	1230	1.52±0.12^{c}	1.06±0.07^{b}	0.89±0.05^{a}
All		**1.52±0.12^{c}**	**1.06±0.07^{b}**	**0.89±0.05^{a}**

LRI= Linear retention index. tr= traces Different superscript lowercase letters in the same row indicate significant differences at $p < 0.05$ among samples by Duncan's multiple range test.

 doi:10.20944/preprints202409.1251.v

As results from the table, a large number of volatiles have been identified, belonging to different classes of substances such as aliphatic acids, alcohols, ketones, esters, and aldehydes; moreover, terpenes, lactones, furans, and aromatic compounds have been identified, too.

The olive cake used in this research has been characterized by high amounts of aldehydes which constituted the 44% about of the volatile fraction, with nonanal the main compounds; free fatty acids followed. The high number of aliphatic aldehydes agrees with the higher level of oleic acid in the olive oil; in fact, aldehydes are common products of the decomposition of hydroperoxides developed by the oxidation of unsaturated fatty acid (UFA).

Comparing the composition of the control fed with that of ECO, an increase of aldehyde compounds resulted in agreement with the olive cake composition; otherwise, a decrease of alcohols mainly due to 3-methyl-1-pentanol resulted in the ECO. A high amount of 3-methyl-1-pentanol is present in the fed control; it is a plant metabolite but could be due to Saccharomyces cerevisiae fermentation.

3.5.2. Milk and Cheese

In Table 6 the volatile percentage composition in single compounds and classes of substances for the milk and cheese both CTR and ECO are reported. The volatiles belonged to different classes of substances, all these well known in the volatile fraction of dairy products, such as aliphatic acids, alcohols, ketones, esters, and aldehydes; moreover terpenes, lactones, and aromatic compounds have been identified, too.

Table 6. Volatile constituents (peak area % ± SD) of CTR and ECO cheese and milk samples.

		Cheese			Milk		
Compounds	**LRI**	**Control**	**Biotrak**		**Control**	**Biotrak**	
Acids							
Acetic acid	1460	0.90±0.06^{b}	0.33±0.02^{a}	**	2.19±0.15^{b}	0.13±0.01^{a}	***
Propanoic acid	1543	tr^{a}	0.46±0.03^{b}	***	-	-	ns
Butanoic acid	1631	3.24±0.16^{b}	1.14±0.07^{a}	**	0.51±0.04^{a}	0.93±0.03^{b}	*
Hexanoic acid	1740	9.09±0.63^{b}	3.90±0.31^{a}	*	1.07±0.05^{a}	1.05±0.04^{a}	ns
Heptanoic acid	1954	0.22±0.01^{a}	0.16±0.01^{a}	*	0.37±0.02^{b}	Tr^{a}	*
Octanoic acid	2062	3.79±0.18^{b}	2.88±0.14^{a}	*	1.21±0.06^{a}	1.65±0.08^{b}	ns
Nonanoic acid	2170	2.02±0.10^{a}	1.96±0.08^{a}	ns	3.49±0.21^{b}	1.09±0.06^{a}	**
(E)-2-Octenoic acid	2184	tr^{a}	0.36±0.02^{b}	**	-	-	ns
Decanoic acid	2276	2.24±0.11^{b}	1.87±0.07^{a}	*	5.21±0.36^{b}	4.00±0.28^{a}	ns
(E)-9-Decenoic acid	2332	-	-	ns	0.08±0.01^{a}	Tr^{a}	ns
Undecanoic acid	2379	0.27±0.02^{a}	0.23±0.02^{a}	ns	0.26±0.02^{a}	0.29±0.02^{a}	ns
(E)-2-Decenoic acid	2399	0.15±0.01^{a}	0.37±0.03^{a}	*	0.24±0.02^{a}	0.32±0.03^{a}	ns
Benzoic acid	2430	-	-	ns	0.32±0.01^{a}	0.29±0.02^{a}	ns
Dodecanoic acid	2488	1.70±0.12^{a}	2.03±0.16^{a}	ns	2.32±0.13^{a}	3.25±0.23^{b}	ns
Tridecanoic acid	2587	tr^{a}	0.23±0.01^{a}	*	0.34±0.02^{a}	0.54±0.04^{b}	ns
Tetradecanoic acid	2698	6.27±0.25^{a}	11.57±0.81^{b}	*	22.69±1.34^{a}	25.67±1.26^{b}	ns
Pentadecanoic acid	2799	2.50±0.15^{a}	3.34±0.23^{b}	ns	3.13±0.10^{a}	3.44±0.08^{b}	ns
Hexadecanoic acid	2810	24.42±1.22^{a}	41.36±1.65^{b}	*	43.50±2.26^{a}	48.72±2.59^{b}	ns
All		**56.81±3.02^{a}**	**72.19±3.66^{b}**	*	**86.93±4.80^{a}**	**91.37±4.77^{b}**	**ns**
Short-Medium		**21.92±1.28^{b}**	**13.66±0.80^{a}**	*	**14.63±0.94^{b}**	**9.46±0.55^{a}**	*
Long		**34.89±1.74^{a}**	**58.53±2.86^{b}**	*	**71.98±3.86^{a}**	**81.91±4.22^{b}**	*
Ratio S-M/L		**0.63±0.74^{a}**	**0.23±0.28^{a}**	*	**0.20±0.24^{b}**	**0.11±0.13^{a}**	*
Alcohols							
Ethanol	938	0.74±0.05^{a}	4.15±0.54^{b}	***	0.16±0.01^{a}	tr^{a}	*
2-Methyl-propanol	1096	0.27±0.02^{a}	0.13±0.01^{a}	*	-	-	ns
Isoamyl alcohol	1206	1.73±0.12^{a}	5.39±0.85^{b}	*	-	-	ns
3-Methyl-butanol	1212	-	-	ns	0.04±0.01^{a}	tr^{a}	ns

