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Effect of feeding fresh or dehydrated sulla forage on microbiological, nutritional, and sensory traits of sheep cheese

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ABSTRACT

Sulla is a forage legume species exploited mainly by grazing ruminants, greatly appreciated for its high protein content and moderate levels of condensed tannins with antioxidant activity. This study was aimed to explore the potential of sulla in relation to its seasonal utilization, as fresh or stored forage, and to improve the quality traits of sulla forage stocks, proposing dehydration as alternative to haymaking. Two experiments were carried out in different seasons, spring and autumn, both involving Valle del Belìce ewes fed different diets evaluated in terms of dairy production. In the spring experiment, diets consisting of fresh sulla forage (SUL) or fresh barley forage (BAR) provided ad libitum were compared using 12 ewes at 70 DIM divided into 2 groups and fed the 2 diets in a 2 × 2 Latin square design. In the autumn experiment, pellets of dehydrated sulla forage (DSF) obtained from an early (April) or a late (May) cutting time were compared with sulla hay (SH) using 9 ewes at 60 DIM divided into 3 groups and fed the following 3 diets in a 3 × 3 Latin square design: 2 kg/d per ewe of April pellets and SH ad libitum (A-DSF); 2 kg/d of May pellets and SH ad libitum (M-DSF); and SH ad libitum (SHL). Concentrate feed was supplied to all ewes (600 or 800 g/d in spring and autumn, respectively). In both experiments, cheeses manufactured from bulk milk of the different groups were sampled at 15 and 30 d of storage. In spring, compared with BAR, SUL improved the milk casein content, and in cheeses increased the level of PUFA, especially rumenic and α-linolenic acids, reduced redness index (a*) and fat content. In autumn cheese, DSF diets enhanced total PUFA, especially due to linoleic and α-linolenic acids, and polyphenol contents, whereas the A-DSF diet tended to improve the cheese antioxidant capacity, regardless of storage time. The microbiological profile of spring and autumn cheeses was almost similar among diets, indicating no negative effect of stored forages on fermentation process. In triangle tests, the panel was able to distinguish cheese from different diets for both seasons and storage times, without recording differences in their acceptance degree. These results highlight the advantages of dehydration as an alternative to haymaking, and confirm the promising potential of DSF in ensuring adequate dairy production during periods of limited fresh forage availability.
Key words: dehydrated pellets, condensed tannins, vitamins, fatty acids, antioxidants stability

INTRODUCTION

A balanced diet is essential to ensure a healthy status to lactating ewes and high quality of their dairy products in terms of nutrients content, oxidative stability and microbiological characteristics. In this context, sulla (*Sulla coronaria* (L.) Medik) stands out as one of the best plant species for ruminant feeding in the Mediterranean region, where it is widely used as a forage crop for grazing, hay, and silage (Ruisi et al., 2011; Annicchiarico et al., 2014).

Sulla forage is particularly appreciated for its positive effect on productivity and quality of animal products (Bonanno et al., 2016; Ponte et al., 2022). The nutritional value of fresh sulla forage is recognized for its high CP content, low fiber incidence, and good level of phenolic molecules, including a moderate concentration of condensed tannins (Piluzza and Bullita, 2010; Piluzza et al., 2014; Tava et al., 2021; Rufino-Moya et al., 2022). At these levels, condensed tannins, by binding to dietary proteins, protect them from degradation in the rumen (Piluzza et al., 2014; Mueller-Harvey et al., 2019), thus enhancing proteins bypass and digestion in the intestinal tract (Ponte et al., 2022) where the derived AA are absorbed to be more efficiently utilized for milk

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casein synthesis in the mammary tissue (Gannuscio et al., 2022). Condensed tannins are also able to protect dietary UFA from a complete biohydrogenation in the rumen, thus promoting their transfer into meat and dairy products that increase their nutritional and health value (Vasta et al., 2019; Frutos et al., 2020; Ponte et al., 2022). Moreover, condensed tannins exert antioxidant activity (Rufino-Moya et al., 2022) that improves the oxidative and immune defenses of animals consuming fresh sulla forage. Phenolic compounds derived from the ruminal degradation of condensed tannins are transferred to various tissues, crossing the intestinal barrier (Gladine et al., 2007; Soldado et al., 2021), and once in the milk, they can display its bioactive properties, with a potential effect on cheese nutritional traits and oxidative stability (Di Trana et al., 2015; Soldado et al., 2021; Ponte et al., 2022), thus also offering benefits to consumer health. However, depending on their chemical structure and concentration, polyphenols can be able to inhibit microorganism activity (Vivas et al., 1997; Reguant et al., 2000), thus affecting the cheese microbiological profile.

Despite the known benefits of fresh sulla-based diets, their effects on dairy production require further investigations to understand the influence of the plants' phenological stage, as well as of the conservation method, on the presence in the forage of nutrients and bioactive molecules (Tibe et al., 2011; Rufino-Moya et al., 2022); in this regard, dehydration and pelleting proved to be a valid alternative to haymaking for preserving the nutrients and functional components of fresh sulla forage (Gannuscio et al., 2022; Piccirillo et al., 2025) that can have a positive effect on nutritional quality, microbiological profile and oxidative stability of sheep cheese.

Under these purposes, the technological, microbiological, nutritional, sensory, and health properties of sheep cheeses, these latter with particular reference to their antioxidant properties and FA profile, were studied in 2 experiments conducted in 2 different seasons, spring and autumn. In spring, corresponding to the period of the largest availability of green forage that the ewes commonly fed on pasture, the experiment was aimed to compare the parameters of cheeses produced from milk of ewes fed with fresh sulla forage or another available fresh resource, such as barley. In autumn, the experiment investigated the same parameters in cheeses produced from the milk of ewes fed with sulla forage dried through dehydration or haymaking. It is known that, in autumn, the forage component of ewes' diet consists of conserved resources, due to the limited or absent availability of green forage at pasture. In this case, the forage stocks used are typically represented by hay obtained from late spring cuts of crops left to grow undisturbed. In this context, dehydrated sulla forage can represent a valid alternative to hay, providing good nutritional and productivity levels and possibly even a higher content of bioactive molecules.

MATERIALS AND METHODS

Animals, Experimental Design, and Feeding Treatments

The experimental activities were conducted on a commercial farm with an attached dairy factory located in Santa Margherita di Belìce (Agrigento, Sicily), using Valle del Belìce breed ewes with more than 2 lambings. The animals were housed indoors in individual straw-bedded pens, equipped with feeders and water troughs. The experimental protocol was approved by the Animal Welfare Body (OPBA) of the University of Palermo (2021-UNPA CLE-0059470), with the assessment that the requirements established by Italian Legislative Decree No. 26/2014, implementing Directive 2010/63/EU, were not applicable.

Two experiments were conducted in different seasons: in spring (April–May) for 40 d and in autumn (November–December) for 55 d, using a Latin square experimental design.

During the spring period, 12 ewes at 70 d of lactation were divided into 2 homogeneous groups based on live weight (53.6 ± 5.9 kg) and milk yield (2,213 ± 277 g/d). After 10 d of adaptation to the experimental conditions, the groups were fed diets based on fresh sulla forage of the Avorio population (SUL) or fresh barley forage (BAR) provided ad libitum, using a 2 × 2 Latin square (2 groups of 6 ewes and 2 experimental phases of 15 d). Daily in the morning, both fresh forages were mowed from respective crops, roughly cut and supplied in amounts ensuring refusals. Each ewe received 600 g/d of a commercial concentrate feed (CP 20.9% on DM), divided into 2 meals (morning and afternoon) after milking.

In the autumn experiment, sulla hay (**SH**) was compared with dehydrated sulla forage (**DSF**) in pellets, produced from fresh sulla forage of the Avorio population cut at 2 different times, April and May, corresponding to partial and full flowering stages, thus differing in their CP content (15.1%, 11.3%, and 11.8% on DM in A-DSF, M-DSF, and SH, respectively; Supplemental Table S1, see Notes). The fresh sulla forage was dehydrated in a small pilot plant consisting of a container into which, for each drying cycle, a forage mass of about 400 kg, previously chopped, was placed on mesh platforms mounted on trolleys and exposed to hot air, generated by a diesel-powered system and blown at a speed of 3 m/s and a temperature not exceeding 50°C, for 18 to 24 h until the forage moisture, measured by a portable hygrometer based on electric conductibility, reached a level of about 15%. Then, the dehydrated forage was ground and passed

through a pelleting machine to obtain pellets (2.5 mm height, 0.6 mm diameter).

Nine ewes at 60 d of lactation (52.3 ± 3.4 kg live weight and 1,812 ± 223 g/d milk yield) were divided into 3 groups and, after a 10-d of adaptation, fed 3 different diets, according to a 3 × 3 Latin square design (3 groups of 3 ewes and 3 experimental phases of 15 d). The diets were SH ad libitum (**SHL**); 2 kg/d per ewe of April pellets and SH ad libitum (**A-DSF**); 2 kg/d of May pellets and SH ad libitum (**M-DSF**). Each ewe received a supplement of 800 g/d of commercial concentrate feed (CP 22.4% on DM) divided into 2 meals supplied after morning and afternoon milking.

At the end of each experimental phase, bulk milk produced over 72 h was collected from each group and processed into pressed-curd type cheese under controlled conditions in the farm's dairy plant.

Measurements and Sampling

In both experiments, measurements of offered feeds and the corresponding refused amounts to calculate feed intake, as well as recording of individual milk yield were performed daily during the last 5 d of each 15-d experimental phase. Forage resources were sampled twice during each phase to be analyzed for DM, CP, ether extract, ash, fibrous fractions, vitamin E, condensed tannins (**CT**) and total polyphenols, as described by Gannuscio et al. (2022); their fatty acid (**FA**) acid profile was determined according to Bonanno et al. (2016), and the values of NEL (Mcal/kg DM) were estimated according to INRA (2018). Data of chemical composition and FA profile of forages and ewes' individual milk yield and feeding intake are reported respectively in Supplemental Tables S1 and S2 (see Notes).

Bulk milk of 6 milkings was collected from each ewes' group over the last 72 h (d 13–15) of each experimental phase, and stored at 4°C to be later sampled for successive analyses and processed for cheese manufacture as described below.

During the cheese-making process and storage, samples of raw milk before and after starter inoculation, curd and cheese stored for 15 and 30 d were collected for microbiological analysis, particularly related to lactic acid bacteria, pathogenic microorganisms, and potential contaminants. The produced cheeses were sampled to assess their chemical traits, antioxidant activity, and lipid oxidation during storage.

Bacterial Strains and Inoculum Preparation

In this study, the experimental cheese-making was carried out by inoculating the selected starter strains *Lactococcus lactis* RC-UNIPASAAFM00094 and RC-UNIPASAAFM00193, which were previously isolated from traditional raw ewe milk cheese productions and characterized by relevant dairy traits (Settanni et al., 2013; Gaglio et al., 2014). Both strains belong to the bacterial culture collection of the Dipartimento SAAF, Università degli Studi di Palermo, Palermo, Italy, and were reactivated from glycerol stocks in ESTY Broth (Condalab, Madrid, Spain) at 30°C for 24 h. The inoculum for cheese production was prepared by centrifuging both broth cultures (RC-UNIPA SAAFM00094 and RC-UNIPA SAAFM00193) at 5,000 × *g* for 5 min; the pelleted cells were washed in sterile Ringer's solution (Thermo Fisher Scientific Inc., Waltham, MA) to remove residual growth medium and resuspended until reaching an optical density at 600 nm of ~1.00, which indicates a cell density of ~10^9 cfu/mL.

Cheese Manufacturing and Sampling

The bulk milk collected over 72 h from each group during each experimental phase was processed under controlled conditions in the farm's dairy plant, following the procedure for pressed-curd type cheese production. The milk was first heated to 40°C in a double-bottomed tank and then inoculated with the dual starter strain lactic acid bacteria (**LAB**) culture at a final density of 10^7 cfu/mL. Lamb's rennet paste (1:10,000), diluted in distilled water (7.5:100), was added to the milk at a ratio of 0.25 g/kg, after checking the pH decrease caused by the starter culture inoculation, which occurred within a few minutes. The milk was then held at 37°C for 40 to 50 min until coagulation was achieved.

