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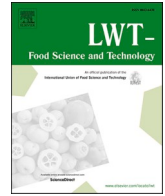
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# Impact of the use of an indigenous starter on microbial community structuring and aromatic profile of rocamadour protected designation of origin cheese

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## ABSTRACT

The use of indigenous strains as starters offers new opportunities that challenge the use of industrial starters, especially to strengthen the link between Protected Designation of Origin (PDO) cheeses and their terroir. This study investigated the effect of two starters, industrial and indigenous, on the microbial ecosystem and volatile organic compounds (VOCs) in Rocamadour PDO cheese. Metabarcoding analysis showed that regardless of the type of starter used, *Lactococcus lactis* and *Geotrichum candidum* largely dominated the microbial communities of Rocamadour (>96 %). The analysis of  $\beta$ -diversity based on the Bray-Curtis indices differentiated the starters according to their bacterial communities. VOC analysis showed that the starters were distinguished by three fatty acids characteristic of the goat cheese aroma ( $p$ -value <0.05): decanoic, hexanoic, and octanoic acids. Hexanoic acid was not present in cheeses produced with the indigenous starter. This fatty acid was positively correlated with the presence of *Paucilactobacillus nenjiangensis*, *Lactiplantibacillus* sp. and *Enterobacteriaceae*, these microorganisms that were also characteristic of cheeses made with the industrial starter. Octanoic and decanoic acid, on the other hand, were suggested to be correlated with the presence of *Leuconostoc mesenteroides*.

Cheeses produced with the indigenous starter differed in bacterial communities and aromatic signatures.

## 1. Introduction

Traditional goat milk products are considered a cultural heritage. They are produced using unique know-how passed down from the previous generations. They reflect their “terroir”, represented by their production environment, such as water availability, location, climate and plant and animal biodiversity (Montel et al., 2014; Sepe & Argüello, 2019).

As of June 2024, 190 PDO cheeses had been granted the PDO label by the European Union, 22 of which were made exclusively from goat's milk. Of these, 15 are produced in France, including Rocamadour PDO cheese (EC, 2024). The Rocamadour cheese is produced mainly in the Lot department of France, using raw goat's milk sourced from this region. It has a soft, dark beige or ivory-colored paste, a white rind and comes in the form of a 35-g puck, 6 cm in diameter and 1.6 cm height.

This cheese has been granted the PDO label and is currently the second most widely consumed PDO goat cheese in France (INAO, 2023).

For many years, this cheese was traditionally produced on farms using the backslopping method, which involved the use of the whey resulting from previous lactic fermentation to initiate a new production (Garofalo et al., 2020). To date, the majority of PDO-labelled goat's cheeses in France (13 out of 15) use this method, including Rocamadour PDO. Producers prefer to use it since it is practical, economical and, above all, gives the product its typical characteristic (Demarigny et al., 2011; Demarigny & Gerber, 2014). However, there are inherent risks, as numerous successive cycles of backslopping can lead to deviations in acidification profiles, resulting in defective products (Demarigny & Gerber, 2014; Gastrow et al., 2020).

In response to these challenges and the ever-growing consumer

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demand, larger volumes of raw goat's milk are now collected from many farms in the Rocamadour region. This milk is subsequently processed in cheese factory, representing artisanal productions where backslipping has been replaced by the use of industrial starters, authorized by the PDO Rocamadour specifications. These industrial starters are not linked to the PDO area and are composed of a small number of well-adapted and genetically related strains, essentially *Lactococcus lactis* strains with similar genetic profiles and phenotypic characteristics (Gibbons & Rinker, 2015; Passerini et al., 2010; van Hylckama Vlieg et al., 2006). This practice leads to the standardization of these PDO cheeses and reduces their natural microbial diversity (Nieto-Arribas et al., 2009; Rasovic et al., 2017).

The specificity of a PDO product lies in its link with its terroir, which means that it must represent its region in its entirety, and in particular the micro-organisms that inhabit it. Recently, and due to the new possibilities presented by using indigenous micro-organisms, industrial starters are being questioned, especially as they do not strengthen the link with the terroir of the designated cheeses (Munekata et al., 2022). The significance of raw milk as a source of microbial diversity on the sensory and organoleptic properties of cheeses has been demonstrated in PDO cheeses, such as Ossau-iraty and Grana Padano cheeses (Feutry et al., 2012; Santarelli et al., 2013).

In a previous work, Couderc et al. (2022) designed an indigenous starter composed of three *Lactococcus lactis* strains isolated from raw goat's milk from the Rocamadour PDO region and well adapted to the production of this cheese.

The selected indigenous starter was successfully tested for its ability to meet the Rocamadour PDO specifications. It has resulted in reproducible productions of this cheese with performances similar to those of an industrial starter but with a different sensory signature.

The aim of this study is to evaluate the impact of the indigenous starter on the microbial communities of Rocamadour PDO cheese and to identify the volatile aromatic compounds that may be affected. Possible correlations between differences in the microbial communities and the production of specific aromatic compounds were also evaluated.

## 2. Material and methods

### 2.1. Rocamadour PDO cheese productions

The production of Rocamadour cheese was carried out as described by Couderc et al. (2022). Briefly, two batches of 180 L each of raw goat's milk collected from the Rocamadour PDO area were used. The first batch, Batch A was inoculated with the industrial starter (CHN19, CHR Hansen, Horsolhm, Denmark). This starter culture was used daily in the cheese factory "Les Fermiers du Rocamadour" (Alvignac, France) for the production of Rocamadour PDO cheese and contained four species: *Lactococcus lactis*, *Lactococcus cremoris*, *Lactococcus lactis* biovar *diacetylactis*, and *Leuconostoc mesenteroides*. The second batch, Batch B was inoculated with the indigenous starter composed of the three selected strains of *Lactococcus lactis* EIP07A, EIP13D, and EIP20B (collection of "Ecole d'Ingénieurs de Purpan", Toulouse, France). These strains were isolated from raw goat milk of the PDO Rocamadour area and selected on the basis of their performance in artisanal cheesemaking to ensure that they match the sensory and quality characteristics of Rocamadour PDO cheese. The concentration of the indigenous starter used was adjusted to 7.7 log cfu/mL in order to obtain optimal acidification profiles and  $V_{max}$  values as recommended for Rocamadour cheese production (Couderc et al., 2022).

