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Amplicon sequence variant–level analysis reveals No stable regional microbial biomarkers in traditional Portuguese PDO cheeses

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ABSTRACT

Protected Designation of Origin (PDO) cheeses are strongly linked to specific geographical areas, where local raw materials, artisanal practices, and microbial communities collectively shape product identity. Metataxonomic-based approaches have been increasingly proposed as tools for food authentication; however, the robustness of taxonomic profiling alone for defining regional biomarkers remains insufficiently tested, particularly in spontaneously fermented cheeses. Here, we conducted a two-year longitudinal analysis of bacterial and fungal communities in Portuguese PDO ewe's milk cheeses from two regions, *Azeitão* and *Nisa*, including four and two producers, respectively, using a metataxonomic analysis to 16 S and ITS.

Alpha diversity analyses revealed no regional differences in bacteria or fungi richness, however some differences at the producer's level were found. Beta diversity highlighted patterns of regional variation in microbial community structures. While permutational analyses supported the idea that producer-specific effects explained a larger proportion of variance than geographical origin. The analysis of differentially abundant amplicon sequence variants (ASVs) identified 36 and 20 bacterial ASVs, and 26 and 4 fungi ASVs, capable of discriminating *Azeitão* and *Nisa* cheeses, respectively. However, further examination showed that these ASVs did not meet the criteria for true regional biomarkers, as their prevalence was uneven across producers within each region.

Our findings suggest that regional identity is not primarily determined by the taxonomic composition of cheese microbiota, but rather by the functional and metabolic activities expressed during ripening. Overall, this study underscores the limitations of taxonomy-based profiling and supports the need for integrated multi-omic approaches to accurately capture PDO-specific signatures.

1. Background

Dairy products, particularly cheeses, are among the most widely consumed foods worldwide. In Europe, the world's largest cheese-consuming region, approximately 9.5 to 9.8 million metric tons were consumed annually between 2024 and 2025 (Cheese Market in Europe—Statistics and Facts),. In Mediterranean countries, traditional cheesemaking is a longstanding practice dating back to Roman times, englobing local heritage and region-specific traditional knowledge (Serrano et al., 2024). Hence, to highlight and safeguard the uniqueness and increase the economic value of these food products, the European Protected Designation of Origin (PDO) status was created (Magliulo et al., 2024; Serrano et al., 2024). According to European Regulation No 1151/2012 (European Parliament, 2012) and Commission Implementing Regulation (EU) No 668/2014 (Commission Implementing Regulation, 2014), PDO labeled products must be produced within a defined geographical region, using traditional manufacturing techniques. In Portugal, 13 cheeses are currently certified under PDO

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scheme. The present study focuses on two producing regions, *Azeitão* and *Nisa*, where cheeses are traditionally manufactured from raw ovine milk, using *Cynara cardunculus* L. as the coagulant agent, and salt. Although those cheeses share similar core ingredients, they differ in some key production parameters: *Azeitão's* cheese is manufactured using milk from non-defined sheep breeds, and undergoes a maturation period of 20 days, whereas *Nisa's* cheeses rely exclusively on the Merina Branca breed sheep and it's cheeses are ripened for around 40 days (eAmbrosia—Queijo de Azeitão; eAmbrosia—Queijo de Nisa).

Cheeses manufactured from raw milk are characterized by rich and heterogeneous microbial ecosystems, which drive biochemical transformations that define physicochemical attributes, sensory qualities, and unique microbial identity (Kamilari et al., 2019). Lactic acid bacteria (LAB) are naturally occurring microorganisms in raw milk and represent core members of the cheese microbiota, as described in previous reports (An et al., 2023; Bintsis, 2018; Coelho et al., 2022; Lorenzo et al., 2018; Zhao et al., 2025). Given the fundamental role of LAB and other microbial groups in PDO cheese production, the study, characterization, and identification of region-specific microbiota are highly relevant. Although conventional culture-dependent methods remain essential for the investigation of microbial populations, they are often labor-intensive and time-consuming, and reliance on selective culture media can limit the detection and recovery of low-abundant microorganisms or those present in a dormant state (Anastasiou et al., 2022). Thus, culture-independent approaches based on molecular techniques have gained interest in microbiome studies. Recent works have demonstrated the potential of metagenomic and metataxonomic approaches for the authentication of food products, such as dairy (Cardin, 2023) or even honey (Sabater et al., 2024). In this context, biomarker selection strategies have been applied to Irish and Eastern Mediterranean cheeses (Kamilari et al., 2022) and Mozzarella di Bufala Campana PDO, using a random forest approach (Magarelli et al., 2024).

In the context of PDO cheese production, the identification of regional biomarkers could complement existing sensory-based certification criteria by detecting ecological shifts associated with panel rejections, while also supporting product authentication. This approach would help protect both consumers and compliant producers by ensuring product consistency and authenticity (reviewed in). To date, metagenomic investigations of Portuguese PDO cheeses remain limited, and, to our knowledge, none have explicitly addressed biomarker identification. Most studies have focused on general metataxonomic profiling and/or cheese overall characterization (Câmara et al., 2019; Cardinali et al., 2022; Coelho et al., 2023; Gonçalves Dos Santos et al., 2017; Gonçalves et al., 2018; Rampanti et al., 2022; Rocha et al., 2021; Salamandane et al., 2024), or comparisons between PDO and non-PDO cheese microbiomes using shotgun metagenomics (Salamandane et al., 2024).

The present study provides a two-year longitudinal characterization of bacterial (16 S rRNA gene) and fungal (ITS) communities in Portuguese PDO ewe's milk cheeses produced in two regions, *Azeitão* and *Nisa*. By integrating taxonomic amplicon profiling, this work aimed to assess the potential of microbial data signatures to: (i) discriminate cheeses according to geographical origin, (ii) support PDO authentication, and (iii) generate robust and transferable lists of region-specific microbial biomarkers. In this context, biomarkers were defined as sequences that differ significantly between regions while remaining stable across production years and independent of individual producer variability. Moving beyond traditional genus-level classifications, which reduce the discriminative power of metataxonomic data, this research introduces a novel framework for biomarker discovery based on amplicon sequence variants (ASVs).

2. Methods

2.1. Sample collection

PDO cheeses from the regions of *Azeitão* (38.5194° N, 9.0138° W) and *Nisa* (39.5180° N, 7.6484° W) were collected between 2021 and 2022. All existing PDO-certified producers were included, comprising four manufactories from *Azeitão* (A1, A2, A3, and A4) and two from *Nisa* (N9 and N10). All cheese samples were collected at the same time point, corresponding to the maximum maturation period defined for each region (20 days in *Azeitão* and 40 days in *Nisa*). Sampling was conducted in 2021 and 2022 and involved obtaining, from each producer, four independent batches, each consisting of four cheeses.