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Pentanol	1250	-	-	ns	0.15±0.01[a]	0.20±0.02[a]	ns
3-Methyl-2-buten-1-ol	1319	tr[a]	0.07±0.01[a]	*	-	-	ns
Hexanol	1350	0.08±0.01[a]	tr[a]	*	0.08±0.01[a]	tr[a]	ns
2-Ethyl-hexanol	1482	0.61±0.04[a]	0.42±0.03[a]	ns	0.61±0.04[b]	0.42±0.03[a]	ns
Octanol	1550	0.13±0.01[a]	0.10±0.01[a]	ns	0.13±0.01[a]	0.10±0.01[a]	ns
Dodecanol	1970	tr[a]	0.72±0.05[b]	*	-	-	ns
Tetradecanol	2174	tr[a]	0.29±0.03[a]	*	-	-	ns
All		**3.56±0.25[a]**	**11.27±1.53[b]**	**	**1.17±0.09[b]**	**0.72±0.06[a]**	**ns**
Aldehydes							
Pentanal	984	-	-	ns	0.31±0.02[b]	0.21±0.01[a]	ns
Hexanal	1085	1.48±0.07[b]	1.06±0.04[a]	ns	1.58±0.13[b]	0.67±0.04[a]	*
Heptanal	1189	0.82±0.04[b]	0.32±0.02[a]	*	-	-	ns
Octanal	1282	-	-	ns	0.35±0.02[b]	tr[a]	*
Nonanal	1394	2.17±0.15[a]	1.99±0.11[a]	ns	1.26±0.11[a]	1.30±0.12[b]	ns
Decanal	1491	-	-	ns	0.78±0.06[b]	tr[a]	*
Undecanal	1598	-	-	ns	0.47±0.03[b]	tr[a]	*
All		**4.47±0.26[b]**	**3.37±0.17[a]**	**ns**	**4.75±0.37[b]**	**2.18±0.17[a]**	*
Ketones							
2-Butanone	910	0.84±0.06[a]	0.52±0.04[a]	ns	1.67±0.11[b]	1.47±0.09[a]	ns
2,3-Butanedione	985	8.76±0.35[b]	1.61±0.08[a]	***	-	-	ns
2,3-Pentanedione	1062	0.24±0.02[a]	0.12±0.01[a]	*	-	-	ns
3-Heptanone	1155	0.08±0.01[a]	tr[a]	*	-	-	ns
2-Heptanone	1185	10.01±0.48[b]	1.54±0.12[a]	***	0.56±0.04[a]	0.70±0.05[b]	ns
Acetoin	1294	9.82±0.59[b]	5.63±0.22[a]	*	-	-	ns
2,5-Octanedione	1316	-	-	ns	0.11±0.01[b]	tr[a]	*
6-Methyl-5-hepten-2-one	1338	0.12±0.01[a]	0.10±0.01[a]	ns	-	-	ns
2-Nonanone	1389	0.48±0.03[a]	0.11±0.01[a]	**	0.16±0.01[b]	0.09±0.07[a]	*
All		**30.35±1.55[b]**	**9.63±0.49[a]**	***	**2.50±0.17[b]**	**2.26±0.21[a]**	**ns**
Esters							
Ethyl acetate	896	0.26±0.03[a]	0.66±0.05[a]	*	-	-	ns
Ethyl propanoate	962	0.04±0.01[a]	0.22±0.01[a]	***	-	-	ns
Ethyl butanoate	1039	0.31±0.02[a]	0.63±0.04[a]	*	0.13±0.01[b]	tr[a]	*
Isoamyl acetate	1122	0.32±0.03[a]	0.09±0.01[a]	**	-	-	ns
Methyl propyl butanoate	1157	0.12±0.01[a]	tr[a]	*	-	-	ns
Butyl butanoate	1218	0.15±0.01[a]	tr[a]	*	-	-	ns
Ethyl hexanoate	1232	tr[a]	0.09±0.01[a]	*	0.05±0.01[a]	0.09±0.01[a]	ns
Isopentyl butanoate	1264	0.32±0.02[a]	tr[a]	**	-	-	ns
Methyl hexadecanoate	2216	-	-	ns	0.23±0.01[a]	0.64±0.04[b]	*
All		**1.52±0.13[a]**	**1.69±0.12[a]**	**ns**	**0.41±0.03[a]**	**0.73±0.05[a]**	*
Aromatic hydrocarbons							
Ethylbenzene	1128	0.18±0.01[b]	0.05±0.01[a]	**	tr[a]	tr[a]	ns
Styrene	1262	0.61±0.04[b]	0.22±0.02[a]	**	tr[a]	tr[a]	ns
All		**0.79±0.05[b]**	**0.27±0.03[a]**	**	**tr[a]**	**tr[a]**	**ns**
Lactones							
δ-Decalactone	2197	tr[a]	0.29±0.03[b]	*	-	0.29±0.02[b]	*
All		**tr[a]**	**0.29±0.03[b]**	*	**-**	**0.29±0.02[b]**	*
Terpenes							
α-Pinene	1024	tr[a]	tr[a]	ns	0.32±0.02[a]	0.55±0.04[b]	*
δ-3-Carene	1146	0.02±0.01[a]	tr[a]	ns	-	-	ns
Myrcene	1160	0.16±0.01[b]	tr[a]	*	-	-	ns
α-Terpinene	1180	0.04±0.01[a]	tr[a]	ns	-	-	ns
Limonene	1201	1.21±0.01[a]	1.38±0.08[a]	ns	0.36±0.03[b]	tr[a]	*