At the same time, a ripening starter composed of the two species *Geotrichum candidum* (0.9 mL of commercial strain Geo 102, Standa laboratoire, Caen, France) and *Debaryomyces hansenii* (0.9 mL of commercial strain Yeast 1002, INRA strain M6F, SAS LIP, Aurillac, France) was also added to both batches.

Milk was then stirred and stored at 20 °C for 2–4 h. At pH 6.3, a commercial rennet (20 mL at 180 mg of chymosin/100 L of milk CHR.

Hansen, Horsolhm, Denmark) was added. The milk was kept at 18 °C until coagulation (22 h). When pH 4.4–4.6 was reached, the coagulated milk was drained in large cloth bags for 12 h at 20 °C to remove the whey. The resulting curd was salted (0.4–0.8 g of salt/100 g of curd), mixed and placed in moulds. The cheeses were then demolded, placed on trays and transferred to a first ripening room at 14 °C and 97 % relative humidity for 24 h. They were then transferred to a second ripening room at 11 °C and 98 % relative humidity for 4 days, during which time they were turned daily before tasting. The cheese production process was made in duplicates (production 1 and production 2) at the "Les Fermiers du Rocamadour" cheese factory and three cheeses from each production were analyzed to determine their microbial communities and aromatic profile. The cheeses were produced on an industrial scale and the cheese factory adheres to Good Manufacturing Practices (GMPs) and HACCP to guarantee the quality and safety of its raw milk cheeses.

### 2.2. Microbial communities of rocamadour PDO cheeses

#### 2.2.1. Total DNA extractions

Total DNA extractions were performed on six cheeses (core and rind separated) from each batch (batch A, CHN19 industrial starter; batch B, indigenous starter), three from production 1 and three from production 2.

Five grams of each cheese were added to 45 mL of sterile peptone water and then homogenized with a stomacher for  $3 \times 1$  min. Five mL of this mixture were centrifuged at 13,000 × g at 4 °C during 3 min and then the supernatant was discarded. The pellet was washed several times with sterile peptone water to remove the fat layer that remained on the sides of the tube. Total DNA was extracted using the DNeasy PowerFood Microbial Kit (Qiagen Hilden, Germany) following the study of *Abi Khalil et al.*, (2022). DNA quality and quantity were verified using a nanodrop spectrophotometer (NanoDrop 2000/2000c, Thermo Scientific, U.S.A) and adjusted to 10 ng/μL for each sample.

#### 2.2.2. Illumina MiSeq sequencing

The DNA metabarcoding amplifications were performed as described by *Abi Khalil et al.*, (2022). The V3-V4 region of the 16S rRNA gene for bacteria (amplicon size, 426 bp) and of the ITS2 region for fungi (amplicon sizes between 187 bp and 367 bp) were amplified.

Sequencing was performed at UMR AGAP platform (INRAe Montpellier, France) using Illumina MiSeq technology generating  $2 \times 300$  bp reads.

#### 2.2.3. Bioinformatics and statistical analysis

The resulting sequences were analyzed using DADA2 and FROGS pipelines. First, the pre-processing steps were carried out using DADA2 package version 1.14.1 in R program version 3.6.1 which permitted obtaining Amplicon Sequence Variants (ASVs) tables. Then, taxonomic affiliations were performed using FROGS version 3.2.2 (Escudé et al., 2018) with DAIRYdb database version 2.0 (Meola et al., 2019) for bacteria and UNITE database version 8.2 (Nilsson et al., 2019) for fungi. Multi-affiliations ASV were resolved manually using the affiliation Explorer (Mariadassou, 2021).

Statistical analysis of diversity was performed using the phyloseq R package implemented in FROGS (FROGSSTAT) (McMurdie & Holmes, 2013). ASVs tables were then normalized by rarefaction before assessing the diversity indices. For the bacterial ecosystem, the number of reads after normalization was 41,628 for the cheese core and 45,096 for the cheese rind. For the fungal ecosystem, the number of reads after normalization was 31,799 reads in the cheese core and 38,998 reads in the cheese rind.

Species diversity within each sample was estimated using the  $\alpha$ -diversity indices (richness, Shannon, Simpson, and inverse Simpson). Bray-Curtis dissimilarity indices were used to estimate species diversity between samples ( $\beta$ -diversity) and were represented using

multidimensional scaling (MDS) plots and clustering. Subsequently, Multivariate Analysis of variance (PERMANOVA) using the Adonis test was used to detect significant differences between communities according to the starter used (industrial, indigenous) as the experimental variable.

### 2.3. Analysis of volatile compounds by headspace SPME and GC-MS

#### 2.3.1. Sample preparation and SPME conditions

The protocol was inspired by Reyes-Díaz et al. (2020) with the following modifications. Volatile compounds were analyzed by placing 3 g of finely grated cheese in 7 mL of a saturated NaCl (28 % w/v) solution, containing 20 µL of an internal standard (4-hydroxy-4-methyl-2-pentanone at 100 µg/L in ethanol) in screw-capped glass vials (20 mL, 22.75 × 75 mm; 18 mm cap) for headspace analysis. The vials were then tightly closed and the samples were allowed to equilibrate at 60 °C (150 rpm) for 10 min. A 75-µm PDMS/DVB/CAR fiber (Supelco Co., Bellefonte, PA) was inserted into the headspace of the vial and left under agitation (150 rpm) at 60 °C for 1 h to extract the volatile compounds.

