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How starter cultures affect the peptidomic profile and bioactive activities of the Asiago-PDO cheese throughout ripening

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ABSTRACT

Our study investigated the chemical, microbiological, and bioactive peptide profiles of Asiago Protected Designation of Origin (PDO) cheese from two dairies (Dairy I and II) produced over two consecutive days (batches) and analysed during three months of ripening. The effect of different starter cultures was evaluated. The microbiome varied between the dairies and batches, with curds post-salting dominated by the starter culture-associated genera. During ripening, there was an increasing trend in the *Lactobacillus* genus, especially for Dairy I, which used an industrial starter. Bioactive peptide intensities differed throughout ripening due to the extent of proteolysis, and their intensity or concentration evolved, modifying, and differentiating profiles. The industrial starter used in Dairy I had the highest relative intensity (average value 76.50%) of bioactive peptides after three months of ripening. In contrast, the cheeses made with natural milk starter (Dairy II) had lower total relative intensity (average value 47.75%) but produced ACE-inhibitory peptides through sub-dominant strains and non-starter lactic acid bacteria. The importance of autochthonous strains of each micro-region even within a delimited PDO production area was highlighted.

1. Introduction

Fermented and ripened dairy products have always been an important pillar of the country's economy and the global market, as well as an essential part of the diet of different countries, people, and communities (Wiley, 2007).

Among cheeses, those with a protected designation of origin (PDO) reflect typical and traditional processes that have a significance linked to economic and social sustainability for the places where they are produced (EC, 2021), with Italy being the country with the highest number of documented PDO cheeses. Asiago is a recognized PDO cheese produced in northern Italy (Padova and Treviso, Veneto Region). It is manufactured exclusively using cows' milk (whole fat or skimmed), using natural (*lattoinnesto*) or industrial starters, and dry or brine salting. Ripening lasts a minimum of 20 days for the fresh variety or can go up to 15 months for the extra ripened one (MIPAAF, 2022).

Industrial cheese starter cultures are generally composed by members of both starter lactic acid bacteria (SLAB) and non-starter lactic acid bacteria (NSLAB) genera, with most contributors being *Streptococcus*, *Lactobacillus,* and *Lactococcus* origin strains, which ensure adequate acidification capacity (SLAB) and flavor development (NSLAB) (Gobbetti et al., 2018). Natural milk starter cultures (*coltura naturale in latte*, *lattoinnesto,* or *lattofermento*), are produced daily from raw cow's milk, using selective heat treatment and incubation at a high temperature (60–65 °C for 15–30 min), with back slopping, followed by a rapid cooling step. *Streptococcus thermophilus* dominates mainly these cultures, but other thermophilic species (*Streptococcus macedonicus*) or genera (*Enterococcus* and *Lactobacillus*) may occur (Gobbetti et al., 2018).

Lactic acid bacteria (LAB) introduced as starter cultures, as well as endogenous bacteria initially present in raw milk or house microbiota contaminants, participate in numerous biochemical reactions during cheese manufacturing and ripening, utilizing the available nutrients, and their metabolites are responsible for the unique sensory and nutritional profile of the cheeses. As well known, proteolysis is the most relevant biochemical event occurring during cheese ripening, in which caseins are broken down contributing to flavor development directly

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through the release of mostly bitter peptides and amino acids, and indirectly by producing substrates for other biochemical reactions. Both the SLAB and NSLAB are significant sources of proteinases and especially peptidases, which result in the synthesis of polypeptides, peptides, free amino acids, and other related compounds during proteolysis (McSweeney, 2017a). Shortly, during proteolysis, caseins are initially hydrolyzed by milk and curd proteolytic enzymes to large- and intermediate-size peptides, which, in turn, are hydrolyzed by SLAB and NSLAB proteinases and peptidases to short peptides and amino acids (Deutsch et al., 2000).

Generally, proteins contain peptide sequences with encrypted biological functions, which can be released when the primary structure is unfolded by enzymatic action. These sequences are called bioactive peptides, of which milk and milk-derived products are the primary sources. The presence of bioactive peptides in dairy products and cheeses has been largely documented throughout the years. Bioactive peptides derived from dairy proteins contain two to twenty amino acids in their structure and can exert several biological functions, including antimicrobial, opioid, angiotensin-converting enzyme inhibition (ACEi), dipeptidyl peptidase-IV inhibition (DPP-IV), and immunomodulatory action (Auestad & Layman, 2021).

The great advances in peptidomics and the use of mass spectrometry assorted with bioinformatics demonstrated that thousands of peptides are released during cheese making and ripening. However, the peptide diversity in each cheese is a result of the enzymatic profile and their activity which can differ largely. In the specific case of ripened cheeses, there are numerous factors affecting the enzymatic profile, its activity, and consequently, the final cheese bioactive peptide profile (Baptista & Gigante, 2021. Namely, these factors can be the heat treatment of milk, starter cultures type, salt content, ripening time, and temperature (Lu et al., 2016).

The focus of our study was to analyse the profile of bioactive peptides of Asiago-PDO cheese made in two distinct dairies within a specific geographic region. The starter utilized (commercial or natural) during the cheesemaking process, the ripening duration, and two consecutive days of production were considered in the study as potential determinants affecting the bioactive peptide profile. Through the use of a variety of analytical methods, we were able to establish a direct and thorough relationship between microbial diversity and bioactive peptides, highlighting the importance of starter cultures.

2. Material and methods

2.1. Samples

Cheese samples were obtained from two PDO Asiago-certified dairies (named Dairy I and II) located in Vicenza (Veneto, Italy) and distant 20 km from each other.

Cheese production was performed according to the official guideline of “Asiago fresco” (Commission Implementing Regulation EU 2020/1300 of 11 September 2020) (MIPAAF, 2022). Briefly, cheese making was carried out on two consecutive days (a total of 2 batches), using cows’ milk from 2 daily milking’s. Raw cows’ milk was heated at 35 °C, and liquid calf rennet (25 mL 100 L^{-1}, 280 IMCU/ml) was added, and coagulation took place within 25 min. After cutting (size of ca. 0.5 to 3.0 cm), the curd-whey mixture was held at 45 °C and pressed for 12 h. The pressed form was left to rest for 36 h at 12 °C and 85% relative humidity and then was brine salted (20%, w/v of NaCl). Ripening occurred in a climatic chamber at ca. 12 °C with a relative humidity of 85% for 90 days. The weight of the cheese was approximately 11 kg. The only difference in the process between the two dairies, allowed by the protocol guidelines, was the type of starter culture used. Dairy I used commercial TCC4-DVS culture (Chr-Hansen, Italy) consisting of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, while natural milk starter, which is usually mainly composed of *Streptococcus thermophilus* (Gobbetti et al., 2018) was used by Dairy II. The presence of the starter-related species was confirmed by culture-based approach (data not shown). Curd after 24 h (post-salting) and cheese samples, one-, two-three- months of ripening were collected from each batch (Fig. 1).

Considering the cheese making from two consecutive days, the time points and dairies, the total number of samples was 16 ($n = 2 \times 2 \times 4$) analyzed in triplicate. Therefore, the samples produced by the same dairy in different batches were treated as a replica of the process, but also analyzed separately to verify possible significant effects of the difference between batches. All samples were transported to the laboratory under refrigerated conditions (ca. 4 °C) and analyzed immediately or frozen (−80 °C). The experimental design comprising the sampling of the cheeses, the sample codes, and the analyses performed are shown in Fig. 1. For all sampling points, the gross composition of curd and cheeses (humidity, fat, and protein) was determined by a FoodScan-Dairy Near Infrared (NIR) analyzer (Foss, Hilleroed, Denmark) (Manuelian et al., 2017).

2.2. Microbiological analyses

2.2.1. Microbial counts

Ten grams of each sample were homogenized with 90 mL of sterile sodium citrate (2% [wt/vol]) solution. Presumptive mesophilic lactobacilli and cocci were enumerated in MRS agar supplemented with cycloheximide (0.1 g/l) and on M17 agar (Oxoid), respectively, at 30 °C for 48 h in anaerobic jars (1 l) with two anaerobic sachets each (Oxoid™ AnaeroGen™, Thermo Fisher Scientific, Waltham, MA, USA). Presumptive thermophilic cocci were enumerated on M17 agar (Oxoid, Basingstoke, Hampshire, United Kingdom) under conditions of anaerobiosis at 42 °C for 48 h.

2.2.2. Next-generation sequencing analysis (NGS) DNA metabarcoding

2.2.2.1. Total microbial genomic DNA extraction. Total genomic DNA was extracted from all homogenized samples 24 h after the milk coagulation (curd) and after one-, two- three- months of ripening and the starter cultures. To reduce the possible contamination of DNA by chloroplast, cheese samples were processed according to the procedure of Minervini et al. (2010). Five grams of cheese sample were homogenized in 50 mL of sterile phosphate-buffered saline (PBS, 50 mM, pH 7.0) in a filter bag using the Stomacher Lab-Blender 400 (PBI International, Milan, Italy), for 5 min at 230 rpm. Five milliliters of the homogenate were centrifuged at 14000 rpm for 10 min at 4 °C to separate the fat present in the samples. The fat layer and the supernatant were discarded, and the pellet was subjected to total DNA extraction by DNeasy PowerFood Microbial kit (Qiagen, Italy). Two independent replicates of each sample were used for DNA extraction and sequencing. The exception for both dairies was the starter culture each sample was extracted from one independent replicate and sequenced once.