To induce syneresis, hot water (1:10 vol/vol) at 70°C to 75°C was added to the curd, which was subsequently broken into small rice-sized grains (3–7 mm in diameter) using a wooden paddle. After removing the whey, the curd was hand-pressed into cylindrical perforated plastic molds with a diameter of 15 cm, forming 2 cheeses weighing ~700 to 900 g per group in each experimental phase.

The cheeses, still inside their molds, were cooked in hot water at 85°C for 2 h. After cooking, the cheeses were placed on a flat surface at room temperature for draining. Twenty-four hours after cooking, the cheeses were salted in saturated brine for a period of 1 h/kg of cheese. After salting, the cheeses were dried with a paper towel, weighed (24 h) and transferred to a storage room set at 16°C and 80% relative humidity.

In both spring and autumn experiments, the 2 cheeses, made with bulk milk from each experimental group and in each phase, were weighed to calculate the cheese yield at 24 h after production and after 15 and 30 d of storage, and sampled, one at 15 d and the other one at 30 d of storage, and then analyzed for microbiological, physical and chemical traits. All samples were frozen at −20°C

Table 1. Microorganisms and growth conditions

Microorganism[1]	Medium	Incubation conditions	Company
TMM	Plate count skim milk agar	30°C for 72 h	Microbiol Diagnostici, Uta, Italy
TPM	Plate count skim milk agar	7°C for 7 d	Microbiol Diagnostici, Uta, Italy
Mesophilic coccus LAB	Medium 17 agar	30°C for 48 h	Oxoid, Basingstoke, UK
Enterococci	Kanamycin esculin azide agar	30°C for 48 h	Oxoid, Basingstoke, UK
Enterobacteriaceae	Violet red bile glucose agar	37°C for 24 h	Condalab, Madrid, Spain
CPS	Baird–Parker agar	37°C for 48 h	Oxoid, Basingstoke, UK
Listeria monocytogenes	Agar *Listeria* to Ottaviani and Agosti	37°C for 24 h	Biolife Italiana, Monza, Italy
Escherichia coli	Chromogenic medium	37°C for 24 h	Condalab, Madrid, Spain
Salmonella spp.	Xylose lysine deoxycholate	37°C for 24 h	Liofilchem, Roseto degli Abruzzi, Italy
Yeasts	Dichloran Rose–Bengal chloramphenicol agar	30°C for 48 h	Microbiol Diagnostici, Uta, Italy
Molds	Potato dextrose agar	25°C for 7 d	Microbiol Diagnostici, Uta, Italy

[1]TMM = total mesophilic microorganisms; TPM = total psychrotrophic microorganisms; LAB = lactic acid bacteria; CPS = coagulase-positive staphylococci.

to be lyophilized (SCAN-VAC Coolfase 55–9, Labogene Aps, Denmark) for subsequent analyses.

Bulk Milk Analyses

Samples of bulk milk for cheese-making were collected and analyzed within 24 h after sampling for lactose, fat, urea, and SCC using infrared spectroscopy (Combi-Foss 6000, Foss Electric), pH with a pH meter (HI 9025, Hanna Instruments), and titratable acidity by the Soxhlet-Henkel method (°SH/50 mL). Bulk milk was also assessed for total bacterial count (**TBC**) using the BactoScan instrument (Foss Electric). Additional analyses were performed for total nitrogen (**TN**), noncasein nitrogen (**NCN**), and NPN following standard Federation Internationale du Lait–International Dairy Federation procedures (FIL-IDF 29, IDF, 1964c; FIL-IDF 20B, IDF, 1993). These values were used to calculate total protein (TN × 6.38), casein [TN − (NCN × 0.994) × 6.38] and whey protein [(NCN − NPN) × 6.38].

Bulk milk samples were also evaluated for their clotting ability by a Formagraph instrument (Foss Electric), according to Zannoni and Annibaldi (1981). The lactodynamometric parameters such as coagulation time (r, min), curd firming time (k_{20}, min), and curd firmness at 30 min (a_{30}, mm) were measured on 10 mL of milk heated to 35°C after addition of 0.2 mL of a diluted solution (1.6:100) of rennet (1:15,000; Chr. Hansen, Parma, Italy).

Microbiological Analysis by Culture-Dependent Approach

All samples collected during the entire cheese production process, from raw ewe's milk to finished products, were microbiologically investigated applying the decimal serial dilution procedure as described by Garofalo et al. (2021). Briefly, liquid (raw milk and inoculated milk) samples were directly serially diluted in Ringer's solution, whereas solid (curd and cheese) samples were first homogenized by stomacher and then serially diluted. The resulting cell suspensions were plated on selective agar medium as reported in Table 1.

All microbial groups were inoculated by spread plating on the agar medium, except mesophilic coccus LAB and members of the *Enterobacteriaceae* family, which were pour plated. Analyses were performed in triplicate.

Total Bacterial Community Analysis by Culture-Independent Approach

Total genomic DNA was extracted from cheese samples collected at 15 and 30 d of ripening and produced in spring and autumn using the DNeasy PowerFood Microbial Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The quality of the extracted DNA was evaluated through gel electrophoresis and UV-visible spectrophotometry. Polymerase chain reaction amplification targeting the V3-V4 regions of the 16S rRNA gene was performed applying the approach reported by Busetta et al. (2025) with the primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACNVGGGTWTCTAATCC-3′). Libraries were purified with the Agencourt AMPure XP system and sequenced on an Illumina MiSeq platform (Run Chemistry 2 × 300 PE) using MiSeq Control Software 2.0.5 and Real-Time Analysis software 1.16.18 (Illumina, San Diego, CA). Raw paired-end FASTQ files were demultiplexed using idemp and imported into Qiime2 (version 2018.2). Sequences underwent quality filtering, trimming, denoising, and merging using the DADA2 method. Chimeric sequences were identified and removed using the consensus method within DADA2 (Callahan et al., 2016). Representative bacterial sequences were aligned using MAFFT and used for phylogenetic reconstruction in FastTree, utilizing the alignment and phylogeny plugins (Price et al., 2009; Katoh and Standley, 2013). Taxonomic and compositional analyses were conducted using the feature-classifier plugin. A pretrained Naive

Bayes classifier, based on the Greengenes 13_8 99% Operational Taxonomic Units database (https://library.qiime2.org/) was used. The data generated by MiSeq Illumina sequencing have been deposited in the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) Sequence Read Archive and are available under the accession number PRJNA1298192.

Cheese Physicochemical Analyses

The color of internal surfaces of cheeses was assessed in duplicate using a Minolta Chroma Meter CR-300 (Minolta, Osaka, Japan) standardized by a white plate, measuring lightness (L* values, range from 0 = black to 100 = white), redness (a* values, range from red = +a* to green = −a*) and yellowness (b* values, range from yellow = +b* to blue = −b*) according to the CIE L*a*b* system (CIE, 1986).

An Instron 5564 tester (Instron, Trezzano sul Naviglio, Milan, Italy) was used to assess the cheese hardness measuring the maximum resistance to compression (compressive stress, N/mm^2) exerted by a cylinder at the point of maximum deformation of a cylindrical sample (2 cm height × 2 cm diameter) held at room temperature (20°C–22°C).

The pH values were measured with a pH meter HI 9025 (Hanna Instruments, Ann Arbor, MI), and the water activity by a HygroPalm water activity indicator (Rotronic, Bassersdrof, Germany), as described by Gaglio et al. (2016).

The cheese samples were lyophilized for successive analyses. International Dairy Federation standards were performed to determine DM (FIL-IDF 4A; IDF, 1982), fat (FIL-IDF 5B; IDF, 1986), protein (N × 6.38; FIL-IDF 25; IDF, 1964a) and ash (FIL-IDF 27, IDF, 1964b).

Lyophilized cheese samples (0.5 g) were analyzed in duplicate for total retinol (vitamin A) and α-tocopherol (vitamin E) using modified reversed-phase HPLC methods (Panfili et al., 1994; Manzi et al., 1996). All vitamin extraction procedures were performed using dark glassware to protect the samples from light exposure. Briefly, 0.5 g of sample was digested with 10 mL of a KOH-ethanol solution (11% wt/vol KOH in 55% ethanol aqueous solution) and 0.2 g of ascorbic acid, which acted as an antioxidant. After vortexing to ensure complete mixing, the suspension was kept in a water bath at 70°C for 30 min, then cooled to room temperature. The vitamin extraction was repeated 3 times. Each time, the sample was shaken for 30 min after adding 5 mL of n-hexane. After shaking, the lower aqueous layer was collected and transferred to a round-bottom flask. The pooled organic layers were evaporated using a rotary evaporator (Buchi Rotavapor Model 461) set to 40°C, and the dry residue was dissolved in 5 mL of methanol and filtered through a 0.20-µm polytetrafluoroethylene filter.

For vitamin determination, a 20-µL sample volume was injected into the HPLC system. The chromatographic system consisted of an Agilent 12100 Series (Agilent Technologies, Milan, Italy) with a 1260 Infinity Quaternary Pump, G1329A High Performance Autosampler, G1316A Thermostated Column Compartment, and a G1315D Diode Array Detector. Separation was carried out using an Agilent Eclipse XBD-C18 column (4.6 × 250 mm, 5 µm). The column temperature was maintained at 25°C, and the flux of the mobile phase was set to 1 mL/min. The gradient program used water (A) and methanol (B) as eluents, following this profile: 96% B until 13 min, 96% to 100% B in 7 min, hold at 100% B for 4 min, 100% to 96% B in 0.5 min, hold at 96% B for 5.5 min. Vitamins A and E were detected at 294 nm and 325 nm, respectively, during the same chromatographic run. Chromatographic data were processed using Open Lab software (Agilent Technologies, Palo Alto, CA). Concentrated standard solutions of 100 µg/mL retinol and 100 µg/mL α-tocopherol were stored at 2°C to 8°C in vials. External calibration solutions were prepared by diluting the concentrated solutions to the range of the material studied (0.1–100 µg/mL). A blank sample was prepared using the solvent of the standard solutions and processed 3 times.

Cheese Antioxidant Properties

Extracts of lyophilized cheese samples were prepared following the method of Rashidinejad et al. (2013), with some modifications. Briefly, 0.5 g of lyophilized cheese was suspended in 25 mL of methanol (95% aqueous solution) containing 1% HCl. The suspension was then homogenized for 30 s using an Art-Miccra D-8 high-speed homogenizer (Art Labortechnik, Müllheim, Germany), followed by incubation in an ultrasonic water bath (LBS1 Sonicator; Falc Instruments, Treviglio, Italy) at 40°C for 30 min. During this time, the mixture was vortexed for a few seconds every 10 min. After cooling, the suspension was filtered through cheesecloth and centrifuged at 7,400 × *g* for 10 min at 9°C. The extract was then stored at −18°C until further analyses.

Each cheese extract was analyzed in duplicate to define its antioxidant properties, by determining total polyphenol content and Trolox equivalent antioxidant capacity (**TEAC**).

Total polyphenols were determined using the Folin-Ciocalteau colorimetric method (ISO, 2005), with gallic acid as standard. Briefly, 100 µL of cheese extract were mixed with 900 µL of distilled water and 500 µL of a 1 N Folin-Ciocalteau reagent solution. Then, 2.5 mL of a 20% (wt/vol) sodium carbonate solution was added, and the mixture was vortexed for 30 s. It was incubated in the dark at room temperature for 40 min. The absorbance was measured at

725 nm using a HACH DR3900 spectrophotometer (Hach, Loveland, CO). A calibration curve (R^2 = 0.99) was prepared using aqueous solutions of gallic acid in concentrations ranging from 0 to 1 mg/mL. Results were expressed as gallic acid equivalents (**GAE**; g GAE/kg DM).