2.2. Sample pre-processing and DNA extraction

Cheeses were sampled (~2 g) for DNA extraction. Composite samples were prepared for each batch. This involved collecting 0.5 g from each of the four cheeses (including both core and rind) and pooling them to represent the entire batch. The remaining cheese material was vacuum-packed and stored at −80 °C for further analysis. Before total DNA extraction, a pre-treatment was performed to remove proteins and lipids, following the methodology adapted from Kuang et al., 2009; Masoud et al., 2012. First, 2 g of cheese were homogenized in 2 mL of 20% (v/v) ethanol and vortexed for 2 min. The suspension was then centrifuged (Hermle Labortechnik Z383 centrifuge, Wehingen, Germany) for 5 min at 5000 rpm and 12 °C. This process was repeated twice. Afterward, the resulting pellet was resuspended in 18 mL of 2% (w/v) sodium citrate (Sigma-Aldrich, Lisbon, Portugal). The suspension was vortexed and incubated at room temperature for 10 min, vortexed for 2 min, and centrifuged for 15 min at 5000 rpm and 12 °C (Hermle Labortechnik Z383, Wehingen, Germany). This cycle was also repeated twice. Subsequently, the pellet was resuspended in 2 mL of TE buffer (10 mM Tris, 1 mM EDTA) supplemented with proteinase K (1 mg/mL, NZYtech, Lisbon, Portugal) and incubated at 55 °C for 2 h in a water bath (Shimaden SR30 Model 3T-21, Japan). Finally, the suspension was centrifuged for 15 min at 12,000 rpm and 4 °C. The resulting pellet was used for DNA extraction with the DNeasy® PowerFood® Microbial Kit (QIAGEN, Maryland, USA), following the manufacturer's instructions. DNA quality and quantification were assessed using a NanoDrop 2000c Spectrophotometer and a Qubit 4 Fluorometer (Thermo Scientific, Massachusetts, USA), according to the manufacturers' protocols. DNA samples were stored at −80 °C.

2.3. Amplicon targeted metagenomics

The extracted DNA was prepared for 16 S rDNA and ITS2 amplification to analyze bacterial and fungal communities, respectively. Sequencing was outsourced to Macrogen Inc (Seoul, Republic of Korea) using the MiSeq 300 bp paired-end platform. For the first PCR, 10 ng of gDNA was amplified in a reaction containing 5× reaction buffer, 1 mM dNTP mix, 500 nM of each primer, and Herculase II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, CA). Cycling conditions were: 95 °C for 3 min, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. The V3–V4 region of the 16 S rDNA was amplified using primers 341 F (CCTACGGGNGGCWGCAG) and 806 R (GGACTACCGGGGTATCT), and the ITS2 region for yeasts using primers ITS3f (GCATCGATGAAGAACGCAGC) and ITS4R (TCCTCCGCTTATTGATATGC). First PCR products were purified using AMPure beads (Agencourt Bioscience, Beverly, MA) and 10 μL were subjected to a second PCR for library construction with Nextera XT Indexed Primers, using the same cycling conditions but with 10 cycles. Purified libraries were quantified with a VICTOR Nivo™ fluorometer (PerkinElmer, Waltham, MA, USA) using PicoGreen reagents and quality-checked on a TapeStation D1000 (Agilent Technologies, Waldbronn, Germany). Libraries were then normalized, pooled, and quantified via qPCR following the KAPA Library Quantification protocol (Roche Sequencing and Life Science, Indianapolis, IN, USA). Sequencing was performed on the MiSeq™ platform (Illumina, San Diego, USA).

2.4. Bioinformatics and statistical analysis

2.4.1. Raw reads processing and amplicon sequence variants (ASVs) inference

Forward and reverse fastq files were processed using the DADA2 pipeline (Callahan, McMurdie, et al., 2016; Callahan, Sankaran, et al., 2016) from *dada2* package (version 1.32.0) in Rstudio version 4.4.0 (R Core Team, 2024). Filtering and trimming were performed according to Callahan et al., (Callahan, Sankaran, et al., 2016; Callahan, McMurdie, et al., 2016). Taxonomic assignment was performed using *dada2*:`assignTaxonomy` function against SILVA database (v138.2) for bacteria and UNITE (v10.0) for fungi (Abarenkov et al., 2024). Metadata, ASV table and corresponding taxonomic classifications have been included as Additional files: 1 – 5.

2.4.2. Data analysis

All data analysis were also performed using Rstudio, a full record of all the analysis performed is included as Additional files 6 and 7, created using *knitr* package in R (Xie, 2014). Data wrangling prior to analysis was conducted using the *tidyverse* R package (Wickham, 2023), all graphical representations, were performed using *ggplot2* (Warnes et al., 2024). Alpha diversity was calculated, using *phyloseq*:`estimate_richness`. Beta diversity, for the study of dissimilarities between samples, based on Weighted and Unweighted UniFrac distance metrics were also calculated also using *phyloseq* and plotted into a Principal Coordinate Analysis (PCoA) using *ggplot2*.

Regional biomarkers were defined as sequences that differ significantly between regions, while remaining stable across production years and independent of individual producer variability, including their consistent occurrence in cheeses from multiple producers within the same region. Biomarkers that did not meet all requirements were considered invalid. Differential abundance analysis was performed using the *DESeq2* package in R (Love et al., 2014). The model accounted for the nested experimental design, including region as the main effect and year of production as a covariate. To handle the overdispersion typical of microbiome count data, *DESeq2* utilized a generalized linear model (GLM) following a negative binomial distribution. Size factors were estimated using the median-of-ratios method to normalize for sequencing depth. Significant differential abundance was determined using the Wald test, with *p*-values adjusted for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) procedure. ASVs were considered differentially abundant if they reached an adjusted *p*-value (p_{adj}) < 0.05 and an absolute log2 fold-change ($|\text{LogFC}|$) > 1.

2.4.3. Statistical analysis

Significant differences between cheese factories for each diversity index were calculated with rstatix (v0.7.2) (Kassambara, 2023). Alpha diversity indices were compared between regions (*Azeitão* vs. *Nisa*) and years (2021 vs. 2022) using the Wilcoxon Rank Sum test. To evaluate changes within individual producers over time (e.g., A1_2021 vs. A1_2022), the Wilcoxon Signed-Rank test was applied to account for the paired nature of the samples. Statistical significance was defined as $\alpha = 0.05$ (statistically significant if *p*-value <0.05). Beta diversity was assessed via Permutational Multivariate Analysis of Variance (PERMANOVA) using the *vegan* package (Oksanen et al., 2024) with 999 permutations.

3. Results and discussion

A total of 6,687,771 raw reads were obtained from 16 S rDNA sequencing (bacteria), and 93,295 raw reads from ITS sequencing (fungi). No agglomeration at a taxonomic rank was performed to maintain the full range of diversity in both communities, therefore all analysis were conducted at the amplicon sequence variant (ASV) level, with the exception of section 3.1. Where the analysis was performed at the genus level in order to characterize the microbial communities in *Azeitão* and *Nisa* cheeses.

3.1. Microbial profiling

The characterization of the microbial communities was performed at the genus for both bacterial and fungi communities. The relative abundancy plots of bacteria and fungi are shown in Fig. 1A and B, respectively.

