# MICROBIAL DYNAMICS OF CACIOFIORE CHEESE COAGULATED WITHCRUDE EXTRACT FROM ONOPORDUM TAURICUM WILLD

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**Abstract** - In recent times there is a growing interest in vegetable coagulants for cheese-making, but only Cynara cardunculus is being effectively used due to lack of standardization of other species with milk-clotting properties. In this study, two experimental coagulants obtained from flower heads of spontaneous or cultivated OnopordumtauricumWilldwere used for the manufacture of Caciofiore cheese and compared to a commercial coagulant from Cynara cardunculus. With the aim of investigating the effect of the coagulants on the cheese microbial dynamics during ripening, a combined approach based on conventional and DNA-based (Illumina sequencing) analyses was adopted. Differences in bacterial biota and mycobiota were found depending on the coagulants, but their effect wasnegligible with respect to initial milk microflora. The similarity between experimental and control cheeses supported the potential use of the experimental coagulants.

**Keywords** - Vegetable coagulants, Cheese-making, Ripening, Microbial dynamics, Illumina sequencing.

## I. INTRODUCTION

Rennet is a key ingredient in cheese production because it contains enzymes responsible for milk coagulation. Animal rennet, particularly the one extracted from calf abomasum, is the most ancient and widely used worldwide, thanks to the presence of chymosin, an aspartic proteinase with strong milk-clotting activity and low proteolytic activity [1]. Several factors, such as abomasashortage, dietary choices (vegetarianism), religious reasonsand rules against GMOs have driven research toward animal rennet substitutes, including vegetable coagulants(VCs) [2]. Flower heads ofCynara cardunculusL. have been used for centuries for small-scale cheese production in the Mediterranean basin and constitute an ingredient in the production of many PDO cheeses [3], but it is not the only thistle species with milk-clotting activity. In this work, Onopordumtauricum flower heads were used to obtain VCsto produceCaciofiore,a typical Central Italian rawewe's milk cheese. Theexperimental and control cheeses, the latter made with a commercial coagulant from Cynara cardunculus, were analyzed during ripeningby culture and DNA-based (Illumina sequencing) techniques to investigate the effect of the coagulant on the bacterial biota and mycobiota.

## II. CRUDE EXTRACTS (CEs) PREPARATION

Flower heads of spontaneous (st) and cultivated (ct) OnopordumtauricumWilld(Ot) were used for the preparation of two different experimental coagulants (CE_st and CE_ct, respectively) by following the process summarized in Figure 1 [4]. The CEs resulting from centrifugation were freeze-dried (VirTis Advantage benchtop freeze dryer, SteroglassS.r.l., Perugia, Italy) and reconstituted in demineralized water (1:10 w/v) at the time of use.

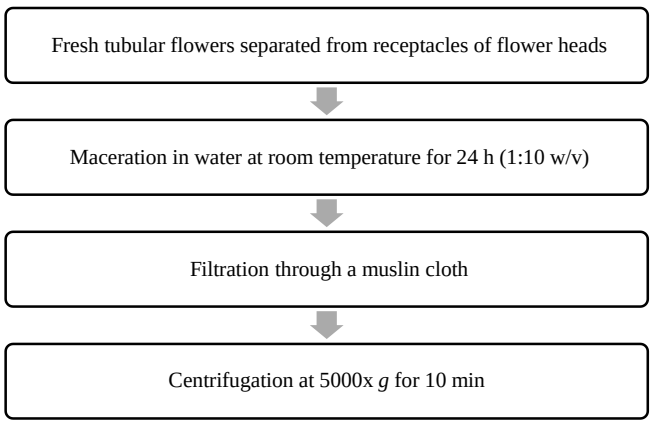



**Figure 1: Steps for CEs preparation**

## III. CHEESE-MAKING

Caciofiore (CF) is an Italian raw sheep milk cheese produced without the addition of any starter cultures. Cheese-makingtrials were conducted in a family-run dairy farm, following an ancient traditional method (Fig.2).The experimental plan (Fig.3) consisted of two Rounds (R) of cheesemaking; the first (R1), conducted between April-July 2021 in which cheeses with CE_st were produced (CF_Ot_st), and the second (R2), between September-November 2021, for cheeses with CE_ct (CF_Ot_ct). For each Round, cheesemaking were repeated twice on two different days (using different batches of milk, B1 and B2, respectively);in parallel to the experimental cheeses, control cheeses (CF_C) were manufacturedthrougha commercial coagulant from Cynara cardunculusL. (Cc)(Dairen, Dairy and Food, Castel San Pietro Terme, BO, Italy).