The antioxidant capacity of cheese extracts was evaluated by the TEAC decolorization assay, following the method of Re et al. (1999) as described by Bonanno et al. (2019). This assay measures the in vitro radical-scavenging activity of the sample against the monocationic radical 2,2-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid (**ABTS●+**), with Trolox as standard. Briefly, the ABTS radical cation was generated by reacting a 14 m*M* ABTS aqueous solution with an equal volume of 4.9 m*M* potassium persulfate and incubating the mixture in the dark at room temperature for 12 to 16 h. During this time, potassium persulfate oxidizes ABTS to the ABTS●+ radical, which gives the solution a blue-green color. The ABTS●+ solution was diluted with 5 m*M* sodium PBS at pH 7.4 in a 1:50 (vol/vol) ratio to give an absorbance of 0.750 (±0.02) at 734 nm. The absorbance of a mixture containing 40 µL of distilled water and 4 mL of the diluted ABTS●+ solution was measured at 734 nm after 6 min of incubation at 30°C. Similarly, 40 µL of each sample extract was mixed with 4 mL of diluted ABTS●+ solution, and the absorbance was read at 734 nm after 6 min of incubation at 30°C. The percentage decrease in absorbance was calculated, reflecting the decolorization of the solution compared with the baseline measurement with distilled water. Trolox solutions in PBS (ranging from 0 to 2.5 m*M*) were used to generate a calibration curve (R^2 = 0.99). Results were expressed as millimole Trolox equivalents per kilogram of cheese DM.

Cheese Oxidative Stability

The oxidative stability of cheese fat was evaluated by primary and secondary lipid oxidation indices. Primary lipid oxidation was measured in duplicate as peroxide value (**POV**) expressed in milliequivalents O_2/kilogram fat (IDF, 1991). Secondary lipid oxidation index was evaluated by measuring the thiobarbituric acid–reactive substances (**TBARS**), according to the methods proposed by Tarladgis et al. (1960) and modified by Mele et al. (2011). The final TBARS values were expressed as micrograms of malonylaldehyde (**MDA**) per kilograms of cheese DM. Briefly, 8 mL of PBS with pH 7 was placed in a 25-mL Sovirel tube and added with the lyophilized cheese extract samples (4 g); the resulting mixture was homogenized using an Art-Miccra D-8 high-speed homogenizer (Art Labortechnik). Subsequently, 2 mL of a 30% (vol/vol) trichloroacetic acid aqueous solution was added to the sample, then vortexed for a few seconds and filtered through Whatman N.1 filter paper into a 25-mL flask. An aqueous solution of 0.02 *M* thiobarbituric acid (5 mL) was added to 5 mL of filtrate; the mixture was placed in a hot water bath (90°C) for 20 min then refrigerated. After centrifugation for 5 min at 4,760 × *g* at room temperature, the absorbance of the supernatant was read at 530 nm using a HACH DR/4000 U spectrophotometer. To evaluate TBARS, 1,1,3,3-tetramethoxypropane solutions at concentrations from 0.016 to 0.165 µg/mL were used for the calibration curve (R^2 = 0.99).

Cheese FA Profile

To determine the FA composition, lyophilized cheese samples (100 mg) were directly methylated by adding 1 mL of hexane and 2 mL of 0.5 *M* sodium methylate ($NaOCH_3$), keeping them at a temperature of 50°C for 15 min, and then adding 1 mL of 5% HCl in methanol and heating them again at 50°C for 15 min, according to the bimethylation procedure described by Lee and Tweed (2008). Fatty acid methyl esters (**FAME**) recovered in 1.5 mL of hexane were injected by an autosampler into a HP 6890 gas chromatograph system equipped with a flame ionization detector (Agilent Technologies, Santa Clara, CA), and separated using a capillary column (100 m long, 0.25-mm internal diameter, 0.25-µm thickness; CP-Sil 88, Chrompack, Middelburg, the Netherlands). The injector temperature was maintained at 255°C and the detector temperature at 250°C, with hydrogen flow of 40 mL/min, air flow of 400 mL/min and a constant helium flow of 45 mL/min. Initially, the thermostatically controlled chamber was maintained at a temperature of 70°C for 1 min, then increased by 5°C/min to 100°C held for 2 min, again increased by 10°C/min to 175°C held for 40 min, and finally increased by 5°C/min until the final temperature of 225°C was reached and maintained for 45 min. Helium was used as the carrier gas at a pressure of 158.6 kPa and a flow rate of 0.7 mL/min (linear velocity 14 cm/sec). A solution of FAME in hexane (Nu Check-Prep, Elysian, MN) was used to identify each FA. Individual standards (Larodan Fine Chemicals AB, Malmö, Sweden) were used to recognize some branched FA, such as C15:0 *iso*, C15:0 *anteiso*, C17:0 *iso* and C17:0 *anteiso*. Isomers of CLA were identified with a standard mixture of rumenic acid (**RU**) and C18:2 c10 t12 methyl esters (Sigma-Aldrich, Milan, Italy) as well as published isomeric profiles (Kramer et al., 2004; Luna et al., 2005). To quantify total FA, C23:0 (Sigma-Aldrich) was used as internal standard (4 mg/g lyophilized cheese). Based on the FA profile, some indexes of the health value of cheese fat were calculated as follows:

$$\text{Thrombogenic index} = (\text{C14:0} + \text{C16:0} + \text{C18:0}) / (0.5 \times \text{MUFA} + 0.5 \times \text{n-6 PUFA} + 3 \times \text{n-3 PUFA} + \text{n-3/n-6})$$ (Ulbricht and Southgate, 1991);

Health promoting index = (n-3 PUFA + n-6 PUFA + MUFA)/(C12:0 + 4 × C14:0 + C16:0) (Chen et al., 2004);

Atherogenic index = (C12:0 + 4 × C14:0 + C16:0)/(n-3 PUFA + n-6 PUFA + MUFA) (Ulbricht and Southgate, 1991); and

Hypocholesterolemic FA to hypercholesterolemic FA ratio = (C18:1 *cis*-9 + C18:2 n-6 + C20:4 n-6 + C18:3 n-3 + C20:5 n-3 + C22:5 n-3 + C22:6 n-3)/(C14:0 + 16:0) (Santos-Silva et al., 2002).

The health value of cheeses was also evaluated using the General Health Index of Cheese (**GHIC**) as proposed by Giorgio et al. (2019), based on the contents of total PUFA, n-3 PUFA and RA, together with polyphenol content and antioxidant capacity by TEAC. The GHIC was calculated by adding the scores attributed to each component in a range from 0 (minimum value) to 10 (maximum value), as reported by Giorgio et al. (2019), with the modifications to express the FA levels as grams per 100 grams FA, and refer the other components to cheese DM. The GHIC-ALA was calculated only replacing n-3 PUFA score with those referred to α-linolenic acid (**ALA**).

Sensory Analysis of Cheeses

The evaluation of sensory properties of cheeses was carried out by means of a descriptive sensory test (ISO, 2003), a hedonic test, and a triangle discriminant test (ISO, 2004), involving a panel of 10 untrained volunteers (4 females, 6 males), selected for their habitual cheese consumption, during 3 sessions for spring cheeses and 4 sessions for autumn cheeses. In each panel session, the evaluation was performed on cheese portions of ~10 g placed in close containers and kept at room temperature (about 20°C) for 30 min before tasting. Sparling water was offered to evaluators after tasting each cheese.

For the descriptive test, before each session the panelists were involved in a training session to define a shared sensorial grid (ISO, 2010). The descriptive evaluation of sensory profile of cheese samples was based on 15 sensory attributes referred to aspect (color, structure uniformity, holes presence), smell (strength of odor, odor of butter, odor of milk, unpleasant odor), taste (salty, sweet, acid, bitter, spicy), and texture (chewiness, solubility, grittiness), as well as overall acceptance. The score given by the evaluators to each descriptor was expressed as the distance (mm) from the anchor point on the left of a 90-mm visual analog scale corresponding to the minimum rating.

The hedonic test was based on rating the overall appreciation of the cheese in terms of taste, texture, mouthfeel, overall appearance, and overall liking; these traits were scored using a 9-point hedonic scale from 1 (extremely dislike) to 9 (extremely like).

For the triangle test, cheeses of 15 and 30 d were evaluated separately. Within the same storage time, 3 samples of cheese, 2 of which belonged to the same dietary treatment, were offered for tasting to each panelist who were asked to identify the odd sample. Comparisons between the experimental theses were made in both triangular combinations, as follows: SUL SUL BAR; SUL BAR BAR; A-DSF A-DSF M-DSF; A-DSF M-DSF M-DSF; A-DSF A-DSF SHL; A-DSF SHL SHL; M-DSF M-DSF SHL; and M-DSF SHL SHL.

Statistical Analysis

Data were statistically analyzed using the SAS 9.2 software (SAS, 2010). The individual ewes' parameters (Supplemental Table S2, see Notes) were analyzed according to a MIXED model including experimental phase (2 levels in spring and 3 levels in autumn) and diet (2 levels in spring = SUL and BAR; 3 levels in autumn = A-DSF, M-DSF, and SHL) as fixed factors, and the ewe was treated as a random factor and used as error term. For bulk milk and cheese, the GLM procedure was performed using linear models including the fixed effects of experimental phase, and only for cheese also storage time (**ST**, 2 levels in both seasons = 15 and 30 d) and the interaction of diet × ST. For the cheese sensory evaluation scores, the fixed effect of the panelist was also considered.

Before analysis, SCC and TBC values were transformed into logarithmic form ($\log_{10}$). When the effect of a factor resulted significant ($P < 0.05$), means were compared using the Tukey–Kramer multiple comparison test with adjusted *P*-values. The significance of the differences between the cheeses compared in the sensory triangle tests was assessed using the standard references tables of Amerine et al. (1965).

RESULTS AND DISCUSSION

Quality Traits of Bulk Milk

Table 2 describes for both experiments the effects of diet on chemical composition and coagulation properties of bulk milk used for cheese-making.

In spring, SUL milk showed a lower fat level. This reduction was likely related to the well-known negative relationship between milk fat concentration and milk

Table 2. Effects of diet on composition and coagulation properties of bulk milk used for cheese-making[1]

	Spring diet				Autumn diet				
Item	SUL	BAR	SEM	*P*-value	A-DSF	M-DSF	SHL	SEM	*P*-value
SCC, log_{10} n/mL	5.29	5.29	0.084	0.9882	5.45	5.00	5.24	0.327	0.6498
Total bacterial count, log_{10} cfu/mL	5.84	5.47	0.225	0.3285	5.91	5.76	5.74	0.122	0.4640
Lactose, %	4.57	4.60	0.015	0.2983	4.58	4.57	4.51	0.090	0.8320
Fat, %	5.57	5.90	0.083	0.0213	6.68	7.10	7.46	0.168	0.0751
Protein, %	5.22	5.07	0.028	0.0052	5.89	6.09	6.11	0.123	0.4410
Casein, %	3.95	3.85	0.020	0.0070	4.52	4.74	4.71	0.108	0.3775
Whey protein, %	0.989	0.895	0.041	0.1399	1.12	1.12	1.16	0.052	0.8413
Nonprotein nitrogen, %	0.041	0.037	0.002	0.1111	0.040	0.037	0.040	0.001	0.1811
Urea, mg/dL	33.92	28.54	0.779	0.0017	27.63	29.33	37.20	2.34	0.0874
pH	6.64	6.63	0.027	0.9314	6.52	6.51	6.51	0.016	0.8464
Titratable acidity, °SH/50 mL	4.93	4.73	0.065	0.0569	6.40	6.00	6.13	0.373	0.7575
Coagulation time (r), min	22.85	19.91	1.65	0.2224	22.17[a]	20.27[ab]	18.70[b]	0.822	0.0330
Curd firming time (k_{20}), min	2.30	1.66	0.183	0.0224	2.22[a]	1.61[ab]	1.27[b]	0.222	0.0287
Curd firmness (a_{30}), mm	50.74	50.80	4.45	0.9923	45.45[b]	53.75[a]	54.41[a]	1.45	0.0012

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

[1]SUL = fresh sulla forage; BAR = fresh barley forage; A-DSF = 2 kg/d dehydrated sulla forage from April cut; M-DSF = 2 kg/d dehydrated sulla forage from May cut; SHL = sulla hay ad libitum.

yield because milk yield was 460 g/d higher in SUL ewes than in BAR-fed ewes (1,777 vs. 1,317 g/d, $P < 0.0001$; Supplemental Table S2, see Notes); in addition, fresh sulla forage contained lower levels of degradable fiber, including cellulose (29.7% vs. 31.8% DM) and hemicellulose (3.7% vs. 21.1% DM), which are fermentation substrates for acetic acid, the precursor of short- and medium-chain FA synthesized in the udder. On the contrary, the SUL diet improved milk content in protein and casein, certainly referable to the effect of dotation in CT (15.2 vs. 1.5 g DE/kg DM; Supplemental Table S1); indeed, binding to dietary proteins, CT protect them from ruminal degradation, enhance their intestinal flow and digestibility and, consequently, improve the efficiency of their utilization for the synthesis of milk proteins in the mammary tissue (Piluzza et al., 2014; Mueller-Harvey et al., 2019; Gannuscio et al., 2022; Ponte et al., 2022). Nevertheless, the SUL diet contributed to a higher content of milk urea, which represents the product of an unbalance between dietary CP and energy for microbial growth in the rumen (Giovanetti et al., 2019); thus, in accordance with other observations (Bonanno et al., 2008; Giovanetti et al., 2019), such a result can be mainly attributed to the higher CP/NEL ratio of sulla forage compared with barley forage (139 vs. 63 g/Mcal, Supplemental Table S1), rather than to the major dietary CP concentration (16.8 vs. 8.6% on DM; Supplemental Table S1) and consequent intake, considering the mentioned effect of CT in limiting the dietary proteins degradation in the rumen. However, the urea level recorded in milk (33.92 mg/dL) can be considered within the values denoting an efficient utilization of dietary proteins, and resulted far lower than the value (56 mg/dL) that Branca et al. (2000) and Giovanetti et al. (2019) indicated as the threshold not to be exceeded to ensure the ewes' reproductive function. Among the coagulation parameters, only the curd firming time (k_{20}) reached the threshold of significance ($P = 0.0224$), being higher for the SUL milk, as an effect of its higher casein content.