2.2.2.2. Preparation of the MiSeq library. Bacterial diversity was analyzed based on the 16S rRNA gene. Primers targeting the 16S rRNA variable region V3-V4 (*Escherichia coli* position 341–805, forward 341F: CCTACGGGNGGCWGCAG and reverse 806R: GACTACNVGGGTWTCTAATCC (Claesson et al., 2010) were used for sequencing (Gardes & Bruns, 1993).

Unique barcodes were attached to the forward primer for facilitating the differentiation of samples. Amplicons were cleaned using the Agencourt AMPure kit (Beckman Coulter) according to the manufacturer’s instructions, to prevent preferential sequencing of the smallest amplicons, and DNA was quantified using the Quant-iT PicoGreen dsDNA kit (Invitrogen). Amplicons 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 the Bioanalyzer-2100 (Agilent). Library preparation and pair-end sequencing were carried out at the Genomic Platform – Fondazione

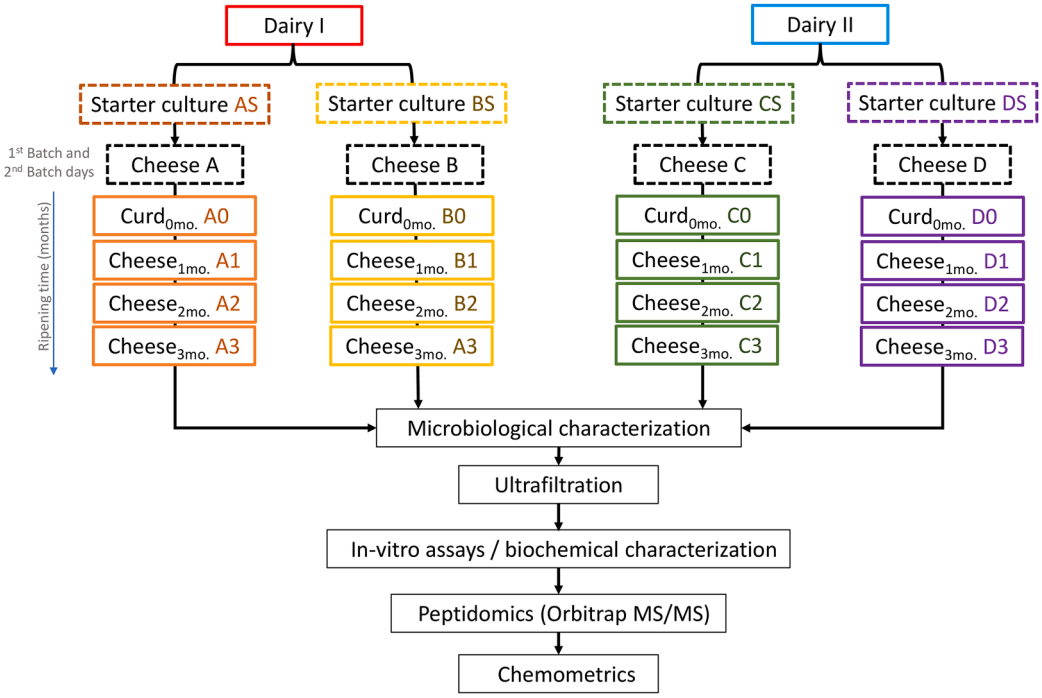

Fig. 1. Experimental design of the study and sample coding.

Edmund Mach (San Michele all'Adige, Trento, Italy) using the Illumina MiSeq system (Illumina, USA) according to standard laboratory procedures.

2.2.2.3. 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 (Callahan et al., 2016).

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 (Katoh & Standley, 2013). The resulting number of OTUs obtained at the genus level was 53, however, only the 23 most abundant genera were selected for the subsequent correlation analysis, with a threshold of relative abundance $\geq$0.1% present in the samples. For the microbiome elaboration and relative abundance calculations, independent replicates of the samples were summed. Statistical analysis was done using XLSTAT 2022.3.1 software (XLSTAT, 2022). Statistical differences were evaluated by the Mann-Whitney test considering a level of significance of 5%.

2.3. Extraction of water-soluble low-molecular-weight peptides and assessment of protein hydrolysis during the cheese ripening

Peptides were extracted from the samples following the protocol described by Martini et al. (2021). After mixing 5 g of each sample with 45 mL of 0.1 mol/L HCL, the mixtures were homogenized by using an Ultra-Turrax homogenizer, centrifuged (4000 *g*; 40 min; 4 °C), and filtered with Whatman filter paper 4. Low-molecular-weight peptide fractions were extracted by ultrafiltration (molecular weight cut-off of 3 kDa) using the Vivaspin®20, 3000 MWCO-PES (Satorius, Italy) from the previously extracted peptide fractions. The peptide content in the peptidic fractions was determined by using the TNBS (Trinitrobenzenesulfonic acid) method as reported in Adler-Nissen (1979) and expressing the results as mmol leucine equivalent/g of cheese. The analyzes were performed on the ultrafiltered and non-ultrafiltered peptide fractions.

2.4. Biological activity analyses

2.4.1. Antioxidant activity

The antioxidant activity of low-molecular-weight peptide fractions extracted from cheese samples was determined using the 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) method as described by Re et al. (1999). ABTS scavenging capacity was expressed as mg of vitamin C/mol of peptides.

2.4.2. Angiotensin-converting enzyme (ACE)-inhibitory activity

The ACE-inhibitory activity was measured by the spectrophotometric assay according to (Ronca-Testoni, 1983) adapted to a microplate reader (Solieri et al., 2022) using the tripeptide, N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) as substrate.

For the calculation of the IC50 value, the ACE assay was carried out in presence of different amounts of the cheese low-molecular-weight peptide fractions extract. IC50 is defined as the concentration of peptides required to inhibit 50% of the enzymatic activity and expressed as mg of peptides/mL. The IC50 values were determined using nonlinear regression analysis and fitting the data with the log (inhibitor) versus the response model generated by GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

2.4.3. α-glucosidase inhibitory activity

The α-glucosidase assay was performed as reported by Martini et al. (2021). Briefly, after mixing 20 μL of water-soluble low-molecular-weight peptide fractions with 5 μL of yeast α-glucosidase 0.2 U/mL, 3.3 μL of glutathione 3 mmol/L and 66.7 μL of potassium phosphate buffer (67 mmol/L, pH 6.8), the reaction mixture was incubated for 20 min at 37 °C before the addition of 5 μL of *p*-nitrophenyl-glucose 5 mmol/L. After additionally 20 min of incubation at 37 °C, the reaction was arrested by adding 150 μL of Na_2CO_3 100 mmol/L. The amount of released *p*-nitrophenol was determined by reading at 405 nm with a microplate reader (Infinite®200 Tecan, Austria).

2.5. Peptidomics

2.5.1. Identification of low-molecular-weight peptides by ultra-high-performance liquid chromatography/high-resolution tandem mass spectrometry (UHPLC/HR-MS/MS)

Low-molecular-weight peptide samples were analyzed by UHPLC/HR-MS. The UHPLC Ultimate 3000 separation module (Thermo Scientific, San Jose, CA, USA) equipped with a C18 column (Acquity UPLC HSS C18 reversed-phase, 2.1 × 100 mm, 1.8 μm particle size, Waters, Milan, Italy) was used for the chromatographic separation of peptides. Mass spectrometry (MS) and tandem MS experiments were performed on a Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, San Jose, CA, USA). The mobile phase composition, the chromatographic conditions as well as the mass spectrometer parameters are fully reported in Martini et al. (2021).

The MASCOT software (Matrix Science, Boston, MA, USA) was used for peptide sequencing after the conversion of the raw data into.*mgf* files. The parameters used for the identification process were: enzyme, none; peptide mass tolerance, ±5 ppm; fragment mass tolerance, ±0.12 Da; variable modification, oxidation (M), and phosphorylation (ST); the maximal number of post-translational modifications permitted in a single peptide, 4 modifications. The assignment procedure was confirmed by the manual verification of MS/MS spectra.

2.5.2. Label-free MS peak quantification and data filtering

The creation of a library of peptides and their fragments, labeling, relative quantification, and data filtering of the identified peptides was performed in Skyline 22.2 software (MacLean et al., 2010) following the parameters described by Dallas and Nielsen (2018).

Peaks that did not meet the criteria or were too close to the noise level to be visually discernible were excluded from the dataset. The criteria used were a mass error of ≤5 ppm and an *idotp* (isotope scalar product) score of ≥80. Through the systematic manual checking of the exported data, peptides whose origin was not from milk proteins were also excluded. In a second step, intensities of isotopically labeled peptides and identical modified peptides (i.e. different protonation patterns and presence of oxidized methionine) were regrouped and the peak area values were summed.

2.5.3. Identification of previously reported bioactive peptides

Peptides with 100% homology with previously reported biologically active peptides were identified by using the Milk Bioactive Peptides Database – MBPDB (Nielsen et al., 2017) and confirmed with the BIOPEP-UWM database of bioactive peptides (Minkiewicz et al., 2019).