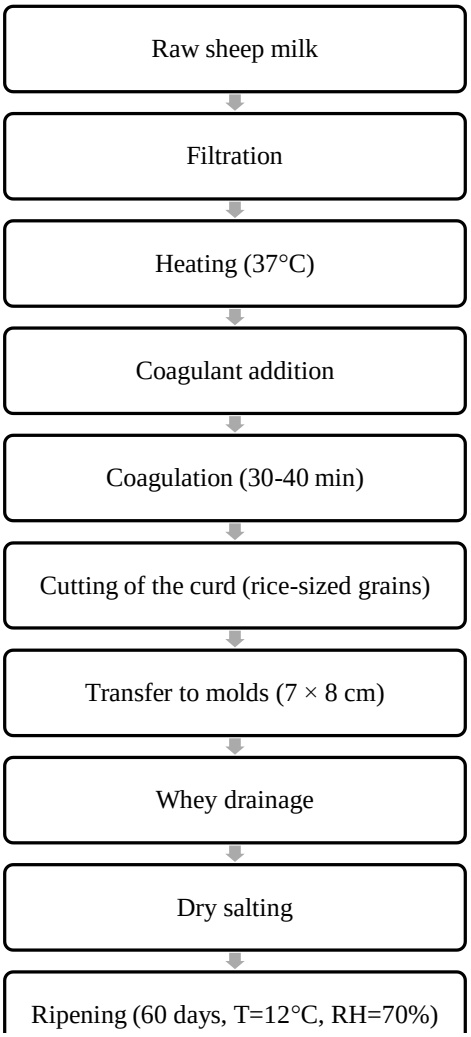



**Figure 2: Flow chart of CF manufacturing process**

Cheese aliquots (about 200 g) were sampled and analyzed after 0, 2, 5, 15, 30, 45 and 60 days of ripening. Analyses were also performed on milk and VCs.

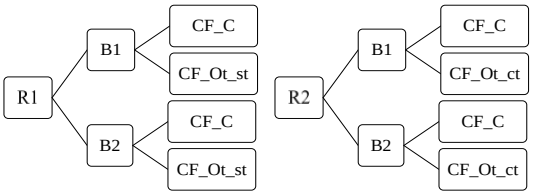



**Figure 3: Experimental plan; R (Round), B (Batch), CF (Caciofiore), C (Control), Ot (Onopordumtauricum), st (spontaneous), ct (cultivated)**

## IV. CHEMICAL AND MICROBIOLOGICAL (CULTURE-DEPENDENT) ANALYSES

pH of milk and cheeses were measured by a pH meter 300 (Hanna Instruments, Padova, Italy) equipped with a solid electrode (HI2031, Hanna Instruments). Total titratable acidity (TTA) was determined on 10 g of sample homogenized with 90 ml of distilled water and expressed as the volume (ml) of NaOH 0.1 N required to reach a pH value of 8.3 [5].For both pH and TTA, measurements were performed in duplicate and the results expressed as mean ± standard deviation. Microbial viable counts were also carried out to determinethe load of presumptive lactococci, thermophilic cocci and lactobacilli, total mesophilicaerobes, Enterobacteriaceae, Pseudomonadaceae, yeasts, and moulds. To this end, 10 g of each sample were homogenized in 90 ml of sterile peptone water(1 g/L) for 2 min at 260 rpm using a stomacher apparatus (400 Circulator, International PBI, Milan, Italy); aliquots of decimal dilutions of milk, VCs and cheeses were inoculated in duplicate on opportune solid media. Viable counts were expressed as log colony-forming units (log cfu) per gram or ml of sample ± standard deviations.Overall means of the results on the cheeses from the two batches were calculated and analyzed by ANOVA and Tukey's test ($\alpha = 0.05$)