In autumn, no significant difference in the content of chemical components emerged among bulk milk samples. However, both diets based on DSF pellets did not negatively affect milk protein and casein contents, despite the higher milk yield by about 350 g/d with A-DSF, and 250 g/d with M-DSF in comparison with SHL (1,667, 1,557, and 1,308 g/d in A-DSF, M-DSF, and SH, respectively, $P < 0.0001$; Supplemental Table S2). Also in these cases, the maintenance of protein and casein levels in milk, despite the increased production, can be attributed to the protective role of CT toward dietary proteins in limiting their degradation in the rumen (Piluzza et al., 2014). The tendency of lower urea emerged in the milk produced with A-DSF denoted how this diet promoted a more efficient utilization of dietary proteins, attributable to both CT content (Piluzza et al., 2014) and the lower ratio CP/NEL (Supplemental Table S1; Giovanetti et al., 2019). Moreover, the lower fat observed at a tendency level in A-DSF milk can be always referred to its inverse relationship with milk yield. The same A-DSF diet was significantly ($P = 0.0330$) responsible of longer time for coagulation and curd firming, and lower curd firmness; this slight worsening in the coagulation efficiency of A-DSF bulk milk may simply reflect a slight delay in coagulation, associated with the lower casein level and the higher SCC, although both these parameters were not significantly affected by the diet.

Table 3. Effects of diet and storage time (ST) on microbial loads (log cfu/g) of samples collected during cheese-making process and storage (spring experiment)[1]

Item	TMM	TPC	Coccus LAB	Enterococci	*Enterobacteriaceae*	Pseudomonads	Yeasts
Raw milk							
SUL	5.81	4.94	5.13	2.91	3.07	5.01	2.84
BAR	5.67	4.61	5.01	2.73	3.02	5.14	2.88
SEM	0.148	0.099	0.109	0.182	0.091	0.110	0.134
P-value: Diet	0.5118	0.0289	0.4571	0.5044	0.6800	0.4057	0.8165
Inoculated milk							
SUL	6.90	6.09	7.40	3.37	2.89	5.14	3.21
BAR	6.82	6.07	7.37	3.25	2.94	5.22	3.03
SEM	0.112	0.121	0.097	0.072	0.112	0.138	0.128
P-value: Diet	0.6232	0.9051	0.8147	0.2339	0.7398	0.6960	0.3489
Curd							
SUL	8.28	6.00	8.20	4.40	3.69	4.17	2.31
BAR	8.12	6.02	8.42	4.34	3.73	4.09	2.43
SEM	0.092	0.093	0.068	0.093	0.105	0.068	0.072
P-value: Diet	0.2313	0.8856	0.0358	0.6173	0.8037	0.4027	0.2447
Cheese							
SUL							
15-d storage	8.17	6.51	8.92	5.37	2.52	2.82[b]	2.86
30-d storage	8.89	6.32	9.22	6.22	1.52	3.46[a]	2.56
BAR							
15-d storage	8.11	6.52	8.96	5.52	2.49	2.40[c]	2.94
30-d storage	8.89	6.38	9.24	6.38	1.67	3.41[a]	2.66
SEM	0.096	0.090	0.063	0.096	0.088	0.078	0.125
P-value: Diet	0.7394	0.6639	0.6792	0.1136	0.4993	0.0045	0.6122
ST	<0.0001	0.0787	<0.0001	<0.0001	<0.0001	<0.0001	0.0415
Diet × ST	0.7589	0.9942	0.8863	0.9760	0.3081	0.0196	0.8037

[a–c]Values within a column with different superscripts differ significantly at $P < 0.05$.

[1]TMM = total mesophilic microorganisms; TPC = total psychrotrophic microorganisms; LAB = lactic acid bacteria; SUL = fresh sulla forage; BAR = fresh barley forage.

Microbial Count Evolution During Cheese Production

Plate count technique was applied to investigate the dynamics of microbial populations from raw milk to ripened cheeses. This approach was essential to evaluate the effect of the ewes' diet on the levels of the different microbial groups. The results of spring and autumn productions are reported in Tables 3 and 4, respectively. A targeted analysis was also conducted to detect key dairy-related pathogenic bacteria commonly associated with global foodborne outbreaks, including *Escherichia coli*, coagulase-positive staphylococci (**CPS**), *Salmonella* spp., and *Listeria monocytogenes* (Lee and Yoon, 2021; EFSA and ECDC, 2022). None of these pathogens were detected in any of the samples analyzed. Consequently, data related to these microorganisms are not included in Tables 3 and 4. Their absence underscores the high hygienic quality of both the raw materials and the production process.

Regardless of season and diet, raw ewe milk was characterized by levels of total mesophilic microorganisms (**TMM**), total psychrotrophic microorganisms (**TPM**), and mesophilic coccus LAB about 10^5 cfu/mL. These levels are commonly found in raw ewe milk used for Pecorino cheese production (Garofalo et al., 2024; Mazzocca et al., 2024; Todaro et al., 2024). A consistent increase in the levels of mesophilic coccus LAB was registered after the addition of *L. lactis* starter culture, which consequently led to the increase in number of TMM and TPM, aligning with the common trend registered for milk inoculated with starter cultures (Guarcello et al., 2016).

Enterococci were found at ~3 Log cfu/mL in raw ewe milk and increased by about 2 Log cycles in cheeses at 30 d of storage. This result confirms the ability of these bacteria to withstand the different stages of cheese production and persist throughout the ripening process (Di Grigoli et al., 2015; Bonanno et al., 2019). Pseudomonads were detected in both raw and inoculated milk samples at ~5 Log cfu/mL, whereas yeasts and *Enterobacteriaceae* were present at lower densities. However, the levels of these microorganisms significantly decreased during ripening, as their growth is hindered by the stressing conditions within the cheese (Todaro et al., 2011). The TMM and mesophilic coccus LAB reached ~8 Log cfu/g in curd samples. During 30 d of storage, mesophilic coccus LAB increased by about 1 Log cycle in all cheese samples, regardless of season or dietary treatment. This behavior was observed by different authors (Randazzo et al., 2006; De Pasquale et al., 2016; Gaglio et al., 2021) in ovine pressed cheeses. The dominance of mesophilic coccus LAB is attributed to the addition of starter LAB cultures during the cheese-making

Table 4. Effects of diet and storage time (ST) on microbial loads (log cfu/g) of samples collected during cheese-making process and storage (autumn experiment)[1]

Item	TMM	TPC	Coccus LAB	Enterococci	Enterobacteriaceae	Pseudomonads	Yeasts
Raw milk							
A-DSF	5.91	4.67	5.13	2.96	2.97[a]	4.47	2.99
M-DSF	5.89	4.26	5.39	3.09	2.49[ab]	4.37	2.95
SHL	5.90	4.44	5.12	2.95	2.28[b]	4.11	2.83
SEM	0.151	0.67	0.172	0.135	0.130	0.104	0.171
P-value: Diet	0.9936	0.2520	0.4765	0.7065	0.0072	0.0780	0.7719
Inoculated milk							
A-DSF	7.05	5.89	7.23	3.60	2.65	4.84[a]	2.78
M-DSF	7.01	5.76	7.23	3.31	2.35	4.75[ab]	2.98
SHL	7.00	5.94	7.33	3.42	2.39	4.30[b]	2.73
SEM	0.087	0.139	0.126	0.122	0.140	0.139	0.142
P-value: Diet	0.9165	0.6481	0.8006	0.2861	0.2817	0.0357	0.4398
Curd							
A-DSF	8.47	6.27	8.02	4.31	3.33	4.24	2.33
M-DSF	8.29	6.15	8.03	4.48	3.60	4.30	2.58
SHL	8.32	6.11	8.21	4.57	3.34	4.34	2.40
SEM	0.087	0.139	0.072	0.118	0.081	0.099	0.082
P-value: Diet	0.3391	0.7288	0.1533	0.3357	0.0636	0.7991	0.1312
Cheese							
A-DSF	8.67	6.28	9.11	5.86	2.17[a]	2.96	2.39
M-DSF	8.43	6.31	9.03	6.02	2.09[ab]	2.76	2.44
SHL	8.59	6.44	9.06	6.04	1.88[b]	2.87	2.36
SEM	0.079	0.089	0.075	0.086	0.068	0.068	0.075
A-DSF							
15-d storage	8.73	6.24	8.83	5.51	2.67	2.63	2.46[ab]
30-d storage	8.61	6.32	9.38	6.20	1.66	3.29	2.32[ab]
M-DSF							
15-d storage	8.47	6.23	8.86	5.77	2.42	2.31	2.38[ab]
30-d storage	8.39	6.38	9.20	6.28	1.76	3.21	2.50[ab]
SHL							
15-d storage	8.66	6.40	8.95	5.53	2.20	2.51	2.62[a]
30-d storage	8.53	6.49	9.17	6.56	1.56	3.24	2.10[b]
SEM	0.112	0.126	0.105	0.122	0.097	0.097	0.107
P-value: Diet	0.1117	0.3975	0.7796	0.2602	0.0161	0.1324	0.7312
ST	0.2291	0.2947	0.0002	<0.0001	<0.0001	<0.0001	0.0425
Diet × ST	0.9728	0.9639	0.3170	0.1124	0.1110	0.4296	0.0183

[a,b]Values within a column with different superscripts differ significantly at $P < 0.05$.

[1]TMM = total mesophilic microorganisms; TPC = total psychrotrophic microorganisms; LAB, lactic acid bacteria; A-DSF = 2 kg/d dehydrated sulla forage from April cut; M-DSF = 2 kg/d dehydrated sulla forage from May cut; SHL = sulla hay ad libitum.

process. The marked increase in their population by d 30 confirms their persistence and central role as fermentative agents. These bacteria are known for their halotolerance, psychrotrophic nature, and both saccharolytic and proteolytic activities. Their metabolic versatility contributes positively to the cheese-ripening process, particularly in the development of characteristic aromas (Akpoghelie et al., 2025). These results clearly demonstrated that no influence of the diet was registered on the evolution of the starter *L. lactis* culture used.

In both production seasons, storage time had a more pronounced effect on the microbial loads of the cheeses than either the diet or the interaction between diet and time. During the spring production (Table 3), the influence of diet and its interaction with storage time was evident only in the case of *Pseudomonas* spp., which showed higher counts in cheeses from ewes fed the SUL diet at 15 d. However, by d 30, *Pseudomonas* levels had equalized across both dietary groups. Enterococci also increased by d 30, whereas spoilage-related microorganisms such as *Enterobacteriaceae* declined by about 1 Log cycle, regardless of diet. In the autumn production (Table 4), the diet's effect was observed only for *Enterobacteriaceae*, which are typically associated with suboptimal hygiene in dairy processing (Claeys et al., 2013). These bacteria were more abundant in cheeses from the SHL diet group, although their levels decreased during storage across all diets, as their growth is hindered by the stressing conditions within the cheese (Todaro et al., 2011). Enterococci again showed an increase by d 30, exceeding 6 log cfu/g in all samples. Conversely, spoilage microorganisms, including *Enterobacteriaceae*, and *Pseudomonas* spp., declined by ~1 Log cycle during storage, independent of dietary treatment.