#### 2.3.2. Detection of volatile compounds

Volatile compounds were analyzed on a Clarus 680 gas chromatograph coupled to a Clarus 600 T quadrupole mass spectrometer (PerkinElmer, Courtaboeuf, France), and separated on BD5-MS (5 % phenyl - 95 % methylpolysiloxane) 30 m × 0.25 mm × 0.25 µm (Agilent, Santa Clara, United States) (Carunchia Whetstone et al., 2003). The fiber was then manually inserted into the injection port of the GC for desorption at 250 °C for 1 min in splitless mode and 9 min in split (ratio 1:20). The carrier gas was hydrogen at a constant flow rate of 3.2 mL/min. The oven temperature program was as follows: 40 °C for 5 min then 5 °C/min to 230 °C finally 20 °C/min to 280 °C (hold for 2 min). The interface and ion source were set at 200 °C. The mass spectrometer was operated in full scan mode with a mass range of m/z 30–300. The MS fragmentation was performed in electron impact ionization mode at 70 eV. Identification of the volatile organic compounds was done by comparing the mass spectra of the samples with the data system of the National Institute of Standards and Technology (NIST 2017; Gaithersburg, Maryland, USA). The confirmation of molecule identity was performed by comparing the retention indices of compounds, determined after injection of a series of n-alkanes (C9-C17; Merck, Darmstadt, Germany) under the analytical conditions previously described, with literature data.

### 2.4. Statistical analyses

Statistical analyses of VOCs data were conducted with XLSTAT (2020) (Addinsoft, France). Relative areas to the internal standard (ratio of compound area/internal standard area) were subjected to a Mann Whitney's test to compare means per starter (industrial starter and indigenous starter). Mean values were considered significantly different at a *p*-value <0.05.

A heatmap was created to reveal potential clusters of cheese samples based on their microbial and VOCs compositions. To do so, the relative abundance of microorganisms (>0.1 % of total sequences) and the VOC values were autoscaled. To order the columns and rows of the data matrix, the correlation similarity metric and the mean link grouping were carried out using the 'heatmap' function in the 'made4' R package.

To gain a better understanding of the possible correlations between microbial communities and the VOCs present, Pearson correlation coefficients were calculated between microorganisms (relative abundances >0.1 %) and the VOCs. Negative and positive correlations with an associated *p*-value below 0.01 were then plotted using R software with the 'corrplot' function from the 'corrplot' package.

## 3. Results

### 3.1. Depending on the acidification starter used, bacterial communities in cheese differ

Bacterial identification was carried out on the cheeses' cores and rinds separately. The sequencing of the 16S rRNA gene permitted the identification of twenty genera (cut-off 95 %) and twenty species (cut-off 97 %) for the cheese cores and nineteen genera and sixteen species concerning the cheese rinds (Fig. A1, Fig. A2 and supplementary data, tables S.A.1 and S.A.2).

The bacterial communities of the cheese cores were largely dominated by the species *Lactococcus lactis*, which was present at 99.24 % (average relative abundance among all samples) in cheeses produced with the indigenous starter and 98.08 % in cheeses produced with the industrial starter.

Among the species with abundances exceeding 0.1 %, regardless of the type of starter used, *Leuconostoc mesenteroides* was consistently present, exhibiting equivalent relative abundances in both types of cheeses (0.32 % for indigenous starter vs. 0.31 % for industrial starter). *Staphylococcus* sp. was also found in both types of cheeses, but it was more prevalent in the cheeses produced with the indigenous starter (0.37 % vs. 0.12 %). Additionally, the indigenous starter appeared to promote the emergence of *Staphylococcus simulans*, representing 0.12 % of the total *Staphylococcus* sp., whereas it fell below the 0.1 % threshold in cheeses made with the industrial starter. Furthermore, *Lactiplantibacillus* sp. and *Paucilactobacillus nenjiangensis* constituted 0.57 % and 0.35 % respectively, of the bacterial population found in the core of cheeses manufactured with industrial starter, whereas they fell below the 0.1 % threshold when the indigenous starter was used.

It was noteworthy that the proportion of Proteobacteria remained below 0.1 % regardless of the type of starter used (Supplementary data, table S.A.1).

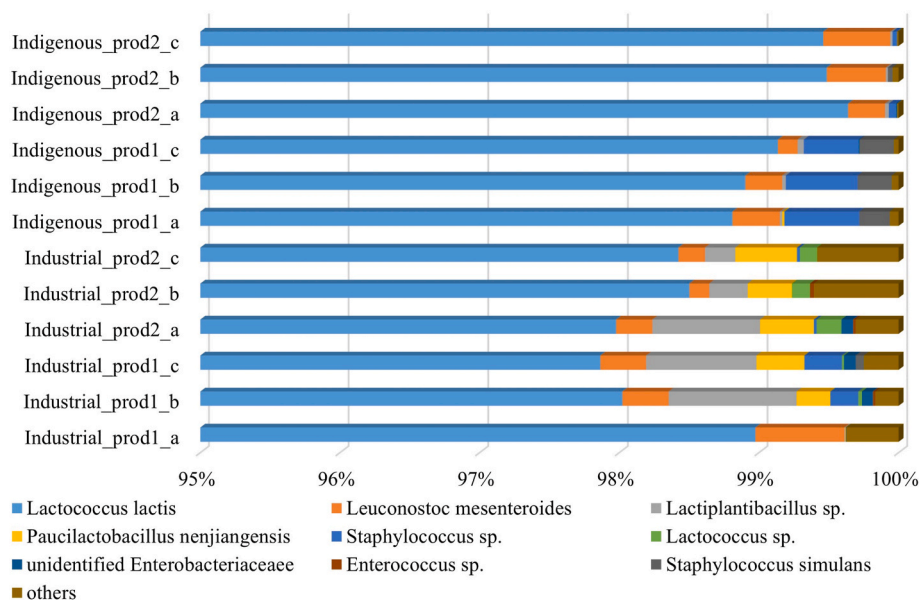
As with the cores, the surface of the cheeses was largely dominated by *Lactococcus lactis* which was present at 99.75 % (average relative abundance among all samples) in the cheeses produced with the indigenous starter and at 97.22 % for the industrial starter. *Leuconostoc mesenteroides* remained the second most abundant species regardless of the type of starter used. However, its abundance was ten times higher on the surface of cheeses made with industrial starter (1.53 %) compared to those prepared with the indigenous starter (0.15 %), while its abundance in the core of the two types of cheese was similar (as indicated above).