## V. CULTURE-INDEPENDENT ANALYSIS: ILLUMINA SEQUENCING

### a. DNA Extraction and Metataxonomic Amplicon Sequencing

Total microbial DNAs were extracted from the cell pellets ofVCs, milk and cheeses homogenates (dilution $10^{-1}$) obtained by centrifugation (14.000 rpm for 10 min). The sample pellets were treated by the E.Z.N.A. soil DNA kit (Omega Bio-tek, Norcross, GA, USA). For each cheese (0, 2, 5, 15, 30, 45, 60 days), pellets from the two batches were pooled to reduce the inter-sample variability.DNAs were quantified using the QUBIT dsDNA Assay kit (Life Technologies, Milan, Italy), and then diluted to 5 ng $\mu L^{-1}$to perform the metataxonomic amplicon sequencing. The amplification of the V3-V4 region of the 16S rRNA gene was carried out to determine the bacterial biota [6], while the amplification of the D1-D2 region of the 26S rRNA gene was carried out to determine the mycobiota [7]. The amplicons were

purified, tagged, and pooled following the Illumina standard protocol. Paired-end reads (2X250 bp) were sequenced by using a MiSeq platform (Illumina, San Diego, California, USA) with v2 chemistry, according to the manufacturer's instructions.

QIIME2 software was used to import and analyze the raw reads (FASTQ format) [8]. Barcode adapters and primers were firstly removed, and the sequences were quality filtered applying DADA2 algorithm to obtain Amplicon Sequence Variants (ASVs) [9].The taxonomic assignment of the bacterial ASVs was executed using the Greengenes 16S rRNA database, whereas a manually build database [10] was used for the taxonomic assignment of the fungal ASVs.The resulting ASVs with a relative frequency > 1.0% in at least two samples were retained. The metataxonomic analysis results were deposited in the Sequence Read Archive of the National Center for Biotechnology Information.

**b. Statistical Analysis**

The QIIME2 diversityplugin was used to calculate alpha and beta diversity of VCs, milk, and cheeses. Significant statistical differences (p-value < 0.05) were evaluated by PERMANOVA. Principal Component Analysis (PCA) aimed at evaluating similarities between experimental variables was performed on ASV relative frequencies using ade4package in R v4.2.0.

## VI. RESULTS

**a. pH, TTA and viable counts**

Raw sheep's milk was characterized by pH and TTA values of 6.74 ± 0.02 and 1.63 ± 0.10, 6.56 ± 0.02 and 1.8 ± 0.18, in R1 and R2 respectively.Figure 4 shows the changes in pH and TTA of the cheeses during ripening.The results of viable countingon cheeses during ripening are shown in Tables 1 (R1) and 2 (R2).Overall, no effect of the coagulant was noticed, as evidenced by the similarity of the results between the control and the experimental cheeses.In both R1 and R2, the same trendwas observed in all the samplesfor total aerobic mesophilic aerobes, presumptive lactococci and presumptive thermophilic cocci, consisting of a major growth in the first two days (by about 3 logs), a maximum value reached between days 5-15 and a slight decrease in the last ripening phase.A same trendwas also seenfor presumptive lactobacilli in R2, while in R1 the growth was slower and the maximum load reached at 30 days, in both CF_C and CF_Ot_st. No significant differences were observed between control and experimental cheeses in the viable counts ofEnterobacteriaceae;the maximum loads at 5 days (7.35-7.99 log ufc $g^{-1}$) were followed by a 4-log and 2-log decrease in R1 and R2, respectively.In R1, the level of Pseudomonadaceae was lower in CF_Ot_stcompared to CF_C (5.09 ± 0.69 vs 6.51 ±0.20) after one month but there were no significant differences at the end of maturation. Finally, except for yeasts in R1, no significant differences were found for eumycetes at 60 days.