Overall, these findings confirm that the different diets did not negatively influence the fermentation process or

the microbiological quality of the cheeses during production and storage.

Characterization of Cheese Microbiota by Culture-Independent Approach

A thorough assessment of the microbiota associated with the cheeses produced in this study was conducted by DNA-based Illumina technology. This approach allows for a more accurate identification of the bacterial communities associated with foods, including subdominant species (Gaglio et al., 2020; Zotta et al., 2022). The results reported in Figure 1 display the taxa with a relative abundance (**RA**) of ≥0.1%, which is considered a threshold level for abundant bacterial communities (Logares et al., 2014). Twenty-three taxonomic groups were identified mainly at the genus level. Lactic acid bacteria, identified as *Enterococcus*, *Lactobacillus*, *Lactococcus*, and *Streptococcus*, were found at consistent RA (81.87%–97.35%) in all cheese samples, regardless of season, diet, and ripening time. *Lactococcus* was the most dominant bacterial group in all cheeses with RA% ranging from 31.19% to 66.51%. The species within this genus are part of the starter LAB group (Grujović et al., 2022), and the presence of *Lactococcus* DNA in the cheeses produced in this study is primarily attributable to the inoculated starter strains *L. lactis* RC-UNIPASAAFM00094 and RC-UNIPASAAFM00193. Comparable results were observed in Pecorino-type ovine cheese after 2 mo of ripening inoculated with the same starter culture (Garofalo et al., 2024). *Enterococcus* was the second group most abundant in all cheeses analyzed (28.79%–43.01% of RA). These bacteria are frequently found in raw ewe milk cheeses (Foka et al., 2024). Although the presence of *Enterococcus* in cheese is controversial for the risks associated with the transfer of antibiotic resistance genes (Abril et al., 2022), they offer a consistent contribution in developing the organoleptic characteristics of the final products (Centeno et al., 2022). For this reason, the European Commission does not impose limits on their presence in cheeses (EC No. 1441/2007; EC, 2007). Lactobacilli (0.29%–2.68% of RA) and streptococci (2.05%–10.31% of RA) were also found in the cheese samples, but the RA% found were less consistent than *Lactococcus* and *Enterococcus* within the LAB group. Their presence is expected, as these bacteria are commonly found in raw ewe milk cheeses (Yeluri Jonnala et al., 2018).

All cheese samples were characterized by the presence of *Acinetobacter* and members of *Enterocacteriaceae* family at low RA% (<6%). The presence of these bacteria at low levels is generally reported in ripened raw milk cheeses and is related to hygiene conditions of the raw material used for the cheese productions (Giammanco et al., 2011; Claeys et al., 2013; Celano et al., 2022). In particular, the lowest RA% of these bacteria was recorded for the cheeses produced with milk obtained from the sheep group subjected to barley forage diet and can be directly imputable to the high microbiological quality of the forage used for this study. Interestingly, the 4 main dairy pathogenic bacteria (*E. coli*, CPS, *Salmonella* spp., and *L. monocytogenes*) were not detected in any cheese, confirming the results observed by plate count.

Cheese Physicochemical Traits, Antioxidant Properties, and Oxidative Stability

The results related to yield and quality traits of cheeses obtained processing the milk from the experimental groups are shown in Tables 5 and 6, respectively, for spring and autumn productions.

In spring, cheese yield, expressed on both fresh and DM, did not differ between diets in each storage time. Diet, ST, and their interaction (diet × ST) revealed significant effects on some physicochemical parameters of cheeses.

The significant effect of the diet emerged for cheese fat and protein. Indeed, the SUL diet was responsible of fat reduction ($P = 0.0213$) and protein increase ($P = 0.0052$), consistent with the milk composition (Table 2). Additionally, SUL forage contributed to reduce the redness index, in accordance with the observations of Ponte et al. (2022); this result, indicating a shift toward a green color, could be linked to the higher level of pigments as chlorophyll metabolites transferred from fresh sulla forage to milk, as suggested by Birkinshaw et al. (2023) for milk from grass-fed dairy cows. The contribution to yellowness from carotenoid pigments, highly represented in fresh sulla (Rufino-Moya et al., 2022), was negligible considering their metabolic conversion into retinol or vitamin A; however, the latter was more abundant in SUL cheeses (on average 3.2 vs 2.6 mg/kg DM), although at not significant level.

The higher content of polyphenols (20.16 vs. 7.22 g GAE/d) and CT (15.22 vs. 1.50 g DE/d) recorded with SUL forage (Supplemental Table S1) did not result in an increase of polyphenols and antioxidant properties (TEAC) in the corresponding cheeses, that were comparable to those of BAR cheeses. In this regard, Ponte et al. (2022) observed a weak transfer in cheeses of polyphenols ingested at higher levels with fresh sulla forage than with sulla hay. The disappearance of polyphenols in cheese can be attributed to their degradation during digestive and cheese-making processes (Gladine et al., 2007) or to their binding with cheese protein, either reducing or avoiding their analytical detection (Rashidinejad et al., 2013).

During storage of cheeses, in addition to the increase in DM, which is foreseeable together with a consequent decrease of water activity, there was also an unexpected

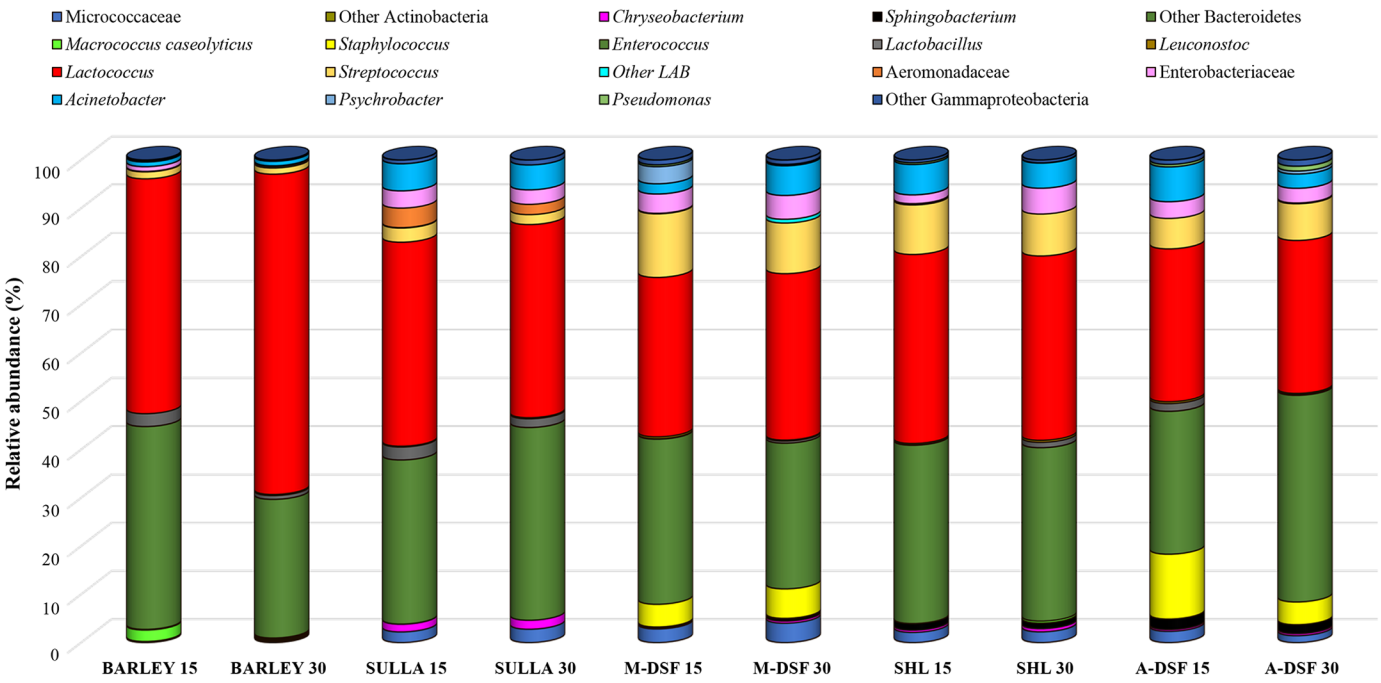

Figure 1. Relative abundances (%) of bacteria identified by MiSeq Illumina. BARLEY 15 = cheese after 15 d of ripening produced with milk obtained from sheep subjected to barley forage diet; BARLEY 30 = cheese after 30 d of ripening produced with milk obtained from sheep subjected to barley forage diet; SULLA 15 = cheese after 15 d of ripening produced with milk obtained from sheep subjected to sulla forage diet; SULLA 30 = cheese after 30 d of ripening produced with milk obtained from sheep subjected to sulla forage diet; M-DSF 15 = cheese after 15 d of ripening produced with milk obtained from sheep subjected to 2 kg/d dehydrated sulla forage from May cut; M-DSF 30 = cheese after 30 d of ripening produced with milk obtained from sheep subjected to 2 kg/d dehydrated sulla forage from May cut; SHL 15 = cheese after 15 d of ripening produced with milk obtained from sheep subjected to sulla hay ad libitum; SHL 30 = cheese after 30 d of ripening produced with milk obtained from sheep subjected to sulla hay ad libitum; A-DSF 15 = cheese after 15 d of ripening produced with milk obtained from sheep subjected to 2 kg/d dehydrated sulla forage from April cut; A-DSF 30 = cheese after 30 d of ripening produced with milk obtained from sheep subjected to 2 kg/d dehydrated sulla forage from April cut.

increasing trend in polyphenols. This result, supported by literature (Gupta and Prakash, 2009; Rashidinejad et al., 2013), could be due to the interaction of certain AA released by proteolysis during cheese maturation with the determination method of polyphenols, leading to the measurement of their false increase. Another explanation is that polyphenols, when interacting with proteins, are not analytically detected in fresh cheeses but are gradually released during aging, thus being detectable in more matured cheeses. Based on this result, the improvement in the antioxidant capacity (TEAC) in 30-d cheeses can be associated with this increase in polyphenols, as no variation emerged in the level of the other compounds with antioxidant action, such as the vitamins A and E. As known, antioxidant capacity is influenced by several factors, among which the presence of β-carotene, vitamin E, phenols, or caseins, as well as the cheese manufacturing and the coagulating agent used (Fardet and Rock, 2018).

Moreover, regarding the oxidative stability of cheeses, the unique significant effect ($P = 0.0214$) was the interaction of POV, due to the better stability to primary oxidation emerged for 30-d stored cheeses produced with BAR; however, this result cannot be attributed to their TEAC value, which was lower than that in SUL 30-d cheese, but presumably to their lower content of compounds more sensitive to oxidation, such as UFA (Table 7). Finally, among the cheese physical parameters, the prolongation of storage time from 15 to 30 d contributed to slightly reduce the yellow color of cheese paste, expressed by the yellowness index, and increase its consistence, measured as resistance to compression or hardness.

In autumn (Table 6), the SHL cheeses at 15 d showed a higher yield than cheeses of other groups, attributable mainly to the higher fat content of the origin milk. However, the differences in yield became less pronounced over time, with no significance after 30 d of storage.

Regarding the physicochemical parameters of autumn cheeses, significant effects of diet and storage time were observed, but there was no significant interaction of their effects (diet × ST).