Cheeses from *Azeitão* and *Nisa* were dominated by lactic acid bacteria. The core bacterial genera shared by both regions were *Lactococcus* and *Leuconostoc*, except for N9, where *Lactococcus* maintained a dominant and stable presence (55–59 %) regardless of the year (Fig. 1A). Other genera contributed significantly to individual producer profiles. *Hafnia* reached up to 41 % in A2, and *Brochothrix*, only abundant in A3 during both sampling periods (16–20 %). *Enterococcus* presence was characteristic of the *Azeitão* producers, where it appeared sporadically in 2021, and was absent in *Nisa*. In contrast, *Nisa* cheeses displayed a broader variety of bacteria, including *Lacticaseibacillus* (up to 13 % in N9), *Pseudomonas*, and *Lactiplantibacillus*. The sporadic presence of *Pseudomonas* and *Acinetobacter* in certain years, suggests that although *Lactococcus* forms a stable core bacterium, these communities are strongly influenced by each facility's environmental and temporal factors. In contrast to the bacteria, the fungal communities exhibited more pronounced differences between regions and individual producers. *Yarrowia* and *Geotrichum* emerged as the core taxa across both PDO cheeses (Fig. 1B). Producer-specific signatures were evident in *Azeitão*: A1 was dominated by *Yarrowia* (66–83%), while A2 was dominated by *Geotrichum* (66–77%). Producers A3 and A4 showed a more complex assembly, including high proportions of *Kurtzmaniella* (up to 54%) alongside the core fungi. In *Nisa*, the genera distribution was particularly dynamic. Producer N9 featured a highly diverse profile in 2021, including *Wickerhamiella* (21%), *Debaryomyces* (18%), and *Cutaneotrichosporon* (4%), before shifting to a *Geotrichum*-dominated profile (60%) in 2022. Similarly, N10 maintained a consistent presence of *Geotrichum* (51–56%) and *Debaryomyces* (11–15%), with additional presence of *Candida* and, in 2022, *Lecanicillium* (9%). These results indicate that while *Yarrowia* and *Geotrichum* form a widespread fungal core, the secondary fungal mycobiota is highly specific to each producer and sensitive to annual variations.

Metataxonomic studies of other Portuguese PDO cheeses made from raw ewe's milk and coagulated with *C. cardunculus* L.—specifically *Serpa*, *Beira Baixa*, and *Serra da Estrela*—reported microbial profiles largely consistent with those observed in cheeses from *Azeitão* and *Nisa* in the present study. For example, *Serpa* PDO cheese was found to have a prevalence of *Lactococcus*, followed by *Leuconostoc* and *Lactobacillus* in both winter and spring, mirroring the patterns observed in *Azeitão* and *Nisa* (Gonçalves et al., 2018). Similarly, 16 S rRNA sequencing of *Beira Baixa* (*Castelo Branco*) and *Serra da Estrela* cheeses showed a core microbiota dominated by LAB. In *Beira Baixa* cheese, *Lactococcus lactis* predominated across three different producers, with lower abundances of *Lactiplantibacillus plantarum* and *Loigolactobacillus coryniformis* detected in all samples (Cardinali et al., 2022). Cardinali et al. (Cardinali et al., 2022) also reported certain genera served as distinguishing factors between producers; for example, *Lactococcus piscium* was unique to producer 1 and *Streptococcus thermophilus* to producer 2, while *Lacticaseibacillus zeae* varied in abundance between sites. *Serra da Estrela* cheese exhibited a microbial composition closely aligned with our findings, with *Leuconostoc mesenteroides* and *Lactococcus lactis* as the primary constituents (Rocha et al., 2021).

The studies on the fungi mycobiota of Portuguese traditional cheeses are not as extensive as for bacteria; however, some studies have been

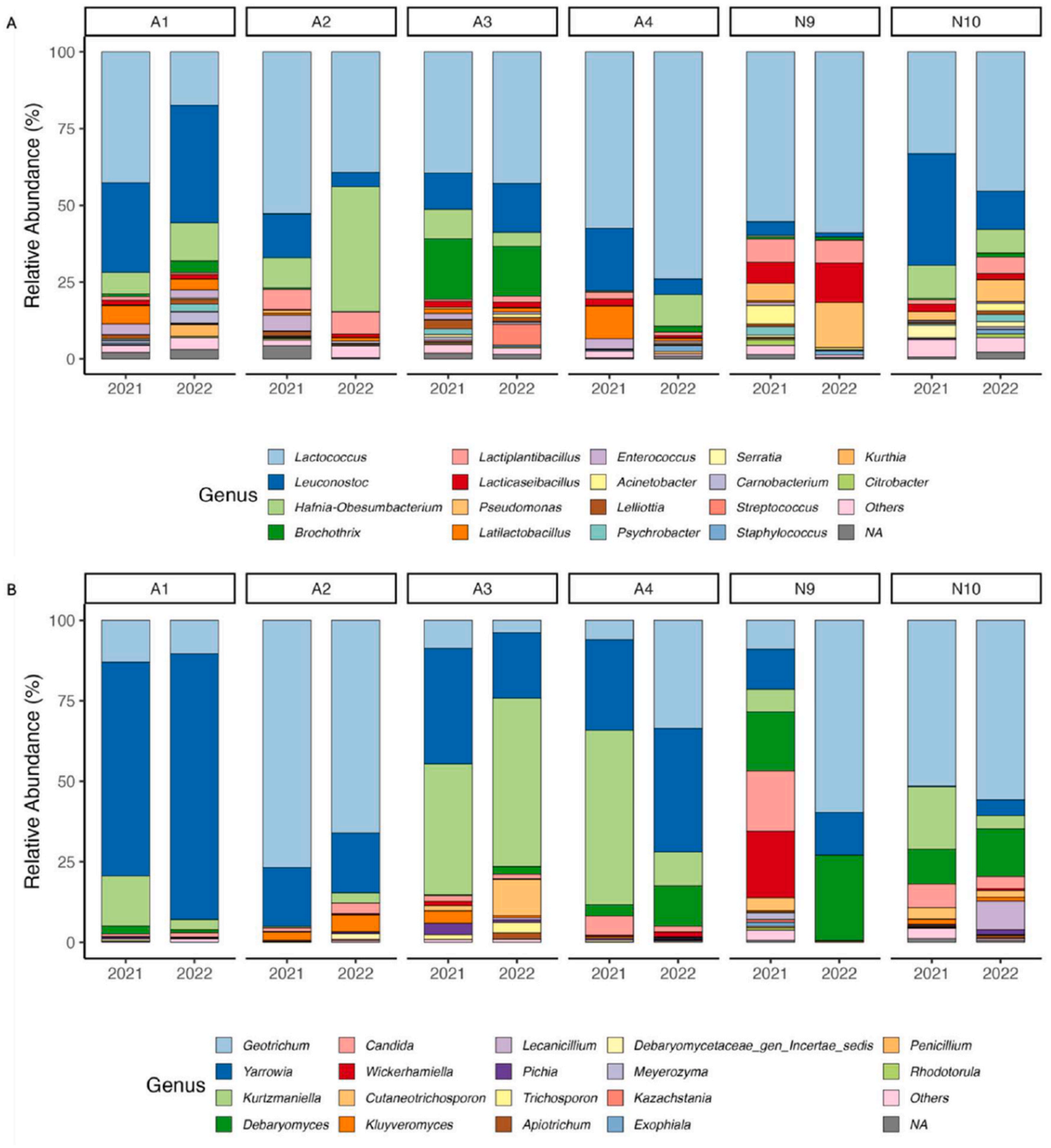

Fig. 1. – Relative abundance (%) of the top 19 bacteria (A) and fungi (B) ASVs identified at the genus level in PDO cheese from *Azeitão* (A1-A4) and *Nisa* (N9 and N10) between 2021 and 2022.

performed to *Serpa*, *Beira Baixa – Castelo Branco* and *Serra da Estrela* cheeses. With regards to *Serpa* cheese, among the three PDO cheese samples tested, the authors identified six different genera, *Debaryomyces*, *Kluyveromyces, Pichia, Candida, Cryptococcus* and *Magnusiomyces*. Among these, the authors detail that most of the PDO cheeses were dominated by *D. hansenii* and *Kluyveromyces* spp. (Gonçalves Dos Santos et al., 2017). In the *Beira Baixa – Castelo Branco* cheese, the following genera were identified *Geometricum, Cladosporium, Candida, Pichia, Proteomyces, Debaryomyces, Ogataea, Ustilago, Starmerella* and *Penicillium* among the three producers studied (Cardinali et al., 2022). In *Serra da Estrela* cheeses there was a predominance of the genera *Candida* and *Debaryomyces* (Rocha et al., 2021).