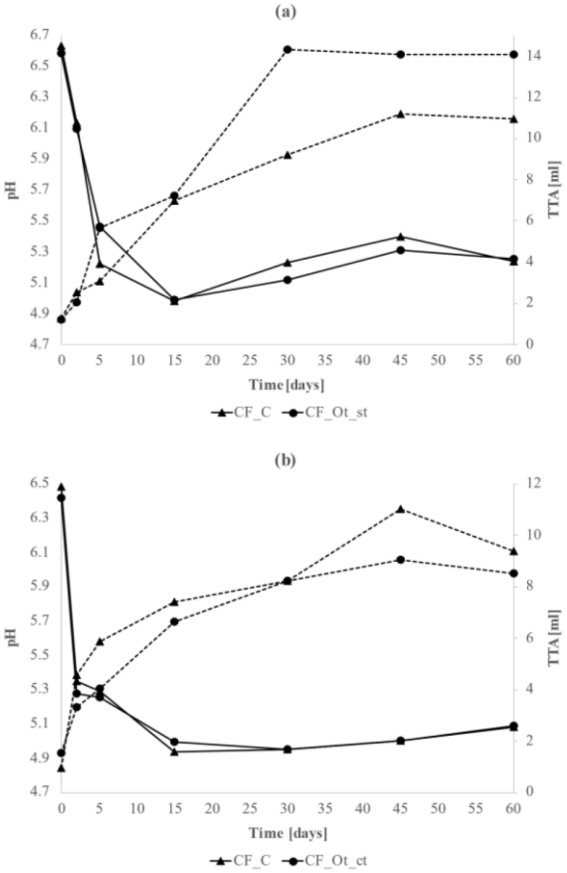



**Figure 4: Results of pH and TTA in Round 1 (a) and Round 2 (b). pH values are indicated in the primary axis and the results represented with a continuous line; in the secondary axis, TTA values are indicated and results represented with a dashed line**

**b. Bacterial Biota and Mycobiota Composition**

The bacterial biota of VCs displayed the common presence of Enterobacteriaceae (6.64, 43.18, and 6.56% of the relative frequencies of Cc, CE_st, and CE_ct, respectively), Erwinia (2.26, 50.65, and 24.58%) and Pseudomonas (50.89, 2.67, and 7.22%). CE_st showed the presence of Lactococcus lactis (1.87%) and Pseudomonas fragi (1.28%), whereas CE_ct comprised Acinetobacter (3.75%) and Acinetobacter johnonii(36.50%). Samples of milk (M) used in cheese-making processes (R1 & R2) shared the presence of Lactococcuslactis (3.03 and 5.62% of the relative frequencies of R1_M and R2_M, respectively), and Acinetobacterjohnsonii (27.56 and 3.49%). R1_M also comprised Staphylococcusaureus (27.96%), Pseudomonas (4.91%), and Serratia (4.56%), whereas R2_M includedStaphylococcus (29.01%), Rummeliibacillus (14.58%), and Corynebacterium (4.74%).As for R1-cheeses (CF_C vs CF_Ot_st), the comparison showed no significant differences (Fig.5a); both productions were characterized by a substantial increase of Lactococcuslactis over time, with initial values of

**Table 1: Results of viable counting (log cfu per gram or ml) of bacteria and eumycetes during ripening of Round 1 (R1) cheeses: control (CF_C) and experimental Caciofiore (CF_Ot_st). Results are expressed as mean values ± st. dev. of the two batches (B1 & B2). Different letters on the same column and time of ripening indicate differences between CF_C and CF_Ot_st according to Tukey's test (P<0.05)**