The diet influenced the polyphenol content, which was higher in cheeses made with A-DSF and M-DSF milk, in line with the higher polyphenol contents in the corresponding forages (13.6, 10.0, and 9.7 g GAE/kg DM

Table 5. Effects of diet and storage time (ST) on cheese yield and physicochemical traits (spring experiment)[1]

	SUL		BAR			*P*-value		
Item	15 d	30 d	15 d	30 d	SEM	Diet	ST	Diet × ST
Cheese weight at 15 d, g	653.8		595.3		27.87	0.1920		
Cheese weight at 30 d, g	618.0		528.7		38.94	0.2031		
Cheese yield at 15 d, g/100 g milk	13.48		13.61		0.113	0.4675		
Cheese yield at 30 d, g/100 g milk	12.79		12.82		0.188	0.9406		
Cheese yield at 15 d, g DM/100 g milk	8.79		8.88		0.104	0.6181		
Cheese yield at 30 d, g DM/100 g milk	8.51		8.60		0.141	0.7274		
pH	5.55	5.59	5.49	5.64	0.070	0.9232	0.2100	0.3945
Water activity, a_w	0.995	0.986	1.00	0.986	0.035	0.2392	0.0043	0.1712
DM, %	63.93	66.51	63.74	67.18	0.423	0.6109	<0.0001	0.3620
Fat, % DM	46.34	46.21	49.19	47.56	0.701	0.0152	0.2629	0.3380
Protein, % DM	43.73	44.24	41.97	41.46	0.776	0.0172	0.9997	0.5563
Ash, % DM	6.07	6.40	6.35	6.65	0.163	0.1604	0.0915	0.9350
Vitamin A, mg/kg DM	3.35	3.13	2.48	2.73	0.523	0.2890	0.9805	0.6850
Vitamin E, mg/kg DM	11.09	9.75	10.40	7.94	2.00	0.5942	0.4172	0.8101
Polyphenols, g GAE/kg DM	3.63	4.04	3.94	4.56	0.242	0.1291	0.0558	0.6815
TEAC, mmol/kg DM	12.47	20.87	13.42	17.81	1.36	0.4855	0.0001	0.1854
Peroxide value, mEq O_2/kg fat	2.44^{ab}	2.74^{a}	2.59^{ab}	2.20^{b}	0.133	0.1834	0.7865	0.0214
TBARS, mg MDA/kg DM	0.523	0.518	0.600	0.614	0.052	0.1370	0.9336	0.8688
Lightness, L*	73.37	72.70	74.74	73.77	0.594	0.6656	0.2065	0.8196
Redness, a*	−3.88	−3.74	−3.30	−3.16	0.130	0.0001	0.3247	0.9842
Yellowness, b*	13.69	13.04	13.36	12.56	0.334	0.2747	0.0489	0.8325
Hardness, N/mm^2	0.611	0.899	0.643	0.858	0.060	0.9466	0.0005	0.5771

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

[1]GAE = gallic acid equivalent; TEAC = trolox equivalent antioxidant capacity; TBARS = thiobarbituric acid–reactive substances; MDA = malonylaldehyde; SUL = fresh sulla forage; BAR = fresh barley forage.

in A-DSF, M-DSF, and SH, respectively; Supplemental Table S1). Instead, the vitamins A and E were not significantly affected by the diet, although showed an increasing trend from SHL to M-DSF and A-DSF that, for α-tocopherol, was consistent with its forage content (14.7, 20.4, and 11.7 mg/kg DM in A-DSF, M-DSF, and SH, respectively; Supplemental Table S1). These trends observed for polyphenols and vitamins correspond to that emerged for TEAC, denoting how the cheese antioxidant capacity also improved from SHL to M-DSF and A-DSF, although only at level of tendency. On this basis, it can be supposed that these molecules present in sulla forage, such as polyphenols and vitamins, or their precursors or metabolites, are little affected by the dehydration process and, consequently, are transferred into the products, improving synergically their antioxidant properties, in accordance with Ponte et al. (2022).

The color parameters, such as lightness (L*; $P = 0.0008$) and redness index (a*; $P = 0.0261$), were also significantly affected by the diet, with lower values in the cheeses made with milk from diets based on dehydrated pellets. The lower red intensity in these cheeses, denoting a shift toward a green color, is consistent with the same reduction observed in spring SUL cheeses, as well as with the findings by Menci et al. (2022) who found a lower redness in the goat cheeses obtained with sainfoin than alfalfa pellets; a similar plausible explanation may be advanced hypothesizing the transfer of greenish-colored compounds as chlorophyll metabolites from the dehydrated pellets, as supported by the findings of Birkinshaw et al. (2023). In this case, an involvement of carotenoid pigments was not detectable in terms of cheese yellowness or conversion into retinol or vitamin A, as these parameters were not different among diets.

The storage time affected cheeses by increasing their DM percentage, as was to be expected, but also increased their polyphenol contents and reduced their vitamins and lightness (L*).

As for the cheeses produced in the spring experiment, the same hypotheses, mentioned before on the basis of findings from Gupta and Prakash (2009) and Rashidinejad et al. (2013), can be advanced regarding the increase of polyphenols in cheese during storage. However, in this case, the first hypothesis, suggesting that certain AA released during proteolysis in cheese matrices might interfere with the polyphenol detection method, leading to a false increase in polyphenols levels, is better supported by the fact that the increase in polyphenols during storage does not correspond to a similar increase in the antioxidant capacity (TEAC) of cheeses.

On the contrary, both vitamin A ($P = 0.0316$) and E ($P = 0.0175$) significantly decreased in cheeses after 30 d of storage, as also occurred in cheeses produced in spring; in this regard, other authors (Marino et al., 2021) observed no change in the level of α-tocopherol in cheese after 14 d of storage, whereas consistent declines of retinol and

Table 6. Effects of diet and storage time (ST) on cheese yield and physicochemical traits (autumn experiment)[1]

					A-DSF		M-DSF		SHL			*P*-value		
Item	A-DSF	M-DSF	SHL	SEM	15 d	30 d	15 d	30 d	15 d	30 d	SEM	Diet	ST	Diet × ST
Cheese weight at 15 d, g	830.9[b]	852.9[ab]	882.2[a]	12.85								0.0439		
Cheese weight at 30 d, g	786.9	818.8	838.4	18.30								0.2485		
Cheese yield at 15 d, g/100 g milk	16.15[b]	16.59[ab]	17.11[a]	0.165								0.0047		
Cheese yield at 30 d, g/100 g milk	15.31	16.20	16.50	0.291								0.0950		
Cheese yield at 15 d, g DM/100 g milk	10.36[b]	10.67[ab]	11.00[a]	0.126								0.0109		
Cheese yield at 30 d, g DM/100 g milk	9.93	10.47	10.68	0.181								0.0950		
pH	5.56	5.66	5.60	0.034	5.57	5.55	5.62	5.69	5.58	5.61	0.047	0.1780	0.5072	0.6097
Water activity, a_w	0.975	0.977	0.977	0.003	0.973	0.976	0.975	0.978	0.977	0.978	0.004	0.7684	0.4873	0.9350
DM, %	64.13	64.30	64.25	0.177	63.39	64.87	63.96	64.65	63.83	64.68	0.251	0.7869	0.0006	0.2963
Fat, % DM	47.21	49.10	47.78	0.846	46.38	48.04	49.76	48.43	47.19	48.38	1.20	0.3142	0.6168	0.4368
Protein, % DM	42.61	42.45	42.04	0.796	43.76	41.46	42.20	42.70	42.53	41.55	1.13	0.8742	0.3349	0.4868
Ash, % DM	5.58	5.43	5.47	0.143	5.63	5.53	5.30	5.57	5.19	5.76	0.203	0.7490	0.1632	0.2912
Vitamin A, mg/kg DM	3.68	3.29	2.64	0.340	4.27	3.08	3.89	2.69	3.01	2.26	0.481	0.1861	0.0316	0.8903
Vitamin E, mg/kg DM	10.36	9.84	8.39	1.04	12.22	8.49	11.07	8.61	10.73	6.04	1.27	0.4206	0.0175	0.7240
Condensed tannins, g DE/kg DM	0.093	0.048	0.069	0.022	0.046	0.141	0.058	0.039	0.075	0.062	0.032	0.3766	0.4355	0.1509
Polyphenols, g GAE/kg DM	4.42[a]	4.24[a]	3.12[b]	0.371	3.55	5.30	3.76	4.73	2.97	3.27	0.525	0.0495	0.0264	0.3024
TEAC, mmol/kg DM	11.29	9.84	8.53	0.877	12.70	9.88	10.56	9.12	8.42	8.63	1.24	0.1017	0.1936	0.4823
Peroxide value, mEq O_2/kg fat	2.13	2.44	1.89	0.173	2.28	1.97	2.74	2.14	1.71	2.07	0.244	0.0963	0.3624	0.1494
TBARS, mg MDA/kg DM	0.243	0.296	0.223	0.038	0.245	0.241	0.252	0.340	0.205	0.242	0.053	0.3888	0.3642	0.6928
Lightness, L*	74.81[b]	75.08[b]	76.98[a]	0.417	75.69	73.92	76.12	74.04	77.24	76.73	0.590	0.0008	0.0038	0.3807
Redness, a*	−2.39[a]	−2.36[a]	−1.95[b]	0.124	−2.27	−2.50	−2.15	−2.56	−1.98	−1.92	0.175	0.0261	0.1764	0.3920
Yellowness, b*	9.40	9.32	9.66	0.246	9.60	9.20	9.21	9.43	9.86	9.46	0.348	0.5930	0.5024	0.5880
Hardness, N/mm^2	0.696	0.724	0.699	0.024	0.720	0.671	0.693	0.754	0.675	0.724	0.034	0.6717	0.4744	0.2213

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

[1]DE = delphinidin equivalent; GAE = gallic acid equivalent; TEAC = trolox equivalent antioxidant capacity; TBARS = thiobarbituric acid–reactive substances; MDA = malonylaldehyde; A-DSF = 2 kg/d dehydrated sulla forage from April cut; M-DSF = 2 kg/d dehydrated sulla forage from May cut; SHL = sulla hay ad libitum.

Table 7. Effects of diet on fatty acid (FA) profile (g/100 g FA) of cheese fat[1]

	Spring diet				Autumn diet				
Item	SUL	BAR	SEM	*P*-value	A-DSF	M-DSF	SHL	SEM	*P*-value
C16:0	24.45	25.37	0.181	0.0047	26.54^{b}	26.60^{b}	28.59^{a}	0.564	0.0450
C18:0	8.66	8.74	0.132	0.7132	4.60^{c}	5.72^{b}	6.44^{a}	0.103	<0.0001
C18:1 *trans*-11, TVA	2.67	1.58	0.040	<0.0001	1.83^{a}	1.58^{b}	1.41^{c}	0.026	<0.0001
C18:1 *cis*-9, OLA	14.93	18.31	0.135	<0.0001	9.90^{c}	11.78^{b}	14.05^{a}	0.260	<0.0001
C18:2 n-6, LA	2.10	1.88	0.019	<0.0001	2.09^{a}	2.22^{a}	1.87^{b}	0.054	0.0033
C18:3 n-3, ALA	1.64	0.631	0.022	<0.0001	1.48^{a}	1.00^{b}	0.455^{c}	0.069	<0.0001
C18:2 *cis*-9,*trans*-11 CLA, RU	1.03	0.721	0.014	<0.0001	0.686	0.605	0.633	0.027	0.1553
Total branched-chain FA	2.21	2.12	0.046	0.2496	1.57^{b}	1.64^{b}	1.89^{a}	0.025	<0.0001
SFA	70.07	71.54	0.272	0.0032	78.07	77.18	76.79	0.495	0.2182
MUFA	22.49	23.71	0.185	0.0006	15.94^{c}	17.44^{b}	19.24^{a}	0.368	0.0003
PUFA	7.00	4.48	0.058	<0.0001	5.75^{a}	5.23^{a}	3.88^{b}	0.144	<0.0001
UFA	29.49	28.19	0.233	0.0026	21.69	22.67	23.11	0.490	0.1620
PUFA/SFA	0.100	0.063	0.001	<0.0001	0.074^{a}	0.068^{a}	0.051^{b}	0.002	<0.0001
n-6 PUFA	3.72	2.76	0.040	<0.0001	3.27^{a}	3.15^{a}	2.47^{b}	0.080	<0.0001
n-3 PUFA	1.94	0.953	0.014	<0.0001	1.66^{a}	1.34^{b}	0.674^{c}	0.072	<0.0001
n-6/n-3	1.91	2.90	0.031	<0.0001	1.98^{b}	2.36^{b}	3.73^{a}	0.182	0.0001
LA/ALA	1.28	3.02	0.063	<0.0001	1.42^{b}	2.23^{b}	4.36^{a}	0.340	0.0003
Thrombogenic index, TI	2.26	2.79	0.026	<0.0001	2.97^{b}	3.08^{b}	3.65^{a}	0.068	<0.0001
Health promoting index, HPI	0.397	0.369	0.011	0.1174	0.239^{a}	0.257^{ab}	0.261^{b}	0.006	0.0444
Atherogenic index	2.54	2.74	0.063	0.0675	4.20^{a}	3.90^{ab}	3.84^{b}	0.089	0.0347
h/H	0.541	0.574	0.008	0.0183	0.344^{b}	0.385^{a}	0.402^{a}	0.005	<0.0001
GHIC	37.25	18.43	0.834	<0.0001	23.00^{a}	18.50^{a}	9.83^{b}	1.71	0.0009
GHIC-ALA	37.25	17.98	0.810	<0.0001	23.83^{a}	18.17^{a}	9.83^{b}	1.70	0.0006

[a–c]Values within a row with different superscripts differ significantly at $P < 0.05$.