Together, these findings support the existence of a conserved core of microorganisms (both bacteria and fungi) across Portuguese PDO ewe's milk cheeses. This shared microbial structure is likely shaped by common raw materials, the use of *C. cardunculus* L., and similar ripening conditions, which may outweigh geographical distance in determining microbial signatures.

3.2. Alpha diversity within cheese samples from Azeitão and Nisa

Alpha diversity analysis was performed to the ASVs, independently to its taxonomic rank, at the region level, combining producers and years of production. The following diversity indices were used: Shannon, Chao1 and Inverse Simpson. Chao1 is a richness estimator, it accounts for rare/unique taxa among samples; Shannon analyses richness and evenness, aside from taking into consideration unique taxa, it combines the proportion of abundance of that rare taxa divided by the full number of individuals in that sample; and Inverse Simpson studies the dominance and evenness, it accounts for unique taxa as well as their relative abundance, in addition it has a higher sensitivity to changes in the abundance of dominant taxa than rare. These metrics were applied separately to bacterial and fungi ASVs.

For bacteria, no significant differences were observed between *Azeitão* and *Nisa* for any of the tested metrics (Fig. 2A). This indicates that the overall complexity and richness of the bacteria community remain consistent across the geographical regions.

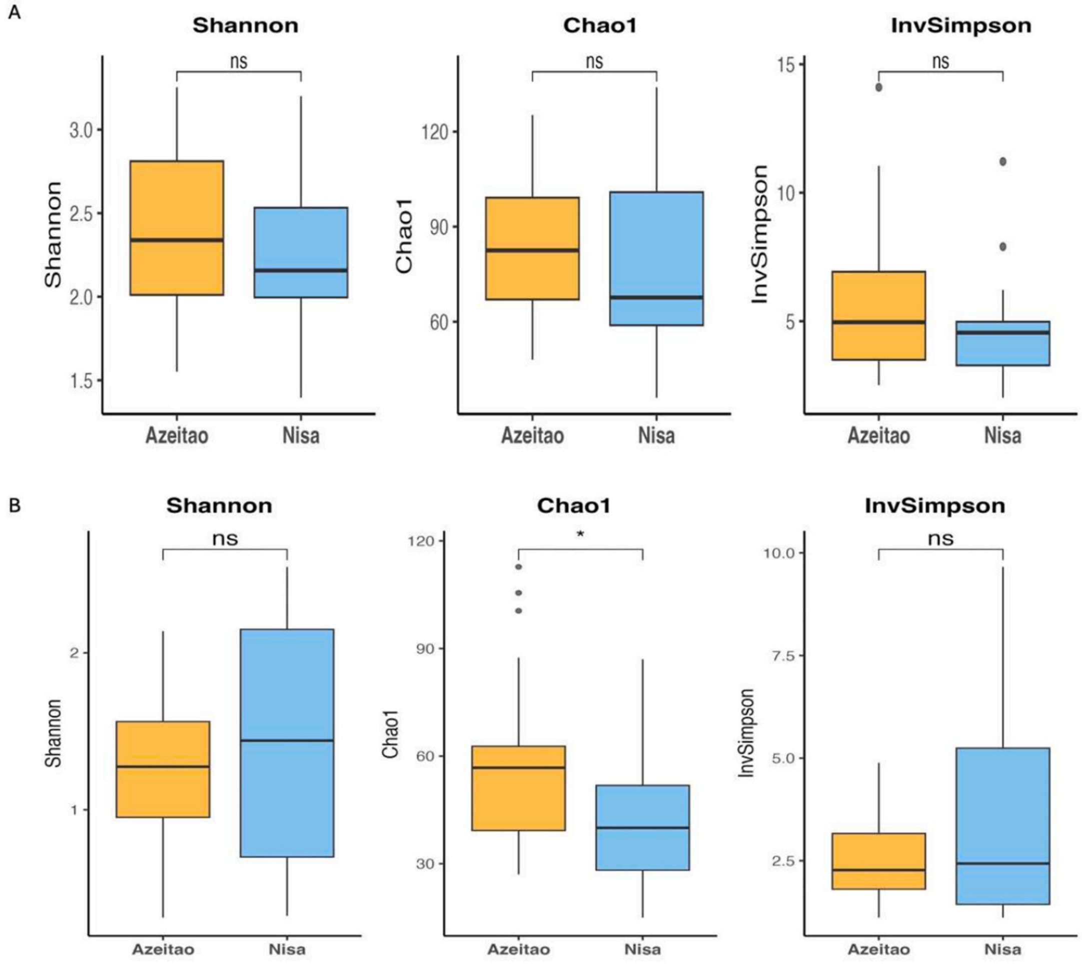

Fig. 2. – Alpha diversity of microbial communities in Portuguese PDO cheeses from *Azeitão* and *Nisa.* Diversity indices Shannon, Chao1 and Inverse Sympson for the bacterial (A) and fungi (B) communities. Boxplots show median, interquartile range, and dispersion across samples from each region. N = X *Azeitão*, n = Y *Nisa*. * Statistical differences *p*-value <0.05; ns – non-significant.

For fungal communities, in the Shannon ($p = 0.477$) and Inverse Simpson ($p = 0.424$) indices no significant differences were observed between regions (Fig. 2B). In contrast, in Chao1 richness differed significantly between *Azeitão* and *Nisa* ($p = 0.007$), with higher fungal richness observed in *Azeitão*. These results show that the core fungi are stable throughout regions, even if there is a higher richness in unique taxa in *Azeitão*.

A statistical analysis to the years of production, as well as producers, was also performed. In the bacterial communities between years of production, no statistic differences were found in any of the regions. Producers were also assessed regarding differences between them across years, and statistical differences were found in two producers, A2 ($p = 0.03$) and A3 ($p = 0.03$) in *Azeitão*, for Chao1. As for *Nisa*, less statistical differences were found, only in N9 for Chao1 ($p = 0.03$) and Inverse Simpson ($p = 0.03$).

In the fungi community the same analysis was performed, no statistical differences were observed across years in *Azeitão*, however, for the Chao1 index in *Nisa,* significant differences were found ($p = 0.04$). Moreover, producers were also compared between years, and no statistical differences were observed, aside from N9 in Chao1 index ($p = 0.03$).

3.3. Beta diversity

Principal Coordinate Analysis (PCoA) was used to visualize bacterial and fungal community structure at the regional level, aggregated by producer and production year. Under weighted UniFrac, the first two axes explained 76.4% and 64% of the total variance for bacteria and fungi, respectively. Unweighted UniFrac captured substantially less variance (approximately 29% for both communities). As the weighted metric — which accounts for relative taxon abundance — explained a considerably higher proportion of total variance than the presence/absence-based unweighted metric, only weighted UniFrac ordinations were retained for further analysis.