| R1 | Total mesophilic aerobes | | Presumptive lactobacilli | | Presumptive lactococci | | Presumptive thermophilic cocci | | *Enterobacteriaceae* | | *Pseudomonadaceae* | | Moulds | | Yeasts | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CF_C | CF_Ot_st | CF_C | CF_Ot_st | CF_C | CF_Ot_st | CF_C | CF_Ot_st | CF_C | CF_Ot_st | CF_C | CF_Ot_st | CF_C | CF_Ot_st | CF_C | CF_Ot_st |
| **Milk** | 5.79 ± 0.20 | | 1.56 ± 1.80 | | 4.75 ± 0.17 | | 4.76 ± 0.15 | | 4.12 ± 0.20 | | 4.77 ± 0.32 | | 2.10 ± 0.14 | | 3.03 ± 0.57 | |
| **Cheese 0 days** | 6.00 ± 0.83$^{a}$ | 6.18 ± 0.69$^{a}$ | 1.46 ± 1.47$^{a}$ | 1.54 ± 1.78$^{a}$ | 5.04 ± 0.20$^{a}$ | 5.08 ± 0.18$^{a}$ | 5.01 ± 0.08$^{a}$ | 4.98 ± 0.09$^{a}$ | 4.62 ± 0.22$^{a}$ | 4.83 ± 0.35$^{a}$ | 4.03 ± 0.51$^{a}$ | 4.29 ± 0.80$^{a}$ | 2.31 ± 0.01$^{a}$ | 2.88 ± 0.58$^{a}$ | 2.99 ± 0.08$^{a}$ | 3.57 ± 1.22$^{a}$ |
| **Cheese 2 days** | 8.68 ± 0.28$^{a}$ | 8.64 ± 0.29$^{a}$ | 3.42 ± 0.90$^{a}$ | 3.37 ± 1.17$^{a}$ | 8.31 ± 0.70$^{a}$ | 8.46 ± 0.68$^{a}$ | 8.53 ± 0.39$^{a}$ | 8.19 ± 0.75$^{a}$ | 7.69 ± 0.58$^{a}$ | 7.71 ± 0.45$^{a}$ | 6.42 ± 0.42$^{a}$ | 6.37 ± 0.39$^{a}$ | 2.73 ± 0.09$^{a}$ | 2.35 ± 0.26$^{a}$ | 3.96 ± 0.04$^{a}$ | 3.35 ± 0.38$^{a}$ |
| **Cheese 5 days** | 9.28 ± 0.04$^{a}$ | 9.21 ± 0.11$^{a}$ | 5.41 ± 0.89$^{a}$ | 5.55 ± 0.69$^{a}$ | 9.24 ± 0.03$^{a}$ | 8.57 ± 0.89$^{a}$ | 8.88 ± 0.28$^{a}$ | 8.73 ± 0.36$^{a}$ | 7.95 ± 0.32$^{a}$ | 7.99 ± 0.17$^{a}$ | 7.09 ± 0.29$^{a}$ | 7.12 ± 0.21$^{a}$ | 4.13 ± 0.76$^{a}$ | 3.78 ± 0.38$^{a}$ | 5.70 ± 0.60$^{a}$ | 6.16 ± 0.56$^{a}$ |
| **Cheese 15 days** | 9.06 ± 0.07$^{a}$ | 9.06 ± 0.06$^{a}$ | 7.53 ± 0.91$^{a}$ | 7.28 ± 0.77$^{a}$ | 9.02 ± 0.08$^{a}$ | 9.07 ± 0.10$^{a}$ | 8.85 ± 0.20$^{a}$ | 8.78 ± 0.20$^{a}$ | 6.62 ± 0.44$^{a}$ | 6.56 ± 0.49$^{a}$ | 7.19 ± 0.13$^{a}$ | 7.38 ± 0.35$^{a}$ | 3.09 ± 0.14$^{a}$ | 3.74 ± 0.56$^{a}$ | 4.14 ± 1.17$^{a}$ | 4.85 ± 1.69$^{a}$ |
| **Cheese 30 days** | 9.00 ± 0.16$^{a}$ | 9.12 ± 0.02$^{a}$ | 8.94 ± 0.15$^{a}$ | 8.83 ± 0.13$^{a}$ | 8.95 ± 0.17$^{a}$ | 8.99 ± 0.05$^{a}$ | 8.59 ± 0.24$^{a}$ | 8.57 ± 0.35$^{a}$ | 5.38 ± 0.66$^{a}$ | 4.76 ± 0.92$^{a}$ | 6.51 ± 0.20$^{a}$ | 5.09 ± 0.69$^{b}$ | 2.78 ± 0.11$^{a}$ | 2.23 ± 0.15$^{a}$ | 3.90 ± 1.51$^{a}$ | 3.15 ± 0.47$^{a}$ |
| **Cheese 45 days** | 8.51 ± 0.39$^{a}$ | 8.75 ± 0.21$^{a}$ | 8.38 ± 0.42$^{a}$ | 8.40 ± 0.36$^{a}$ | 8.30 ± 0.53$^{a}$ | 8.56 ± 0.12$^{a}$ | 7.31 ± 0.29$^{a}$ | 7.70 ± 0.16$^{a}$ | 4.36 ± 0.61$^{a}$ | 4.11 ± 0.43$^{a}$ | 4.96 ± 0.58$^{a}$ | 4.73 ± 0.98$^{a}$ | 2.16 ± 0.36$^{b}$ | 3.86 ± 0.49$^{a}$ | 3.89 ± 0.67$^{a}$ | 3.82 ± 0.91$^{a}$ |
| **Cheese 60 days** | 8.72 ± 0.61$^{a}$ | 8.63 ± 0.20$^{a}$ | 8.51 ± 0.39$^{a}$ | 8.27 ± 0.08$^{a}$ | 8.64 ± 0.60$^{a}$ | 8.46 ± 0.18$^{a}$ | 6.88 ± 0.23$^{a}$ | 7.00 ± 0.22$^{a}$ | 3.32 ± 1.53$^{a}$ | 3.46 ± 1.11$^{a}$ | 4.52 ± 0.70$^{a}$ | 3.99 ± 0.87$^{a}$ | 2.77 ± 0.68$^{a}$ | 3.07 ± 0.53$^{a}$ | 3.54 ± 0.10$^{b}$ | 4.80 ± 0.79$^{a}$ |

