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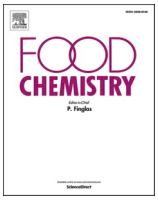

Effectiveness of modified atmosphere and vacuum packaging in preserving the volatilome of Stelvio PDO cheese over time

Bruno Domingues Galli [a], Elisabetta Trossolo [a], Ilaria Carafa [a], Simone Squara [b], Andrea Caratti [b], Pasquale Filannino [c], Chiara Cordero [b], Marco Gobbetti [a], Raffaella Di Cagno [a,*]

[a] Libera Università di Bolzano, Faculty of Agriculture, Environmental and Food Sciences, Bolzano, BZ, Italy
[b] Università di Torino, Dipartimento di Scienza e Tecnologia del Farmaco, Turin, TO, Italy
[c] University of Bari Aldo Moro, Department of Soil, Plant and Food Sciences, Bari, BA, Italy

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ABSTRACT

We aimed to assay the effectiveness of vacuum or modified atmosphere packaging in preserving the organoleptic characteristics of already ripened slices of Stelvio Protected Designation of Origin cheese during 3 months of storage. A multi-omics panel, including metagenomic and metabolomic analyses, was implemented together with physicochemical and sensory analyses.

Among the 177 volatiles identified, 30 out of the 50 potent odorants were found to be prevalent, regardless of packaging. Isovaleric acid showed the highest relative intensity in all samples. Caproic and caprylic acids always increased during storage, while metabolites such as dodecane and 2,3-butanediol always decreased. Slow proteolysis occurred during storage, but did not differentiate cheese samples. The type of packaging differentiated the microbiota and volatile profile, with modified atmosphere packaging keeping the volatilome more stable. Out of the 50 potent odorants, 9 were relevant to sample discrimination, with 8-nonen-2-one, 2-nonanone, and caproic acid being more abundant in stored samples.

1. Introduction

Stelvio or Stilfser is a cows' milk semi-hard and smear-ripened cheese with Protected Designation of Origin (PDO) produced in some territories of the Bolzano province (South Tyrol). After a ripening lasting at least two months, the cheese has an intense and peculiar aroma and taste. The cheese paste is firm and elastic, with a light-yellow straw color and uneven holes. The rind has a typical color that ranges from yellow-orange to brown-orange (Gobbetti et al., 2018). The large size of Stelvio wheels (8 to 10 kg in weight, 34 to 38 cm in diameter and 8 to 10 cm in height) and the current consumer orientation towards ready-to-eat and packaged food have led to an increase in demand for already sliced and packaged cheeses.

The main preservation methods for portioned cheeses include vacuum packaging (VaP) and modified atmosphere packaging (MAP), which consists of eliminating oxygen or replacing it with other gases in the headspace of the packa (e.g., carbon dioxide, oxygen, and nitrogen) (Albisu et al., 2023). VaP and MAP can promote the physical, chemical and biochemical stability of cheese, for instance by counteracting the growth of mesophilic and psychotropic microorganisms, weight loss due to moisture loss and lipid oxidation. Therefore, these preservation techniques have been applied to different categories of cheeses, albeit with conflicting results, especially with regard to sensory properties and their evolution during storage (Singh et al., 2012).

The efficacy of VaP and MAP is highly dependent on several factors, including the type of cheese and microbiome, ripening and storage conditions, the composition of the gas atmosphere inside the package, and the type of material used as a barrier (Possas et al., 2021). An inert anaerobic atmosphere could have a substantial impact on the overall features of smear-ripened cheeses such as Stelvio, where the metabolism of the surface microbiome is crucial for the development of the distinctive organoleptic characteristics (Brown et al., 2018). Indeed,

Abbreviations: PDO, protected designation of origin; VaP, vacuum packaging; MAP, modified atmosphere packaging; PCA, plate count agar; PDA, potato dextrose agar; LAB, lactic acid bacteria; RAPD-PCR, randomly amplified polymorphic DNA- polymerase chain reaction; Qiime2, quantitative insights into microbial ecology; GPA, generalized procrustes analysis; PLS-DA, partial least squares discriminant analysis; sPLS-DA, sparse partial least squares discriminant analysis; VIP, variable importance in the projection index; NSLAB, non-starter lactic acid bacteria.

* Corresponding author.
E-mail address: raffaella.dicagno@unibz.it (R. Di Cagno).

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Brevibacterium linens, micrococci (including *Micrococcus luteus*), staphylococci (including *Staphylococcus equorum)*, and coryneform bacteria (such as *Arthrobacter* and *Corynebacterium)*, are the main bacteria that reside on the surface of smear-ripened cheeses. Thanks to their complex portfolio of enzymes, including proteinases, peptidases, and lipases, these bacteria release amino acids and fatty acids that serve as the building blocks or precursors of numerous flavor compounds (Beresford et al., 2001). On the other hand, starter cultures or autochthonous milk bacteria that benefit from a more anaerobic environment, such as lactococci and streptococci, act more intensely on the inside of cheeses (Choi et al., 2020). The high heterogeneity of cheese varieties necessitates the design of *ad hoc* packaging systems, adapted to the intrinsic characteristics of each cheese (e.g., moisture and salt gradients, redox potential, production process, microbial dynamics, type of starter, etc.). To the best of our knowledge, there is no information in the literature on the evolution of the volatilome and microbiome of Stelvio cheese stored under VaP and MAP.

The aim of this study was to verify the effectiveness of storing already ripened Stelvio PDO cheese slices under VaP and MaP in order to preserve their organoleptic characteristics during 3 months of storage. A multi-omics panel including metagenomic and metabolomic analyses will be implemented together with physicochemical and sensory analyses. In light of the peculiar microbial dynamics that characterize Stelvio PDO cheese, the study was conducted by distinguishing the cheese core and the under-rind fractions. Multivariate statistics were also applied to chemical fingerprints in order to highlight discriminating patterns of volatiles. The study is innovative because of the scarcity of literature data on the microbial dynamics and biochemical events that take place during the storage of PDO Stelvio cheeses. Therefore, it could be considered as a pioneering work to provide a comprehensive basis of potential biomarkers useful for the overall quality control of PDO Stelvio cheeses.

2. Material and methods

2.1. Experimental design, cheese sampling, fractioning, and storage

Cylindrical Stelvio-PDO cheeses of 8.7 kg were produced by the company Mila Latte Montagna Alto Adige Soc. Ag coop. (Bolzano, Italy) in 3 different batches (A, B, and C) and ripened for 3 months. After this period, the wheels were fractionated into wedges of approximately 1 kg and immediately packed under vacuum packaging (encoded as VaP) or modified atmosphere packaging (encoded as MAP) with 80/20 % CO_2/N_2. Cheeses were packaged into 0.300 m × 0.400 m polymeric double-layer bags made of polyamide and polyethylene (PA/PE), with a thickness of 80 μm, and with double welding after filling. The packaging conditions were chosen taking into account the recommendations for semi-hard cheeses according to Khoshgozaran et al. (2012) and Albisu et al. (2023). Packaged samples were stored in a refrigerated chamber at 4–8 °C for a further 3 months in the dairy processing plant of the Micro4food laboratory at the Free University of Bolzano (Bolzano, Italy).

Cheese samples from three production batches (e.g., A, B, and C) were analyzed in triplicate at time 0 (unpacked) and after one, two, and three months of storage under VaP or MAP (Fig. 1).

The under-rind fraction of the cheese just below the surface (coded as "r") and its core central part (coded as "c") were defined as the fraction just below the surface of the entire outer layer of the 2 cm thick piece with the exception of the rind (approximatively 1 mm thick) and the remaining homogenized core of the cheese, respectively. The rind was discarded.

Therefore, the experimental design included 3 biological replicates corresponding to three independent cheese-making processes, with 3 variables (packaging mode, storage time, and the cheese section) for a total of 42 samples analyzed in triplicate (Fig. 1).

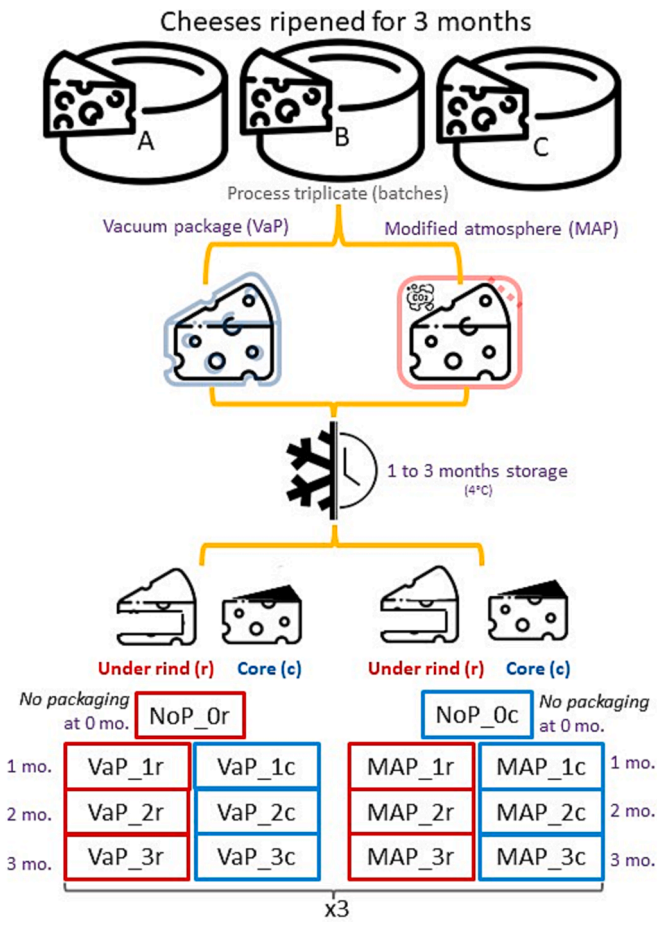

Fig. 1. Schematic representation of the experimental design of the research project (n = 14 x 3).

2.2. Gross composition

The gross composition of the samples referring to the content (%) of moisture, protein, fat, ashes, and NaCl was determined on 5.00 g of a homogenized sample, using a FoodScanTM2 equipment (Foss Analytics, Hilleroed, Denmark), pre-calibrated with artificial neural network (ANN) calibration and database nationally certified for the determination of key parameters, in accordance with the manufacturer's instructions (Garbowska et al., 2020).

2.3. Microbiological enumeration

The Stelvio PDO cheese samples were subjected to the culture-dependent approach for the counting of mesophilic aerobic bacteria, mesophilic and thermophilic anaerobic bacteria, yeasts, and molds as described by Amato et al. (2012).