The bacterial community ordination revealed a high similarity between the two regions (Fig. 3). Samples from *Azeitão* and *Nisa* overlapped substantially, and the proximity of group centroids together with the overlapping 95% confidence ellipses suggest a largely conserved bacterial composition across PDO labeled cheeses.

A Permutational Multivariate Analysis of Variance (PERMANOVA) was performed. When accounting for the nested structure of the dataset (producers nested within region), the model explained a significant proportion of the total bacterial variance ($R^2 = 0.46$, $p = 0.001$). This confirms that while regionality plays a role, the microbial identity of these PDO cheeses is strongly dictated by the specific “producer effect”, englobing the unique environmental conditions, in-house microbiota and handling practices of individual producers. The influence of temporal factors was also evaluated to determine the stability of these microbial profiles. A small yet statistically significant interaction was detected between region and production year ($R^2 = 0.14$, $p = 0.015$), which may indicate that the two regions may respond differently to inter-annual environmental fluctuations.

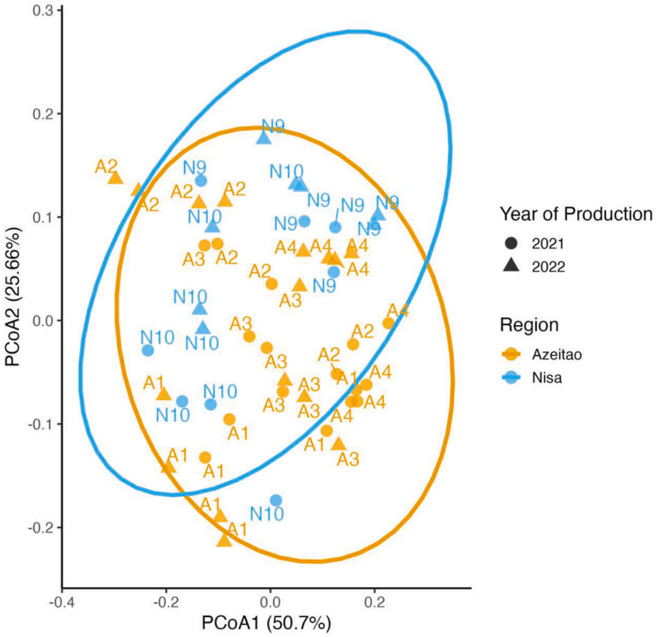

Fig. 3. Principal Coordinates Analysis (PCoA) of bacterial community composition in PDO cheeses using weighted unifrac. Points represent producers from *Azeitão* (yellow, A1 – A4) and *Nisa* (blue, N9 – N10). The axes indicate the percentage of total variation explained by the first two principal coordinates (PCoA1: 50.7 %; PCoA2: 25.66 %). Ellipses represent 95% confidence intervals around each regional group, illustrating differences in community structure between *Azeitão* and *Nisa* cheeses.

The fungal community ordination (Fig. 4) revealed complete regional overlay, it is possible to see that within the *Nisa* samples there is a high variability, that is mostly explained along the PCoA2 axis, whereas in *Azeitão* there is bigger dispersion of producers along PCoA1. Overlapping 95% confidence ellipses indicates shared core fungal mycobiota, yet distinct centroid positions support a regional effect. The nested PERMANOVA model confirmed producer identity as the main determinant of fungal composition ($R^2 = 0.415$, $p = 0.001$). Region × year interaction was small but also statistically significant ($R^2 = 0.137$, $p = 0.008$), suggesting region-specific temporal shifts.

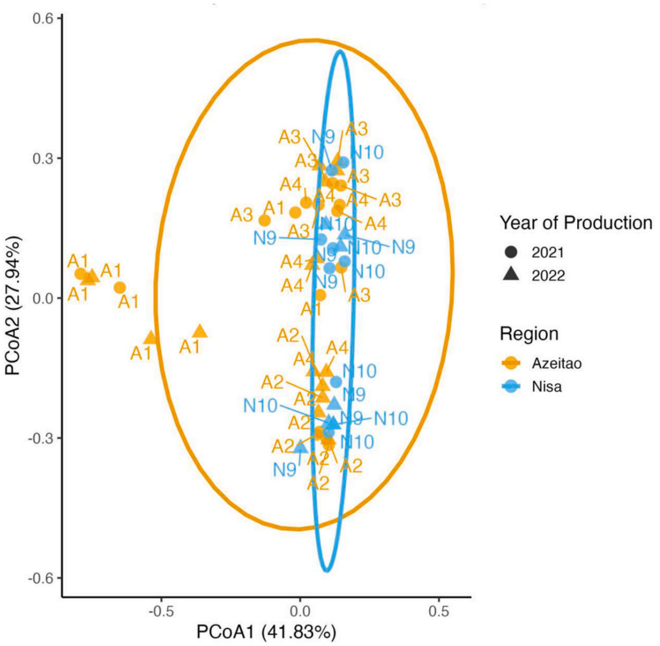

Fig. 4. Principal Coordinates Analysis (PCoA) of fungal community composition in PDO cheeses using weighted unifrac. Points represent producers from *Azeitão* (yellow, A1 – A4) and *Nisa* (blue, N9 – N10). The axes indicate the percentage of total variation explained by the first two principal coordinates (PCoA1: 41.83 %; PCoA2: 27.94%). Ellipses represent 95% confidence intervals around each regional group, illustrating differences in community structure between *Azeitão* and *Nisa* cheeses.

Overall, both bacterial and fungal communities were primarily influenced by the ‘producer effect’, reflecting stable, producer-specific microbiota across years. However, significant region × year interactions indicate that regional signatures fluctuate between years, complicating the identification of consistent region-specific biomarkers.

3.4. *Identification of microbial biomarkers*

3.4.1. *Selection of differently abundant ASVs*

To identify robust regional biomarkers, a differential abundance analysis was performed, using region as the main explanatory variable. Given the significance of inter-annual fluctuations observed in beta diversity (section 3.3), production year was incorporated as a fixed effect to control temporal confounding variance. This analytical framework was applied to both bacterial and fungal communities.

Volcano plots (Fig. 5A B) show the differentially expressed ASVs for bacteria and fungi, respectively. For bacteria, over 36 ASV were selected for *Azeitão* and 21 ASV for *Nisa* (Additional files 8 and 9). An asymmetrical distribution of differentially abundant ASVs (Fig. 5A), was observed, with *Azeitão* displaying a broader set of region-specific features as compared to *Nisa*. Among the identified features, ASV_13, ASV_34, ASV_37, ASV_49, ASV_59, ASV_62, ASV_100, ASV_126, ASV_130, ASV_143 and ASV_156 were selected as the most discriminative for *Azeitão*, characterized by the highest effect sizes (log2 fold-change) and strongest statistical significance ($p_{adj} < 0.05$). Conversely, for *Nisa*, ASV_38, ASV_63, ASV_64, ASV_71 and ASV_77 emerged as the primary taxa with the highest discriminative power.

Fungal communities exhibited fewer differentially abundant ASVs overall, with 26 ASVs enriched in *Azeitão* and 4 in *Nisa* (Additional files 10 and 11). As shown in Fig. 5B, a more profound asymmetrical distribution to that observed in bacteria, again indicating clear regional differentiation. Consistent with the bacterial patterns, *Azeitão* exhibited a more diverse collection of regional fungi ASVs. Among the identified features, ASV_20, ASV_21, ASV_27, ASV_29, ASV_36, ASV_38, ASV_40, ASV_43, ASV_44, ASV_52, ASV_56, ASV_65 and ASV_67 emerged as the most discriminative taxa for *Azeitão*. For *Nisa*, ASV_8 and ASV_35 represented the primary region-specific biomarkers.