[1]TVA = trans vaccenic acid; OLA = oleic acid; LA = linoleic acid; ALA = α-linolenic acid; RU = rumenic acid. Thrombogenic index = (C14:0 + C16:0 + C18:0)/(0.5 × MUFA + 0.5 × n-6 PUFA + 3 × n-3 PUFA + n-3/n-6) (Ulbricht and Southgate, 1991). Health-promoting index = (n-3 PUFA + n-6 PUFA + MUFA)/(C12:0 + 4 × C14:0 + C16:0) (Chen et al., 2004). Atherogenic index = (C12:0 + 4 × C14:0 + C16:0)/(n-3 PUFA + n-6 PUFA + MUFA) (Ulbricht and Southgate, 1991). h/H, hypocholesterolemic FA to hypercholesterolemic FA ratio = (C18:1 *cis*-9 + C18:2 n-6 + C20:4 n-6 + C18:3 n-3 + C20:5 n-3 + C22:5 n-3 + C22:6 n-3)/(C14:0 + 16:0) (Santos-Silva et al., 2002). GHIC = General Health Index of Cheese (Giorgio et al., 2019). SUL = fresh sulla forage; BAR = fresh barley forage; A-DSF = 2 kg/d dehydrated sulla forage from April cut. M-DSF = 2 kg/d dehydrated sulla forage from May cut. SHL = sulla hay ad libitum.

α-tocopherol were detected in cheeses ripened until 60 d (Gutiérrez-Peña et al., 2021).

These opposite trends of polyphenols and vitamins A and E during storage can explain the negligible changes that occurred in terms of antioxidant capacity between 15-d and 30-d cheeses.

Finally, no variation due to the effect of diet or storage time emerged in the oxidative stability of cheeses, except for the tendency for higher peroxide values in cheeses from both A-DSF and M-DSF pellets, presumably dependent on their high content in UFA (Table 7). Also, hardness of cheeses, expressed as compressive stress (N/mm^2) that reflects the consistency of their paste, was found without significant differences, probably depending on the slight moisture variations recorded among cheeses, despite the significant effect ($P = 0.0006$) of storage time in increasing cheese DM.

Cheese FA

The Table 7 shows the FA profile of cheese fat, reporting for both experiments the means of main FA and the related indexes significantly affected by the diet, without discussing the effects of storage time and the interaction diet × ST, as they were never significant. The complete FA composition and the significance of the influencing effects are shown in supplemental tables, which report, for spring (Supplemental Tables S3, S4, and S5, see Notes) and autumn (Supplemental Tables S6, S7, and S8, see Notes) experiments, the short, medium- and branched-chain FA, the long-chain FA, and the FA profile and health indexes, respectively.

In spring, among the short- and medium-chain FA (Supplemental Table S3, see Notes), the SUL diet reduced the presence of palmitic acid (C16:0) and some branched FA, such as C13:0 *anteiso*, C15:0 *iso*, and C15:0 *anteiso*, whereas it increased the level of C17:0 *iso* and C17:0 *anteiso*, so that the total amount of branched-chain FA was comparable between diets. Because the branched-chain FA are derived by the synthesis activity of ruminal bacteria, favored by diets with high fiber level and forage:concentrate ratio, as reported by Vlaeminck et al. (2006), their reduction with the SUL diet can be associated with the higher intake of more degradable fiber. The same authors (Vlaeminck et al., 2006) suggested how the increase of C17:0 *anteiso* can be related to a deficit of degradable protein in the rumen, in this case attributable by the binding of dietary protein to CT of sulla forage. The

effect of diet emerged for almost all the long-chain FA (Table 7 and Supplemental Table S4, see Notes). Among FA with 18 carbon atoms, SUL diet increased the levels of the main FA with health benefits, such as LA, ALA, that is the precursor of long-chain n-3 FA, RU, the most prevalent isomer of CLA with recognized antitumoral activity (Parodi, 2009), and trans vaccenic acid (**TVA**), precursor of RU in udder tissues; whereas the SUL diet reduced oleic acid (**OLA**) and did not change the stearic acid (C18:0). These results can be attributed to the role of CT of sulla forage in inhibiting the complete biohydrogenation of dietary UFA in the rumen, thus increasing their level in the final products (Vasta et al., 2019; Frutos et al., 2020; Ponte et al., 2022).

Indeed, the cheeses from the SUL diet were characterized by a substantial increase in PUFA, which contribute to improve their FA profile from a health perspective (Parodi, 2009). In particular, the ratio of PUFA to SFA showed a favorable increase, and the n-6/n-3 and LA/ALA ratio decreased in the SUL cheeses, primarily due to the higher levels of ALA. Moreover, it can be observed as most indexes calculated to evaluate the health quality of cheese fat (Table 7 and Supplemental Table S5, see Notes), such as thrombogenic index (Ulbricht and Southgate, 1991), ratio of hypocholesterolemic FA to hypercholesterolemic FA (**h/H**) (Santos-Silva et al., 2002), and both GHIC and GHIC-ALA (Giorgio et al., 2019) significantly improved in the cheeses produced from the SUL diet.

In autumn, the data reveal that dehydrated sulla pellets contributed to an increase in most short and medium-chain SFA, and a reduction in palmitic acid (C16:0) and some odd- (C15:0 and C17:0) and branched-chain FA (C15:0 *iso*, C15:0 *anteiso*, C17:0 *iso*) in comparison with SHL (Supplemental Table S6, see Notes), as also observed by Ponte et al. (2022).

It is well known that the short- and medium-chain FA are de novo synthetized in the udder tissues from acetic acid produced by the ruminal fermentation of cellulose and hemicellulose. Thus, the increase of de novo FA in cheese can be certainly related to the higher forage intake with the DSF based diets (2,872, 2,876, and 2,244 g/d of DM for A-DSF, M-DSF, and SH, respectively; $P < 0.0001$; Supplemental Table S2), and the consequent availability of cellulose and hemicellulose, and thus, a more consistent microbial degradation of this fiber fractions to acetic acid.

Whereas the lower levels of some single and total branched-chain FA in DSF diets than in the SHL diet can be linked to the lower content of lignified and less digestible fiber (Supplemental Table S1), this less digestible fiber, as previously mentioned, favors their synthesis by ruminal bacteria (Vlaemink et al., 2006).

With regard to long-chain FA (Table 7 and Supplemental Table S7, see Notes), both pellets led to the reduction in stearic acid (C18:0) and OLA, but increased the levels of TVA, LA, and especially the amount of ALA; on the contrary, RU did not show a similar increase, even though the level of its precursor TVA significantly rose up ($P < 0.0001$).

The reduction of stearic acid observed in the DSF cheeses was able to balance the higher presence of the short- and medium-chain SFA, up to maintaining analogous amount of SFA. However, among the SFA, the contribution to hypercholesterolemia and the consequent risk or cardiovascular diseases is considered relevant only for the myristic acid (C14:0), null for the stearic acid (C18:0), and low for lauric (C12:0) and palmitic (C16:0) acids (Santos-Silva et al., 2002).

Moreover, the dehydrated sulla pellets contributed to increase the level of total PUFA, thus improving the health profile of the cheese fat, in accordance with Ponte et al. (2022). Indeed, it is possible to notice the improvement of A-DSF and M-DSF cheeses in terms of PUFA/SFA ratio, as well as of n-6/n-3 ratio and the corresponding one based on their precursors LA/ALA, mainly due to the increased levels of ALA. However, with all diets the values of n-6/n-3 ratio were greatly below the recommended threshold (<5) for human diet to prevent and treat chronic diseases (FAO/WHO, 1994).

Among the other calculated health-related indicators (Table 7 and Supplemental Table S8, see Notes), the significant effect of diet emerged only for thrombogenic index and both GHIC and GHIC-ALA, which improved progressively from SHL to M-DSF and A-DSF. The GHIC values of A-DSF and M-DSF cheeses were within the range of sheep cheeses (16–23) observed by Di Trana et al. (2022), whereas the GHIC level of A-DSF cheeses was quite close to that estimated by Ponte et al. (2022) for cheese from ewes fed DSF (25.6), confirming the ability of DSF to maintain the health properties of cheeses.

Cheese Sensory Profile

The cheeses were also evaluated for their sensory traits assessed through descriptive sensory test, hedonic test, and triangle discriminant test.

Tables 8 and 9 report for spring and autumn experiments, respectively, the mean scores for each trait in descriptive test and hedonic test in relation to diet, storage time, and their interaction, and Figure 2 displays the results in spider graphs.

In spring, the diet had a tendency ($P < 0.10$) to influence the traits of descriptive test related to the strength of odor (weaker in SUL cheeses) and the acid and bitter tastes (slightly less pronounced in BAR cheeses). There was a greater differentiation involving many descriptors due to storage time; accordingly, the cheeses stored for 30 d achieved higher scores for bitter and

Table 8. Effects of diet and storage time (ST) on sensory traits of cheese (spring experiment)[1]

	SUL		BAR			*P*-value		
Item	15 d	30 d	15 d	30 d	SEM	Diet	ST	Diet × ST
Color	5.20	5.15	5.05	4.84	0.270	0.3727	0.6137	0.7527
Structure uniformity	5.07	3.04	5.41	3.84	0.380	0.1231	<0.0001	0.5293
Holes	3.05	3.39	2.25	3.26	0.463	0.2904	0.1325	0.4536
Strength of odor	4.98	4.18	5.00	5.17	0.277	0.0574	0.2395	0.0690
Odor of butter	4.09	3.48	4.29	4.07	0.285	0.1473	0.1362	0.4610
Odor of milk	2.17	1.87	2.03	2.00	0.250	0.9769	0.4967	0.5711
Unpleasant odor	0.645	0.804	0.622	0.404	0.171	0.1987	0.8577	0.2530
Salty	2.23	1.64	2.17	1.64	0.248	0.9762	0.0134	0.7616
Sweet	1.30	0.94	1.60	1.21	0.242	0.2192	0.1157	0.9490
Acid	0.927	0.959	0.573	0.809	0.138	0.0597	0.3187	0.4411
Bitter	0.431	1.154	0.157	0.808	0.185	0.0837	0.0002	0.8382
Spicy	0.573	0.799	0.395	0.861	0.122	0.6232	0.0042	0.3062
Chewiness	5.18	4.45	5.61	4.74	0.285	0.1909	0.0048	0.7809
Solubility	3.88	3.92	3.89	3.99	0.341	0.9168	0.8373	0.9307
Grittiness	4.56	3.80	5.12	3.95	0.295	0.2169	0.0011	0.4607
Overall acceptance	6.09	4.17	5.90	4.42	0.301	0.9251	<0.0001	0.4408
Hedonic test								
Taste	7.34	6.35	7.21	6.48	0.272	0.9890	0.0015	0.6214
Texture	7.29	6.34	7.07	6.76	0.216	0.6466	0.0032	0.1232
Mouthfeel	7.26	6.33	7.37	6.50	0.261	0.5797	0.0005	0.9102
Overall appearance	7.34[a]	6.15[b]	7.10[a]	6.90[a]	0.224	0.2434	0.0019	0.0226
Overall liking	7.41	6.14	7.11	6.55	0.254	0.7983	0.0003	0.1482

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

[1]SUL = fresh sulla forage; BAR = fresh barley forage.

spicy taste, and lower scores for structure uniformity, salty and sweet taste, chewiness, grittiness, overall acceptance, and all traits assessed in hedonic test. The interaction reached significance ($P = 0.0226$) only for the overall appearance, which was slightly lower in 30-d cheeses from SUL diet. In the triangle discriminant test (Table 10), the differences between the diets were significantly ($P < 0.0001$) perceived, although at a more moderate level in 15-d cheeses (52.5% of corrected responses) than in 30-d cheeses (59.4% of corrected responses).