In short, ten grams of each sample were homogenized with 90 mL of sterile sodium citrate (2 % w/v) solution. The enumeration was carried out using the pour plate technique. Total bacterial growth was calculated on Plate count Agar (PCA, Oxoid, Basingstoke, United Kingdom) supplemented with 1 % (w/v) skimmed milk, at 30 °C for 24 h. Presumed mesophilic lactobacilli were counted on MRS agar (Oxoid, Basingstoke, United Kingdom) supplemented with cycloheximide (0.1 g/L), under anaerobic conditions at 30 °C for 48 h. Presumed mesophilic lactococci and thermophilic streptococci were counted on M17 agar (Oxoid) supplemented with 0.5 % lactose, under anaerobic conditions for 24 and 48 h at 30 °C and 45 °C, respectively (Celano et al., 2022). Presumptive yeast and molds were counted on Potato Dextrose Agar media (PDA, Oxoid, Basingstoke, United Kingdom) supplemented with

0.01 % chloramphenicol and incubated at 25 °C for three and 3–7 days (Settanni et al., 2021). Pure cultures were subjected to microscopic observation and stored at −80 °C in glycerol (15 %, v/v) stocks.

2.4. Isolation, genotyping, and identification of mesophilic lactic acid bacteria

From each sample at time zero, at least fifteen colonies were isolated on MRS and M17 30 °C agar plates and were then purified by sub-cultivation. Seventy-five colonies (corresponding to 10 % of total colonies) were subjected to the biotyping.

Genomic DNA from presumptive lactic acid bacteria (LAB) was extracted from the pellet of 1 mL of pure culture using the DNeasy blood and tissue kit (Qiagen, SA, Courtaboeuf, France), according to the manufacturer's instructions. Two oligonucleotides, *P*4 (5′-CCGCAGCGTT −3′) and M13 (5′- GAGGGTGGCGGTTCT −3′), were used for biotyping the isolates by RAPD-PCR as described by Nikoloudaki et al. (2021). RAPD-PCR profiles were analyzed through the Unweighted Pair Group Method Arithmetic averages (UPGMA) using the GelCompar II-BioNumerics software 8.0 (package version; Applied Maths, Sint-Martens-Latem, Belgium). The calculation of the similarity of the PCR fingerprinting profiles was based on the Pearson product-moment correlation coefficient. Isolates with a similarity coefficient higher than 90 % were considered to belong to the same biotype. Genotypic identification of different LAB biotypes was carried out by partial 16S rRNA gene sequencing and species-specific PCRs. LpigF/LpigR primers (5′-TACGGGAGGCAGCAGTAG-3′ and 5′-CATGGTGTGACGGGCGGT-3′) were used to amplify the 16S rRNA gene fragment of presumptive lactic acid bacteria (Nikoloudaki et al., 2021). For species assignment, sequences were compared using the BLAST algorithm of the National Center for Biotechnology Information (NCBI, Bethesda, USA). All amplifications were performed using a TurboCycler 2-thermal-Cycler (Bluray Biotech, Taiwan).

2.5. Microbiota analysis by culture independent approaches: Next-generation sequencing analysis

2.5.1. Total microbial genomic DNA extraction

Total genomic DNA was extracted from all samples (both core and under-rind sections) according to Galli et al., (2023). Quantification of total DNA was performed with Qubit™ dsDNA HS Assay Kit (ThermoFisher Scientific, Rodano, Italy). Two independent replicates of each sample were used for DNA extraction and mixed, and pooled.

2.5.2. Preparation of the MiSeq library

Bacterial and fungal diversity was analyzed based on the 16*S* and internal transcribed spacer (ITS), respectively. Primers targeting the 16S rRNA variable region V3-V4 (*Escherichia coli* position 341–805, forward 341F: CCTACGGGNGGCWGCAG and reverse 806R: GACTACNVGGGTWTCTAATCC of the 16S rRNA gene were used for bacteria (Galli et al., 2023), while primers (forward ITS1: CTTGGTCATTTAGAGGAAGTAA and reverse ITS2: CTGCGTTCTTCATCGATGC) targeting the ITS1 region between 18S and 5.8S rRNA genes were used for fungi (Gardes and Bruns, 1993).

To simplify sample distinction, barcodes were affixed to the forward primer. To prevent preferential sequencing of the smallest amplicons, the amplicons were cleaned using the Agencourt AMPure kit (Beckman Coulter, Brea, CA, USA) in accordance with the manufacturer's instructions. DNA was then quantified using the Quant-iT PicoGreen dsDNA kit (Invitrogen, Milano, Italy). They were mixed and combined in equimolar ratios, and the quality and purity of the library were evaluated with the High Sensitivity DNA Kit (Agilent, Palo Alto, CA, USA) by Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA). Library preparation and pair-end sequencing were carried out at the Genomic Platform – Fondazione Edmund Mach (San Michele a/Adige, Trento, Italy) using the Illumina MiSeq system (Illumina, San Diego, USA) according to standard laboratory procedures.

2.5.3. Illumina data analysis and sequences identification by QIIME2

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights Into Microbial Ecology (Qiime2, version 2018.2). Sequences were quality filtered, trimmed, de-noised, and merged using DADA2 (Galli et al., 2023).

Chimeric sequences were identified and removed via the consensus method in DADA2. Representative bacterial sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugin alignment and phylogeny (Galli et al., 2023). The core diversity plugin in QIIME2 and Emperor were used to calculate the alpha and beta diversity measurements (Pontonio et al., 2021). The Bray-Curtis distance matrix was used to determine beta diversity. Bacteria taxonomic and compositional analyses were carried out by using a plugin feature classifier (https://github.com/qiime2/q2-feature-classifier). Using the q2-feature-fit-classifier-naive-Bayes classifier's method, a naive Bayes taxonomy classifier was trained on the Silva (Pontonio et al., 2021) r132 reference sequences (clustered at 99 % similarity), trimmed to the V3-V4 region of 16S rDNA, and applied to paired-end sequence reads to create taxonomy tables. Fungi sequences were classified to the species level with a 97 or 99 % threshold (based on which is more accurate for certain lineages of fungi) by using UNITE v.8.0 Dynamic Classifier (UNITE Community 2019). Data on bacterial and yeast communities were subjected to one-way ANOVA and pair comparison of treatment means was obtained by Tukey's procedure at $p < 0.05$ using the statistical software R 3.6.1 (Pontonio et al., 2021).

2.6. Volatile organic compounds (VOCs) analysis

VOCs were profiled via headspace solid-phase microextraction sampling followed by comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometric detection (HS-SPME-GC × GC-TOFMS) as described by Cordero et al. (2019). Cheese samples were finely grounded with an analytical mill equipped with stainless-steel blades and in the presence of liquid nitrogen to avoid overheating (IKA Analytical Mill, IKA-Werke GmbH & Co, Staufen, Germany). The resulting powder (1–2 mm particle size) was accurately weighted (1.00 ± 0.01 g) in a 20 mL headspace vial before HS-SPME.

Sampling was carried out with a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME device d_f 50/30 μm – 2 cm for 50 min at 50 °C. Sampling conditions were optimized to achieve the highest HS information capacity while limiting artefact formation and not exceeding the run time duration

The GC × GC analyses were performed on an Agilent 7890B gas chromatograph (Agilent Technologies, Wilmington DE, USA) coupled to a Markes BenchTOF Select™ mass spectrometer (Markes International, Bridgend, UK). The system was equipped with a two-stage KT 2004 loop-type thermal modulator (Zoex Corporation, Houston, TX) cooled with liquid nitrogen controlled by Optimode v2.0 (SRA Instruments, Cernusco sul Naviglio, Milan, Italy). Column settings and operative conditions were as follows: ^{1}D polar capillary column Heavy-Wax (100 % Polyethylene Glycol) (30 m × 0.25 mm d_c × 0.25 μm d_f) coupled with a ^{2}D medium polarity column OV17 (86 % polydimethylsiloxane, 7 % phenyl, 7 % cyanopropyl) (1.0 m × 0.10 mm d_c × 0.10 μm d_f) from J&W (Agilent, Palo Alto, CA, USA); additional 0.80 m 0.10 mm d_c deactivated silica were wrapped in the modulator slit as modulator capillary (e.g., the loop capillary). Columns were placed in the same oven and no temperature offset was applied to the two separation dimensions. The carrier gas was helium at 1.3 mL/min - constant flow. The modulation period (P_M) was 2.5 s, the hot jet pulse time was 250 ms, and the cold jet flow was reduced from 40 % to 5 % of the total mass flow controller flow-rate (max 40 L/min) across the analytical run duration. The oven temperature ramp was as follows: 40 °C (1 min) to 260 °C (10 min) at 3.5 °C/min. The injector temperature was kept at 250 °C operating in

split mode with a split ratio of 1:20. The TOF MS acquisition parameters were as follows: EI operating in single ionization mode at 70 eV with an acquisition rate of 100 Hz within the mass range 40–350 *m/z*; the filament voltage was set at 1.7 V. The ion source and transfer line were set at 280 °C and 250 °C respectively. Data were acquired by TOF-DS software (Markes International, Bridgend, UK) and processed using GC Image GC × GC Software ver. 2021r2 (GC Image, LLC, Lincoln, NE, USA). The following parameters were used to analyze the *n*-alkanes liquid sample solution for linear retention indices (I^T) determination: split/splitless injector in split mode, split ratio 1:50, injector temperature 250 °C, and injection volume 1 μL (50 mg/L *n*-alkanes final concentration).

Pure reference compounds for identity confirmation of potent odorants listed in Table 1, *n*-alkanes (*n*-C9 to *n*-C25) for I^T determination, and α/β-tujone for internal standardization were obtained from Merck Millipore (Milan, Italy). HS-SPME sampling repeatability was verified by internal standard (IS) pre-loading onto the SPME device before each sampling on Stelvio cheese aliquots. In details, 5.0 μL of α/β-thujone IS solution (0.100 g/L in dibutyl phthalate) were placed in a 20 mL headspace vial and sampled at a temperature of 50 °C for 5 min before fiber exposure to the sample. Additionally, quality control samples (QCs) of a different type of cheese (Fontina di Aosta, PDO cheese, Aosta, Italy) were used to confirm system stability over time and to normalize features response data in each analytical session (Stilo et al., 2021; Wang et al., 2005).

Analyte identification was by combining retention data (experimental $I^T \pm 10$ unit tolerance *vs.* tabulated ones) and comparing EI-MS spectral signature with reference data in commercial and in-house libraries by the NIST identity search algorithm and direct match factor (DMF) and reverse match factor (RMF) scores threshold ≥ 900.

2.7. Aqueous fraction extraction and proteolysis analysis

The cheese aqueous fraction soluble at pH 4.6 was obtained as described by Celano et al. (2022). The 70 % ethanol soluble extract was diluted in 20 mL of ultra-pure water (Milli-Q, Merck, Darmstadt, Germany) after the total evaporation of ethanol, gently mixed to resuspend the extract, and freeze-died for 24 h at −60 °C.