*Staphylococcus* sp. and *Paucilactobacillus nenjiangensis* were no longer found in the cheeses, or at least in proportions below 0.1 %. On the other hand, *Lactiplantibacillus* sp. persisted (0.25 %) in cheeses made with the industrial starter. The emergence of *Enterococcus* sp. (0.11 %) and unidentified *Enterobacteriaceae* (0.16 %) in this type of cheese should also be noted (supplementary data, table S.A.2).

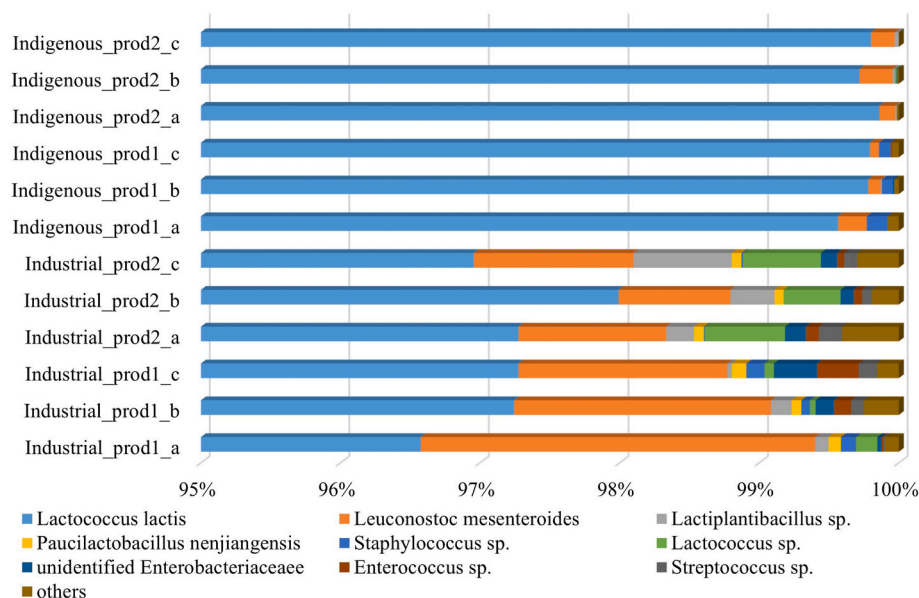
Species richness for each sample was assessed using the  $\alpha$ -diversity indices Richness, Chao1, Shannon, and inverse Simpson (supplementary data, table S.B). The comparison between the Richness and the Chao1 indices (the estimated richness) revealed that all the bacterial diversity of the cheese core and the rind was identified (supplementary data, table S.B).

The values of the Shannon and inverse Simpson indices were low, indicating that the bacterial community on the rind and in the core was largely dominated by a single species, as shown in Fig. A1 and Fig. A2. This trend was confirmed by the low number of ASVs found in all cheeses (ranging from five ASVs in the samples indigenous starter\_prod2\_c and indigenous starter\_prod2\_c to 34 ASVs in the sample industrial starter\_prod2\_a) (supplementary data, table S.B).

The analysis of  $\beta$ -diversity was based on Bray-Curtis indices. The multidimensional scaling (MDS analysis) allowed grouping the samples according to the type of starter used, indigenous or industrial (Fig. B1 and Fig. B2). This meant that the type of starter has an impact on the



**Fig. A.1.** Analysis of the bacterial communities in the core of Rocamadour cheeses produced with the two types of starter (indigenous or industrial) by sequencing the V3-V4 region of the 16S rRNA gene. Three biological replicates (cheeses; a, b and c) per production (production 1 and production 2), i.e. n = 6, were analyzed.



**Fig. A.2.** Analysis of the bacterial communities in the rind of Rocamadour cheeses produced with the two types of starter (indigenous or industrial) by sequencing the V3-V4 region of the 16S rRNA gene. Three biological replicates (cheeses; a, b and c) per production (production 1 and production 2), i.e. n = 6, were analyzed.

microbial populations that have emerged during cheese production.

A PERMANOVA statistical analysis applied to Bray-Curtis dissimilarity indices confirmed significant differences with a  $p$ -value  $< 0.001$  between bacterial communities according to the starter used (supplementary data, tables S.C.1 and S.C.2).

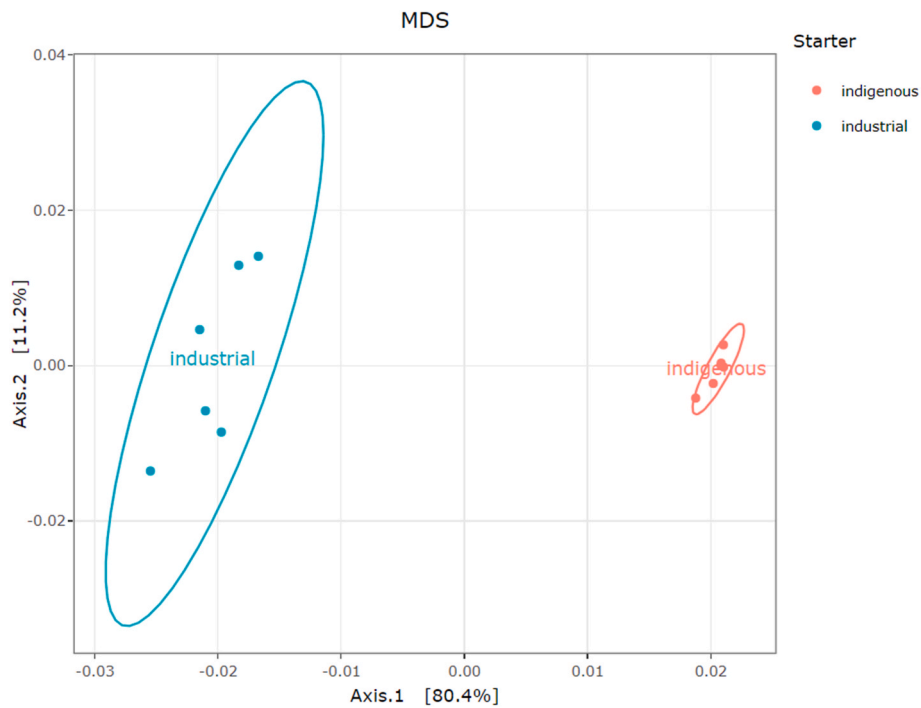
The DESeq2 analysis showed that out of the 103 ASVs present in the cheese cores, 10 ASVs were affected by the type of starter used (supplementary data, table S.D.1). Two ASVs of *Staphylococcus simulans* (ASV274 and ASV290) were significantly more abundant in the cheeses produced with the indigenous starter, and two ASVs of *Paucilactobacillus nenjiangensis* (ASV96 and ASV260) and one ASV of *Lactiplantibacillus sp.* (ASV4) were significantly less abundant in the cheeses produced with the indigenous starter.

