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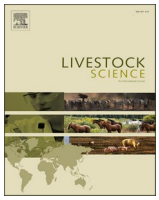

Short communication: Comparing the microbiota diversity from the core, middle part and rind of six Spanish commercial goat cheeses

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HIGHLIGHTS

- Cheese core and rind provide different ecosystems for microbial growth.
- We have sequenced the microbiome of the core, middle part, and rind from 6 Spanish cheeses.
- Lactic acid bacteria were predominant in the cheese microbiome.
- We detected psychrophilic taxa often associated with post-pasteurization contamination.
- The magnitude of microbial diversity in the core, middle part and rind was very similar.

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ABSTRACT

The cheese core has a lower oxygen saturation and salinity and a higher acidity than the rind, but there is controversy about the incidence of such factors on the magnitude of microbial diversity. The goal of the current work was to investigate the existence of differences in α-diversity between the core, middle part, and rind of six Spanish commercial cheeses through a sequencing approach. To this end, we have collected rind, middle part, and core samples from fresh (H and M), soft semi-ripened (C and P), hard semi-ripened (B) and semi-hard aged (G) goat cheeses. After purifying deoxyribonucleic acid from these 18 samples, the V3-V4 ultravariable region of the 16S rRNA gene was sequenced. The analysis of microbial composition revealed that lactic acid bacteria from the genera *Lactococcus, Lactobacillus, Streptococcus*, and *Leuconostoc* are predominant in all six goat cheeses. Furthermore, we identified several psychrophilic taxa often associated with the post-pasteurization contamination of refrigerated milk. Comparison of three α-diversity estimators (Chao1, Shannon and Faith's phylogenetic diversity indices) of microbiota in the core, middle part, and rind of all six goat cheeses did not reveal substantial differences, being only significant (at the nominal level) the comparison of rind vs middle part for the Shannon index (P-value = 0.031). Moreover, the construction of a dendrogram based on Aitchison distances revealed that cheese samples cluster according to their manufacturing characteristics, with a clear distinction between fresh vs semi-ripened or aged cheeses. We conclude that the magnitude of microbial α-diversity in the cheese core is similar to that in the rind despite their different physicochemical attributes. This result could be because physicochemical differences between cheese compartments are often attenuated during cheese ripening.

1. Introduction

The complex network of interactions between enzymes, microorganisms and milk components determines, to a remarkable extent, the technological and organoleptic properties of cheese (Fox et al., 1995). Cheese core has a lower pH and NaCl content and less oxygen availability than cheese surface, which is more exposed to environmental cues, particularly oxygen saturation (Montel et al. 2014). While Montel

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et al. (2014) and Choi et al. (2020) reported that microbial diversity is generally higher in the cheese rind than in the core, O'Sullivan et al. (2015) described the opposite trend in continental-type cheese: initially microbiota diversity was higher in the rind, but this pattern was reversed over time. Because of these controversial results, we aimed to investigate whether the magnitude of microbiota diversity differs amongst three compartments (core, middle part and rind) sampled in six Spanish commercial goat fresh and ripened cheeses with different textures (soft, semi-hard and hard).

2. Materials and methods

This study was conducted on commercial cheeses, so no ethical approval was needed.

2.1. Sequencing the microbiome of six Spanish cheese samples

We acquired six commercial Spanish goat cheeses, i.e. two fresh cheeses (H and M), three semi-rippened cheeses (P, B and C) and one aged cheese (G). Further information about their elaboration characteristics is available in **Supplementary Table 1**. Cheeses were stored at 4°C until processing. Three samples were retrieved from the rind, middle part, and core of each cheese by using sterilized instruments in a UV-irradiated cabinet to avoid external microbial contamination. Cheese sample homogenization and deoxyribonucleic acid (**DNA**) extraction were performed with the DNeasy PowerFood Microbial Kit (Qiagen, Redwood) following the instructions of the manufacturer with a slight modification, i.e. the lysate was heated at 56°C before vortex homogenization.