The proteolysis of cheeses was obtained using the RP-FPLC-ÄKTA-pure equipment (General Electric Healthcare Systems, Milwaukee, USA). The reversed phase chromatography column used was the RESOURCE-RPC 3 mL based on rigid monosized 15 μm diameter polystyrene/divinyl-benzene beads. The elution solutions used were: A) Aqueous solution containing 5 % acetonitrile and 0.05 % Trifluoroacetic acid; B) Pure acetonitrile solution containing 0.65 % Trifluoroacetic acid. The chromatography method was set up as follows: sample injection at a flow rate of 1 mL/min filling the injection loop with at least 1.25 mL of sample; Constant elution flow rate of 1 mL/min starting with 100 % eluent A; First gradient elution from 0 to 46 % eluent B in 43.5 min; second gradient elution from 46 to 100 % eluent B in 9 min; A constant flow of 100 % eluent B for 9 min; A last gradient from 0 to 100 % of eluent A in 9 min; A constant flow of 100 % of eluent A for 5 min; UV detector operating at 214 nm. Right before the injection step, 1.5 g of the lyophilizate sample extract was dissolved in 1.5 mL of eluent A, filtered (0.22 μm) and injected entirely into the equipment (Celano et al., 2022).

The total area of the chromatograms was divided into five equal parts according to the elution time of the peaks, the first corresponding to the very-low molecular weight peptides eluted from 0 to 15 min, the second to the peptides eluted from 15 to 30 min described as low molecular weight peptides, the third to the medium molecular weight peptides eluted from 30 to 45 min, the fourth to peptides eluted from 45 to 60 min described as high molecular weight peptides, and the fifth and final fraction corresponding to the very-high molecular weight peptides eluted from 60 to 75 min. The area of each fraction was integrated into the total area and analyzed by Principal Component Analysis (PCA).

2.8. Descriptive sensory analysis

The descriptive approach known as Flash-Profile method was used to conduct the sensory analysis, following the method provided by Dairou and Sieffermann (2002). A panel of 20 people (12 women and 8 men aged 23–38), previously familiarized with the product, conducted the sensory evaluation. The experiments were carried out individually in a white-light room and without environmental or external sensory interference. In order to prevent the participants from being influenced, samples were presented concurrently in 10 g portions, at a regulated ambient temperature, on white randomly-coded plastic trays. During the examination, crackers and mineral water were provided to cleanse the palate. A brief preliminary training was conducted in order to get the members ready for the Flash-Profile test. Additionally, the sensory assessment technique was provided, including how to assess any features perceived after swallowing, including appearance, aroma, taste, and texture. The assessors were given instructions to use an unstructured 9 cm scale, anchored by extremes for quantitative descriptive analysis to award a score according to the intensity of each characteristic. The absolute scale values were not used, only ordinal values were considered. The Commandeur method of Generalized Procrustes Analysis (GPA) was used to analyze the Flash-Profile sensory analysis findings. Assessors with analysis residue higher than 25 % were considered outliers and excluded up to a maximum of 10 excluded Assessors. Analyses were performed on 6 samples of each category analyzed (non-packed, VaP at 3 months of storage, and MAP at 3 months of storage) due to the impossibility of analyzing more than 6 samples simultaneously without causing sensory fatigue and data with high residue, high variation, and little agreement between tasters. Therefore, the data obtained are exploratory and not comparative.

2.9. Statistical and chemometric analysis

Statistical analysis was performed using the XLStat software – version Premium 2023.1.1. Data on the composition and relative responses of the volatile metabolites of interest (features) were statistically analyzed by ANOVA and Tukey's honest significance test (HST) at a 5 % significance level. The proteolysis data obtained by calculating the chromatographic area of 5 different regions of the chromatograms obtained by FPLC-AKTA were analyzed through PCA with 5 % of significance. Additionally, the correlation of raw data from 50 potent odorants, relative microbial abundance (Illumina 16S and ITS), microbiological plate count, and proteolysis profile were analyzed by PCA and by Pearson correlation matrix combined with Tuckey HST at the level of 5 % significance. Variables with high correlation were those with an index greater than 0.6 in modulus.

Using chemometric tools, the absolute response of the 50 potent odorants by the GC × GC-TOF MS was explored to extract biological information related to the storage time and type of packaging. The chemometric analysis was performed using the MetaboAnalyst 5.0 software, normalizing the data set by the median, without data transformation, using Pareto scaling, and with 95 % confidence ellipses. For the analysis of the effect of storage time, the Partial Least Squares Discriminant Analysis (PLS-DA) models were created. The Variable Importance in the Projection index (VIP-score) of the metabolites was also evaluated, using as high-importance threshold metabolites with a VIP-score greater than 1 for the PLS-DA analysis. In order to study the effect of the type of packaging, given the numerous explanatory classes, the Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was used in order to obtain discrimination with a good explanation of the data (sum of the main components higher than 50 %). A loading score of 0.1 was used as a threshold value in the sPLS-DA.

Table 1
Normalized responses % of the 50 most potent odorants identified in Stelvio-PDO cheese samples, their respective molecular formulas, and sensory quality descriptors. Equal lowercase letters on the same line indicate that there is no significant difference ($P < 0.05$) in the relative response of the analyte between samples. Data are mean ± SD of three 3 biological replicates (A, B, C) analyzed in triplicate.