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Eucalyptol	1211	0.64±0.05b	0.13±0.01[a]	**	0.17±0.01[a]	0.10±0.01[a]	ns
p-Cymene	1272	0.31±0.03[a]	0.33±0.02[a]	ns	0.72±0.06[b]	tr[a]	*
All		**2.38±0.12[b]**	**1.84±0.11[a]**	**ns**	**1.57±0.12[b]**	**0.65±0.05[a]**	*

LRI = Linear Retention Index, tr= traces, -= not detected, Different superscript lowercase letters in the same row indicate significant differences at $p < 0.05$ among samples by Duncan's multiple range test; Statistically significant differences among CTR and ECO cheese and milk at $p < 0.001$ (***), $p < 0.01$ (**) or $p < 0.05$ (*), ns=not statistically significant ($p > 0.05$).

Aliphatic acids are the main classes of substances in the milk and cheese samples. The short-chain acids are responsible for the sour taste while long-chain acids are odorless; the unsaturated such as (E)-2-Decenoic acid have a more intense odor. Short-chain FFA are associated with strong sweaty, cheesy, and lipolysis notes, and considering their lower flavor thresholds, they are mainly responsible for dairy products' aroma. In particular, butanoic acid (cheesy, dairy, buttery) has been demonstrated to be the marker aroma compound of raw cow milk.

On the other hand, free fatty acids such as C_{10} and C_{12} are characterized by higher flavor thresholds, and they contribute less to the aromatic profile of dairy products. Comparing the acid amount in CTR and ECO milk, a higher amount resulted in the ECO which had the highest amount of long-chain free fatty acids while medium and short resulted highest in the control. Thus, the ECO cheese showed a healthier free fatty acid profile.