To construct the libraries for sequencing, an initial PCR was performed by using the KAPA HiFi PCR Mix (Kapa Biosystems, Cape Town) to amplify the hypervariable V3-V4 region of the bacterial 16S ribosomal ribonucleic acid (**rRNA**) gene with the set of universal primers 5′-CCT ACG GGN GGC WGC AG-3′ and 5′-GAC TAC HVG GGT ATC TAA TCC-3′. Amplicons were purified with the AMPure XP beads (Beckman Coulter, Indianapolis). Afterwards, a second PCR was carried out to add Nextera adapters, with barcodes for multiplexed sequencing, to amplicons. Subsequently, the concentration of the PCR product was normalized and purified with the SequalPrep kit (ThermoFisher, Barcelona, Spain). The sequencing of the libraries was performed on an Illumina MiSeq equipment to generate 2 × 301 bp reads. All library construction and sequencing tasks were performed at the Centre de Regulació Genòmica (Barcelona, Spain).

2.2. Taxonomical and microbial diversity analyses

Data quality control was conducted with the QIIME2 version 2021.4 software (Bolyen et al. 2019). Demultiplexed sequences were retrieved as FASTQ files. Subsequently, primers were removed with the *cutadapt* plugin in QIIME2 (Martin 2011). Singletons and sequences with length below 260 bp and a Phred score < 20, were removed from the dataset. Reads were denoised and grouped into amplicon sequence variants (**ASVs**) using the DADA2 protocol (https://benjjneb.github.io/dada2/tutorial.html), and chimaeras were removed with the consensus method (Callahan et al. 2016). Filtered reads were aligned with MAFFT to generate phylogenetic trees (Katoh and Standley 2013).

A taxonomical classifier was created using as reference DAIRYdb 2.0 (Meola et al. 2019), a manually curated database optimized for dairy product environments. The classifier was trained exclusively on the target reads generated with primers V3-V4 to improve accuracy (Werner et al. 2012). Taxonomy was assigned to the identified ASVs using the feature-classifier *classify-sklearn* command of QIIME2 (Pedregosa et al. 2012; Bolyen et al. 2019). The unassigned features and those categorized as eukaryotes, archaea, mitochondria, or bacteria with a taxonomy depth inferior to family were removed. Barplots depicting the relative abundance of bacterial families and genera were built with R (R Core Team, 2022). Bacterial taxa with relative abundances below 0.1 % were catalogued as "Other".

For performing microbial diversity analyses, the feature table, phylogenetic tree, and metadata were exported to R as a *phyloseq* object using the QIIME2R package (https://github.com/jbisanz/qiime2R). Sequences were rarefied to a depth of 60,000 to avoid library size bias. Chao1, Shannon and Faith's phylogenetic diversity (**Faith's PD**) α-diversity indices were estimated with the R packages Microbiome (https://bioconductor.org/packages/release/bioc/html/ microbiome.html) and Picante (Kembel et al. 2010). Bray-Curtis, unweighted Unifrac and weighted Unifrac β-diversity indices were estimated with the Vegan R package (Oksanen et al., 2022).

2.3. Statistical analysis of the data

In our experimental design, each one of the three groups under comparison (rind, middle part and core) includes six experimental units drawn from different types of cheese, with each group having exactly the same cheese composition. Including samples from different types of cheese in each group ensures that all experimental units within groups are completely independent. More importantly, this design captures, in an efficient way, random biological variation associated with each group, making the results more generalizable than a design in which all samples are drawn from the same type of cheese. The non-parametric Wilcoxon signed rank test (Wilcoxon 1945) was used to compare α-diversities between core, middle part and rind of all six cheeses in a pairwise manner. The Wilcoxon signed rank test is particularly suitable when comparing groups with matched samples and it does not assume normality. The null hypothesis of this test is that groups under comparison have equal medians. To correct for multiple testing we used the method of Bonferroni (1936), which controls the family-wise error rate by assuming that the false positive rate α is equal to the desired value of α (in our case $\alpha = 0.05$) divided by the number of hypotheses (9 comparisons in our study). In consequence, in our experiment, the statistical threshold of statistical significance is $\alpha = 0.0055$.