Compound	Chemical Formula	Odor quality	NoP_0r	NoP_0c	VaP_1r	VaP_2r	VaP_3r	VaP_1c	VaP_2c	VaP_3c	MAP_1r	MAP_2r	MAP_3r	MAP_1c	MAP_2c	MAP_3c	Pr > f
Acids																	
Isovaleric acid	$C_5H_{10}O_2$	Cheesy/Sweaty	41.42	42.79	36.82	35.92	28.28	41.74	38.44	38.60	33.94	33.82	30.97	38.08	37.27	39.04	0.794
Caproic acid	$C_6H_{12}O_2$	Goat/Barn	6.73^{d}	8.56 ^{cd}	9.56 ^{cd}	13.36^{abc}	17.40^{ab}	8.00 ^{cd}	10.60^{bcd}	11.45^{bcd}	14.97^{abc}	16.93^{ab}	17.76^{a}	9.11 ^{cd}	10.69 ^{cd}	12.13^{bcd}	**<0.001**
Isobutyric acid	$C_4H_8O_2$	Sweaty	11.62	10.41	9.00	9.65	6.36	11.12	10.50	9.58	7.08	6.58	5.26	11.48	9.21	9.77	0.377
Acetic acid	$C_2H_4O_2$	Vinegar/sour	10.42^{ab}	8.6^{abc}	7.22^{c}	6.81^{abc}	7.11^{abc}	8.76^{abc}	8.16^{ab}	7.96^{abc}	7.14^{bc}	6.96^{abc}	6.77^{ab}	8.77^{a}	8.86^{ab}	8.35ab	**0.003**
Caprylic acid	$C_8H_{16}O_2$	Oily/acid	1.63^{c}	2.00^{c}	2.36^{bc}	4.15^{abc}	9.49^{ab}	1.89^{c}	2.65^{bc}	2.95^{bc}	5.84^{abc}	7.13^{abc}	10.55^{a}	2.44^{bc}	2.97^{bc}	3.38^{bc}	**<0.001**
Capric acid	$C_5H_{12}O$	Feet	0.86^{b}	0.7^{b}	0.79^{b}	0.96^{ab}	2.84^{ab}	0.72^{b}	0.94^{b}	0.97^{ab}	1.45^{ab}	1.74^{ab}	3.12^{a}	0.87^{b}	1.11^{ab}	1.00^{ab}	**0.005**
Valeric acid	$C_9H_{18}O$	Erbal	1.12	1.06	1.12	1.00	0.98	1.01	1.00	1.04	0.99	0.91	0.90	1.08	1.04	0.98	0.283
Propionic acid	$C_3H_6O_2$	Rancid	0.31	0.34	0.30	0.32	0.25	0.33	0.35	0.30	0.30	0.25	0.21	0.44	0.34	0.31	0.564
Ketones																	
Acetoin	$C_4H_8O_2$	Buttery	3.74	3.65	3.94	3.93	3.69	3.21	3.39	3.07	4.42	3.6	3.58	3.44	3.84	3.18	0.060
2-Pentanone	$C_5H_{10}O$	Woody	1.42	1.45	1.57	1.49	1.36	1.33	1.55	1.38	1.36	1.40	1.34	1.44	1.60	1.46	0.122
2-Nonanone	$C_5H_{10}O_2$	Cheesy/Sweaty	0.42^{c}	0.47^{c}	0.93^{abc}	1.11^{abc}	1.37^{ab}	0.47^{c}	1.41^{ab}	0.81^{abc}	1.34^{abc}	1.54^{a}	1.44^{a}	0.58^{bc}	1.37^{abc}	1.08^{abc}	**<0.001**
2,3-Butanedione	$C_4H_6O_2$	Butter	0.94	0.86	0.91	0.77	0.76	0.76	0.73	0.67	0.91	0.79	0.67	0.78	0.85	0.69	0.110
2-Heptanone	$C_7H_{14}O$	Banana	0.57^{d}	0.57 ^{cd}	0.93^{abc}	0.87^{a}	0.74^{abc}	0.49^{d}	0.76^{abcd}	0.57^{bcd}	0.79^{abc}	0.89^{a}	0.66^{abc}	0.57^{bcd}	0.93^{ab}	0.84^{ab}	**<0.001**
Acetone	C_3H_6O	Solvent	0.47^{b}	0.56^{ab}	0.80^{ab}	0.59^{ab}	0.53^{ab}	0.58^{ab}	0.62^{ab}	0.49^{ab}	0.65^{ab}	0.62^{ab}	0.63^{a}	0.61^{ab}	0.64^{ab}	0.65^{ab}	**0.022**
2-Butanone	C_4H_8O	Sharp sweet	0.32^{b}	0.38^{ab}	0.58^{ab}	0.51^{b}	0.43^{ab}	0.50^{ab}	0.49^{ab}	0.43^{ab}	0.41^{ab}	0.44^{ab}	0.46^{ab}	0.45^{ab}	0.48^{ab}	0.51^{ab}	**0.045**
8-nonen-2-one	$C_9H_{16}O$	Fruity baked	0.07^{d}	0.10^{d}	0.27^{bcd}	0.32^{abcd}	0.48^{abc}	0.08^{d}	0.36^{abcd}	0.21 ^{cd}	0.39^{abcd}	0.57^{ab}	0.56^{a}	0.14 ^{cd}	0.47^{abcd}	0.30^{abcd}	**<0.001**
2-Undecanone	$C_{11}H_{22}O$	Floral rose	0.18^{b}	0.18^{b}	0.23^{b}	0.21^{b}	0.42^{a}	0.18^{b}	0.34^{ab}	0.21^{b}	0.26^{b}	0.39^{ab}	0.44^{a}	0.21^{b}	0.28^{b}	0.23^{b}	**0.048**
2-Tridecanone	$C_{13}H_{26}O$	Slightly spicy	0.06^{c}	0.06^{c}	0.08^{bc}	0.06^{abc}	0.17^{a}	0.07^{bc}	0.06^{bc}	0.07^{abc}	0.07^{abc}	0.09^{abc}	0.14^{ab}	0.07^{abc}	0.07^{bc}	0.06^{bc}	**0.003**
Alcohols																	
2,3-Butanediol	$C_4H_{10}O_2$	Butter/Creamy	2.48	2.29	2.41	2.19	1.86	2.30	2.38	2.35	1.95	1.68	1.53	2.48	2.34	2.26	0.356
3-Methyl-1-butanol	$C_{10}H_{20}O_2$	Barn/Goat	1.55^{ab}	1.43^{ab}	2.40^{a}	1.62^{ab}	1.05^{b}	1.62^{ab}	1.55^{ab}	1.66^{ab}	1.24^{ab}	1.17^{ab}	0.84^{b}	1.54^{ab}	1.68^{ab}	1.43^{ab}	**0.010**
Isovaleraldehyde	$C_5H_{10}O$	Peach like	0.90	0.91	1.18	0.91	0.85	0.91	0.83	0.93	1.03	0.88	0.77	0.89	0.76	0.78	0.059
Ethanol	C_2H_6O	Alcohol	0.82	0.81	1.24	0.75	0.72	1.15	0.82	0.78	0.79	0.59	0.62	1.02	0.80	0.81	0.051
2-Butanol	$C_4H_{10}O$	Fruit alcoholic	0.50	0.44	0.55	0.50	0.40	0.51	0.49	0.43	0.43	0.36	0.33	0.52	0.43	0.46	0.051
1-Propanol	C_3H_8O	Apple, cognac, rum	0.41	0.40	0.47	0.35	0.35	0.43	0.41	0.39	0.35	0.30	0.30	0.44	0.40	0.39	0.161
2-Propanol	C_3H_8O	Strong alcoholic	0.42^{c}	0.28^{c}	0.72^{a}	0.32^{bc}	0.29^{c}	0.58^{ab}	0.33^{bc}	0.34^{bc}	0.38^{bc}	0.26^{c}	0.26^{c}	0.41^{bc}	0.36^{c}	0.38^{bc}	**<0.001**
Benzeneethanol	$C_9H_{12}O$	Unknown	0.18^{e}	0.23^{cde}	0.31^{abcd}	0.24^{abcd}	0.33^{abcd}	0.18^{e}	0.24^{abcd}	0.40^{ab}	0.31^{abcd}	0.34^{abc}	0.35^{a}	0.18^{de}	0.25^{bcd}	0.19^{d}	**<0.001**
2-Heptanol	$C_7H_{16}O$	Fresh lemon	0.23^{ab}	0.15^{b}	0.41^{ab}	0.35^{a}	0.32^{ab}	0.23^{ab}	0.29^{ab}	0.31^{ab}	0.20^{ab}	0.21^{ab}	0.23^{ab}	0.27^{ab}	0.25^{ab}	0.34^{ab}	**0.019**
1-Butanol	$C_4H_{10}O$	Solvent	0.11^{c}	0.12^{abc}	0.26^{a}	0.19^{ab}	0.14^{abc}	0.14^{abc}	0.19^{abc}	0.16^{abc}	0.12^{abc}	0.11^{abc}	0.09^{bc}	0.17^{abc}	0.16^{abc}	0.18^{abc}	**0.004**
2-Pentanol	$C_5H_{12}O$	Fermented	0.08^{b}	0.08^{b}	0.11^{b}	0.13^{ab}	0.09^{ab}	0.10^{b}	0.14^{ab}	0.14^{ab}	0.06^{b}	0.09^{b}	0.11^{ab}	0.12^{ab}	0.12^{ab}	0.18^{a}	**<0.001**
1-octen-3-ol	$C_8H_{16}O$	Mushroom	0.05	0.04	0.06	0.05	0.04	0.05	0.05	0.05	0.05	0.06	0.04	0.05	0.05	0.04	0.595
Aldehydes																	
Nonanal	$C_9H_{18}O$	Floral citrus	1.37	1.45	1.97	1.74	1.20	1.11	1.49	1.63	1.51	1.30	1.26	1.54	1.61	1.18	0.767
Benzaldehyde	C_7H_6O	Bitter nutty	0.83^{c}	1.06^{abc}	1.13^{abc}	0.76^{abc}	1.07^{a}	0.80^{bc}	0.73^{bc}	0.96^{abc}	1.11^{ab}	1.02^{a}	0.71^{abc}	0.67^{bc}	0.82^{bc}	0.62^{c}	**<0.001**
Benzeneacetaldehyde	C_8H_8O	Grassy floral	0.69^{c}	0.68^{bc}	0.73^{bc}	0.55^{bc}	0.65^{ab}	0.69^{abc}	0.61^{bc}	0.79^{a}	0.58^{bc}	0.56^{abc}	0.56^{abc}	0.66^{abc}	0.68^{bc}	0.58^{bc}	**<0.001**
Esters																	
Ethyl hexanoate	$C_8H_{16}O_2$	Fruity	0.55^{b}	0.56^{ab}	1.01^{ab}	0.86^{a}	0.90^{a}	0.65^{ab}	0.69^{ab}	0.76^{ab}	0.95^{ab}	0.69^{ab}	0.81^{a}	0.75^{ab}	0.71^{ab}	0.68^{ab}	**0.005**
Ethyl decanoate	$C_{12}H_{24}O$	Wine/yeast	0.54^{c}	0.52^{bc}	0.40^{c}	0.54^{abc}	0.91^{a}	0.57^{bc}	0.57^{abc}	0.68^{abc}	0.64^{abc}	0.64^{abc}	0.77^{ab}	0.62^{abc}	0.68^{abc}	0.60^{abc}	**0.002**
Ethyl octanoate	$C_{10}H_{20}O_2$	Fruity/floral	0.21^{c}	0.27^{bc}	0.55^{abc}	0.52abc	0.86^{a}	0.27^{bc}	0.31^{abc}	0.39^{abc}	0.60^{abc}	0.51^{abc}	0.70^{ab}	0.32^{abc}	0.36^{abc}	0.31^{abc}	**0.005**
Ethyl butyrate	$C_6H_{12}O_2$	Pineapple	0.46^{c}	0.40^{c}	0.68^{ab}	0.57a	0.49^{abc}	0.42^{bc}	0.44^{abc}	0.41^{bc}	0.56^{abc}	0.40^{abc}	0.40^{abc}	0.42^{abc}	0.46^{abc}	0.43^{abc}	**<0.001**
Ethyl Acetate	$C_4H_8O_2$	Sweet fruit	0.21^{ab}	0.21^{ab}	0.32^{ab}	0.18ab	0.18^{ab}	0.27^{ab}	0.22^{ab}	0.22^{ab}	0.20^{ab}	0.13^{b}	0.12^{b}	0.29^{a}	0.20^{ab}	0.20^{ab}	**0.007**
Lactones																	
δ-Decalactone	$C_{10}H_{18}O_2$	Creamy/coconut	0.98^{b}	0.92^{ab}	0.92^{ab}	1.07ab	0.94^{ab}	1.04^{ab}	1.03^{ab}	0.98^{ab}	0.87^{ab}	0.81^{ab}	0.83ab	1.24^{a}	1.02^{ab}	0.96^{ab}	**0.047**
δ-Octalactone	$C_8H_{14}O_2$	Coconut/mint	0.35	0.35	0.33	0.35	0.45	0.36	0.28	0.32	0.34	0.31	0.41	0.42	0.32	0.28	0.103
δ-Dodecalactone	$C_{10}H_{18}O_2$	Butter/Cream	0.38	0.26	0.27	0.22	0.37	0.32	0.22	0.32	0.25	0.30	0.34	0.31	0.35	0.27	0.153
γ-Decalactone	$C_{10}H_{18}O_2$	Fruity/Peach	0.04	0.04	0.05	0.04	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.352
γ-Dodecalactone	$C_{12}H_{22}O_2$	Fruity/Musky	0.03^{c}	0.03^{c}	0.04^{bc}	0.04^{abc}	0.05^{ab}	0.04^{bc}	0.03^{bc}	0.04^{bc}	0.04^{bc}	0.04^{abc}	0.05a	0.04^{abc}	0.04^{bc}	0.03^{bc}	**<0.001**
Miscellaneous																	
Dodecane	$C_{12}H_{26}$	Gasoline	1.29^{abcd}	1.22^{abcd}	1.45^{abcd}	0.81^{abc}d	0.71^{bcd}	1.92^{ab}	1.21^{abcd}	1.58^{abc}	1.07^{abcd}	0.67^{bcd}	0.26d	1.95^{a}	1.05^{abcd}	0.59 ^{cd}	**<0.001**
I-menthol	$C_{10}H_{20}O$	Mint	0.61^{b}	0.58^{ab}	0.57^{b}	0.53^{ab}	0.70^{a}	0.64^{ab}	0.62^{ab}	0.74^{ab}	0.57^{ab}	0.55^{ab}	0.57ab	0.61^{ab}	0.69^{ab}	0.62^{ab}	**0.014**
Tetramethylpyrazine	$C_8H_{12}N_2$	Musty/coffee	0.45^{e}	0.51^{cde}	0.54^{cde}	0.45^{bcd}	0.52^{abc}	0.41^{de}	0.39^{de}	0.43^{de}	0.58^{abc}	0.57^{ab}	0.57a	0.41^{de}	0.46^{de}	0.41^{de}	**<0.001**
Limonene	$C_{10}H_{16}$	Lemon/Citrus	0.56	0.51	0.74	0.65	0.41	0.58	0.63	0.63	0.38	0.34	0.26	0.58	0.49	0.45	0.075
Dimethyl disulfide	$C_2H_6S_2$	Garlic	0.23	0.24	0.31	0.18	0.17	0.19	0.18	0.19	0.24	0.15	0.15	0.21	0.21	0.19	0.070
Methanethiol	CH_4S	Rotten cabbage	0.08^{c}	0.17^{bc}	0.31^{a}	0.16^{ab}	0.13^{bc}	0.1^{bc}	0.12^{bc}	0.1^{bc}	0.15^{bc}	0.13^{bc}	0.13bc	0.15^{bc}	0.13^{bc}	0.10^{bc}	**<0.001**
Dimethyl trisulfide	$C_2H_6S_3$	Foul/Putrid	0.08	0.11	0.16	0.16	0.14	0.12	0.08	0.09	0.12	0.11	0.12	0.11	0.07	0.10	0.075

Pr > F = p-value associated with the F statistic (ANOVA's probability of observing a difference larger than the observed one, if the null hypothesis were true).

3. Results and discussion

3.1. Compositional and microbiological characteristics

Table S1 shows the gross chemical composition of the cheese samples during storage. The core composition of the samples remained stable during the storage, with no significant difference between the packaging technology used. Despite small variations, the moisture, protein and fat content of under-rind fractions remained nearly stable.

As estimated by the plate counts, mesophilic lactobacilli (8.2–8.9 log CFU/g) and mesophilic lactococci (7.6–8.6 log CFU/g) were the predominant microbial groups in all samples at time zero.