2.6. Statistical analysis

The entire set of data obtained by the microbiological, instrumental, chemical, and enzymatic analyzes were evaluated using XLSTAT 2022.3.1 software (XLSTAT, 2022). To evaluate differences in the gross chemical composition and microbial counts between dairies and batches (processing days), ANOVA was used. In case of difference, the averages were compared by the Tukey test considering a level of significance of 5%. The correlation between the data was evaluated through a Principal component analysis (PCA) generating a Pearson correlation matrix. The significant difference was evaluated by Analysis of Variance (ANOVA). In case of difference, the averages were compared by the Tukey test considering a level of significance of 1 or 5%. Three analytical replicates for each sample were used for all analyses.

The peptide profile obtained by Orbitrap UHPLC/HR-MS/MS and its correlation with the studied variables were evaluated by chemometrics. Data from the mass spectra and a list of each peptide mass-charge ratio with corresponding absolute intensities (peak area) was exported to a digital spreadsheet, where the data matrix was assembled. All the replicates were used for each sampling point. The online software MetaboAnalyt 3.0 was used for the chemometric analyses (Xia, Sinelnikov, Han, & Wishart, 2015), in which a mass tolerance of 0.025 *m/z* was used, outliers were filtered out using an interquartile range, and data were normalized by median and Pareto scaling. The chemometric tool used was the principal component analysis (PCA) and mainly the partial least square discriminant analysis (PLS-DA) with a confidence ellipse of 95%, a multivariate analysis that was validated by multiple correlation coefficients (R2) and cross-validation (Q2). The significance of the biomarkers was ranked using the projection variable importance score (VIP score >1) of the PLS-DA.

3. Results

3.1. Gross chemical composition

Moisture, fat, and protein content of the Asiago cheeses produced by Dairy I and Dairy II were as follows: moisture content 35 ± 4%, protein content 28 ± 4%, and fat content 31 ± 4%. Between the dairies, curd samples differed significantly ($P < 0.05$) for their fat content, while cheeses throughout ripening time differed significantly for all chemical attributes analyzed (Supplementary Table S1). The exception was at 90 days of ripening where cheeses did not differ in their fat content. Within the dairies, and different batches (processing days), samples differed statistically ($P < 0.05$) mostly for Dairy II at 1 month and 2 months of ripening for all chemical parameters, while for Dairy I, batches differed for their protein content at 2 months of ripening, and at 3 months for their humidity and protein content.

3.2. Microbiological analyses

Presumptive mesophilic lactobacilli were present in curds (post-dry salting, ca. 6 ± 0.02 log CFU g-1 for both dairies) and attained the highest level after one month of ripening for Dairy I (9.57 ± 0.02 log CFU g-1) and after two months for Dairy II (9.61 ± 0.01 log CFU g^{-1}). The number of lactobacilli remained elevated throughout ripening, even though it progressively decreased (ca. 0.3 log cycle for Dairy I and 1 log cycle after 90 days for Dairy II). Between the two dairies, the cell number of presumptive mesophilic lactobacilli were significantly different throughout ripening ($P < 0.05$). However, no significant difference was found in the curd samples. Within the same dairy and between the batches, significant differences were found only for the curd samples ($P < 0.05$). Compared to presumptive mesophilic lactobacilli, curds contained a slightly lower number of mesophilic cocci (5.29 ± 1.4 log CFU g^{-1} for Dairy I, and 6.91 ± 1.4 log CFU g^{-1} for Dairy II), which had already markedly increased (ca. 3.5 log cycles) at 30 days of ripening (post-dry salting) for Dairy I, and at 60 days of ripening for Dairy II (ca. 1.1 log cycles), then decreased to ca. 7.2 log CFU g^{-1} at 90 days of ripening for both dairies. Between dairies, the number of mesophilic cocci was significantly different ($P < 0.05$) for all types of samples apart from the cheeses ripened for 90 days, while within dairies and different batches significant differences ($P < 0.05$) were found between curds of Dairy I, and between curds and cheeses ripened for two and three months for Dairy II. High numbers of thermophilic cocci were found throughout ripening (6.85 to 8.21 log CFU g^{-1}) with an increasing trend after post-salting. Between the dairies, the difference in thermophilic cocci was about 1 log CFU g^{-1} which was significant ($P < 0.05$) but at 3 months of ripening the samples didn't differ. Within dairies and different batches significant ($P < 0.05$), differences were found between cheeses after 90 days of ripening for both dairies.

The microbiological analyses of the starter cultures showed that they differ significantly ($P < 0.05$) in their composition of cocci (thermophilic-mesophilic) and lactobacilli (mesophilic). As expected, Dairy I, using the industrial starter, was characterized by high numbers of thermophilic cocci (10.07 ± 0.12 log CFU g^{-1}), mesophilic cocci (9.17 ± 0.06 log CFU g^{-1}), and mesophilic lactobacilli (9.54 ± 0.56 log CFU g^{-1}). On the contrary, Dairy II, using the natural-fermented milk starter, had significantly ($P < 0.05$) lower numbers of thermophilic cocci (6.85

± 0.46 log CFU g^{-1}), mesophilic cocci (7.51 ± 0.01 log CFU g^{-1}), and mesophilic lactobacilli (5.57 ± 0.05 log CFU g^{-1}).

3.2.1. 16S-metabarcoding microbiota profile

The distribution and evolution of the relative abundances of the 23 most relevant genera are shown in Fig. 2. The microbial community diversified during the manufacture and ripening of Asiago cheese. A total of 53 OTUs were identified at the genus level including the starter culture (Supplementary Table S2). The 23 most abundant genera were selected based on their relative abundances (≥0.1%) across all samples using the Qiime2 (version 2018.2). In agreement with the cell number of presumptive thermophilic cocci, *Streptococcus* was the most dominant genus in all curd samples (67.4 to 90.3%), while during ripening remained dominant, together with *Lactobacillus*. Except for the curds from Dairy II (C0 and D0), *Lactobacillus* was identified in curds A0 and B0 (ca. 8%) and in all cheeses (>24 to 55.5%) of both dairies. This genus was also flanked by the *Lactococcus* genus (ca. 22%) in the curd sample of the second batch of Dairy I (curd B0) and remained at low abundance (0.27 to 1.79%) throughout ripening. Cheeses from Dairy II (C and D) had a richer microbial profile during ripening compared to those from Dairy I. In addition to *Streptococcus* and *Lactobacillus*, other genera were identified such as *Bifidobacterium* (3.07 to 7.24%), *Chryseobacterium,* (ca. 0.1 to 7%), *Enhydrobacter* (0.1 to 11.2 %), *Acinetobacter* (0.3 to 6.3%), *Leuconostoc* (0.6 to 2.0%). These genera have been identified in both dairies but were much more prevalent in Dairy II except for *Enterococcus* (0.17 to 1.6%), *Rothia* (0.01 to 0.8%), *Pediococcus* (0.30 to 0.5%), *Enterobacter* (0.18 to 0.37%) and *Citrobacter* (0.13 to 0.2%) that were only present in Dairy I. Similarly, for Dairy I exceptions were *Stenotrophomonas* (0.01 to 0.7%), and *Clostridium sensu stricto 12* (1.5%), while *Escherichia-Shigella* was present with very low relative abundances (0.13 to 0.2%) in curds B0 and D0. Other genera sporadically present (in one sample or two within one dairy) with low relative abundances (0.1–0.2%) were *Macrococcus*, *Paracoccus*, *Pseudomonas*, *Staphylococcus*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Sphingobacterium*, and *Kurthia*.

Excluding the genera of lactic acid bacteria, the remaining genera had higher relative abundances in curds with a reducing trend until the end of ripening for both dairies. Regarding the starter cultures, for both dairies, differences were found in the presence of identified genera between the batches, but mostly relevant for Dairy I. In Dairy I, batch one (AS), the starter was characterized by the presence of *Streptococcus* (56.1%) and *Lactobacillus* (43.8%). Instead in batch two of the same dairy, the starter was characterized by the presence of *Streptococcus* (35.4%), *Lactobacillus* (24.1%), and *Lactococcus* (40.6%). In Dairy II, batch one (CS), *Streptococcus* was the most dominant genera (99.1%) as well as in batch two (99.6%). However, other milk contaminants such as *Chryseobacterium*, *Enhydrobacter, Rothia,* and *Acinetobacter* were found but with very low relative abundances (0.01 to 0.46%). Statistical ($P < 0.05$) differences were found for the relative abundances of *Streptococcus* between the starter cultures of Dairy I and Dairy II, while for Dairy I differences were found for *Lactobacillus* abundances between batches. Generally, the relative abundances of *Streptococcus* differed significantly ($P < 0.05$) for the curd samples of the dairies, but not for the cheese samples throughout ripening. On the other hand, *Lactobacillus* relative abundances differed statistically ($P < 0.05$) between dairies for all sample types.