The amount of free fatty acids in the cheese samples reflects the milk composition with the highest amount in the ECO and a different ratio between short, medium, and long chain fatty acid amounts.

The differences in the volatile free fatty acids here reported are in agreement with Calabrese et al. [5], who demonstrated an increase of unsaturated volatile free fatty acids in cheese when olive cake was added to the feed. Of interest resulted the differences in the ketone amount and, in particular that of 2-ketones. Their amount was lower in the ECO cheese. They arise from the β-oxidation of fatty acids to β-ketoacids and decarboxylation to alkan-2-ones with one less C-atom. Alkan-2-ones may be reduced to the corresponding secondary alcohols, a step that is reversible under aerobic conditions. A similar behavior was shown by acetoin and diacetyl (2,3 butanedione) with the lowest amount in the ECO cheese. Moreover, the ECO cheese was characterized by higher number of alcohols mainly ethanol and isoamyl alcohol; the methyl-branched alcohols may derive through reduction of aldehydes formed from amino acid via Strecker degradation. The terpene fraction seems to be less influenced by the addition of olive cake to the feed.

3.5. Sensory Analysis and Acceptability

Figure 3 reports the results of the QDA regarding the cheese CTR and ECO.

 doi:10.20944/preprints202409.1251.v

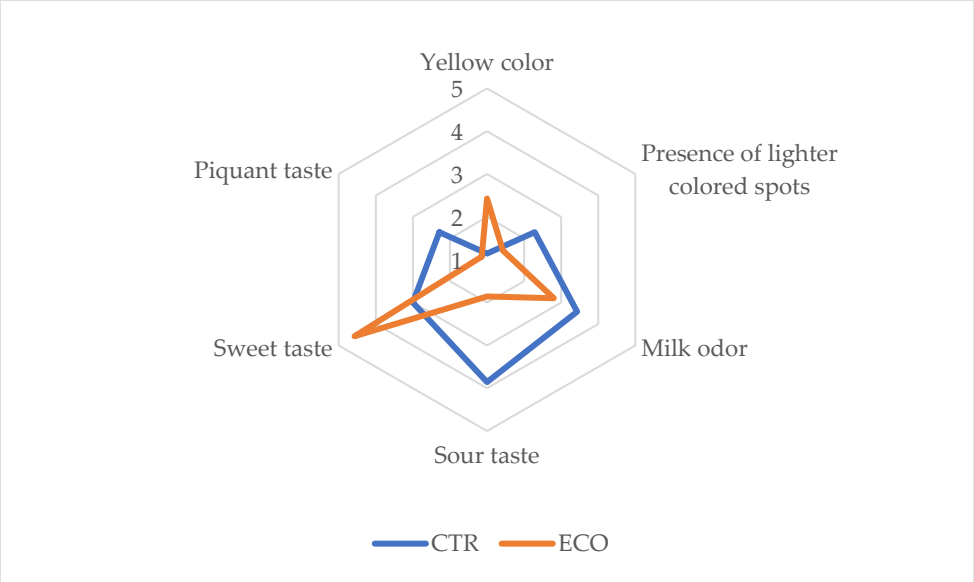

Figure 3. QDA spider plot of significant descriptors of Caciocavallo cheeses.

Among the identified and considered descriptors, only a few significant statistical differences according to the fed composition were displayed. As regards appearance, ECO resulted in a more "yellow color" and in a lower "presence of lighter colored spots"; the "milk odor" and the "sour and piquant taste" intensity resulted higher in the control cheese while the "sweet taste" in the ECO one. The differences in the color and appearance of the ECO samples could be due to the presence of carotenoids in the olive cake since it has been demonstrated to be an important source of these substances; as regards the CTR cheeses, the higher intensity of "milk odor" could be related to the amount of 2-ketones in the volatile fraction, while that of piquant and sour taste to the highest amount of short-chain free fatty acids [55]. Figure 4 shows the results of the consumer's acceptability test.