This was expected since mesophilic cultures were added as natural starters into milk after pasteurization, as stated in the Stelvio-PDO disciplinary, and were probably able to survive during the first months of ripening. Furthermore, the presence of non-starter lactic acid bacteria (NSLAB) is extremely common after three months of ripening. Indeed, starter bacteria undergo autolysis during ripening and release enzymes and metabolic by-products that favor the development of NSLAB. For several months, they grew in the extremely selective cheese environment, where they eventually take over and contribute to flavor development (Coelho et al., 2022). Biotyping by RAPD-PCR with UPGMA analysis clustered lactobacilli in 20 biotypes and lactococci in 15 biotypes at a similarity level of 90 % (Figure S1). Each cluster grouped isolates proceeding from the same cheese sample, except for those belonging to the cluster n. 14. All biotypes belonged to *Lacticaseibacillus casei*, *Lacticaseibacillus paracasei*, *Lacticaseibacillus rhamnosus,* and *Lactococcus lactis.* The presence of a large number of strains belonging to *L. casei, L. paracasei, L. rhamnosus, and Lc. lactis* species suggests that the above-mentioned species are dominant, which could proceed from milk added with mesophilic starters during the manufacturing process. The population of mesophilic lactobacilli and lactococci decreased significantly ($p < 0.05$) after three months of storage in both VaP and MAP, reaching a cell density between 6.2 and 7.9 log CFU/g. Indeed, previous research has indicated that the quality of cheese and packaging influences both aerobic and anaerobic bacteria in cheese, depending on the degree of airtightness of the packaging (Barone et al., 2017). VaP and MAP result in a strong reduction or controlled presence of oxygen, only obligate or facultative anaerobic bacteria to grow. However, if the cheese is stored at 4 °C, the growth of mesophilic lactobacilli is also slowed down. Total mesophilic counts roughly reflected the trend of mesophilic lactobacilli and lactococci, with high values at time zero (8.2 to 8.9 log CFU/g) and a progressive reduction during storage (7.1 – 8.1 log CFU/g after 3 months).

Presumptive thermophilic streptococci ranged between ca. 3 and 4 log CFU/g at time zero in all samples and remained stable throughout preservation under VaP and MAP. Their presence at low cell densities was likely due to their ability to survive after pasteurization and cooking treatment, which reaches a temperature of 40 °C. This value corresponds to the optimal temperature of thermophilic cultures, and the final cooking temperature used after cutting the curd in the manufacture of Stelvio-PDO. In addition, it has been previously demonstrated that some *S. thermophilus* strains were able to respond to heat stress by modulating the production of various heat proteins, such as chaperonins and proteases (Zotta et al., 2008).

Yeasts showed a variable trend. They were always present, but under MAP, their cell density remained mostly constant, while they tended to decrease under VaP, especially in the core of cheese. Apparently, molds were not found in any sample and section as determined by the culture-dependent analysis.

3.2. MiSeq Illumina data analysis, alpha and beta diversity

Total (5,506,608 and 6,473,492) and quality-trimmed (4,108,125 and 5,043,409) sequences for the 16S rRNA gene and ITS1 region explained the entire bacterial and fungal composition of the Stelvio-PDO cheese samples. Furthermore, the quantity of observed OTU number, Chao1, and Shannon indices were estimated (Table S2). Overall, the diversity indices for both bacteria and yeasts were similar across all samples.

The relative abundance of the bacterial *meta*-community showed that in all cheese samples the genus *Lactococcus* corresponded to more than 85 % of the total abundance (Fig. 2A). The genus *Leuconostoc* was the second most abundant in all samples apart from the used packaging method. A slight difference in the sub-dominant genera between the core and under-rind fractions was found. Indeed, *Enterococcus* and *Corynebacterium* genera were identified under-rind fraction stored under VaP, while the genera *Pseudomonas* and *Stenotrophomonas* in the surface fraction stored under MAP. There was also an apparent difference in the diversity of genera considered sub-dominants or satellites, these being more present in the samples stored in MAP, mainly in the under-rind fraction. The predominance of the genera *Lactococcus* and *Leuconostoc* found in all samples was expected as mesophilic starter cultures of Italian cheeses such as Stelvio-PDO generally include *Lactococcus* spp., whereas NSLAB comprise *Leuconostoc* and *Enterococcus* (Gobbetti et al., 2018). The dominant presence of the genera *Pseudomonas* and *Stenotrophomonas* in samples stored under MAP, both composed of mostly aerobic bacteria, may be a decisive variable for the sensory differentiation of the product due to the packaging technology used (Wisplinghoff, 2017). Although the genus *Pseudomonas* is considered an aerobic species, there are studies showing how species of this genus are able to grow in foods with very low oxygen availability and under modified atmosphere packages. Therefore, it is assumed that 80 % CO_2 atmosphere as used under the conditions of this study is probably not as efficient for the inhibition of these two genera as VAP (Stoops et al., 2012).

The relative abundance of yeasts and molds *meta*-community showed an evolution throughout storage for all samples. A relevant difference of the unpackaged samples concerning the core and under-surface fractions was found (Fig. 2B). The under-surface unpacked samples showed a high predominance of the genus *Geotrichum*, whereas the core unpacked samples showed a predominance of the genus *Debaryomyces*. The behavior during storage was similar between samples stored under VaP, with a constant increase of the genus *Debaryomyces* throughout storage to the detriment of the reduction of the genus *Geotrichum*. A relevant difference among the VaP samples was the greater presence of the genus *Malassezia* in the third month of storage in the surface fraction. On the contrary, the samples stored under MAP showed a greater abundance of the genus *Debaryomyces* after the first month of storage. We can observe a relevant reduction of this genus after the second month of storage, accompanied by an increase in the abundance of the genera *Geotrichum* and *Malassezia*, and again an increase in the genus *Debaryomyces* after three months of storage. It is worth noting, therefore, that the main difference between the packaging methods observed here was the greater abundance of the genus *Debaryomyces* under MAP. *Debaryomyces* is reported as the dominant genus of most surface-ripened cheeses (Beresford et al., 2001). Due to its capacity to adapt to the cheese environment, which is characterized by low pH, high NaCl concentrations, and the use of lactate as a major carbon source, *D. hansenii* is able to proliferate during the early stage of ripening. By assimilating lactate and producing alkaline metabolites as ammonia, the growth of *D. hansenii* helps raise the pH of the cheese surface, which favors the development of the smear bacteria (Gori et al., 2012). The production of ammonia by *Debaryomyces* coordinates and favors the growth of other yeast species by the quorum sensing mechanism (Gori et al., 2012), for instance favoring the growth of *Malassezia*.

3.3. Proteolysis assessment

The PCA of the 5 chromatographic fractions of the cheeses allowed us to evaluate their proteolysis profile (Fig. 3).

In the score plots at the 95 % confidence level, there was no

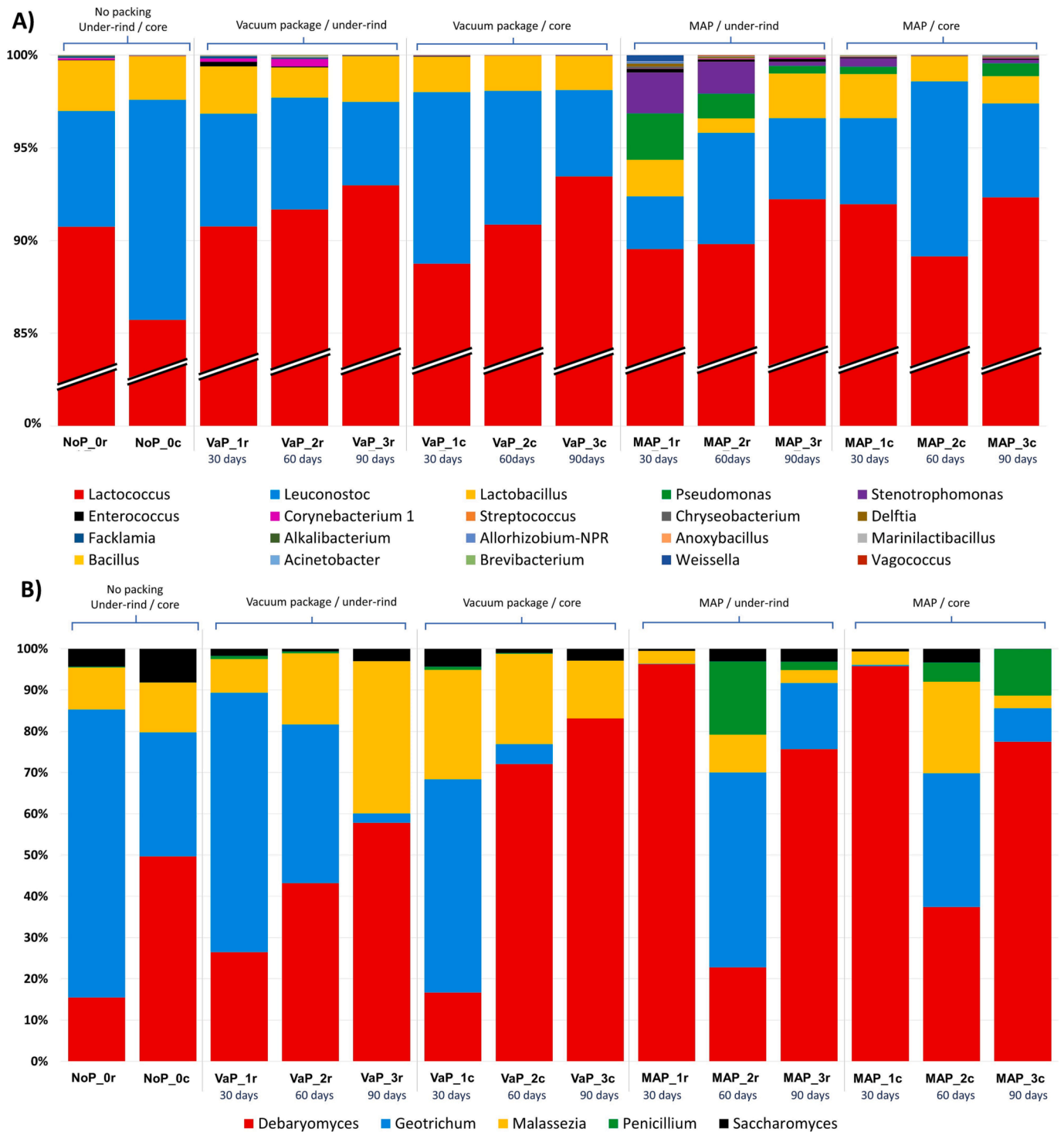

Fig. 2. Relative abundances of top 20 bacterial genera (A) and top 5 yeast & molds (B) of the cheese samples.

difference among the proteolysis profiles based on the type of packaging or the storage time (Fig. 3A and 3C). The loading plots showed that, as expected, the high and very high molecular weight peptides were in completely opposite quadrants and directions to the variables of low and very low molecular weight peptides, which confirms the degradation kinetics of the peptides during the cheese storage, even if not in such a way as to significantly change the proteolysis profile of the cheeses (Fig. 3B and 3D). When we further analyzed the loading plot in relation to the type of packaging (Fig. 3B), we found a high dispersion of the samples between the quadrants, which corroborates the similarity of the proteolytic profile concerning the type of packaging. On the other hand, analyzing the loading plot in relation to the storage period of the samples, a trend towards clustering of the samples in the second month of storage, relative to the high molecular weight peptides, was found. However, it does not differ significantly from the other time points, confirming the relative stability of proteolysis in all samples during storage. This finding was expected since the cheese wheels were fractionated at 3 months of ripening, packaged, and stored at 4–8 °C. It was well-established that proteolysis in hard and semi-hard cheeses occurs almost entirely during the first 30 to 90 days of cheese ripening (Zhao

