Food Microbiology 82 (2019) 349–362

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Microbial shifts in Minas artisanal cheeses from the Serra do Salitre region of Minas Gerais, Brazil throughout ripening time

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ABSTRACT

The Minas artisanal cheese is a traditional product in its way of producing. Produced in the Minas Gerais state, Brazil, this cheese is made using raw cow's milk with the addition of an endogenous starter culture called "pingo", responsible for inoculating specific microorganisms that could enhance flavor and sensorial aspects. There are seven regions able to produce and commercialize this product - *Araxá, Campo das Vertentes, Canastra Cerrado, Serra do Salitre, Serro and Triângulo Mineiro*. This study aimed to assess the bacterial community of raw milk, endogenous starter culture and to uncover possible shifts in the bacterial community of the rind and core of cheeses at sixty days of ripening located in the *Serra do Salitre* region by Illumina MiSeq 16S rRNA gene amplicon sequencing. Raw milk and starter culture are responsible for inoculating specific bacteria into the cheese, with *Planococcaceae* and *Streptococcaceae* being prevalent throughout ripening time. The *Planococcaceae* family seems to develop strong interactions with the *Leuconostocaceae* family on the surface of these cheeses, and is associated with environmental aspects of the region, probably leading to a microbial signature of these products. Additionally, abiotic factors such as geographical location, moisture and acidity are major drivers in the microbial shift.

1. Introduction

Minas Gerais state is the largest cheese producer in Brazil, and it is known for various types of cheeses, especially the Minas artisanal cheese, made from raw cow's milk. The local tradition of cheese making consists in the transmission of both ability and wisdom from elderly producers to new generations in the family. There are seven regions in the state able to produce and commercialize the Minas artisanal cheese: *Araxá*, *Campo das Vertentes*, *Cerrado*, *Serra da Canastra*, *Serra do Salitre*, *Serro* and *Triângulo Mineiro*.

Raw milk with an endogenous starter culture and a long ripening time grants the cheese unique flavor and sensorial aspects. The endogenous starter culture, also known as *pingo*, origins from drained whey of previously made cheeses and is stored at room temperature (approximately 25 °C) to be used in the cheese making process during the next day (Castro et al., 2016). The Minas artisanal cheese is manufactured in round shapes measuring approximately 40 cm. Its rind is usually whitened, tending to transform into a thin yellowish layer during the ripening process, usually without moulds. The cheese core is whitened, homogenous and sometimes friable. Due to the natural fermentation processes, the cheese usually presents small eye formations and a typical and markedly acid flavour. The "way of producing" the Minas artisanal cheese has been recognized as an immaterial heritage of Minas Gerais state by IPHAN (National Institute of Historical and Cultural Heritage, 2008). Recently, the Minas artisanal cheese has been awarded with several prizes, including the "Super Gold" medal at the *Mondial du Fromage* 2017, held in France.

By high throughput sequencing, some bacterial communities of artisanal products were characterized in different foods, especially cheeses and meats, revealing microbial communities ranging from simple to complex microbial structures which are quite specific for each product (e.g. Masoud et al., 2012; Quigley et al., 2011; Alegría et al., 2012; De Pasquale et al., 2014; Schornsteiner et al., 2014; Irlinger et al., 2015; Połka et al., 2015). Several factors such as raw material source, manufacturing practices and environmental characteristics of the region may determine the diversity and dynamics of microbial

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https://doi.org/10.1016/j.fm.2019.02.016
Received 17 October 2018; Received in revised form 12 February 2019; Accepted 28 February 2019

communities, as well as sensory properties and the quality of the final product.

As production regions have individual *terroirs,* the understanding and the definition of the bacterial microbiota in Minas artisanal cheese may contribute to establish geographical indications such as Protected Denomination of Origin (P.D.O) in the European Union. So far, studies regarding the Minas artisanal cheeses managed to identify a local lactic acid bacteria (LAB) population, spoilage microorganisms and foodborne pathogens using culture-dependent techniques (Perin et al., 2017; Castro et al., 2016; Luiz et al., 2017; Arcuri et al., 2013; Resende, 2010; Leite et al., 2009; Lima et al., 2009; Borelli, 2006). However, these techniques cover only a small fraction of the complexity of microbial communities (Hugenholtz et al., 1998). Thus, culture-independent methods such as high throughput sequencing (HTS) targeting the 16S rRNA gene may appear more suitable to define the microbiota and its associated shifts during cheese ripening at high precision.