**Table 2:: Results of viable counting (log cfu per gram or ml) of bacteria and eumycetes during ripening of Round 2 (R2) cheeses: control (CF_C) and experimental Caciofiore (CF_Ot_ct). Results are expressed as mean values ± st. dev. of the two batches (B1 & B2). Different letters on the same column and time of ripening indicate differences between CF_C and CF_Ot_st according to Tukey's test (P<0.05)**

| R2 | Total mesophilic aerobes | | Presumptive lactobacilli | | Presumptive lactococci | | Presumptive thermophilic cocci | | *Enterobacteriaceae* | | *Pseudomonadaceae* | | Moulds | | Yeasts | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CF_C | CF_Ot_ct | CF_C | CF_Ot_ct | CF_C | CF_Ot_ct | CF_C | CF_Ot_ct | CF_C | CF_Ot_ct | CF_C | CF_Ot_ct | CF_C | CF_Ot_ct | CF_C | CF_Ot_ct |
| **Milk** | 5.26 ± 0.45 | | 4.76 ± 0.97 | | 5.05 ± 0.71 | | 4.94 ± 0.94 | | 3.16 ± 0.47 | | 3.85 ± 0.25 | | 3.08 ± 0.47 | | 3.63 ± 0.28 | |
| **Cheese 0 days** | 5.41 ± 0.12$^{a}$ | 5.41 ± 0.16$^{a}$ | 5.16 ± 0.29$^{a}$ | 4.89 ± 0.34$^{a}$ | 5.27 ± 0.29$^{a}$ | 5.22 ± 0.30$^{a}$ | 5.29 ± 0.28$^{a}$ | 5.96 ± 1.30$^{a}$ | 4.31 ± 0.33$^{a}$ | 3.53 ± 1.38$^{a}$ | 3.75 ± 0.12$^{a}$ | 3.82 ± 0.06$^{a}$ | 2.81 ± 0.38$^{a}$ | 2.75 ± 0.32$^{a}$ | 3.34 ± 0.31$^{a}$ | 3.42 ± 0.18$^{a}$ |
| **Cheese 2 days** | 8.97 ± 0.07$^{a}$ | 8.83 ± 0.10$^{a}$ | 8.20 ± 0.07$^{a}$ | 8.12 ± 0.16$^{a}$ | 9.09 ± 0.17$^{a}$ | 8.79 ± 0.20$^{a}$ | 9.02 ± 0.14$^{a}$ | 8.83 ± 0.21$^{a}$ | 7.36 ± 0.14$^{a}$ | 7.64 ± 0.09$^{a}$ | 6.59 ± 0.36$^{a}$ | 6.88 ± 0.08$^{a}$ | 2.92 ± 0.11$^{a}$ | 3.03 ± 0.03$^{a}$ | 4.49 ± 0.02$^{a}$ | 4.24 ± 0.31$^{a}$ |