3. Results and discussion

A total of 3831,251 reads were obtained by sequencing the 18 cheese samples. After applying quality control procedures and removing undesired taxa, we retained 1639,816 reads assigned to 283 ASVs for downstream analyses. Cheese microbiota was characterized at the genus level because the sequencing of marker gene regions (such as 16S rRNA) allows a good taxonomical resolution up to such a level (Knight et al. 2018). Like data reported in cow and ewe (Choi et al., 2020; Walsh et al., 2020), main genera identified in all goat cheese samples corresponded to lactic acid bacteria (LAB) commonly used as starters in cheesemaking e.g., *Lactococcus, Lactobacillus, Leuconostoc* and *Streptococcus* (Fig. 1). A list of minority genera (< 0.1 % abundance) can be found in **Supplementary Figure 1**.

Non-LAB microorganisms frequently used as starters were also identified in the six goat cheeses under study. As shown in Fig. 1**A**, a high abundance of bacteria from the genera *Pseudomonas* and *Pseudoalteromonas* was detected in fresh soft goat cheeses H (13.92 – 39.80 % *Pseudomonas*, 5.63 – 57.47 % *Pseudoalteromonas*) and M (3.86 – 21.87 % *Pseudomonas*, 0.44 – 4.32 % *Pseudoalteromonas*), while *Pseudomonas* (but not *Pseudoalteromonas*) was abundant in soft semi-ripened cheeses P and C (0.84 – 18.85 % (Fig. 1). *Pseudomonas, Pseudoalteromonas* and *Shewanella* are psychrophilic/psychrotolerant genera, being able to grow in refrigerated milk as post-pasteurization contaminants (De Jonghe et al. 2011).

The non-parametric Wilcoxon signed test (Wilcoxon 1945) was used to compare α-diversities between core, middle part, and rind of all six cheeses in a pairwise manner. As shown in Fig. 1**B** and Table 1, when comparing the diversity of microbial taxa between the cheese rind and core, we did not detect any significant difference in terms of richness