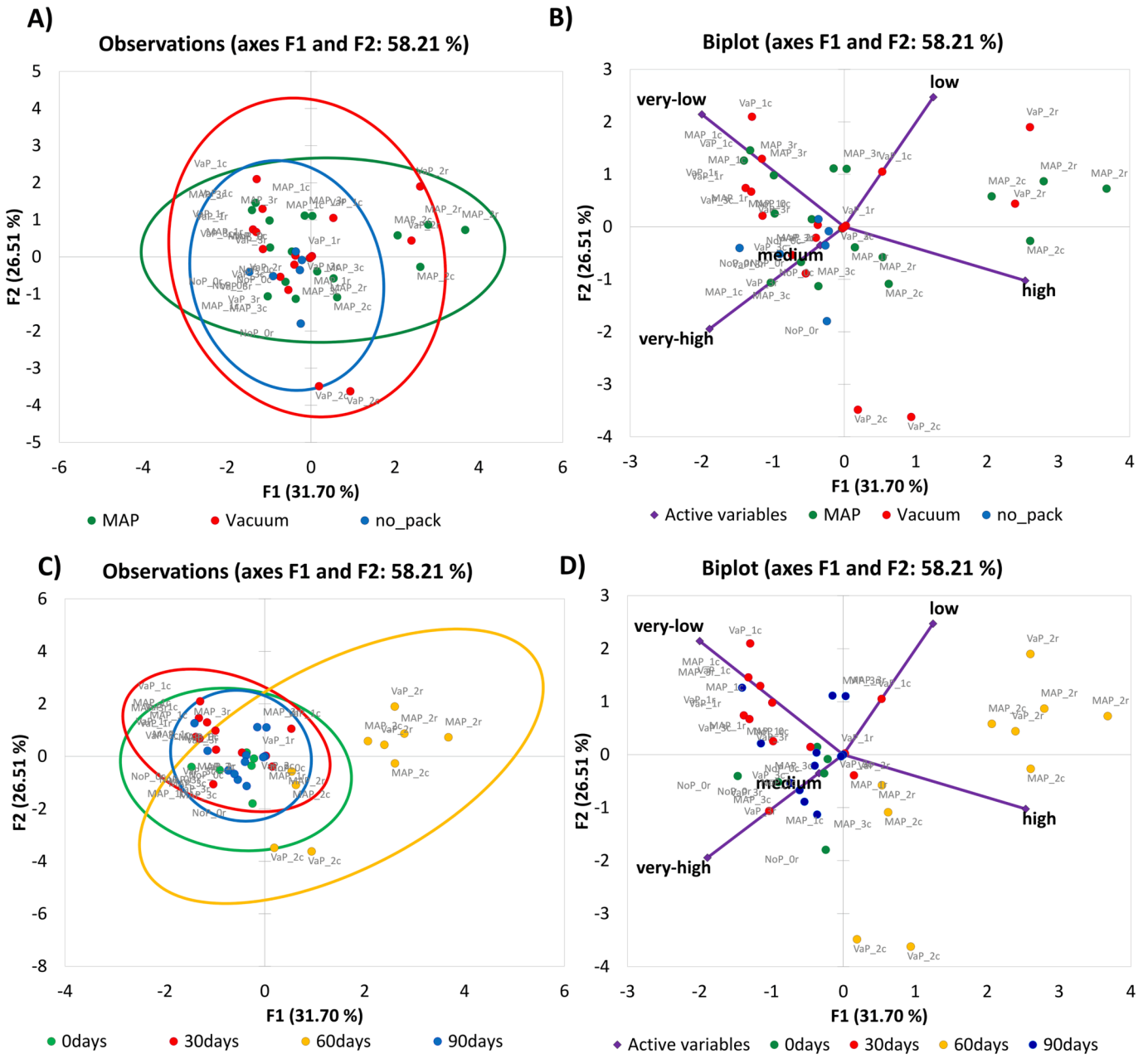

Fig. 3. PCA score plot of the proteolysis profile of the cheeses over the 3 months of storage regarding the type of package (A) and the storage time (C), and biplots representing the spatial distribution of the samples in relation to the chromatographic regions corresponding to peptides of very-high molecular weight (lower proteolysis occurred) to peptides of very-low molecular weight (greater proteolysis occurred) regarding the type of package (B) and the storage time (D). The underrind and core fractions of the cheese were distinguished were coded as "r" and "c", respectively.

et al., 2019). Therefore, some small and slow proteolysis happens in cheeses stored under refrigeration, which may have a minor impact on the volatile profile of the product but does not differentiate the proteolysis profile of the cheeses.

3.4. Volatilome of Stelvio PDO cheese: Combined untargeted and targeted fingerprinting (UT fingerprinting) results

The combined Untargeted and Targeted fingerprinting approach (Cordero et al., 2019; Reichenbach et al., 2019) was applied to all samples' VOCs fingerprints to highlight the most discriminative patterns in relation to samples storage conditions and time. A total of 895 reliable 2D peaks (i.e. peak features that match 50 % of the analyzed patterns – Stilo et al., 2021), 1,250 peak regions (i.e. chromatographic areas delineating the contour of detectable peaks), 1,073 untargeted features, and 177 targeted features were highlighted in the collected samples fingerprints. Fifty among all 177 targeted features (Table S3), were reported in the literature as commonly found in cheese (Kilcawley & O'Sullivan, 2017), with a description of their individual sensory quality and molecular formulas (Table 1). Therefore, the subsequent steps of chemometric analysis took into account only these 50 volatile compounds, defined as potent odorants. The five most abundant components (isovaleric acid, caproic acid, isobutyric acid, acetic acid, and caprylic acid) are short-chain fatty acids directly correlated to the type of milk used in cheese making, they are considered key-odorants (Dunkel et al. 2014), and on their own add up to more than 50 % of the total VOCs response. As shown in Table 1, 30 of the 50 potent odorants evaluated had higher ($p < 0.05$) normalized responses during cheese storage,

regardless of the packaging method used. Isovaleric acid showed the highest relative response in all samples. The most ($p < 0.05$) abundant volatiles (>1%) among the odorants were the caproic, caprylic, and acetic acids for acids; 2-nonanone for ketones; 3-methyl-1-butanol for alcohols; benzaldehyde for aldehydes; ethyl hexanoate for esters; δ-decalactone for lactones; and dodecane for alkanes. With the exception of the alkane, all these metabolites have sensory characteristics expected or already extensively reported in the literature for ripened cheeses such as *cheesy*, *sweaty*, *rancid*, *nutty*, *fruity*, and *creamy* notes.

The effect of packaging and storage time on the volatilome of under-rind fractions is shown in Fig. 4. Samples stored under VaP and MAP had a different evolution of the volatile profile. Under-rind VaP cheese samples were grouped in 3 clusters based on their volatile profiles (Fig. 4A); the first referring to the samples at time 0 (NoP_0r), the second referring to the samples after one month of storage (VaP_1r), and the third group referring to the samples after two and three months of storage (VaP_2r and VaP_3r), with the ellipses of confidence slightly overlapping. The VIP-score plot of these samples allowed to observe that 23 out of 50 potent odorants were relevant to differentiate these profiles, in addition to explaining that compounds such as 2-nonanone and 9-nonen-2-one have an ever-increasing response intensity throughout storage, while compounds such as 1-propanol and dodecane have ever-decreasing trend during storage (Fig. 4B).

Regarding the under-rind MAP samples, greater dispersion of the data was observed, and only 2 groups of volatile profiles were found (Fig. 4C), one composed of the unpacked samples at time 0 (NoP_0r), and another grouping with all the other samples (MAP_1r, MAP_2r, and MAP_1r). Regarding these samples, the VIP-score plot of Fig. 4D shows that 20 of the 50 targets were relevant for the discrimination of samples. Analytes such as caproic and caprylic acids were always increasing throughout storage, whereas dodecane and 2,3-butanediol isomers were always decreasing throughout storage.

In the same way, the effect of storage time on the profile of volatile metabolites in the core fraction of the cheeses was evaluated (Fig. 4E-H). The score plot indicates two macro clusters, the first one consisting of the unpacked samples at time 0 of storage (NoP_0c), and another group with all the VaP samples (VaP_1c, VaP_2c, and VaP_1c) (Fig. 4E). The VIP-score plot of this class of samples (Fig. 4F) showed a smaller number of analytes relevant for the discrimination of samples, with only 8 of the 50 targets being important for such distinction. Metabolites such as caprylic acid showed an ever-increasing intensity throughout storage, while acetoin showed an ever-decreasing intensity over the three months evaluated.

Regarding the core of the samples stored under MAP, their profile can be visualized in Fig. 4G. Overall, less discrimination than VaP was observed. The higher intra-group variability is responsible for greater data dispersion, which does not allow group discrimination with a 95 % level of confidentiality, with a tendency to differentiate samples during the second and third months of storage. In the VIP-score plot referring to these samples (Fig. 4H), 9 of the 50 analytes were the ones with the highest discriminative capabilities. By closely examining the score plot, samples belonging to the latest storage months were characterized by positive values on the first component; this is due to the greater intensity of compounds such as caproic and caprylic acids, as well as the absence (at least below the method's detection capability) of isobutyric acid and dodecane.

A sPLS-DA model was created by using the type of packaging as explanatory variable instead of storage time (Fig. 5A-B). A separation into 3 different groups was observed in the first score-plot (Fig. 5A), the first consisting of the samples at time 0 that were not packaged (NoP_0c and NoP_0r), the second grouping of the sample from the core of the cheese at the third month under VaP (VaP_3c), and a third grouping composed of all other samples to the third month of storage. It is worth highlighting the tendency to separate samples from the core portion stored for 3 months in MAP since only a small region of its confidentiality ellipse intersects only one of the other groupings. In Fig. 5B related to this analysis, 9 of the 50 analytes are relevant for the separation of the samples. Compounds such as 8-nonen-2-one, 2-nonanone, and caproic acid are present at greater abundances in packed samples than in unpackaged ones, and compounds such as 1-propanol and valeric acid differentiated the unpackaged samples from the others. It was also observed that compounds with higher loadings are more abundant in the unpackaged samples, which corroborates the more pronounced differentiation of these samples from the others.