In autumn, the scores of individual descriptors, overall acceptance, and traits of hedonic test showed a certain overlap among cheeses, as can be also observed in the spider graph displayed in Figure 2. Indeed, no statistical difference emerged among diets, whereas the prolongation of storage time was responsible of some significant variations, contributing to the increase in scores attributed to color, holes, salty, overall acceptance, and most of hedonic traits, and reduced the scores for structure uniformity and odor of milk. The only significant interaction ($P = 0.0289$) emerged for bitter, due to the higher score of 30-d SHL cheese in comparison with the 30-d A-DSF cheese. In the analysis of the results obtained in the triangle test (Table 10), the differences among 15-d cheeses attributable to the diet were all perceived, although at moderate levels; indeed, the percentages of corrected responses were only slightly greater than 50%. Among cheeses at 30 d, only the difference between cheeses made with the 2 types of pellets was noted, with about 50% of corrected responses.

These results denote how the sensory properties of the cheeses were minimally affected by the ewes' diet, whereas the traits related to cheese liking were influenced by storage time with an opposite trend in the 2 experiments; indeed, the maximum appreciation was expressed for the cheeses stored for the shorter 15-d period in spring, and for the cheeses stored 30 d in autumn. This discrepancy can be associated with a presumable better oxidative stability of more matured cheeses produced in autumn, as shown by their lower peroxide values (although at not significant levels; Table 6), and attributable to their lower content in UFA (Table 7) and major antioxidant protection derived by their higher levels of polyphenols and vitamin E (Table 6).

CONCLUSIONS

This study proved the validity of dehydration as an alternative to haymaking, especially when applied to early forage cuts, and confirms the potential of both fresh and dehydrated sulla forage in ensuring adequate production levels and good nutritional, technological, and health properties of milk and cheese. Fresh sulla forage, due to its moderate content in CT, increased milk casein content, and in cheese improved the level of main PUFA, as RU and ALA, and induced a good microbiological profile and sensory properties. Dehydrated sulla contributed

Table 9. Effects of diet and storage time (ST) on sensory traits of cheese (autumn experiment)[1]

					A-DSF		M-DSF		SHL			*P*-value		
Item	A-DSF	M-DSF	SHL	SEM	15 d	30 d	15 d	30 d	15 d	30 d	SEM	Diet	ST	Diet × ST
Color	4.25	4.08	3.82	0.192	3.78	4.72	4.08	4.10	3.39	4.26	0.263	0.2521	0.0062	0.1463
Structure uniformity	4.39	4.56	4.93	0.248	4.69	4.10	5.13	4.00	5.50	4.36	0.341	0.2701	0.0011	0.6430
Holes	3.73	3.58	3.10	0.260	3.69	3.76	2.87	4.30	2.37	3.84	0.357	0.1844	0.0011	0.0798
Strength of odor	5.39	5.13	5.45	0.128	5.16	5.61	5.07	5.19	5.39	5.51	0.176	0.1538	0.1086	0.5496
Odor of butter	3.82	3.82	3.64	0.123	3.96	3.68	3.88	3.76	3.83	3.44	0.170	0.4627	0.0616	0.7258
Odor of milk	1.54	1.67	1.59	0.108	1.79	1.30	1.89	1.47	1.78	1.40	0.148	0.6567	0.0007	0.9172
Unpleasant odor	0.285	0.385	0.527	0.105	0.319	0.252	0.412	0.358	0.445	0.609	0.144	0.2354	0.9046	0.6626
Salty	1.82	1.78	2.07	0.142	1.64	2.00	1.51	2.05	1.82	2.33	0.195	0.2537	0.0045	0.8758
Sweet	0.751	0.841	0.806	0.081	0.797	0.704	0.907	0.775	0.836	0.775	0.111	0.7102	0.3048	0.9499
Acid	0.608	0.572	0.645	0.086	0.641	0.576	0.458	0.686	0.461	0.829	0.118	0.8195	0.0741	0.1666
Bitter	0.551	0.489	0.649	0.092	0.694[ab]	0.407[b]	0.360[b]	0.617[ab]	0.484[ab]	0.814[a]	0.127	0.4372	0.3443	0.0289
Spicy	0.353	0.215	0.408	0.076	0.464	0.242	0.227	0.202	0.507	0.309	0.104	0.1553	0.0907	0.5792
Chewiness	5.53	5.81	5.60	0.170	5.52	5.54	5.84	5.77	5.59	5.61	0.233	0.4719	0.9574	0.9749
Solubility	3.78	3.89	3.91	0.196	3.34	4.22	3.81	3.97	3.84	3.99	0.269	0.8620	0.0803	0.3002
Grittiness	4.64	4.93	4.54	0.180	4.54	4.74	4.92	4.94	4.65	4.43	0.247	0.2512	0.9850	0.6831
Overall acceptance	5.36	5.17	5.03	0.207	5.03	5.68	4.75	5.60	4.87	5.18	0.284	0.4969	0.0115	0.6229
Hedonic test														
Taste	6.17	6.10	5.93	0.170	5.95	6.39	5.95	6.25	5.75	6.12	0.233	0.5807	0.0602	0.9589
Texture	6.42	6.47	6.31	0.139	6.28	6.57	6.21	6.74	6.11	6.50	0.191	0.6647	0.0130	0.8257
Mouthfeel	6.13	6.20	5.96	0.159	5.81	6.45	5.94	6.45	5.84	6.09	0.218	0.5364	0.0109	0.6391
Overall appearance	6.46	6.52	6.37	0.148	6.19	6.73	6.45	6.59	6.29	6.46	0.203	0.7557	0.0941	0.5436
Overall liking	6.18	6.18	5.98	0.161	5.98	6.38	5.85	6.51	5.88	6.08	0.222	0.5745	0.0246	0.5657

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

[1]A-DSF = 2 kg/d dehydrated sulla forage from April cut. M-DSF = 2 kg/d dehydrated sulla forage from May cut. SHL = sulla hay ad libitum.

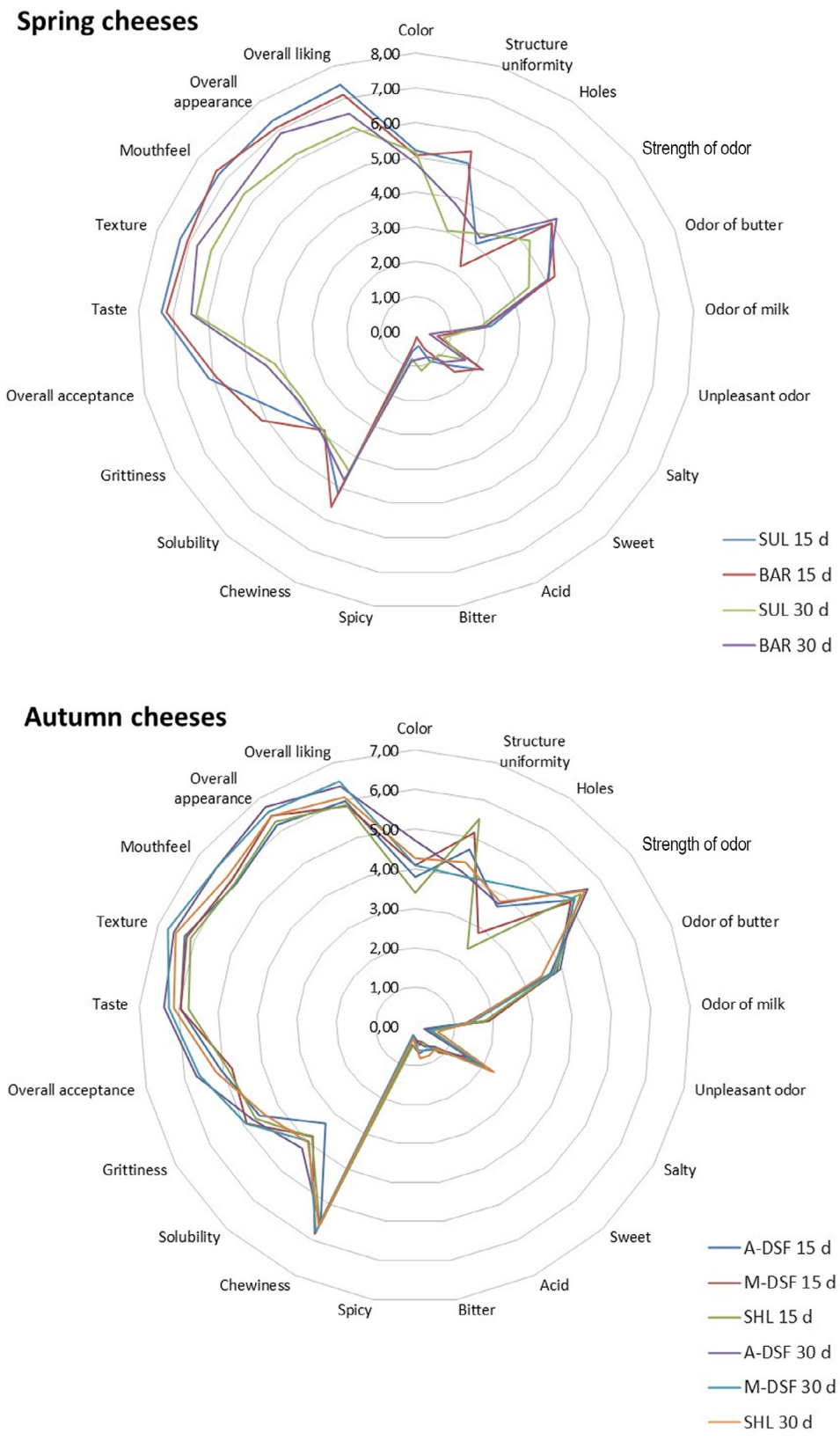

Figure 2. Spider graphs displaying the scores of sensory evaluations of cheeses produced in spring and autumn experiments by descriptive test and hedonic test. SUL = fresh sulla forage; BAR = fresh barley forage; A-DSF = 2 kg/d dehydrated sulla forage from April cut; M-DSF = 2 kg/d dehydrated sulla forage from May cut; SHL = sulla hay ad libitum.

Table 10. Comparisons at triangle test between cheeses from different diets in relation to storage time[1]

Storage time, d	Comparison		Corrected response, % (n/total)	*P*-value
15	SUL	BAR	52.5 (21/40)	<0.01
	A-DSF	M-DSF	54.8 (17/31)	<0.01
	A-DSF	SHL	51.7 (15/29)	<0.05
	M-DSF	SHL	53.3 (16/30)	<0.05
30	SUL	BAR	59.4 (19/32)	<0.01
	A-DSF	M-DSF	50.0 (15/30)	<0.05
	A-DSF	SHL	40.0 (12/30)	ns
	M-DSF	SHL	46.7 (14/30)	ns

[1]SUL = fresh sulla forage; BAR = fresh barley forage; A-DSF = 2 kg/d dehydrated sulla forage from April cut; M-DSF = 2 kg/d dehydrated sulla forage from May cut; SHL = sulla hay ad libitum; ns = not significant.

to maintaining high levels of milk casein and increasing polyphenols content and antioxidant capacity in cheese. Thus, the dehydration process of fresh sulla forage seems to preserve its content in nutrients and bioactive molecules, which are beneficial for animal productivity and health status, as well as interesting for the potential benefits on consumers' health.

NOTES

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Nonstandard abbreviations used: ABTS = 2,2-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid; A-DSF = 2 kg/d dehydrated sulla forage from April cut; ALA = α-linolenic acid; CPS = coagulase-positive staphylococci; CT = condensed tannins; DSF = dehydrated sulla forage; FA = fatty acid; FAME = fatty acid methyl esters; GAE = gallic acid equivalent; GHIC = General Health Index of Cheese; LAB = lactic acid bacteria; MDA = malonylaldehyde; M-DSF = kg/d dehydrated sulla forage from May cut; NCN = noncasein nitrogen; ns = not significant; OLA = oleic acid; POV = peroxide value; RA = relative abundance; RU = rumenic acid; SH = sulla hay; SHL = sulla hay ad libitum; ST = storage time; TBARS = thiobarbituric acid–reactive substances; TBC = total bacterial count; TEAC = Trolox equivalent antioxidant capacity; TMM = total mesophilic microorganisms; TN = total nitrogen; TPM = total psychrotrophic microorganisms; TVA = trans vaccenic acid.

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