The taxonomic profile of the discriminative ASVs suggests a clear distinction in community complexity between the two regions (Table 1). In *Azeitão*, the ASVs represent a broader taxonomic spectrum of LAB. The high log2 fold-change of ASV_13 (*Lactiplantibacillus*), ASV_49 and ASV_143 (*Weissella*), and ASV_34/37 (*Lactococcus*) highlights a robust community responsible for both primary fermentation and early-stage flavor development (Yadav & Shukla, 2017). The inclusion of ASV_59/62 (*Brochothrix*) and ASV_126 (*Carnobacterium*), both spoilage bacteria, as significant biomarkers in *Azeitão* further suggests a more diverse initial microbiota that persists during its shorter ripening cycle. In contrast, the biomarkers for *Nisa* are more specialized. The presence of ASV_38 (*Lactiplantibacillus*) and a strong cluster of *Lacticaseibacillus* (ASV_63, 64, and 77) indicates a transition toward a more mature, acid-tolerant microbial population. Interestingly, ASV_71 (*Lactococcus*) also appears as a significant biomarker for *Nisa*.

These differences likely reflect the distinct ripening periods of the two cheese-products. The shorter maturation of *Azeitão* (approx. 20 days) allows for the detection of a wide array of primary fermenters and environmental bacteria like *Weissella* and *Brochothrix*. In contrast, *Nisa's* prolonged ripening (up to 40 days) creates a selective environment that favors late-stage Non-Starter Lactic Acid Bacteria (NSLAB), specifically *Lacticaseibacillus* species, which are better adapted to the lower pH and

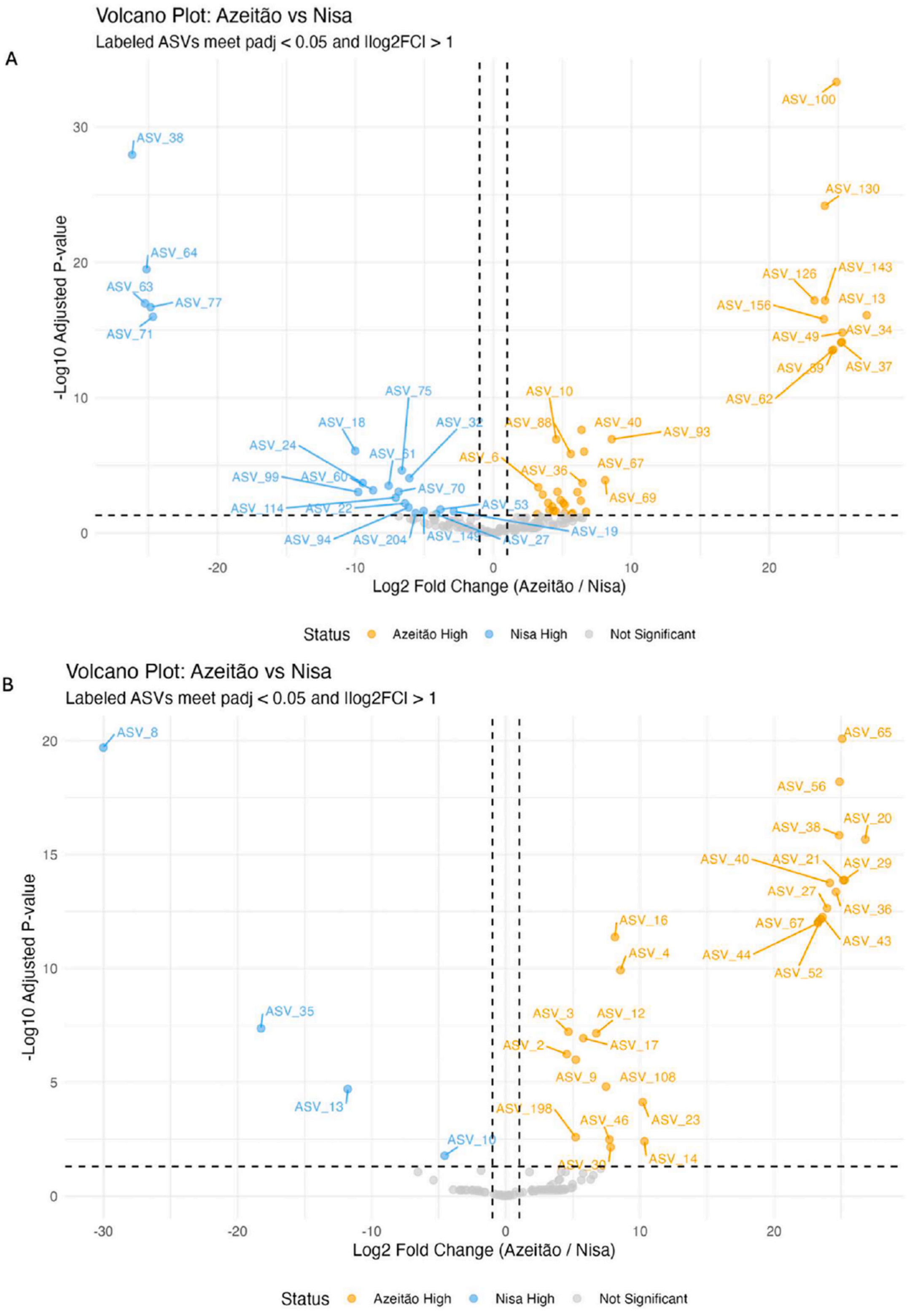

Fig. 5. Differential abundance of bacteria (A) and fungi (B) ASVs between *Azeitão* and *Nisa* regions. The volcano plot displays the relationship between the magnitude of change (log2 fold-change, x-axis) and statistical significance (-log10 adjusted p-value, y-axis). Each point represents a unique ASV. Points colored in yellow represent taxa significantly enriched in *Azeitão*, while those in blue indicate enrichment in *Nisa* ($p_{adj} < 0.05$ and $|LFC| > 1$). Gray points represent ASVs with non-significant regional differences.

moisture levels of aging curd (Renes et al., 2021).

Regarding the fungal community, *Azeitão* was characterized by a diverse assembly of ASVs associated with active surface ripening and early-stage proteolysis (Table 2). The presence of ASV_29 (*Geotrichum candidum*) remained a dominant species; however, it is joined by several yeast from the order Saccharomycetales, including ASV_66 (*Kluyveromyces marxianus*), ASV_56 (*K. lactis*), and ASV_20/44 (*Kurtzmaniella*). These taxa play key roles in lactate metabolism and curd deacidification,

Table 1
Taxonomic identification of discriminative ASVs in the bacterial communities from the regions of *Azeitão* and *Nisa*. FC – *fold change;* p_{adj} – adjusted *p*-value.