Although the *Lactococcus* population was higher within the cheeses

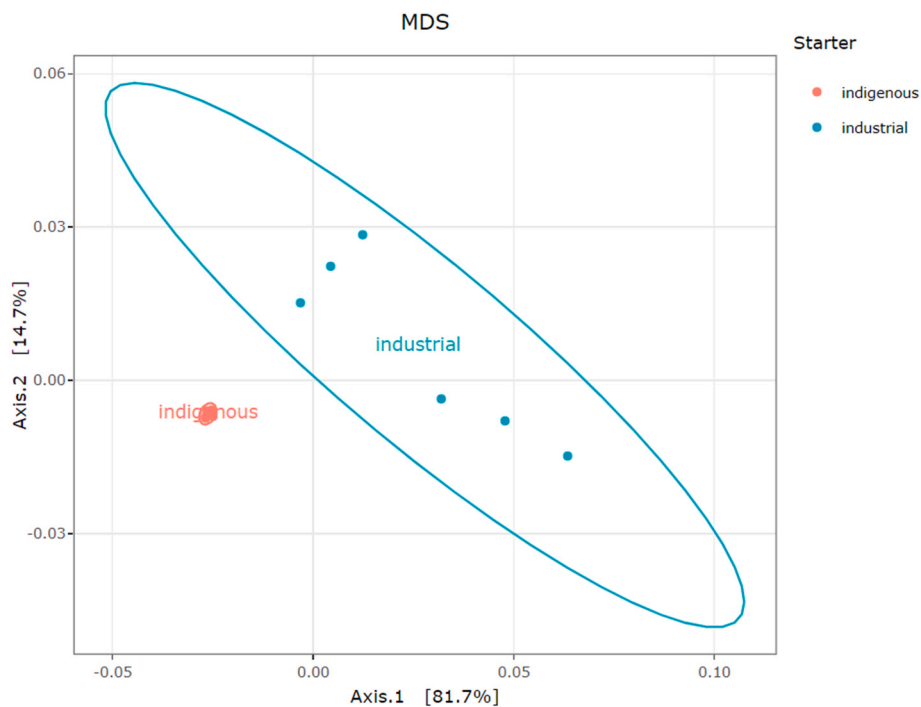
produced with the indigenous starter, four ASVs of *Lactococcus lactis* (ASV33, ASV21, ASV81, and ASV100) and one ASV of *Lactococcus sp.* (ASV117) were found in relatively higher abundances within the cheeses produced with the industrial starter.

On the rinds of the cheeses, out of the 103 ASVs present, five ASVs were significantly different (supplementary data, table S.D.2). One *Leuconostoc mesenteroides* ASV (ASV24), one *Lactiplantibacillus sp.* ASV (ASV4) and one *Staphylococcus sp.* ASV (ASV99) were significantly more abundant in the cheeses produced with the indigenous starter. Two *Lactococcus* ASVs were affected by the type of starter used; ASV1 was significantly more abundant, while ASV33 was significantly less abundant within the cheeses produced with the indigenous starter.

DESeq2 analysis confirmed the results observed between the relative abundance differences of the cores vs. rinds and industrial vs.



**Fig. B.1.** Multidimensional scale (MDS) based on the relative abundance of ASVs (>0.1%) in the 16S rRNA-targeted bacterial microbiota of the cheese core. The two types of starters are indicated as follows: indigenous starter (orange) and industrial starter (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)



**Fig. B.2.** Multidimensional scale (MDS) based on the relative abundance of ASV in the 16S rRNA-targeted bacterial microbiota of the cheese rind. The two types of starters are indicated as follows: indigenous starter (orange) and industrial starter (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

indigenous modalities.

A similar study was carried out on the fungal population. This study showed that cheeses produced with both types of starters were largely dominated by the *Geotrichum candidum* species, both on the rind and in the core of the cheese (>99.30% of total sequences; supplementary data

tables SE.1 and SE.2). This species was present in the ripening starter and developed in a dominant manner, masking any effects of the acidification starters. The MDS failed to provide a distinct clustering amongst the samples based on the Bray-Curtis dissimilarity indices (supplementary data, fig. S.A.1 and S.A.2).

Analysis of the microbial ecosystem of the cheeses showed that the two types of starter (industrial and indigenous) differed in terms of their bacterial communities.

### 3.2. The VOC signature of cheeses varies according to the acidification starter used

Fifteen volatile compounds were identified by HS SPME GC-MS and semi-quantified by using the internal standard 4-hydroxy-4-methyl-2-pentanone: four alkanes (tridecane, tetradecane, pentadecane, hexadecane); two ketones (methyl isobutyl ketone, 2-octanone); one aldehyde (2,4-nonadienal); four fatty acids (butanoic acid, hexanoic acid, octanoic acid, and decanoic acid) and four esters (ethyl butanoate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate) (supplementary data, table S.E).