| **Cheese 5 days** | 9.15 ± 0.29$^{a}$ | 8.98 ± 0.07$^{a}$ | 9.15 ± 0.21$^{a}$ | 8.97 ± 0.05$^{a}$ | 9.19 ± 0.15$^{a}$ | 9.13 ± 0.07$^{a}$ | 9.20 ± 0.18$^{a}$ | 9.11 ± 0.17$^{a}$ | 7.35 ± 0.16$^{a}$ | 7.67 ± 0.16$^{a}$ | 6.13 ± 0.18$^{a}$ | 6.30 ± 0.12$^{a}$ | 5.43 ± 0.73$^{a}$ | 5.31 ± 0.39$^{a}$ | 6.72 ± 0.87$^{a}$ | 5.84 ± 0.73$^{a}$ |
| **Cheese 15 days** | 9.05 ± 0.20$^{a}$ | 8.98 ± 0.05$^{a}$ | 9.01 ± 0.27$^{a}$ | 8.85 ± 0.04$^{a}$ | 9.06 ± 0.19$^{a}$ | 8.84 ± 0.06$^{a}$ | 8.87 ± 0.09$^{a}$ | 8.78 ± 0.22$^{a}$ | 6.90 ± 0.11$^{a}$ | 7.16 ± 0.09$^{a}$ | 6.13 ± 0.45$^{a}$ | 6.23 ± 0.28$^{a}$ | 3.51 ± 1.18$^{a}$ | 4.47 ± 0.11$^{a}$ | 6.16 ± 0.63$^{a}$ | 5.09 ± 0.19$^{b}$ |
| **Cheese 30 days** | 8.53 ± 0.48$^{a}$ | 8.60 ± 0.24$^{a}$ | 8.55 ± 0.53$^{a}$ | 8.59 ± 0.18$^{a}$ | 8.64 ± 0.51$^{a}$ | 8.07 ± 0.50$^{a}$ | 8.30 ± 0.55$^{a}$ | 7.98 ± 0.50$^{a}$ | 6.11 ± 0.17$^{a}$ | 6.52 ± 0.24$^{a}$ | 3.58 ± 0.50$^{a}$ | 3.97 ± 1.12$^{a}$ | 2.93 ± 1.03$^{a}$ | 2.89 ± 0.89$^{a}$ | 4.77 ± 0.55$^{a}$ | 4.55 ± 0.22$^{a}$ |
| **Cheese 45 days** | 8.94 ± 0.23$^{a}$ | 8.69 ± 0.11$^{a}$ | 8.57 ± 0.28$^{a}$ | 8.60 ± 0.06$^{a}$ | 8.37 ± 0.24$^{a}$ | 8.21 ± 0.07$^{a}$ | 8.33 ± 0.36$^{a}$ | 8.01 ± 0.55$^{a}$ | 6.06 ± 0.54$^{a}$ | 6.47 ± 0.67$^{a}$ | 3.29 ± 0.13$^{a}$ | 3.63 ± 0.83$^{a}$ | 2.12 ± 0.49$^{a}$ | 3.19 ± 0.72$^{a}$ | 4.88 ± 0.36$^{a}$ | 5.46 ± 0.34$^{a}$ |
| **Cheese 60 days** | 8.42 ± 0.12$^{a}$ | 8.12 ± 0.13$^{a}$ | 8.49 ± 0.27$^{a}$ | 8.26 ± 0.04$^{a}$ | 8.19 ± 0.19$^{a}$ | 7.81 ± 0.17$^{a}$ | 8.16 ± 0.15$^{a}$ | 7.70 ± 0.15$^{a}$ | 5.53 ± 0.68$^{a}$ | 5.50 ± 0.42$^{a}$ | 2.77 ± 0.14$^{a}$ | 2.11 ± 0.00$^{a}$ | 2.50 ± 0.06$^{a}$ | 2.49 ± 0.13$^{a}$ | 4.48 ± 0.35$^{a}$ | 4.55 ± 0.37$^{a}$ |