3.3. Evolution of protein hydrolysis and peptide content throughout cheese ripening

The degradation of milk proteins throughout the cheese ripening was compared by measuring the amount of released free amino groups using the TNBS assay (Table 1). As expected, in curd samples for both unfiltered and filtered counterparts, a very low level of free amino groups

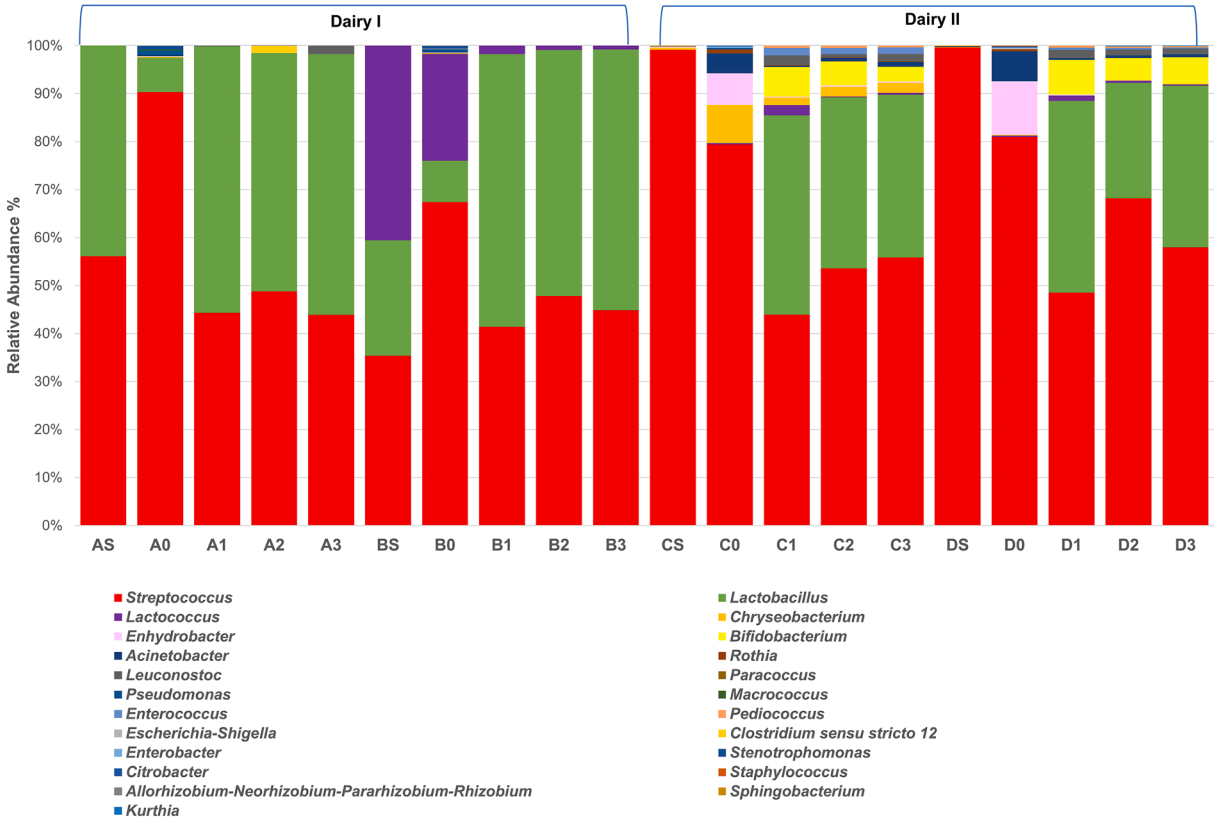

Fig. 2. Relative abundances of bacterial genera, of the starter cultures (AS, BS, CS, and DS), of curds (A0, B0, C0, and D0), and cheeses during ripening (1, 2, and 3 months [A1, B1, C1 and D1 to A3, B3, C3, and D3]). Only bacterial genera present with relative abundance above 0.1% per sample are shown.

Table 1
Concentration (mmol Leu/g) of peptides of the aqueous extracts soluble at pH 4.6 of Asiago curds (A0, B0, C0, and D0), and cheese during ripening (1, 2, and 3 months [A1, B1, C1 and D1 to A3, B3, C3, and D3]). Extracts were analyzed before and after ultrafiltration with a cut-off of 3 kDa. Mean values ± standard deviations for two batches of each type of cheese, analyzed in triplicate. Different uppercase letters (A to F) mean a significant difference between unfiltered samples ($P < 0.01$) and different lowercase letters (a to h) mean a significant difference between ultrafiltered samples ($P < 0.01$).

	mmol leucine/g cheese			mmol leucine/g cheese	
Dairy I	Unfiltered	Filtered (<3 kDa)	Dairy II	Unfiltered	Filtered (<3 kDa)
A0	$10.14^{F} \pm 0.22$	$3.53^{h} \pm 0.84$	C0	$10.16^{F} \pm 0.41$	$2.56^{h} \pm 0.19$
A1	$44.91^{E} \pm 2.62$	$21.47^{g} \pm 2.17$	C1	$49.30^{E} \pm 3.16$	$27.04^{f} \pm 0.43$
A2	$72.59^{CD} \pm 1.80$	$39.08^{de} \pm 1.85$	C2	$76.60^{C} \pm 4.12$	$52.26^{c} \pm 1.18$
A3	$65.80^{D} \pm 2.81$	$37.07^{e} \pm 0.53$	C3	$117.18^{AB} \pm 3.86$	$80.78^{a} \pm 2.63$
B0	$11.01^{F} \pm 1.37$	$2.73^{h} \pm 0.59$	D0	$8.77^{F} \pm 1.80$	$1.95^{h} \pm 0.40$
B1	$77.79^{C} \pm 3.05$	$19.48^{g} \pm 0.94$	D1	$43.36^{E} \pm 7.55$	$28.20^{f} \pm 0.62$
B2	$122.86^{A} \pm 9.36$	$49.09^{c} \pm 2.57$	D2	$81.38^{C} \pm 2.57$	$44.33^{d} \pm 0.58$
B3	$108.66^{B} \pm 2.27$	$39.62^{de} \pm 1.11$	D3	$117.40^{AB} \pm 4.52$	$73.97^{b} \pm 0.66$

was found. Starting from the first month of ripening, there was an increasing trend in the peptide content in the samples from both dairies, which was significantly ($P < 0.01$) higher than in the curd. The only difference found between the two dairies was the ripening time corresponding to the highest concentration of peptides, which was two months for Dairy II and three months for Dairy I.

The amount of released amino groups found in unfiltered and ultrafiltered samples showed the same trend throughout the cheese ripening. The only exception was the reduction of ca. half of the peptide content after ultrafiltration, demonstrating that ca. half or most of the peptides were low molecular weight peptides.

3.4. Determination of biological activities throughout cheese ripening

Regardless of the dairy, the ACE-inhibitory activity showed a significant ($P < 0.05$) increase throughout the ripening of the cheeses (Fig. 3A). No ACE-inhibitory activity was found in the low-molecular-weight peptide fractions from all the curds, whereas appeared in the other samples starting from the first month of ripening. The evolution of ACE-inhibitory activity was similar among samples collected from the same dairy, and in all cases, the highest inhibitory activity (therefore, the lowest IC50 value) was found after the first month of ripening. At the same time point of ripening, cheeses from Dairy I showed significantly ($P < 0.05$) greater inhibitory activity compared to the samples of Dairy II.

The antioxidant activity determined by the ABTS assay showed a significant ($P < 0.05$) difference throughout the ripening of cheeses with a similar profile among all samples (Fig. 3B). Cheeses after one month of ripening and especially those from Dairy I showed the highest activity followed by a reduction during the ripening. This trend was observed for both dairies with Dairy I, showing the highest antioxidant activity among all samples analyzed ($P < 0.05$).

No α-glucosidase-inhibitory activity was found in the low-molecular-weight peptide fractions from both the dairies at any time point of ripening (data not shown).

3.5. Peptidomics

3.5.1. General peptide profile

Peptidomics analysis allowed the identification of 1337 unique peptides, with exception one sample which had only 10 peptides present (EAMAPKHK; EEEKNR; KTVDMESTEVFTKKTKLTEEEKNRLNFLKKISQR; LGYLEQLLRL; PLR; PVLGPVRGPF; QNIPPL; TVDMESTEVFTKKTKLTEEEKNRLNFLKKISQR; VFGKEKVNE; VKEAMAPKHK), whereas others were found in two or more samples at different intensities. Most peptides came from the β-casein fraction (39.4%), followed by peptides from αs1-casein (28.7%), αs2-casein (24.0%), k-casein (6.7%), β-lactoglobulin (0.8%), and α-lactalbumin (0.2%). The peptide with the highest intensity among all 1337 peptides identified was the αs1-casein-derived peptide ENLLRF (f18-23).

The origin of the peptides found as well as the total amount of peptides identified is shown in Table 2. Peptides from the δ-casein fraction were the most present in both dairies and at any sampling time during ripening, where curd samples contained already between 67.2 and 81.5% of the total peptides identified through the ripening. The complete list of identified peptides together with the mass spectrometry data is reported in Supplementary Table S3–S18.

Regardless of the dairy, the lowest number of peptides was found in the curd samples whereas the highest number was detected in cheeses after two or three months of ripening. Chemometric analyses (PCA and PLS-DA) were done using as variables the relative intensities for all peptides (Fig. 4). The PCA results showed that the samples tend to group according to the batches, especially for the curd (A0, B0, C0, and D0), and during ripening (one-, two-, and three months) the samples tend to be closer together but separated by dairy. The first two PCs have 77.4% of the total variance explained (Fig. 4 A and B). The loading plot also showed that the peptide ENLLRF (m/z 791.4410) was the variable of greater positive loading together with NENLLRF (m/z 905.4839), hence with higher relative intensities for these peptides passing from fresh curd to ripened cheese.