The ratio of the five most intense metabolites in the evaluated samples – isovaleric acid, caproic acid, isobutyric acid, acetic acid, and caprylic acid – is closely related to the expected sensory characteristics of matured cheeses, respectively: *cheesy*, *waxy* or *goaty*, *sweaty*, *acidic*, and *oily* aromas. The dehydrogenase complex that has already been identified, for example, in the *Bacillus* genus consists of three catalytic components, a ketoacid dehydrogenase, dihydrolipoyl transacylase, and a lipamide dehydrogenase. The oxidative decarboxylation of α-ketoacids by this complex in LAB is relevant for the development of flavor in cheese since carboxylic acids such as isovaleric acid produced by this metabolic pathway are among the main aroma compounds typical of matured cheeses. The reaction also occurs in lactococci, propionibacteria, and micrococci (Smit et al., 2005). In addition, it was reported that although to a lesser extent than propionic bacteria, NSLAB also contribute to the production of isovaleric acid in cheeses by the direct conversion of leucine (Thierry et al., 2004). This metabolism may be relevant for those cheeses that already underwent ripening with medium or intense proteolysis, with a consequent higher amount of free amino acids (Thierry et al., 2004). The action of yeasts and molds, due to their intense lipolytic action, are also equally or more important than LAB for the development of aroma compounds in cheeses. Indeed, yeasts are able to convert milk fats into amino acids and free fatty acids (FFA), which are the precursors of many aroma and flavor compounds (e.g., butyric, isobutyric, and caproic) (Geronikou et al., 2020).

Under our experimental conditions, MAP was more effective in stabilizing the volatilome of Stelvio cheese as shown by the non-separation of the groups (Figs. 4 and 5). Several authors have previously reported that there is a slight difference in the growth of bacteria and yeasts in foods stored under VaP or MAP, with storage in MAP being more efficient to slow down the growth of microorganisms in general, dependent however on the concentration and type of gas used (Atallah et al., 2021). Additionally, several studies have documented that one of the main differences between the storage under VaP or in MAP is related to the stability of the gaseous composition inside the packaging throughout the storage, with foods packed under MAP being more stable or even with increasing levels of CO_2 during storage due to the cellular respiration of microorganisms. On the other side, VaP products have a higher oxygen content after a few days of storage due to the permeability of the packaging and the gas exchange with the environment, probably favored by the negative pressure difference inside the packaging (Atallah et al., 2022). Thus, we hypothesized that the difference in the profile of volatiles over time observed only for samples stored under VaP and in MAP was due to the slight difference in the concentration of oxygen inside the packages during storage, which in turn may favor the microbial activity as well as gas exchange with the external environment.

The sensory characteristics described for the Stelvio cheese (Fig. 6) are consistent with the most important compounds for the differentiation and characterization of the volatile profiles observed in the VIP-scores plot, such as isovaleric and caproic acids with *rancid* and *sweaty* aroma, 2-nonanone with its *fruity* and *cheesy* aroma, and 2,3-butanediol isomers with their *buttery* aroma. All these descriptors were highly cited among the samples. The variation in metabolites, which distinctly defines core and under-rind fractions of the cheese, can be attributed to the prominent abundance of ketones and alcohols in the under-rind fraction as depicted in the VIP-score plot. Conversely, the core fraction is characterized by a higher presence of carboxylic acids. This difference is probably the result of the strong correlation observed between volatiles, yeasts and moulds. Interestingly, the number of these correlations is

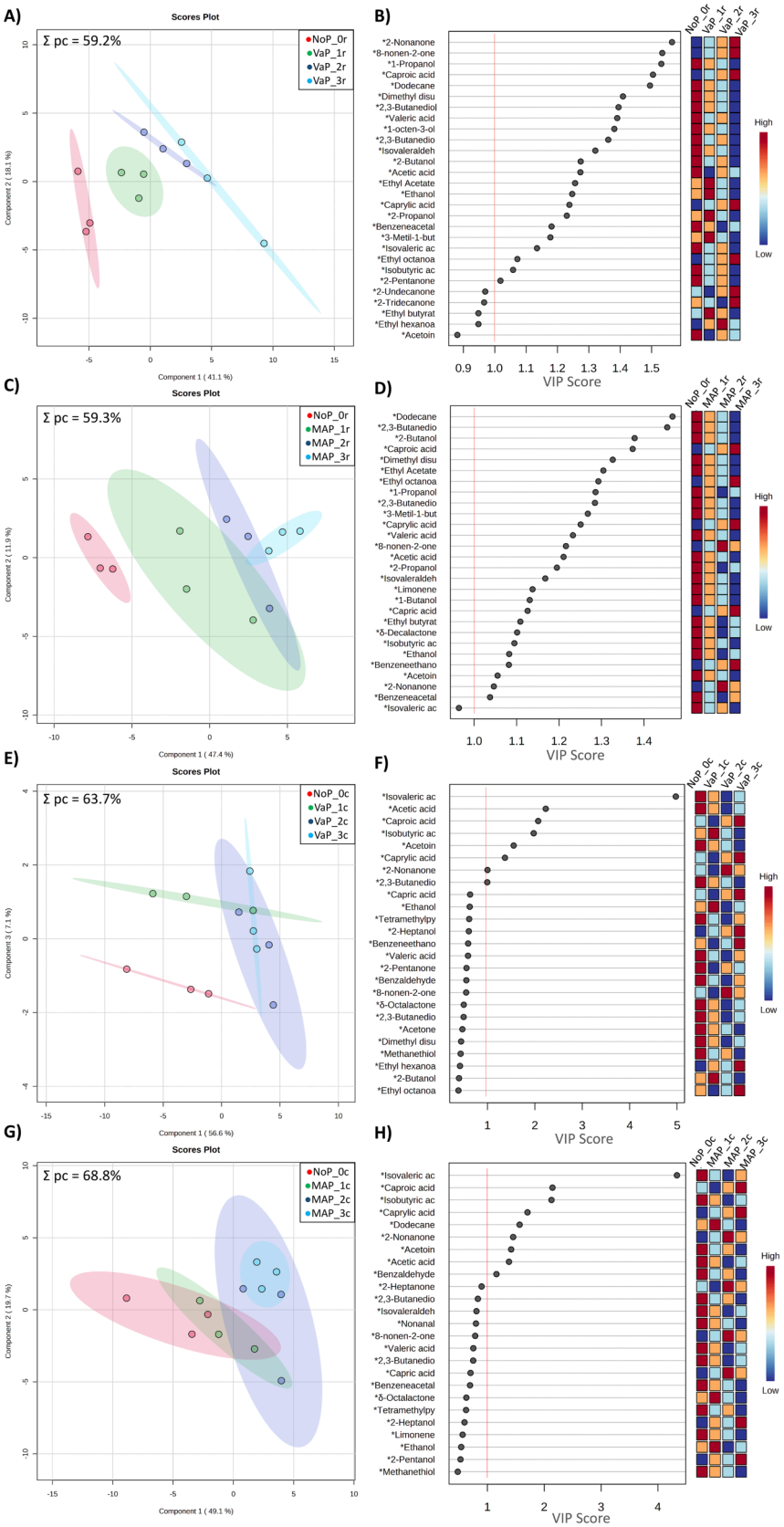

Fig. 4. PLS-DA score plot evaluating the storage time effect in VaP (A) and MAP (C) under-rind samples and their VIP scores metabolites (B and D, respectively); PLS-DA score plot evaluating the storage time effect in VaP (E) and MAP (G) core samples and their VIP scores metabolites (F and H, respectively). The under-rind and core fractions of the cheese were distinguished were coded as "r" and "c", respectively.

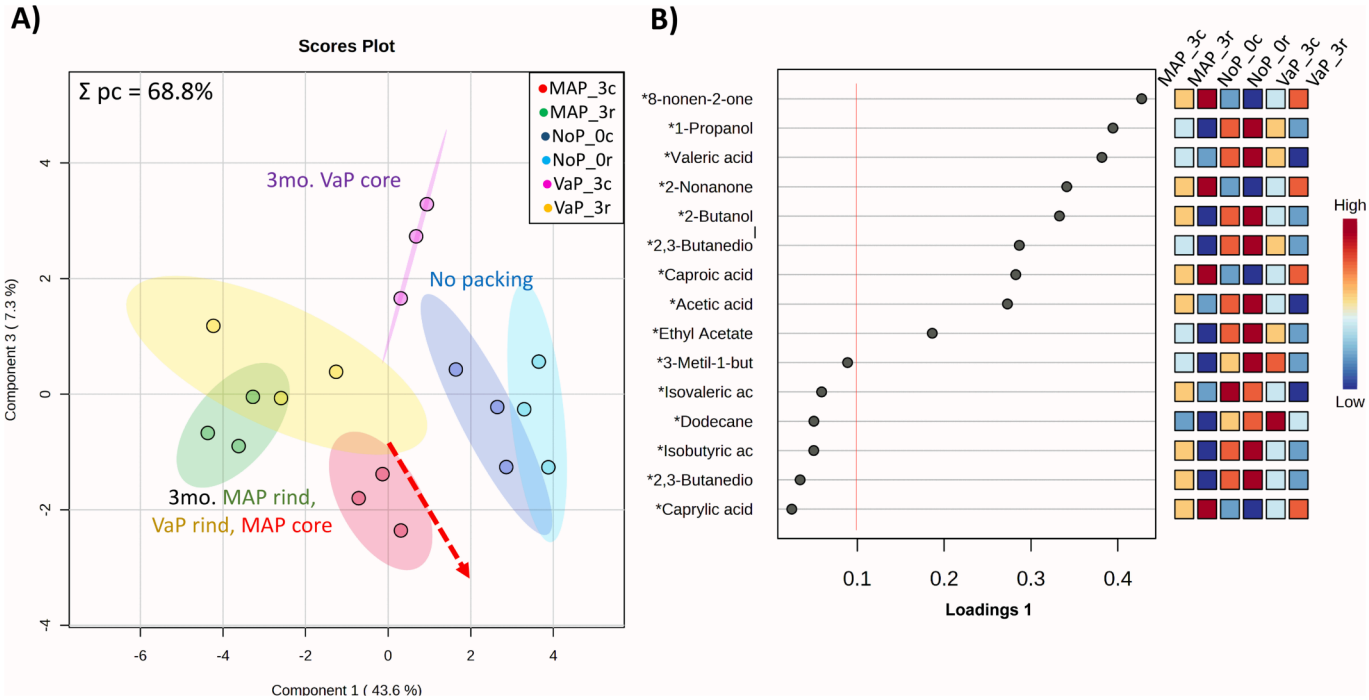

Fig. 5. Spls-da score plot (a) and loadings plot (b) evaluating the type of package effect at 0 and 3 months of storage. The loadings plot shows the variables selected by the sPLS-DA model for a given component. The variables are ranked by the absolute values of their loadings. The under-rind and core fractions of the cheese were distinguished were coded as "r" and "c", respectively.

greater than that with lactic acid bacteria, which underlines the strong influence of microorganisms other than lactic acid bacteria on the volatilome. The surfaces or under-rind of smear-ripened cheeses like the Stelvio support intricate microbiological populations. This surface microbiota, which is primarily in charge of the distinctive flavor and appearance, is dominated by salt-tolerant yeasts (i.e. *Debaryomyces hansenii* and *Geotrichum candidum*) and bacteria from the Actinobacteria phylum, including the families *Corynebacteriaceae, Brevibacteriaceae*, and *Micrococcaceae* (Mounier and Coton, 2022).