Azeitão			
ASV_ID	FC	p_{adj}	Genus/Species
ASV_13	27,05	7,98E-17	*Lactiplantibacillus*
ASV_49	25,29	1,55E-15	*Weissella oryzae*
ASV_34	25,26	7,53E-15	*Lactococcus*
ASV_37	25,18	8,47E-15	*Lactococcus*
ASV_100	24,86	4,63E-34	*Vagococcus*
ASV_59	24,64	2,77E-14	*Brochothrix*
ASV_62	24,54	3,34E-14	*Brochothrix*
ASV_143	24,03	6,56E-18	*Weissella*
ASV_130	24,01	6,49E-25	*Enterococcus*
ASV_156	23,95	1,56E-16	*Latilactobacillus*
ASV_126	23,27	6,46E-18	*Carnobacterium*
Nisa			
ASV_38	−26,18	1,08E-28	*Lactiplantibacillus*
ASV_63	−25,25	1,06E-17	*Lacticaseibacillus*
ASV_64	−25,15	3,23E-20	*Lacticaseibacillus*
ASV_77	−24,84	2,02E-17	*Lacticaseibacillus*
ASV_71	−24,67	1,03E-16	*Lactococcus*

creating conditions that facilities secondary ripening (Fröhlich-Wyder et al., 2019). The detection of ASV_67 (*Mucor*) and ASV_43 (*Penicillium paneum*) in *Azeitão* indicates a more complex, multi-kingdom surface colonization typical of young, softer cheeses. In contrast, the discriminative fungi for *Nisa* were significantly more restricted, dominated by ASV_8 (*Yarrowia lipolytica*) and ASV_35 (*Penicillium paneum*). The high negative log2 fold-change of ASV_8 (−30.00) is particularly significant. *Y. lipolytica* is known for its potent lipolytic and proteolytic activities, which likely influence the development of the intense organoleptic profile associated with *Nisa*'s cheeses. Interestingly, while *Penicillium paneum* appears in both regions, its specific association with *Nisa* (ASV_35) compared to *Azeitão* (ASV_43) may suggest strain-level selection driven by the different environmental conditions of the two production sites.

The differentially abundant ASVs reveal distinct multi-kingdom successional pattern associated to the maturation timelines of *Azeitão* and *Nisa* cheeses. *Azeitão*'s microbial signature is characterized by a high diversity of both bacteria and fungi associated with early fermentation, rind development, and environmental colonization. The co-occurrence of ASV_34/37 (*Lactococcus*) and ASV_29 (*Geotrichum candidum*), alongside environmental taxa such as ASV_49/143 (*Weissella*) and ASV_67 (Mucor) indicates a dynamic primary ripening stage in which lactic acid bacteria rapidly acidify the curd while fungi drive surface deacidification (Fröhlich-Wyder et al., 2019). Conversely, the *Nisa* ASVs suggest a more specialized ecosystem adapted to prolonged maturation. The selection of NSLAB, most notably ASV_63, 64, and 77 (*Lacticaseibacillus*), alongside the potent lipolytic yeast ASV_8 (*Y. lipolytica*), aligns with the physiological demands of a 40-day maturation cycle. While *Azeitão* retains a broad community of primary fermenters and diverse surface yeasts like *Kluyveromyces* (ASV_65/57) and *Kurtzmaniella* (ASV_20/44), the *Nisa* profile shifts toward a ripening-specialist community.

Overall, these patterns underscore the technological influence of ripening time: short-ripened *Azeitão* supports a diverse and heterogeneous early microbiota, whereas extended aging in *Nisa* selects for specialized taxa that drive late-stage biochemical transformations and define its distinct organoleptic identity.

3.4.2. Verification of selected ASVs as possible regional biomarkers

To identify robust regional biomarkers, candidate ASVs were filtered based on three stringent criteria: (i) consistent detection across multiple production years, (ii) presence across all producers within a specific region and (iii) stability over time. ASVs that failed to meet all requirements were excluded from further analysis. The resulting candidates were classified either as prevalence-based (unique presence/absence pattern) or abundance-based (higher read counts in one region). Applying these filters ensured that the selected ASVs represent stable microbial signatures of the PDO regions rather than inter annual or producer-specific fluctuations.

The filtration and analysis of the top-performing ASVs, revealed a highly fragmented microbial distribution. For both bacteria and fungi, statistical significance observed in the DESeq2 analysis appears to be driven by producer exclusivity and localized occurrences rather than a consistent, region-wide core microbiota.

In the bacterial community, the *Azeitão* biomarkers exhibited significant producer and temporal bias. ASV_13 and ASV_49 were exclusively associated with producer A2 across both sampling years, whereas ASV_34 and ASV_37 were restricted to A2 only during 2022. Similarly, ASV_59 and ASV_62 were specific to producer A3 in 2022, while ASV_156 was unique to producer A4 in 2021. Other taxa, such as ASV_100, ASV_130, ASV_143, were found to be specific to producers A1 and A2, respectively, though at lower abundance. The high *fold-changes* reported for these taxa resulted from their total absence across all *Nisa* samples. A similar pattern was observed in *Nisa*, where only ASV_38 showed consistency across years, yet remained specific to producer N9. The remaining *Nisa* biomarkers were detected only sporadically in certain replicates, primarily from N9, with ASV_71 being the exception

Table 2
Taxonomic identification of discriminative ASVs in the fungi communities from the regions of *Azeitão* and *Nisa*. FC – *fold change,* p_{adj} – adjusted *p*-value.

Azeitão					
ASV_ID	FC	p_{adj}	Order	Family	Genus/Species
ASV_20	26,79	1,09E-16	Saccharomycetales	Debaryomycetaceae	*Kurtzmaniella*
ASV_29	25,25	6,79E-15	Saccharomycetales	Dipodascaceae	*Geotrichum candidum*
ASV_21	25,16	6,79E-15	Saccharomycetales	Debaryomycetaceae	
ASV_65	25,08	4,22E-21	Saccharomycetales	Saccharomycetaceae	*Kluyveromyces marxianus*
ASV_56	24,87	3,26E-19	Saccharomycetales	Saccharomycetaceae	*Kluyveromyces lactis*
ASV_38	24,84	7,28E-17	Saccharomycetales	Dipodascaceae	*Yarrowia alimentaria*
ASV_36	24,61	2,23E-14	Saccharomycetales	Debaryomycetaceae	
ASV_40	24,15	8,92E-15	Saccharomycetales	Saccharomycetaceae	*Kazachstania unispora*
ASV_27	23,94	1,16E-13	Trichosporonales	Trichosporonaceae	*Trichosporon ovoides*
ASV_43	23,60	2,85E-13	Eurotiales	Aspergillaceae	*Penicillium paneum*
ASV_52	23,48	3,56E-13	Saccharomycetales		
ASV_67	23,32	4,29E-13	Mucorales	Mucoraceae	*Mucor*
ASV_44	23,26	5,25E-13	Saccharomycetales	Debaryomycetaceae	*Kurtzmaniella*
Nisa					
ASV_ID	FC	padj	Order	Family	Genus /Species
ASV_8	−30,00	1,02E-20	Saccharomycetales	Dipodascaceae	*Yarrowia lipolytica*
ASV_35	−18,25	2,19E-08	Eurotiales	Aspergillaceae	*Penicillium paneum*

as it was found in most replicates from N10.

The fungal ASV analysis mirrors this fragmentation, as none of the 13 selected fungal taxa met the criteria for a universal regional biomarker. Their presence was largely confined to specific production sites or years: ASV_20, ASV_21, and ASV_40 were exclusive to producer A2, while ASV_38 was unique to A1. Temporal restrictions were also evident, with ASV_27 (A2), ASV_52 (A4), and ASV_56 (A2) appearing only in the 2022 sampling period. As with the bacteria, these fungi biomarkers achieved high significance because they were completely absent from the *Nisa* region. In *Nisa*, the primary biomarkers ASV_8 and ASV_35 were identified only in specific replicates from producer N9 and were entirely absent from producer N10 and the *Azeitão* region.