By classifying the samples according to their ripening time, through a supervised PLS-DA method (Fig. 4 C and D), a high explanation of the variables in components 1 and 2 was obtained (76.6 %). From the PLS-DA score plot is evident that samples separate according to the ripening time. There was a clear differentiation in the profile of the samples, with the formation of three distinct clusters depending on the peptide profiles. The first and most dispersed cluster included the curd samples, the second cluster is much less dispersed and was composed separately of cheeses after one month of ripening, followed by a third cluster also with low dispersion composed by the samples after 2 and 3 months of ripening.

A linear differentiation behavior along the ripening time was ascertained that culminated in similar profiles at the end of ripening. Peptide ENLLRF (m/z 791.4410) was the variable of greater positive loading together with NENLLRF (m/z 905.4839) with VIP score >1 as previously shown in the PCA, hence with higher relative intensities for these peptides passing from fresh curd to ripened cheese.

3.5.2. Profile of bioactive peptides

Of the 1337 peptides found in the general peptide profile, 101 (7.5%) were identified as bioactive peptides, with 100% homology with previously reported bioactive peptides. Most of these (63.4%) are from the β-casein, followed by αs2-casein (18.8%), αs1-casein (11.9), k-casein (3%), β-lactoglobulin (2 %), and α-lactalbumin (1%).

The peptides found, according to the databases, have 22 different biological activities reported in the literature, the main ones having

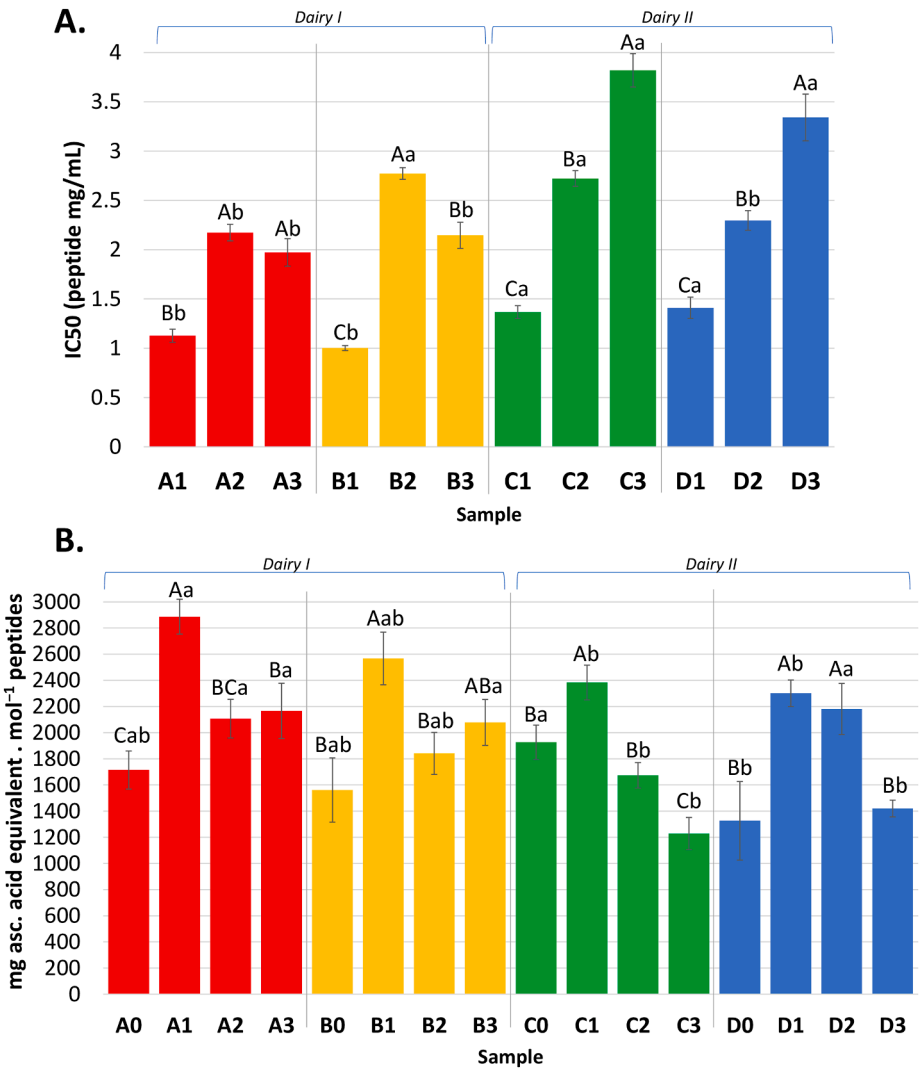

Fig. 3. Bioactivity found in the ultrafiltered aqueous extract soluble at pH 4.6 of curds (A0, B0, C0, and D0), and cheese during ripening (1, 2, and 3 months [A1, B1, C1, and D1 to A3, B3, C3, and D3]). Data were corrected and normalized by the concentration of peptides found in each sample obtained with the TNBS assay. Evolution of ACE (Angiotensin-converting enzyme) inhibitory activity (A), and ABTS (azino-bis-3-ethylbenzothiazoline-6-sulfonic) radical scavenging antioxidant activity (B). Mean values ± standard deviations for two batches of each type of cheese, analyzed in triplicate. Means with different uppercase letters (A to C) mean a significant difference between all the time points for the same samples (ANOVA, $P < 0.05$), whereas means with different lowercase letters (a and b) mean a significant difference between the same time point for all samples (ANOVA, $P < 0.05$).

Table 2
The absolute and relative (%) number of peptides and their predecessor protein fractions found in curds (A0, B0, C0, and D0), and cheese during ripening (1, 2, and 3 months [A1, B1, C1 and D1 to A3, B3, C3, and D3]) (β_{CN} = beta casein fraction; $\alpha s1_{CN}$ = alfa s1 casein fraction; $\alpha s2_{CN}$ = alfa s2 casein fraction; k_{CN} = kappa casein fraction; β_{LG} = beta-lactoglobulin whey protein fraction; α_{LA} = alfa lactalbumin whey protein fraction).

Sample	β_{CN}	%	$\alpha s1_{CN}$	%	$\alpha s2_{CN}$	%	k_{CN}	%	β_{LG}	%	α_{LA}	%	Total
A0	404	41.6	298	30.7	213	21.9	51	5.3	3	0.3	2	0.2	971
A1	470	38.6	353	29.0	299	24.5	84	6.9	9	0.7	3	0.2	1218
A2	473	38.7	359	29.4	294	24.1	83	6.8	9	0.7	3	0.2	1221
A3	467	38.4	355	29.2	299	24.6	84	6.9	8	0.7	3	0.2	1216
B0	373	40.2	268	28.9	227	24.5	55	5.9	3	0.3	2	0.2	928
B1	460	38.4	348	29.0	297	24.8	82	6.8	9	0.8	2	0.2	1198
B2	469	38.5	360	29.6	296	24.3	82	6.7	9	0.7	2	0.2	1218
B3	481	38.9	363	29.3	300	24.3	82	6.6	9	0.7	2	0.2	1237
C0	414	41.3	292	29.1	236	23.6	52	5.2	6	0.6	2	0.2	1002
C1	496	40.2	349	28.3	307	24.9	71	5.7	9	0.7	3	0.2	1235
C2	497	40.2	358	28.9	298	24.1	72	5.8	10	0.8	2	0.2	1237
C3	483	39.3	364	29.6	300	24.4	72	5.9	8	0.7	2	0.2	1229
D0	310	38.0	261	32.0	191	23.4	45	5.5	5	0.6	3	0.4	815
D1	480	40.0	341	28.4	297	24.8	71	5.9	9	0.8	2	0.2	1200
D2	479	40.5	345	29.2	280	23.7	68	5.7	8	0.7	3	0.3	1183
D3	480	39.6	358	29.6	291	24.0	72	5.9	8	0.7	2	0.2	1211

ACE-inhibitory (59 peptides), antimicrobial (25), and antioxidant (21) activities. No peptides with α-glucosidase inhibitory activity were found. The complete list of identified bioactive peptides, their respective bioactive functions, reference, and relative intensities can be found in the Supplementary Material (Supplementary Table S19).

The relative intensity of the identified bioactive peptides, focusing on the 20 peptides with the highest intensity, as well as the evolution of their total intensity during cheese ripening was showed in Fig. 5.