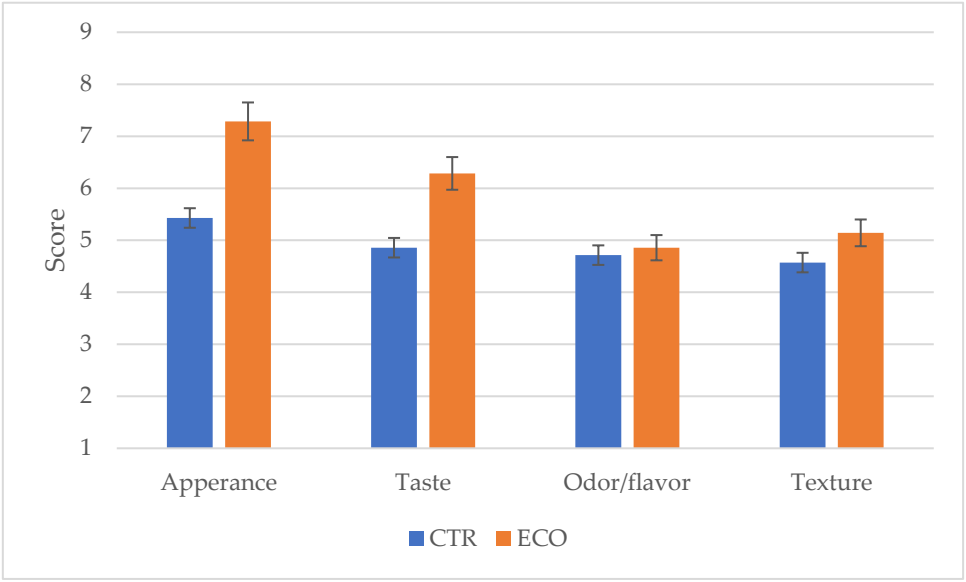

Figure 4. Consumer acceptability of Caciocavallo cheeses.

The consumers mostly appreciated the ECO cheese, especially for their appearance and taste. A more intense yellow color, together with a sweeter and less sour and piquant taste is related to the consumer's acceptability.

4. Conclusions

 doi:10.20944/preprints202409.1251.v

Local cheeses are characterized by unique flavors which reflect the environment where the cheese is made, which include the animal feed, the climate, the environment and the traditional processing method that make the product unique. The production of a local cheese as Caciocavallo Ragusano, with the integration of olive cake enriched in polyphenols in the diet of dairy cows, can constitute a valid opportunity to enrich nutritionally the cheese, thanks to the presence of polyphenols and unsaturated fatty acids positively related to human health; moreover, the phenolic component of olive cake did not affect negatively the microbial composition of Caciocavallo Ragusano. Furthermore, this study showed a good acceptability of ECO cheese for the consumers. With the growing awareness of consumer of health and quality, the acceptability of cheese provides to an important economic impact for the local economy, since in a competitive market, consumer acceptability drives innovation. This study suggests that enriched olive cake not only boosts the sustainability, but also the quality of Ragusano cheese.

Author Contributions: Conceptualization, L.L..; methodology, V.L..; investigation, C.R., C.G. and A.V.; resources, A.A.; data curation, F.C., N.R., F.L., M.M.; writing—original draft preparation, F.L., F.C, N.R., M.M.; writing—review and editing, A.A., C.C., F.L., N.R., F.C.; visualization, L.L., C.G., V.L.; supervision, L.L., C.R., A.V.; project administration, L.L. and V.L.; funding acquisition, L.L.

Funding: This study was funded by P.O. FESR SICILIA 2014/2020. Obiettivo Tematico 1—Ricerca, Sviluppo Tecnologico e Innovazione Obiettivo specifico 1.1—Incremento dell'attività di innovazione delle imprese Azione 1.1.5—Sostegno all'avanzamento tecnologico delle imprese attraverso il finanziamento di linee pilota e azioni di validazione precoce dei prodotti e di dimostrazione su larga scala. Project BIOTRAK. Grant No. 08SR1091000150–CUP G69J18001000007.

Data Availability Statement: Data available on request from the authors.

Acknowledgments: The authors wish to thank Feed Manufacturing Industry Dipasquale Srl. (Avola, SR, IT) and Oil Mill Industry Consoli (Adrano, Catania, IT) for producing and providing the olive cake and integrate complete feed; Lissandrello and Tumino dairy farms for guesting the in-field trials; Natura & Qualità and Ragusa Latte dairy company for the milk analysis and supplying the cheese samples.

Conflicts of Interest: The authors declare no conflicts of interest.

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