Ultimately, these results suggest that the identified biomarkers represent sporadic, localized drivers of diversity rather than a stable, region-wide biological signature. These likely reflect individual producer practices, specific in-house microbiota, or inter-annual environmental fluctuations rather than a standardized regional microbial identity for either *Azeitão* or *Nisa* cheeses. Therefore, the identification of prevalence-based biomarkers appears to be impossible, and a search for abundance-based biomarkers was pursued by selecting ASVs with lower *fold-changes*.

For bacteria in *Azeitão*, ASV_6 (*Brochothrix thermosphacta*) and ASV_10 (*Latilactobacillus*) were identified as possible abundance-based biomarkers. ASV_6 (FC = 3.2, $p_{adj} = 4.21 \times 10^{-4}$) is present in both regions but shows significantly higher read counts in producer A3, while in producers A1, A2, and A4, the counts are lower and mirror those found in *Nisa*'s producer N9. Similarly, ASV_10 (FC = 4.5, $p_{adj} = 1.21 \times 10^{-7}$) was detected across all *Azeitão* producers and in producer N10 from *Nisa*; it maintained high read counts in A1 and A4 but appeared in lower counts in A2 and A3, where the levels were often similar to those found in specific *Nisa* replicates.

Regarding fungi, ASV_2 (*Kurtzmaniella*), ASV_3 (*Yarrowia lipolytica*), and ASV_6 (*Candida parapilosis*) were selected as potential biomarkers for *Azeitão*. ASV_2 (FC = 4.5, $p_{adj} = 2.99 \text{ x } 10^{-7}$) is widely present across all *Azeitão* producers, despite some inter-annual differences in A1 and A2, whereas its appearance in *Nisa* is more sporadic and absent in several replicates. However, when ASV_2 is present in *Nisa*, the read counts are high and comparable to those in A3 and A4. ASV_3 (FC = 4.7, $p_{adj} = 3.09 \text{ x } 10^{-8}$) follows a similar trend, showing very high read counts in all *Azeitão* producers but also appearing at high levels in certain *Nisa* replicates. Finally, ASV_6 (FC = 1.76, $p_{adj} = 4.48 \text{ x } 10^{-2}$) exhibited the lowest abundance and *fold-change* among these candidates; its presence in *Nisa*, particularly in producer N10, reached levels similar to those in *Azeitão,* which accounts for the lower fold-change and higher adjusted *p*-value.

Identifying robust regional biomarkers for PDO *Azeitão* and *Nisa* cheeses proved highly challenging. The spontaneous fermentation and inherent variability across years and producers created microbial profiles dominated by producer rather than region-specific signatures. Despite efforts to identify prevalence-based biomarkers, the most discriminative ASVs consistently failed to meet the criteria for region-wide stability.

Even when focusing on abundance-based biomarkers, no viable candidates emerged for *Nisa*, and the apparent candidates for *Azeitão* displayed substantial overlap with *Nisa* in terms of presence and abundance. As such, none of the selected ASVs can be confidently assigned as definitive regional biomarker.

Given the inherent diversity associated with the producer, the next logical step toward identifying possible biomarkers could be to combine metataxonomic data with other “omics” approaches, such as volatilomics, metabolomics, or even functional metagenomics. Studies combining volatile compound content and metataxonomic data have been performed on other Portuguese PDO cheeses, such as *Beira Baixa – Castelo Branco* cheese. The authors reported a very diverse microbial content but very similar dominant chemical classes (carboxylic acids, esters, carbonyls, and alcohols). Moreover, they suggested that this link between metataxonomic information and volatile profiling could provide a clearer picture of cheese identity and, therefore, contribute to its authenticity (Cardinali et al., 2022).

In *Serra da Estrela* PDO cheese, amino acid profiling was used as a tool to identify different producers within the region, with a classification accuracy of 96%, showing that this method could potentially be applied to select regional biomarkers (Reis Lima et al., 2019); however, further testing is required since no testing was performed with cheeses from different PDO producing regions to assess if these distinct amino acid profiles are found and maintained throughout production years. Furthermore, also within the *Serra da Estrela* PDO cheeses, Salamandane et al., 2024 successfully distinguished PDO from non-PDO cheeses using a shotgun metagenomic approach, basing the distinction on functional gene. Analogous to studies conducted on Portuguese traditional cheeses, multi-omics work on Mozzarella di Bufala Campana PDO using metagenomics and volatile compound profiling was able to establish a link between geographical origin, microbiome structure, and associated metabolic outputs (Mangliulo et al., 2024).

These studies have shown that exploring other omics might aid in the selection of regional biomarkers that remain stable across years and producers. Thus, for the selection of biomarkers should focus on the byproducts of bacterial metabolism and combine this information with metataxonomic data.

4. Conclusion

This study presents the first ASV-level investigation aimed at identifying microbiological biomarkers capable of distinguishing traditional Portuguese PDO cheeses by geographical origin. Unlike previous cheese microbiome studies, which typically rely on genus- or species-level taxonomic assignments, we applied a taxonomy-independent, ASV-based analytical framework. To our knowledge, this approach has not previously been used in cheese research for biomarker discovery and provides the highest resolution achievable with amplicon sequencing.

Across two production years and all certified producers from *Azeitão* and *Nisa*, our results consistently show that producer-specific microbial signatures dominate over regional patterns, regardless of microbial kingdom. While statistical analyses of beta diversity detected measurable regional shifts, the ASVs driving these differences were not shared across all producers or years. Both bacteria and fungi ASVs exhibited strong producer-level and inter-annual variability, preventing any ASV from meeting the strict criteria required to function as a stable, region-wide biomarker.

These findings indicate that, in spontaneous fermentation systems such as these PDO cheeses, regional identity is not encoded in the consistent presence or abundance of specific microbial taxa, even at the ASV level. Instead, typicity may emerge from functional and metabolic activities (“what microbes do”) rather than taxonomic composition (“who is there”). Consequently, metataxonomic data alone are insufficient to capture the biological basis of Portuguese PDO regionality.

By explicitly testing the limits of taxonomy-based authentication, this study introduces a novel high-resolution ASV framework and highlights its constraints. Future authentication efforts will likely require integrated multi-omic approaches, combining metagenomics, metabolomics, proteomics, or volatilomics, to capture the biochemical processes that ultimately drive ripening, sensory profiles, and the distinctive identity of traditional Portuguese PDO cheeses.

CRediT authorship contribution statement

Susana Serrano: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Catarina Araújo:** Writing – original draft, Investigation. **Francisco Nascimento:** Writing – review & editing, Formal analysis, Data curation. **Eugenio Parente:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Lisete Sousa:**

Writing – review & editing, Validation, Methodology, Data curation. **Maria Teresa Barreto-Crespo:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Teresa Semedo-Lemsaddek:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Declarations

4.1. Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of supporting data

The raw read data of amplicon sequencing fastQ have been deposited in NCBI SRA under BioProject PRJNA1430741 and BioSamples with the accession numbers SAMN56290959 to SAMN56291054. Metadata, ASV tables and taxonomic classifications have all been included as Additional files 1 – 5. A full record of the data analysis is also included in Additional file 6.

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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.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2026.109191.

Data availability

FastaQ files have been deposited in SRA-NCBI. Scripts have been added as supplementary files.

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