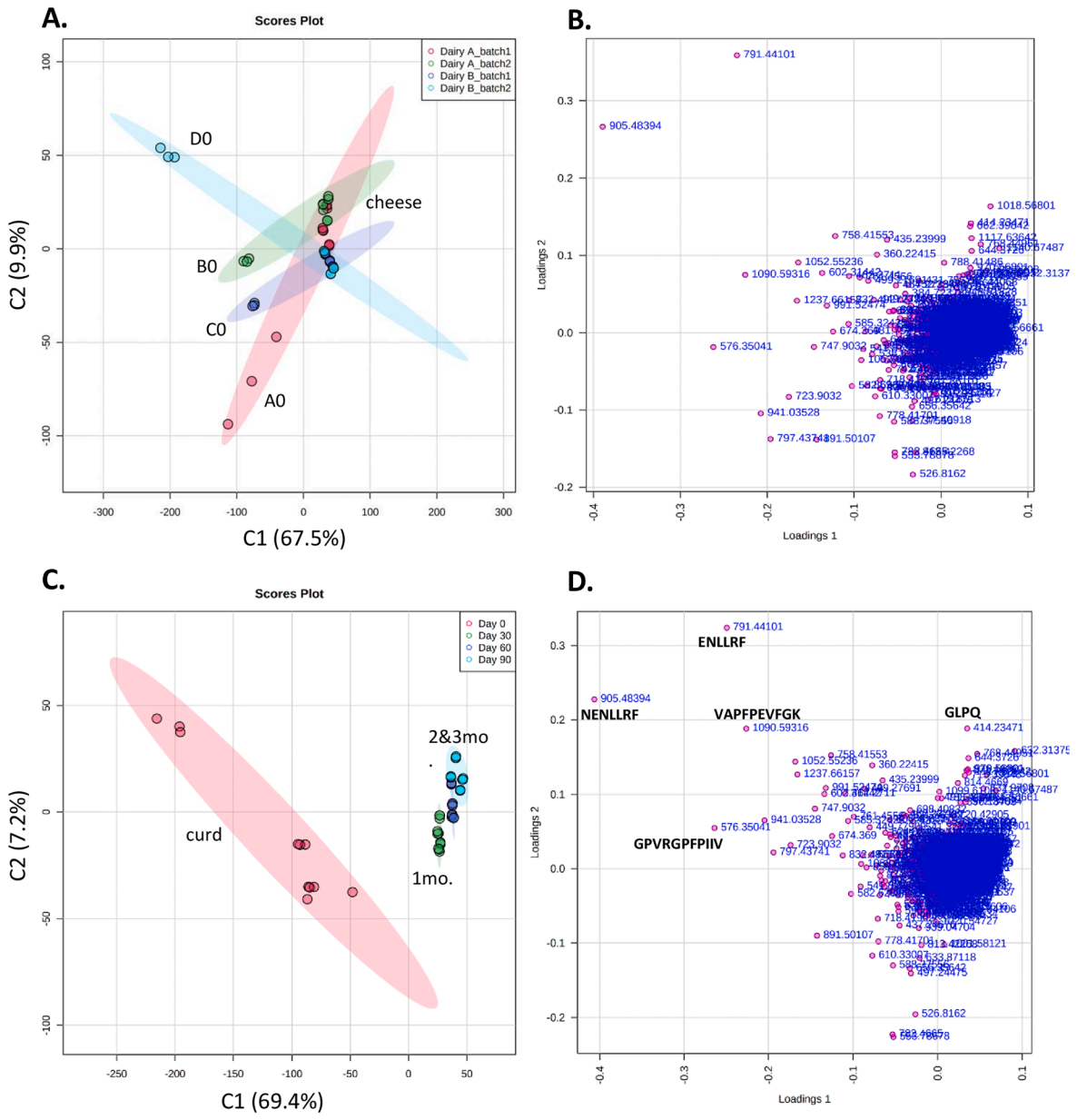

Fig. 4. Chemometric analysis of the full peptide profile obtained from curds (A0, B0, C0, and D0), and cheese during ripening (1, 2, and 3 months [A1, B1, C1 and D1 to A3, B3, C3, and D3]). Score plot (A) and loading plot (B) of the principal component analysis (PCA) of the peptide profile of all samples from two batches per dairy. Score plot (C) and loading plot (D) of the Partial Least-Squares Discriminant Analysis (PLS-DA) analysis of the peptide profile found throughout ripening. In panel D peptides with their name present had VIP score $\geq$1.

A clear differentiation in the production behavior of bioactive peptides during ripening was found, being similar between samples from the same dairy but different between samples from different dairies. Samples from Dairy I showed a peak production of bioactive peptides after 3 months of ripening, while samples from Dairy II after two months of ripening. Despite the similar behavior, there were significant ($P < 0.05$) differences among the sum of bioactive peptides intensity for all samples. There was also a significant ($P < 0.05$) difference between samples from Dairy I after one and two months of ripening, in which the samples from the second batch (samples B1 and B2) remained with a constant total intensity of the peptides, while the samples from the first batch (samples A1 and A2) suffered a reduction in the total intensity. The highest significant intensity of bioactive peptides was found in samples from Dairy I after three months of ripening. With respect to the intensity or relative quantity of the individual bioactive peptides identified during ripening, the ACE-inhibiting peptide ENLLRF was constant and the most abundant, corresponding alone, in some cases, to >50% of the sum of the intensity of all the bioactive peptides found. Furthermore, of the six peptides with the highest intensity, four (ENLLRF, RPKHPIKHQ, VLNENLLR, RELEEL) have reported ACE-inhibitory activity, one (VAPFPE) antioxidant activity, and one (FPEVFGK) antimicrobial activity.

The chemometric analyzes (PCA and PLS-DA) made it possible also to visualize clusters between the samples regarding the bioactive peptide profile. In the PCA scores and loadings plot, in which all sample times were analyzed together, separating the two dairies (I and II) and batches (Fig. 6 A and B), we obtained a high explanation (62.9%) of the variables in components 1 and 2.

There was no clear separation or grouping, however, the peptide with the highest positive loading was ENLLRF (*m/z* 791.4410).

On the other hand, classifying the samples according to ripening time (PLS-DA) resulted in a high explanation (60.5%) of the variables in

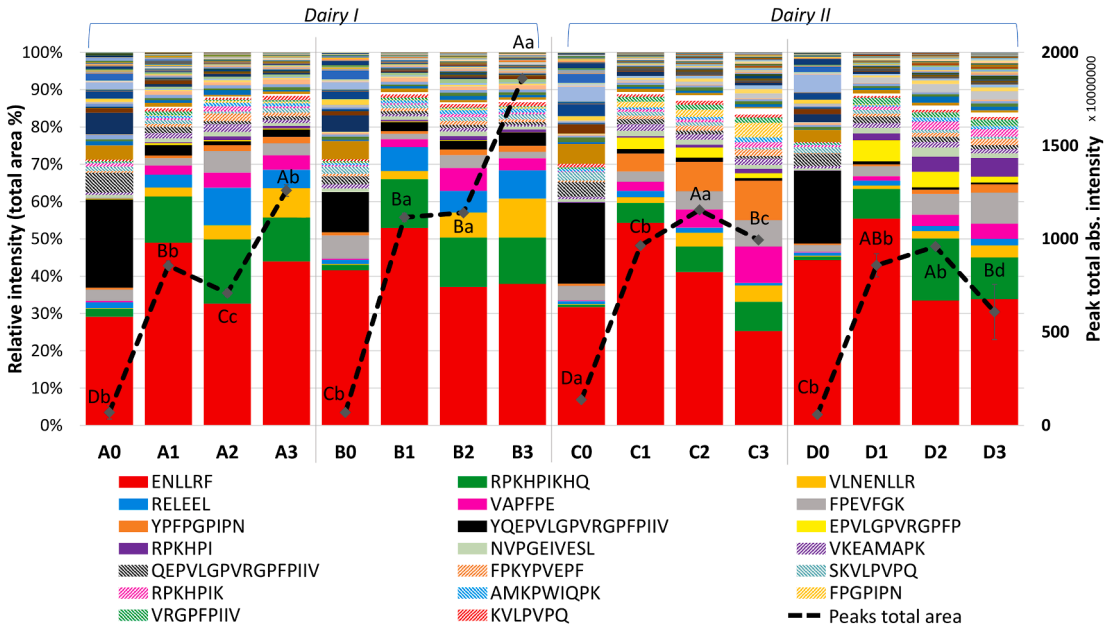

Fig. 5. The relative intensity (%) of identified bioactive peptides and evolution of their total intensity during Asiago cheese ripening (dotted line). Curds (A0, B0, C0, and D0), and cheese during ripening (1, 2, and 3 months [A1, B1, C1 and D1 to A3, B3, C3, and D3]). Mean values ± standard deviations for two batches of each type of cheese, analyzed in triplicate. Means with different uppercase letters (A to C) mean a significant difference between the total intensity of all time points for the same samples (ANOVA, $P < 0.05$), whereas means with different lowercase letters (a and b) mean a significant difference between the total intensity of the same time points for different samples (ANOVA, $P < 0.05$).

components 1 and 2, with a major weight of component 1 (horizontal plane) where the greatest differentiation of the samples occurred. As observed for the general peptide profile, there was a clear difference among the bioactive peptide profiles with the presence of the same three clusters, one grouping the curd samples, a second cluster included the peptide profiles from cheeses after one month of ripening, and a third cluster grouped cheeses after two and three months of ripening.

The VIP-score graph generated by the PLS-DA analysis shows the 15 peptides with a VIP-score >1.0, which allowed the differentiation of the samples and those in which these peptides were more concentrated. The ACE-inhibiting peptide YQEPVLGPVRGPFPIIV, found mainly in curds that tended to degrade at the end of ripening, contributed mainly to the differentiation of the samples. The concentration of other peptides such as VLNENLLR and RELEEL, with antimicrobial and antioxidant activity, respectively, constantly increased throughout ripening, while peptides such as the ACE-inhibitory ENLLRF showed the highest concentration at the intermediate period of ripening.