A significant difference between the two types of starters used for three of the four acids was detected: octanoic acid, decanoic acid, and hexanoic acid were found in different amounts, depending on the starter used (Man-Whitney test  $p < 0.05$ , Fig. C and supplementary data, table S.E). The differences were as follows: no hexanoic acid was observed in the cheeses produced with the indigenous starter; octanoic and decanoic acids were present 1.4 times more in the industrial starter compared to the indigenous starter. No significant differences were observed for the other twelve compounds (supplementary data, table S.E).

### 3.3. A correlation between the microbiota of cheeses and their VOCs profile can be highlighted

The relative abundances of the fifteen volatile compounds and the relative abundances ( $>0.1\%$ ) of microorganisms present in the cheeses' cores and rinds were used to create a heatmap with sample clustering (Fig. D).

The cheeses could be classified according to the type of starter used. Branch 1 consisted exclusively of cheeses made with the indigenous starter and branch 2 with the industrial starter, which confirmed the results obtained from the analysis of the bacterial community (MDS) (Fig. D).

Cheeses produced with the indigenous starter were characterized by a pattern (group I) composed of *Lactococcus lactis* and *Staphylococcus* sp. and *Staphylococcus simulans* and most alkanes (3/4), esters (3/4), and all the ketones (2/2). Other microorganisms and volatile compounds were associated with Group II, corresponding to the cheeses produced with the industrial starter (Fig. D).

To further investigate the potential links between the microorganisms and VOCs that differentiate the indigenous and industrial starters, we performed a correlation analysis (Fig. E). Only those correlations were discussed that had biological significance, i.e., followed a proportional (linear) trend, and whether the underlying distribution of the data was normal (supplementary data, fig. S.B.1, S.B.2, and S.B.3).

A highly significant negative correlation was observed between the

presence of *Lactococcus lactis* in the cheese cores and rinds and the presence of the three volatile fatty acids specific to goat flavor in cheese, i.e., hexanoic acid ( $r = -0.92$  for rind and  $r = -0.97$  for core), decanoic acid ( $r = -0.73$  for rind and  $r = -0.74$  for core) and octanoic acid ( $r = -0.77$  for rind and  $r = -0.65$  for core) (supplementary data, fig. S.B.1). Conversely, the presence of *L. mesenteroides* in the rinds of cheeses was positively correlated with these acids (hexanoic acid  $r = 0.89$ ; octanoic acid  $r = 0.70$  and decanoic acid  $r = 0.70$ ) and negatively correlated (*L. mesenteroides* on the core) with the 4-nonadienal ( $r = -0.85$ ) (supplementary data, fig. S.B.2 and S.B.3).

Hexanoic acid was also positively correlated with *Lactiplantibacillus* sp. and *Paucilactobacillus nenjiangensis* present in the core of the cheeses ( $r = 0.89$  and  $0.91$  respectively) and *Enterobacteriaceae* present in the rind of the cheeses ( $r = 0.80$ ). A positive correlation was observed between *Lactiplantibacillus* sp. (rind) and tetradecane ( $r = 0.89$ ) (supplementary data, fig. S.B.3).

The correlations between bacterial species and volatile compounds showed that, firstly, the presence of *Leuconostoc mesenteroides* correlated with the dominance of *Lactococcus lactis* species, which appeared to be markers for differentiating the type of starter used and, secondly, the presence of *Enterobacteriaceae*, *Lactiplantibacillus* sp. and *Paucilactobacillus nenjiangensis* seemed to be discriminating markers specific to hexanoic acid and therefore associated with the industrial starter.

## 4. Discussion

This work assessed the impact of using an indigenous starter, composed exclusively of three strains of *Lactococcus lactis* previously selected (Couderc et al., 2022), on the microbial population and flavor development of Rocamadour PDO cheese.

The metabarcoding results showed that the core of Rocamadour PDO cheese was dominated by lactic acid bacteria (*Lactococcus lactis*, *Leuconostoc mesenteroides*, *Lactiplantibacillus* sp. and *Paucilactobacillus nenjiangensis*). These species were also found on the cheese rind, except for *Paucilactobacillus nenjiangensis*. These results are consistent with those found in the literature on the microbial population of cheeses. Indeed, *Lactococcus lactis* was also identified as the dominant bacterial species in several PDO cheeses such as the Greek PDOs Feta, Anevato and Kalathaki (Michailidou et al., 2021; Spyrelli et al., 2020), the Italian PDO Canestrato Pugliese (De Pasquale et al., 2014) and the French PDOs Pelardon (Penland et al., 2021) and Bleu d'Auvergne (Ferroukhi et al., 2022), but with low relative abundances, except for the PDO Pelardon for which similar lactococcal abundances were found.

*Leuconostoc mesenteroides* is the second most abundant species in the core of Rocamadour cheeses, regardless of the type of starter used. Consistent with findings in other studies, such as those conducted on Pelardon PDO cheese, *Leuconostoc mesenteroides* was identified as the second most prevalent species after *Lactococcus lactis* (Penland et al., 2021). *Leuconostoc* species are mainly used for their aromatic properties, in particular to produce diacetyl and acetoin, compounds appreciated