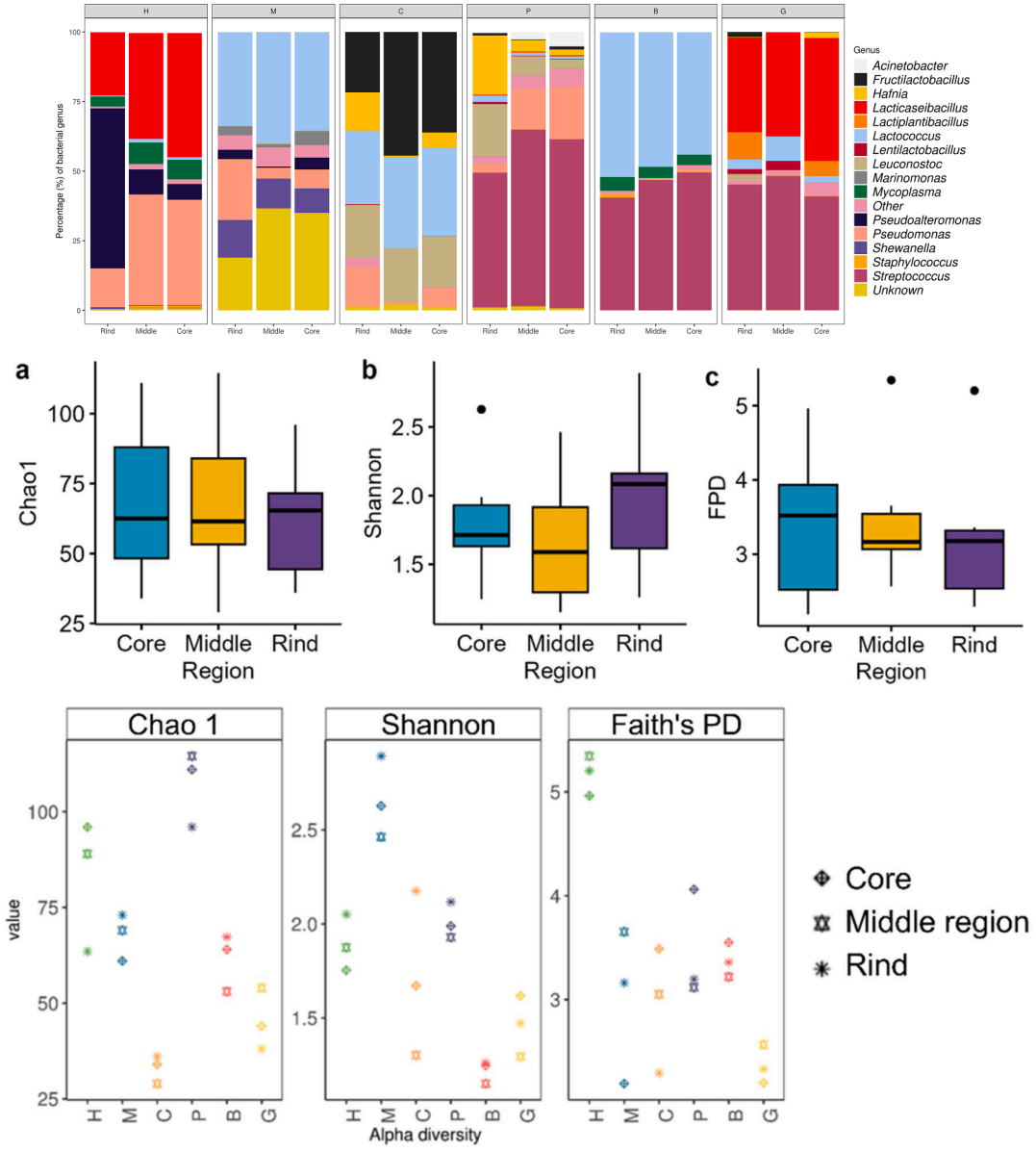

Fig. 1. A. Relative abundances of bacterial genera identified in the rind, middle part and core samples from soft fresh (H and M), soft semi-ripened (C and P), hard semi-ripened (B) and semi-hard aged (G) goat cheeses. Bacterial genera with relative abundances below 0.1 % have been catalogued as "Other". **1B** Boxplot depicting the α-diversity in the rind, middle part, and core microbiota of six commercial goat cheeses. Panels correspond to indices (a) richness (Chao1), (b) evenness (Shannon) and (c) phylogenetical diversity (Faith's PD). **1C.** α-diversity from three sampled regions of soft fresh (H and M), soft semi-ripened (C and P), hard semi-ripened (B) and semi-hard aged (G) goat cheeses. All contrasts are non-significant after correction for multiple testing.

(Chao1), evenness (Shannon) or phylogenetical diversity (Faith's PD). Indeed, by inspecting the boxplots displayed in Fig. 1**B** we can see that the distribution of observations for these three parameters are mostly overlapping when rind, middle part and core groups are compared. Indeed, the only significant difference was that between the middle part and the rind for the Shannon index (Wilcoxon test p-value = 0.031, Fig. 1**B** and Table 1). However, after correction for multiple testing with the method of Bonferroni (1936) such difference became non-significant. Moreover, when visualizing diversity by type and cheese region (Fig. 1**C**), we could not identify any spatial trend affecting microbial diversity. However, we found, in general, lower diversity for all indices in hard and semi-hard cheeses (B and G) compared to the semi-cured and fresh ones (Fig. 1**C** and Table 1).

The results obtained when calculating β-diversity indices, which reflect differences between microbial communities among samples, are depicted in **Supplementary Figure 2**. The analysis based on non-phylogenetic Bray-Curtis distances (**Supplementary Figure 2**) showed that samples clustered according to the cheese type and not to the portion of the cheese sampled (*i.e.* core, middle part and rind). The principal coordinates analysis (PCoA) of this metric made evident two defined clusters: one formed by soft fresh H and M samples, and the other one comprising soft semi-ripened P and semi-hard aged G cheeses, while soft-semi-ripened C and hard semi-ripened B cheeses were placed in separate locations in the plot (**Supplementary Figure 2**). The PCoA plot based on phylogenetic Unifrac distances, both weighted and unweighted, showed a consistent affinity between hard semi-ripened B and soft semi-ripened P cheeses (**Supplementary Figure 2**). Samples from the soft semi-ripened C cheese made from raw milk did not cluster with the other cheese types in all three analyses.