3.6. Data correlation

Chemical, enzymatic, microbiological data (thermophilic cocci, mesophilic cocci and bacilli, and top 23 bacterial genera), and the intensity of the 101 bioactive peptides were analyzed through a correlation matrix generated from a PCA independently for each dairy. In Dairy I, 70.9% of the variables were explained by the first two components.

Positive significant ($P < 0.01$) correlations (correlation index >0.8) were found between factors such as peptide concentration and ACE-inhibitory activity, and between *Lactobacillus* relative abundances and ABTS radical scavenging antioxidant activity (Supplementary Table S20). *Lactobacillus* was the only genus positively correlated with 12 bioactive peptides, while *Streptococcus* was negatively correlated with the same bioactive peptides as long as the genus of *Lactobacillus* ($P < 0.01$). In Dairy II, 68.0 % of the variables were explained by the first two components. Positive significant correlations were found between thermophilic cocci and 29 bioactive peptides, while 15 bioactive peptides were positively linked with mesophilic bacilli, of which seven were in common with the thermophilic cocci (Supplementary Table S21). The *Lactobacillus* genus was positively correlated with eight bioactive peptides, the same of which *Streptococcus* was negatively correlated with. In addition in Dairy II, *Lactobacillus* was positively correlated with *Enterococcus* and *Pediococcus*, which were the only genera positively correlated with up to 20 peptides in total. Merging the data from both dairies, only 59.5% of the variables could be explained by the first two components. Positive significant ($P < 0.01$) correlations (correlation index >0.8) were mostly found between peptides and mesophilic lactobacilli counts and *Lactobacillus* genus, with six and nine peptides, respectively (Supplementary Table S22). *Bifidobacterium* was correlated with *Enterococcus* and *Pediococcus*, which were also significantly correlated with nine peptides each, while *Bifidobacterium* with five peptides.

4. Discussion

We analyzed Asiago-PDO cheese from two different dairies and days of production, produced using different starter cultures, for their chemical composition, microbiological and peptide profile, throughout their ripening time (up to three months). Peptidomic analysis included the characterization of bioactive peptides and their biological function relevant to the ACE-inhibitory, α-glucosidase-inhibitory, and antioxidant activity.

Differences in the major chemical parameters (fat, moisture, and protein content) were more pronounced between dairies ($P < 0.05$) throughout the ripening process and not for the curds, while within the dairies, Dairy II had the highest variability between the different batches (processing days). The major difference between the dairies lies in the type of starter culture used. Dairy I is using industrial starter constituted by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, while Dairy II is using natural milk starter, which is usually mainly composed of *Streptococcus thermophilus*. As known, differences in cheese chemistry and biochemistry during ripening can be associated with the use of different starters (Hayaloglu et al., 2005, Jia et al. 2021).

Clearly, the differences between dairies were also prominent for the microbiome analyses. Cell densities of mesophilic lactobacilli were statistically ($P < 0.05$) different between dairies during ripening, with Dairy I, reaching the highest cell number at one month while Dairy II was at two

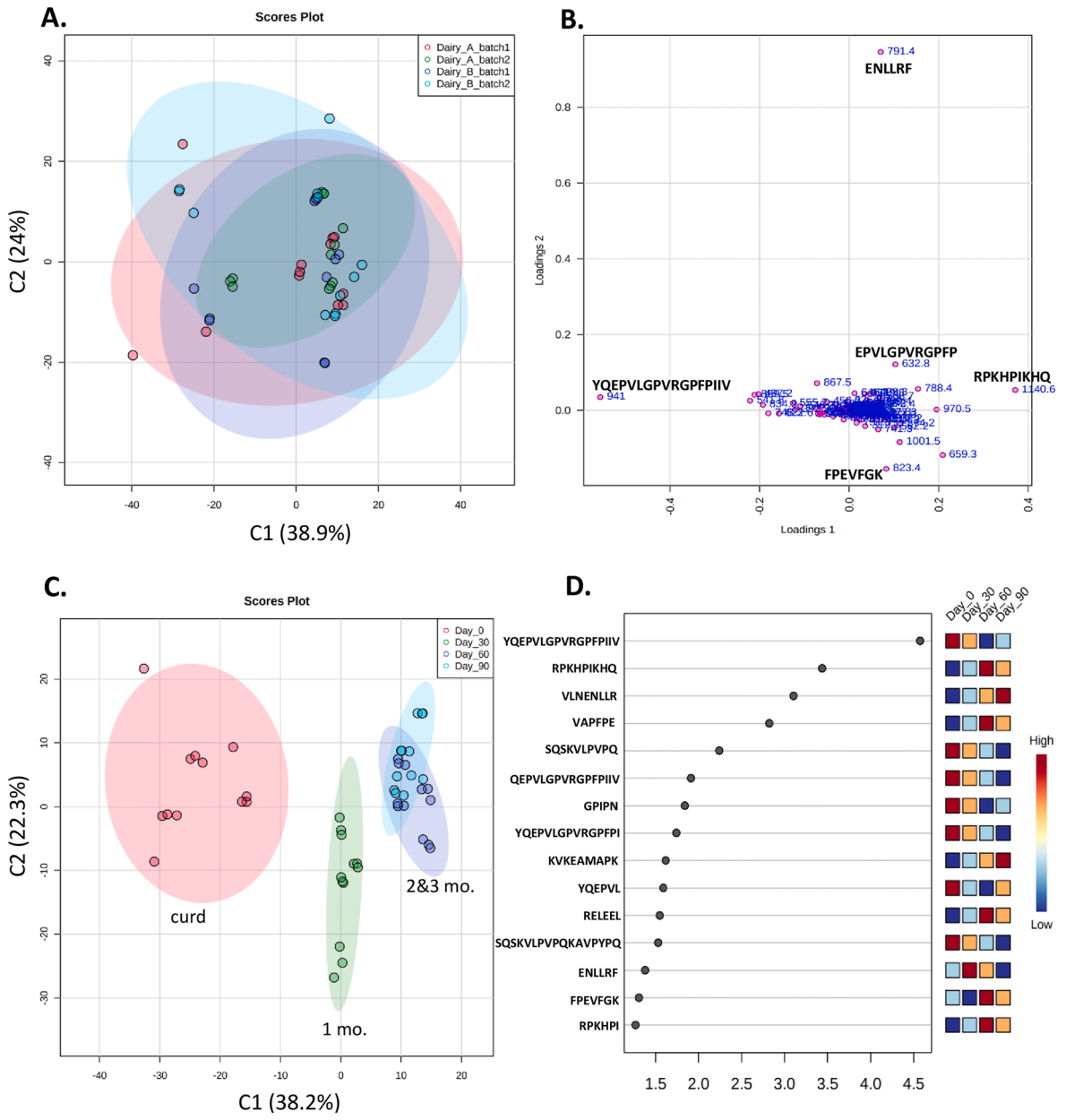

Fig. 6. Chemometric analysis of the bioactive peptide profile from curds (A0, B0, C0, and D0), and cheese during ripening (1, 2, and 3 months [A1, B1, C1 and D1 to A3, B3, C3, and D3]). Score plot (A) and loading plot (B) of the principal component analysis (PCA) analysis of the bioactive peptide profile of all samples from two batches per dairy. Score plot (C) and loading plot of peptides with VIP score >1 (D) of the Partial Least-Squares Discriminant Analysis (PLS-DA) analysis of the bioactive peptide profile of cheeses throughout ripening.

months. The same trend was found for the counts of mesophilic cocci, while thermophilic cocci started with the highest cell densities post-salting and progressively were reduced for both dairies. Industrial starter cultures include strains with specific metabolic traits relevant to the cheese-making process and have generally very high cell densities (9–10 log CFU g^{-1}), which was confirmed by the cell densities found in the starter culture from Dairy I (Parente & Cogan, 2004). On the contrary, the natural milk starter, which is a natural undefined starter culture had moderate cell densities of mesophilic cocci and bacilli, and thermophilic cocci (5.6–7.5 log CFU g^{-1}). These significant differences in the microbial composition between the starter cultures evidently played a key role in the microbiome differences found in the cheeses during ripening (Coelho et al., 2022). This was double confirmed with 16S metabarcoding analysis, where *Lactobacillus* genus relative abundances were significantly different ($P < 0.05$) between the dairies' curds and cheeses throughout ripening, with higher abundances found for Dairy I. Generally, for Dairy I, the microbiota composition of the curds and cheeses was influenced by the starter culture, with main players the genus *Lactobacillus* and *Streptococcus*, while in Dairy II the microbiota composition was more complex and included milk contaminants such as *Chryseobacterium*, *Macrococcus*, *Kurthia,* and others. Relative abundances of *Streptococcus* also differed between dairies, being more abundant in Dairy II. The results reflected the known composition of the industrial starter culture and the cell densities found, likewise for the milk starter.

The differences in peptide concentration found during ripening were mainly related to the extent of proteolysis. The lower concentration of low molecular weight peptides when compared to the total concentration of peptides was expected after ultrafiltration (Roslan et al., 2021), showing an evolution during ripening similar to that found by Dimitrov and Gotova (2014) where peptides with molecular weight lower than 3

kDa increased in concentration during ripening, while high molecular weight peptides were degraded. In fact, it is well-recognized that proteolysis gives rise to the gradual formation of low molecular weight peptides due to intense exogenous and endogenous bacterial proteases of active and lysed bacteria (Chen et al., 2012; Fox, 1989).