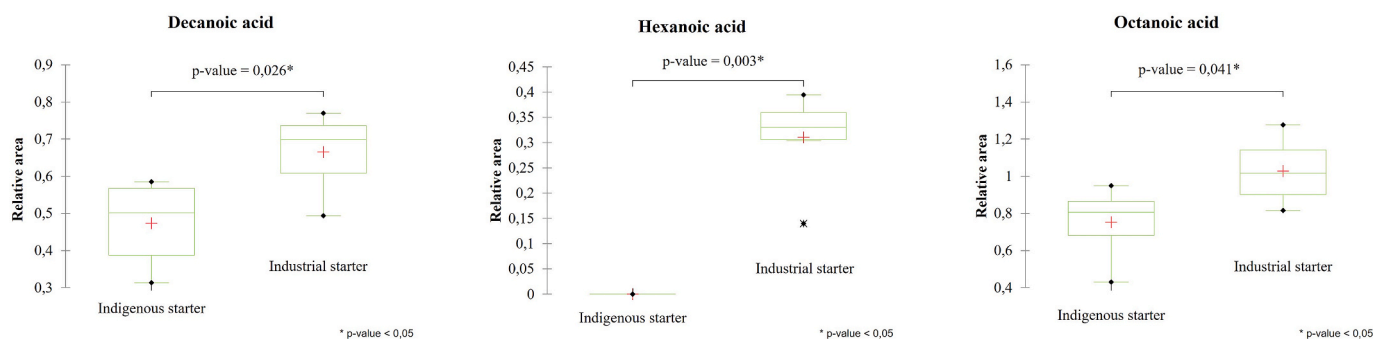
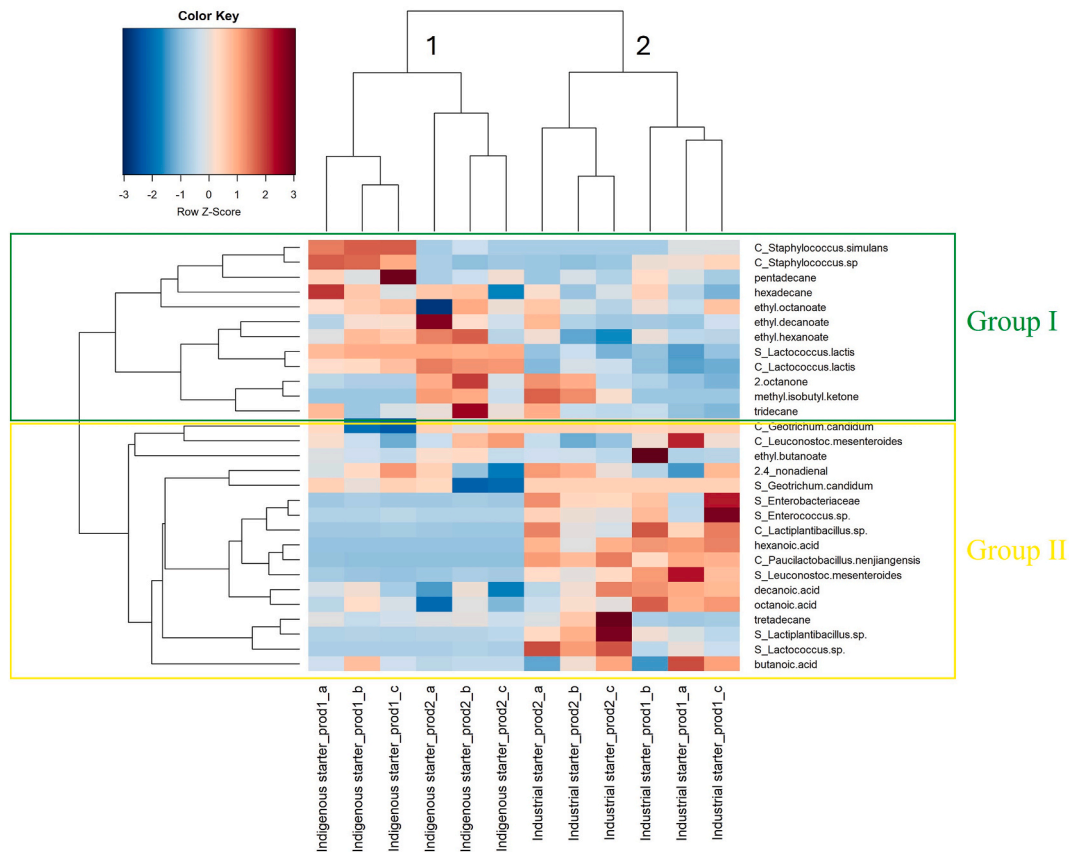
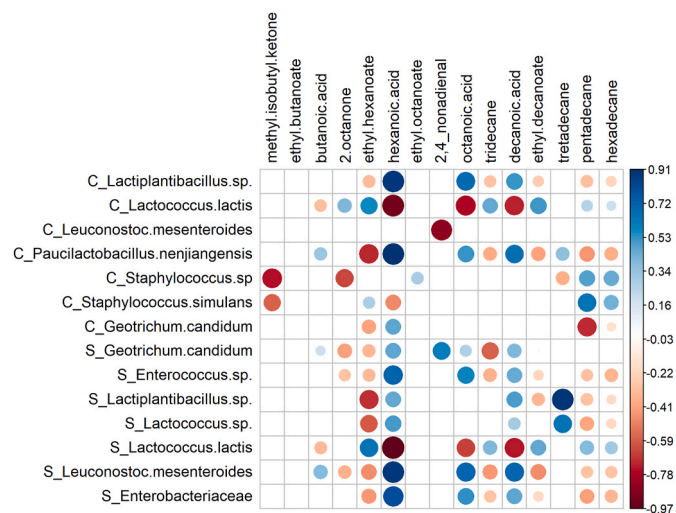


Fig. C. Relative areas of the three discriminating volatile compounds (decanoic, hexanoic and octanoic acids) as a function of the type of starter used, industrial or indigenous. The results were expressed in relative area. The symbol + represented the mean and the line represented the median.



**Fig. D.** Clustered Heatmap: Each column represented a cheese sample and each row represented the microorganisms and VOCs. The relative abundance values were centered and scaled in the direction of the rows. The sample nomenclature was formulated as follows: Type of starter used - Production number - Number of repetitions, e.g. Industrial\_Prod1\_a). For the nomenclature of bacteria and fungi, the symbols C for core and S for surface (rind) were used.



**Fig. E.** Pearson's correlation between microbial communities (ASVs >0.1 %) and volatile aroma compounds in Rocamadour cheeses. Significant associations between ASVs and VOCs are shown in bold ( $p < 0.05$ ). The color intensity represents the degree of correlation, where blue represents a positive degree of correlation and red a negative correlation. For the nomenclature of bacteria and fungi, the symbols C for core and S for surface (rind) were used.

for their buttery and nutty notes (Cardamone et al., 2011).