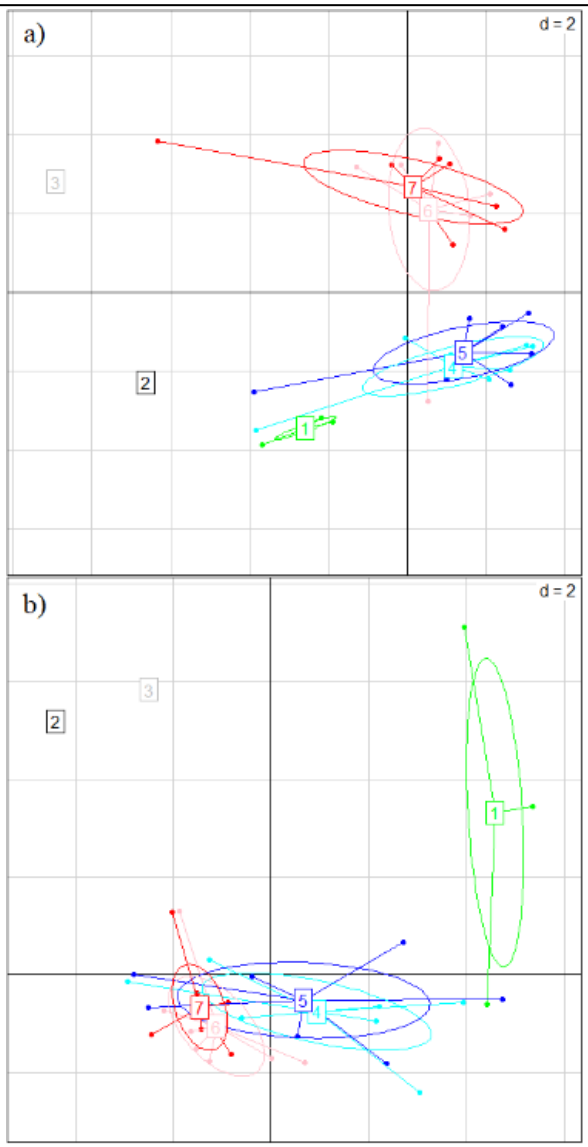



**Figure 4: PCA based on ASV relative frequencies of VCs, milk and cheeses for bacterial biota (a) and mycobiota (b). 1-green, VCs; 2-black, R1_M; 3-gray, R2_M; 4-cyan, R1_CF_C; 5-blue, R1_CF_Ot_st; 6-pink, R2_CF_C; 7-red, R2_CF_Ot_ct**

3.71 (R1_CFC_t0) and 3.41% (R1_CF_Ot_st_t0)of the relative frequency, and a clear dominance at the end of ripening with 62.54 (R1_CFC_t60) and 48.15% (R1_CF_Ot_st_t60). Staphylococcusaureus and Acinetobacterjohnsonii were prevalent only at the beginning of the cheese-making processes. After five days of ripening, Lactobacillus and Latilactobacillussakei displayed a progressive increase, reaching up to 7.77 and 4.37% of the relative frequency in sample R1_CFC_t60, and 6.66 and 7.61% of the relative frequency in sample R1_CF_Ot_st.Also, in R2-cheeses (CF_C vs CF_Ot_ct) no significant differences were highlighted (Fig. 5a). Lactococcus lactisdrove the fermentation over time, with a final relative frequency of 54.38 (R2_CFC_t60) and 56.57% (R2_CF_Ot_ct), respectively. Staphylococcus was detected among the prevalent taxa in both productions in the first five days of ripening and decreased below 1% of the relative frequency after 60 days. Lactobacillus showed a gradual increase after 5 days, with a relative frequency of 20.85 and 20.03% in R2_CFC_t60 and R2_CF_Ot_ct_t60, respectively.

The mycobiota of Cc was dominated by Debaryomyceshansenii (99.51%). CE_st and CE_ct shared the presence of Saccharomycescerevisiae (7.22 and 55.30% of the relative frequency, respectively), Aerobasidiumproteae (15.24 and 2.96%), Aspergillus (5.82 and 3.36%), Alternaria (4.85 and 0.96%) and Debaryomyceshansenii (2.36 and 0.46%).As for the milkused in the cheese-making processes, the common presence of Geotrichumsilvicola (24.82 and 45.22% in samples R1_M and R2_M, respectively), Cladosporium (10.21 and 21.93%), Yarrowialipolytica (20.26 and 4.24%), Candidaparapsilosis (3.61 and 10.01%) and Wickerhamiellapararugosa (4.42 and 2.44%) was highlighted.R1-cheeses(CF_C vs CF_Ot_st)showed no significant differences (Fig. 5b); Debaryomyceshansenii was predominant in both the productions, reaching up to 85.96 (R1_CFC_t60) and 73.14% (R1_CF_Ot_st) of the relative frequency at the end of ripening, respectively. Penicillium was detected with variable levels in almost all cheese samples, with an overall mean relative frequency of 19.94%. A decreasing trend was registered for Geotrichumsilvicola in both the cheese-making processes. Similar results (Fig. 5b) were found for R2-cheeses (CF_C vs CF_Ot_ct); in both productions, Debaryomyceshansenii increased during time up to 49.58 (R2_CFC_t60) and 73.97% (R2_CF_Ot_ct_t60) of the relative frequency. On the contrary, Geotrichumsilvicola and Candidaparapsilosis decreased over time.

## VII. CONCLUSIONS

The combination of conventional analysis and Illumina sequencing allowed a comprehensive investigation of the microbial dynamics during ripening of Caciofiore cheese. Despite the differences found among the VCs, the results of this study showed that raw milk was the most affecting factor in the bacterial biota and mycobiota of the cheeses. The experimental cheeses at the end of ripening were comparable to the control cheeses, supporting the potential use of the experimental coagulants from both spontaneous and cultivated Onopordumtauricum Willd for cheese-making.

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