Regarding the general peptide profile, the greater presence of β-casein peptides is basically because this casein fraction is more susceptible to hydrolysis. This is attributed to its open conformation, mainly to the high content of proline in its primary chain (Bhat et al., 2016).

The presence of most of the peptides identified during the entire cheese ripening process already in the curd (24 h) after cheesemaking confirms the importance of the initial phase of cheese fermentation. The action of the rennet, and mainly of the starters, play a major role in primary proteolysis, thus defining to a large extent the peptide content of the samples and the subsequent peptide fractions that will be produced during ripening by non-starter cultures and by the endopeptidases released from the lysed primary starter cells (McSweeney, 2007b).

The chemometric analysis allows us to show that, although the diversity of identified peptides did not change much during ripening, there was an evolution in the intensity or concentration of these peptides, modifying and differentiating profiles during ripening, which is probably due to the secondary proteolysis, carried out mainly by starter cultures and especially NSLAB and sub-dominant genera, after the first weeks of cheese making (Sousa et al., 2012).

The data also lead us to the observation that although there was no differentiation between the peptide profile of the cheese manufactured at the two dairies, which corroborates the high standard of quality and process standardization of PDO cheeses, there is a relevant differentiation of the profile throughout the ripening, possibly due to the intense action of the microbiota in the first months of ripening that produces more low molecular weight peptides from predecessor peptides initially produced by the action of rennet or starter cultures. Similar peptide profile behavior was found for Camembert cheese (Galli et al., 2019) and ripened Prato-cheese (Baptista et al., 2020). Likewise, for longer ripening periods of Parmigiano-Reggiano cheese (up to 24 months), similar trends in the bioactive peptide release were observed and were even possible to relate ripening time with specific bioactivity (Martini et al., 2020, Rocchetti et al., 2021). Based on the profile of bioactive peptides, we found that β-casein was the protein fraction with the greatest potential for producing bioactive peptides (Guha et al., 2021). However, the main identified bioactive peptide (ENLLRF), derived from the αs1-casein fraction, has been reported in high-concentration in Parmigiano-Region cheese (Martini et al. 2021), Spanish semi-hard cheese (Sánchez-Rivera et al., 2013), but interestingly not in Asiago cheese (Lignitto et al., 2010). However, this last study monitored the release of bioactive peptides after six months of ripening and since this peptide corresponds to the primary site of cleavage of αs1-CN by chymosin during primary cheese proteolysis, its absence likely results from further breakdown into inactive peptides as a result of the prolonged action of endo– and/or exo-peptidases.

The biological activities found in our samples fitted with the antioxidant and ACE-inhibitory activities. Although the difference between the antioxidant capacity was not so relevant among the samples, the samples coded “A” showed the highest activity and the highest concentration of the antioxidant peptide RELEEL. However, the relationship between the behavior of the antioxidant activity measured in the samples and the concentration of antioxidant peptides was imprecise, probably because milk and its derivatives have other fat-soluble antioxidant agents in large quantities, such as carotenoids, retinol, and α-tocopherols that might interfere with antioxidant chemical analysis (Khan et al., 2019).

According to Gobbetti et al. (2002), the production of bioactive peptides is higher in cheeses with greater proteolysis but not in cheeses with excessive proteolysis. The total intensity of peptides here seems to be affected by the microbiological profile. The reduction in intensity at the end of the ripening period in cheeses from Dairy II is probably due to the fact that NSLABs have greater proteolytic activity during secondary proteolysis and because of a greater diversity of peptidases, producing intense amounts of free amino acids in the late-ripening, therefore degrading peptides faster (Williams & Banks, 1997; Di Cagno et al., 2006). In fact, the cell densities of presumptive non-starter lactobacilli for Dairy II reached the highest number after two months of ripening, likewise, the relative abundances of *Lactobacillus* genus by 16S metabarcoding, while in Dairy I this trend was observed within one month of ripening.

The low correlation between the *Streptococcus* genus and bioactive peptides, and the high correlations between the *Lactobacillus* genus and most of the high intense bioactive peptides (as well as the internal correlation between these peptides), confirmed the active role of lactobacilli in the production of bioactive peptides. Since the *Lactobacillus* genus includes fastidious species with a high demand for amino acids, their proteolytic system is very active, thus most of the bioactive peptides found in dairy products are produced by *Lactobacillus* species and were mainly antihypertensive tripeptides, mainly IPP, IPA and VPP, these last two identified in our cheese samples (Fitzgerald et al., 2004; Raveschot et al., 2018).

Principal component analyses done for each dairy using microbiological, bioactive peptides and measured bioactivity results, depicted several strong correlations. Generally, in Dairy I, bioactive peptides were mostly correlated with the cell density of mesophilic lactobacilli and relative abundances of the *Lactobacillus* genus, confirming the significance of NSLAB for the formation of bioactive peptides. In Dairy II, several correlations were associated with thermophilic cocci and mesophilic lactobacilli cell densities, while the *Lactobacillus* genus was linked to other NSLAB genera (*Pediococcus* and *Enterococcus*), which were strongly correlated with bioactive peptides. Cumulatively considering the results from both dairies, the unique high correlation of some high-intensity peptides such as EPVLGPVRGPFP with the genus *Bifidobacterium* and *Pediococcus*, and VKEAMAPK with the genus *Enterococcus,* as well as the correlation of lower-intensity peptides such as EAMAPK, HPHPHLSF, and VRGPFP with the genera *Enterococcus* and *Pediococcus*, leads us to the conclusion that NSLABs and sub-dominant genera were the second most potential agent for the production of bioactive peptides after lactobacilli. The production of bioactive peptides was previously found in cheeses where streptococci were combined with *Bifidobacterium* (Li et al., 2020) and the production of ACE-inhibitory peptides was associated with *Pediococcus* (Daliri et al., 2018).

The correlation of the highest intensity peptide ENLLRF (αs1-casein 18–23) found in ripened cheeses, corroborates the leading role of the *Lactobacillus* genus in proteolysis and in the production of bioactive peptides. A possible explanation for the high concentration of this peptide may be the symbiotic action of the chymosin at the beginning of ripening that generates peptides predecessors of ENLLRF as well as the RPKHPIKHQGLPQEVLNENLLRF (αs1-casein 1–23), a bioactive one, of low intensity and weakly correlated (correlation index = 0.618) with the *Lactobacillus* genus (Baptista & Gigante, 2021).

5. Conclusions

Our study confirmed that the standardized protocol adopted to produce Asiago-PDO cheese ensures a product with acceptable chemical attributes. However, the use of different starter cultures can affect the microbiome composition post-salting and during ripening and consequently, deviate the time and extend that biochemical processes take place.

The microbiome composition and evolution from curd to ripened cheese were distinguishable between the dairies and fully driven by the starters used. The combination of starter lactic acid bacteria (SLAB) and NSLAB mainly lactobacilli is a prerequisite for an industrial starter, but natural milk starters are primarily dominated by SLAB of genus *Streptococcus* and milk microbial contaminants with higher diversity. Thus,

the use of industrial starter is linked to reproducibility and efficient transition from fast acidification and action of SLAB to initiation of proteolysis and flavor development by NSLAB, but natural starters due to their increased unknown diversity, even though effective, may be unpredictable (Carminati et al., 2010). Our study proved that even though there are no discernible peptide profiles between cheeses made with different starters referring to general and bioactive peptide diversity, there is a significant difference in the concentration of the peptide during ripening. This not only influences the number of bioactive peptides found but will also have sensory and nutritional relevance even if it does not de-characterize the product.

In this scenario, we reaffirmed the probable direct relationship between the genus *Lactobacillus* and ACE-inhibitory peptides as well as the importance of the starter culture for their temporal release. The industrial starter used in Dairy I reassured the highest relative intensity of bioactive peptides after three months of ripening, while the cheeses made with natural fermented milk starter culture (Dairy II) at two months, but with lower total relative intensity compared to Dairy I. However, the sub-dominant strains and NSLABs present in Dairy II are responsible for the production of part ACE-inhibitory peptides, reaffirming the importance and relevance of autochthonous strains of each micro-region even within a delimited PDO production area.

The integration of various analytical techniques resulted in a comprehensive and detailed understanding of the relationship between microbial diversity and bioactive peptides, underscoring the significance of starter cultures. Artisanal cheese producers of Asiago could utilize such knowledge not only to produce cheeses with consistent chemical attributes but also with greater functionality and potential health-promoting properties.

Data availability

Data of sequencing are available in Sequence read archive (SRA) under the submission number SUB12360532 (https://submit.ncbi.nlm.nih.gov/subs/sra/SUB12360532/overview).

CRediT authorship contribution statement

Bruno Domingues Galli: Conceptualization, Investigation, Formal analysis, Validation, Writing – original draft, Visualization. **Olga Nikoloudaki:** Data curation, Writing – review & editing, Visualization. **Stefano Tonini:** Investigation. **Ahmed Helal:** Methodology, Formal analysis. **Raffaella Di Cagno:** Writing – review & editing, Supervision. **Marco Gobbetti:** Supervision. **Davide Tagliazucchi:** Project administration, Methodology, Formal analysis, Writing – review & editing, Resources, 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

Raw sequences are deposited in SRA archive. All other raw data are provided in supplementary material.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112743.

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