In this study, *Leuconostoc mesenteroides* was part of the composition of the industrial starter, so it was not surprising to find it in the microbial ecosystem of cheeses made with this starter. Interestingly it was found at

very similar relative abundances in the cores of the cheeses made with the indigenous starter, which contained only strains of *Lactococcus lactis*.

The growth of *Leuconostoc* in cultures containing *Lactococcus* is complex and strain-dependent. It could be described as a trophic interaction (Erkus et al., 2013). In fact, *Leuconostoc* is only able to produce aromatic compounds after *Lactococcus* has acidified the milk (Hemme & Foucaud-Scheunemann, 2004).

In the current literature, *Paucilactobacillus wasatchensis* is the most common *Paucilactobacillus* species isolated from cheese and is implicated in cheese spoilage (Oberge et al., 2021; Chiu, Green, & Zhu, 2023; Berthoud, Wechsler, & Irmeler, 2022). To our knowledge, the species *Paucilactobacillus nenjiangensis* has never been identified in the microbiota of a cheese. It was isolated for the first time from traditional pickles and raw milk may have been contaminated by plants on which it was present (Gu, Li, Yang, & Huo, 2013). This species was found in the core of Rocamadour PDO cheeses made with the industrial starter. It may originate from raw milk in very low proportions and emerge due to trophic interactions with the industrial starter. It can be reasonably assumed that the strains of *Leuconostoc mesenteroides* present in the industrial starter are capable of metabolising complex plant sugars, such as xylans and arabinoxylans, which are naturally present in raw milk. This degradation would result in the release of pentoses such as xylose and arabinose, which would favour the growth of *Paucilactobacillus* sp. such as *Paucilactobacillus nenjiangensis*.

*Staphylococcus* sp., a potentially pathogenic bacteria, especially for immunocompromised people, was identified with low relative abundances in the core of cheeses. These results were consistent with the literature. Indeed, *Staphylococcus* sp. is known to be part of the dominant flora of raw goat milk (Tormo et al., 2011) and subdominant in cheese microbiota, as shown by studies on Silter raw milk cheese



(Silvetti et al., 2017) and Serra da Canastra artisanal cheese (Kamimura et al., 2020). *Staphylococcus* sp. was also regularly detected in cheeses (78 % of cheeses in this study) in the review by Irlinger et al. (2015).

*Enterobacteriaceae* species were detected on the surface of cheeses produced with the industrial starter (0.16 %). These gram-negative bacteria are commonly found in raw milk and traditional cheeses, most commonly contaminating milk via animal feces or teats during milking (Montel et al., 2014; Papadakis et al., 2021). Their presence is commonly used as a microbiological indicator of poor hygiene (Martin et al., 2016), but some strains of *Enterobacteriaceae* have high flavor potential as they can catabolize citrate, lipids and proteins (Pangallo et al., 2014; Zago et al., 2007).

Regarding the fungal microbiota, the cheese cores and rinds of the cheeses were largely dominated by *Geotrichum candidum* (>99.30 %), limiting the development of other fungal species. Penland et al. (2021) showed similar relative abundances in Pelardon PDO cheeses, (above 99.50 % in all cheeses tested), and no difference between the core and rind of cheeses. This species is often deliberately inoculated in cheeses for its technological properties, as is the case with the Rocamadour PDO cheese. *G. candidum* plays an important role in cheese ripening, flavor and aroma development and rind formation (Boutrou & Guéguen, 2005; Maldonado et al., 2016).

The composition of cheese flavor compounds is particularly important for artisanal cheeses, as it contributes significantly to their typicity. The “volatilome” study showed that Rocamadour cheeses were characterized by high abundances of ketones, in particular 2-octanone and three medium-chain fatty acids, namely hexanoic, decanoic, and octanoic acids, also known as caproic, capric, and caprylic acids.

These three free fatty acids (FFAs) were found to be significantly different between the two types of starter used and are known to be flavor markers in goat’s cheese (Delgado et al., 2011; Poveda & Cabezas, 2006). The production of these acids is a consequence of the hydrolysis of milk fat, due to the lipolytic activity of the starter (Vénica et al., 2018). This suggests that the industrial starter exhibits a higher lipolytic activity than the indigenous one. In contrast, hexanoic acid was only detected in the cheeses produced with the industrial starter. Indeed, Carunchia Whetstine et al., (2003) showed that hexanoic acid contributed to a bad sour/sweaty taste in goat cheeses.

Methyl isobutyl ketone was found in all cheeses analyzed. Terminal oxidation of free fatty acids (FFAs) produces methyl ketones, compounds associated with flavor intensity, giving fruity, floral and musty notes such as 2-octanone. The presence of these volatile compounds can be considered positive to flavor goat cheese (Delgado et al., 2011; Mondello et al., 2005). *G. candidum* and *D. hansenii* were shown to have played an important role in the formation of methyl ketones in mature cheeses (Boutrou & Guéguen, 2005; Padilla et al., 2014).

## 5. Conclusion

This study described for the first time the impact of an indigenous starter on the microbial ecosystem of Rocamadour PDO cheese. The indigenous starter permitted to reproduce the dominant ecosystem of Rocamadour PDO cheese, with distinctive bacterial communities and aromatic richness. This work shows that an indigenous starter could represent a viable alternative to the use of industrial starters. This is advantageous because it could strengthen the link of Rocamadour PDO cheeses with their terroirs. A similar approach could be interesting to develop for other regional cheeses.

## CRedit authorship contribution statement

**Christel Couderc:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Reine Abi Khalil:** Writing – original draft, Formal analysis. **Helene Tormo:** Writing – review & editing, Funding

acquisition. **Charlotte Paës:** Writing – original draft, Formal analysis. **Michael Mourez:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Marie-Line Daveran-Mingot:** Writing – review & editing, Validation, Supervision, Project administration, Conceptualization.

## Declaration of competing interest

The authors declare that they have no conflict interests in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lwt.2024.116813>.

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