Some authors have already observed significant changes in the sensory profile and volatilome of cheeses stored under MAP and VaP. For instance, it has been verified that the CO_2 and N_2 treatments exerted significant changes in all groups during the storage of Domiati cheese (Atallah et al., 2021). Samples packaged under 100 % N_2 showed the significantly highest levels of all the volatile ketones after 75 days of storage, particularly 2-pentanone, acetoin, methyl isobutyl ketone, 2-heptanone, 2-nonanone, and 2-undecanone. Some important compounds contributing to the pleasant flavor of cheese are acetic acid, butanoic acid, pentanal, benzaldehyde, acetoin, and 2,3-butanedione, the same compounds found here in the Stelvio-PDO but without overall significant difference during storage. However, Cheddar cheeses stored under CO_2 contained higher concentrations of aldehydes and fatty acids and lower concentrations of alcohols and esters than cheeses stored under nitrogen (an environment similar to the VaP) (Colchin et al., 2001). Carbon dioxide atmospheres potentiated light-induced oxidation in shredded Cheddar cheeses, as evidenced by aldehyde and fatty acid headspace volatiles measured following storage. Finally, other authors (Akpınar et al., 2017) similarly found that packaging under different conditions had made a significant difference between Sepet cheese samples regarding volatile compounds, fatty acid compositions, and microbiological characteristics. They stated that the percentage of aldehydes cheeses in the MAP was higher than in the control cheese.

3.5. Sensory analysis

We implemented the flash profiling as a flexible method for quickly profiling products based on their most salient sensory attributes. Flash profiling is a variant of free choice profiling combined with a comparative ranking of products based on the simultaneous presentation of the entire sample set, and has proven to be as satisfactory as conventional profiling in many applications (Dairou & Sieffermann, 2002). A general cut-off limit of 25 % was established for the residue analysis, excluding tasters with a high degree of non-consensus with the group or no agreement between their own sensory assessments. The flash-profile sensory analysis of the unpackaged samples and the samples after the third month of storage under VaP or in MAP raised between 25 and 42 different sensory descriptors (Fig. 6). The GPA statistical analysis did not report differences or sample clusters between the sensory profile of the biological replicates ($p < 0.05$). The unpackaged samples at time 0 (Fig. 6A), not only generated the highest number of sensory descriptors (42 in total) but also the highest number of aroma-related descriptors (9). Among these, 4 were cited more than once by the group of tasters: *cheesy*, *milky*, *feet*, and *cow*.

The VaP samples with 3 months of storage (Fig. 6B) generated 29 sensory descriptors, 8 of which related to the aroma of the product. In this category, the attributes most frequently cited by tasters were *cheesy* and *butter* aroma.

Finally, the samples stored for 3 months in MAP (Fig. 6C) produced the lowest number (25 descriptors) of sensory descriptors among the evaluated samples, 5 of which are related to the aroma of the product. The most cited were *butter*, *moldy*, *ripened*, and *cheesy* aromas.

3.6. Data correlation

Analyzing the correlation matrix of the data referring to the under-rind samples (Table S4), there were more high correlations between volatiles with very-low and medium molecular weight peptides. Additionally, it was s observed that 4 compounds (isovaleric-acid, dodecane, 2–3-butanedione, and 2-propanol) out of the 50 primarily targeted had a high correlation with the total count of mesophilic bacteria, mesophilic lactobacilli, and thermophilic streptococci, whereas 16 compounds (*e.g.*, caproic acid, acetoin, 2-pentanone, nonanal, valeric-acid, δ-decalactone,

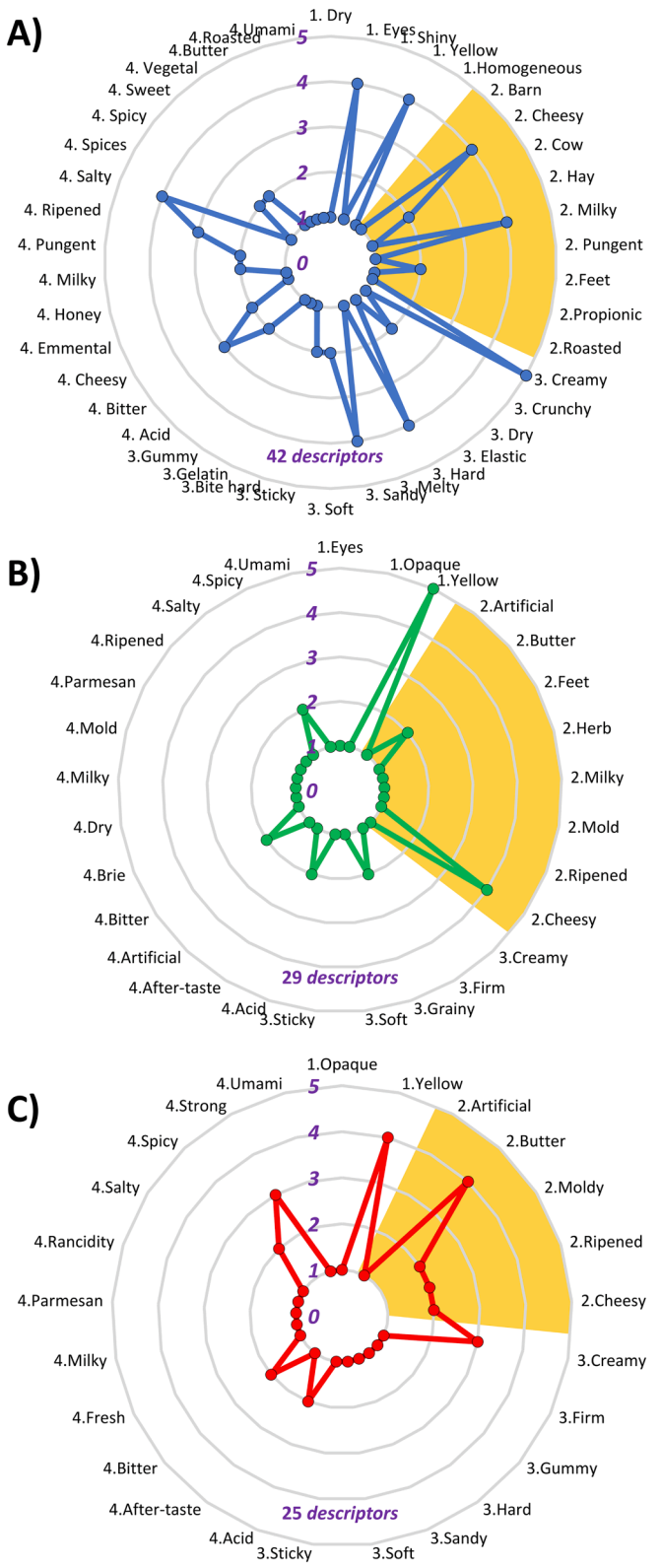

Fig. 6. Frequency of citation of sensory descriptors in the flash-profile sensory analysis for Stelvio-PDO cheese samples not packed at the beginning of storage (A), vacuum packed (VP) after 3 months of storage (B), and packed in MAP after 3 months of storage (C). The area highlighted in yellow represents the descriptors related to Aroma. (1 = appearance attributes; 2 = aroma attributes; 3 = texture attributes; 4 = taste attributes). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ethyl-hexanoate, etc.) were correlated with yeast and mold counts. Regarding the genera identified through Illumina 16 s and ITS, a stronger correlation ($r > 0.6$) was observed between volatiles and genera of NSLAB and subdominant cultures (i.e., *Pseudomonas* and *Stenotrophomonas*). Several correlations between VOCs having the highest total intensity (*e.g.*, valeric acid, caproic acid, isobutyric acid, acetic acid) and bacteria (*e.g.*, *Leuconostoc*, *Streptococcus*, *Corynebacterium*) were found. The genus *Debaryomices* was the variable with the highest number of high correlations with 13 volatile compounds The PCA that originated the Pearson matrix related to the under-rind data explained 57.2 % of the data analyzed in the first two principal components.

The PCA that originated the core data matrix explained 50.6 % of the data analyzed in the first two principal components (Table S5). The isovaleric acid had a high correlation with the genera *Pseudomonas*, *Stenotrophomonas*, *Chryseobacterium*, *Delftia*, *Acinetobacter*, and *Debaryomyces*, in addition to an extremely high correlation (>0.9) with the genus *Streptococcus*. Caproic acid had a high correlation with the genera *Weisella* and *Debaryomyces*. Isobutyric acid had a high correlation with very small and medium peptides, in addition to a high correlation with the *Bacillus* genus. Acetic acid was highly correlated with thermophilic streptococci counts and with the genera *Stenotrophomonas*, *Delftia*, *Bacillus*, and *Debaryomyces*. Caprylic acid was highly correlated only with the genus *Weissella*. The variable correlated with the highest number of metabolites was the genus *Bacillus*, which correlated well with 20 of the 50 metabolites, namely: isobutyric acid, acetic acid, acetoin, 2,3-butanediol isomers, dodecane, valeric acid, δ-decalactone, 2,3-butanedione, ethyl hexanoate, 2-butanol, 1-propanol, δ-octalactone, δ-dodecalactone, propionic acid, ethyl acetate, dimethyl disulfide, dimethyl trisulfide, 2-tridecanone, γ-decalactone, and γ-dodecalactone.

4. Conclusions

This study confirmed that the packaging method of already ripened and sliced Stelvio-PDO cheese does not negatively alter the overall quality of the cheese and stabilizes its gross compositional characteristics throughout the storage period. It was also observed that, although there were few differences in the microbiological cell density between the samples in relation to the type of packaging used, there is a significant difference in the profile of volatile compounds during storage and due to the type of packaging used, MAP being the one that maintains the volatile profile most unchanged throughout the storage period. Furthermore, it was observed that despite the predominance of the same main volatile compounds in all samples, minor compounds important for cheese characterization and differentiation are also present, which also differ between the regions of the cheese analyzed. Out of the 50 potent odorants, 9 were relevant to sample separation. For instance, compounds such as 8-nonen-2-one, 2-nonanone, and caproic acid were in a greater abundances in samples stored under VaP and MAP than in unpackaged ones. Finally, the study elucidates part of the metabolome of Stelvio-PDO cheese, listing for the first time the volatile compounds peculiar to this product and associating them with reported and original sensory descriptors and microbiological data.

CRediT authorship contribution statement

Bruno Domingues Galli: Investigation, Formal analysis, Data curation, Visualization, Writing – original draft. **Elisabetta Trossolo:** Investigation, Writing – review & editing. **Ilaria Carafa:** Conceptualization, Investigation, Formal analysis, Data curation, Writing – review & editing, Project administration. **Simone Squara:** Investigation, Formal analysis, Writing – review & editing. **Andrea Caratti:** Investigation, Formal analysis, Writing – review & editing. **Pasquale Filannino:** Writing – review & editing. **Chiara Cordero:** Investigation, Supervision, Resources, Writing – review & editing. **Marco Gobbetti:** Supervision, Resources. **Raffaella Di Cagno:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Illumina raw sequences have been deposited in GenBank under Bioproject number PRJNA1069711.

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Ethical statement

Informed consent of all participants was obtained prior to participating in sensory analysis. Appropriate protocols for protecting the rights and privacy of all participants were utilized during the execution of the sensory analysis.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.138544.

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