In accordance with PCoA analyses, the clustering of the cheese samples in a dendrogram based on Aitchison distances and taking into account only the most abundant taxa (relative abundance > 1 %) made

Table 1
Averages and standard deviations of three α-diversity parameters measured in 6 Spanish cheeses and statistical contrast of their magnitudes in three compartments with the non-parametric Wilcoxon signed test.

Type of cheese	Sampled region	Chao1	Shannon	Faith's PD[1]
M	Rind	73	2.89	3.16
H	Rind	63.50	2.05	5.20
C	Rind	36	2.18	2.29
P	Rind	96	2.12	3.19
B	Rind	67.25	1.26	3.36
G	Rind	38	1.47	2.33
Average ± SD		62.30 ± 22.60	1.99 ± 0.58	3.25 ± 1.06
M	Middle	69	2.46	3.65
H	Middle	89	1.87	5.34
C	Middle	29	1.30	3.05
P	Middle	114.50	1.93	3.12
B	Middle	53	1.15	3.22
G	Middle	54	1.29	2.57
Average ± SD		68.08 ± 30.15	1.66 ± 0.50	3.49 ± 0.97
M	Core	61	2.63	2.19
H	Core	96	1.75	4.96
C	Core	34	1.67	3.49
P	Core	111	1.99	4.06
B	Core	64	1.25	3.55
G	Core	44	1.62	2.20
Average ± SD		68.33 ± 29.76	1.81 ± 0.46	3.40 ± 1.07
Nominal significance (*P*-value) of the contrast				
Contrast		**Chao1**	**Shannon**	**Faith's PD1**
Core vs rind		0.560	0.156	0.840
Core vs middle		1	0.156	1
Middle vs rind		0.440	0.031[2]	0.160

[1] PD, phylogenetic distance,.
[2] Non-significant after correction for multiple testing.

possible to distinguish two principal groups (**Supplementary Figure 3**): one containing soft semi-ripened mouldy rind cheeses (C and P) and semi-hard aged cheese (G), and another group encompassing soft fresh (H and M) and hard semi-ripened (B) cheeses. The inclusion of the less common taxa (< 1 %) in the analysis led to a pattern of clustering that was perfectly coherent with the classification of the cheese varieties portrayed in **Supplementary Table 1** (**Supplementary Figure 3**). One of the clusters comprised soft fresh cheeses (H and M), while the other one encompassed semi-ripened and ripened cheeses (B, G, C and P). The two soft semi-ripened cheeses (C and P) grouped in a secondary node. These findings are consistent with the key impact of the ripening process on the composition of the cheese microbiome.

4. Conclusions

In contrast with previous reports (Montel et al. 2014; Choi et al. 2020), our results do not support differences in microbial diversity between the cheese rind and core. Although differences in oxygen saturation, pH, salt, water activity and redox potential exist between the core and the rind, some of these differences are mitigated during ripening. (Van den Tempel et al., 2002; Leclercq-Perlat et al. 2004; Wemmenhove et al. 2016). Indeed, O'Sullivan et al. (2015) studied the microbial dynamics during the manufacture of brine-salted continental-type cheese and found that diversity was higher in the rind (when compared to the core) at 1 day postproduction, but this trend was reversed at all subsequent time points. Increased diversity in the rind could have been due to the higher temperatures sustained by the core during the manufacturing process, or to the presence of halophilic bacteria in the rind because of brining (O'Sullivan et al., 2015).

CRediT authorship contribution statement

Maria G. Luigi-Sierra: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Yuliaxis Ramayo-Caldas:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Dailu Guan:** Writing – review & editing, Resources, Methodology, Investigation. **Marcel Amills:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

No potential conflict of interest is reported by the authors.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.livsci.2024.105496.

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