Currently, the Minas artisanal cheese faces several changes in the legal procedures of its production, e.g. the optimal ripening time, which is not yet fully elucidated in order to obtain harmless products regarding potential pathogen overgrowth. Because of this, according to federal law, sixty days are considered the ideal ripening period for these cheeses, which raises a lot of questions from the scientific community and cheese producers, since sixty days of ripening can lead to several changes in the structure, physical-chemical, flavor and subsequent acceptance of this cheese on the market. Therefore, this study aimed i.) to assess the bacterial community of raw milk and of the endogenous starter culture, and ii.) to uncover possible shifts in the bacterial community of the rind and core of Minas artisanal cheeses produced in the *Serra do Salitre* region throughout the ripening time of 60 days in the dry season, via HTS by Illumina MiSeq amplicon sequencing.

2. Material and methods

2.1. Cheese making process

All cheeses were made from the same batch of milk in each farm, whereas the starter culture was obtained from previous cheese making and stored at room temperature for natural fermentation. The general process of cheese making is shown in Fig. 1.

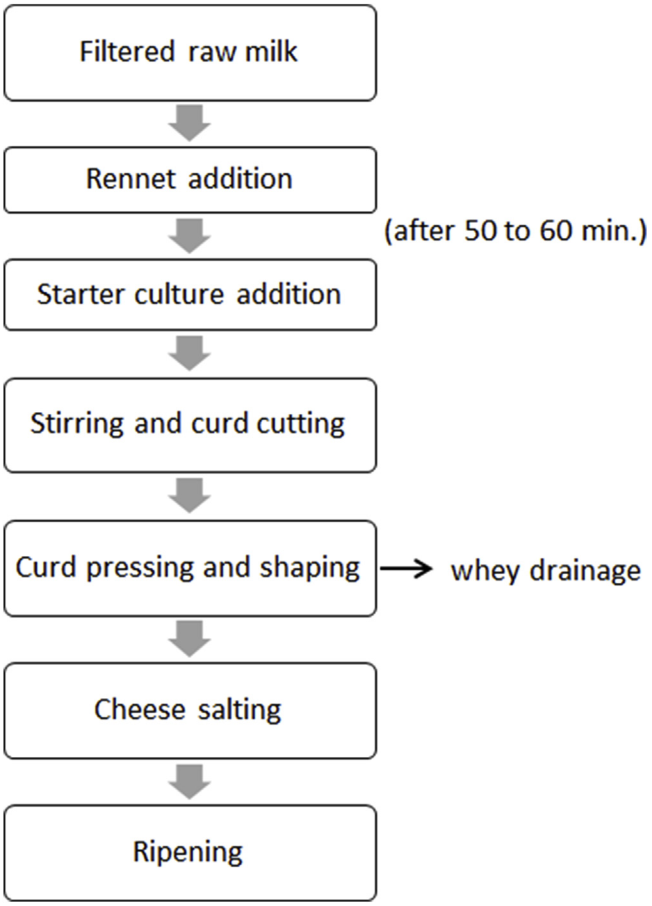


Fig. 1. Minas artisanal cheese making process.

2.2. Experimental design and sampling

A total of 14 cheese producers were accounted in the *Serra do Salitre* region at the time of this study, of which six producers were randomly selected for sampling. Cheeses from these producers were sampled at days 1, 7, 14, 21, 28, 45 and 60 of ripening. Raw milk and endogenous starter culture samples were also collected together with day one cheeses. The experimental design was: 6 (producers) x 2 (milk and starter culture); 6 (producers) x 2 (rind and core) x 7 (sampling time points during ripening), one negative control sample, resulting in a total of 97 samples for MiSeq sequencing.

Raw milk, endogenous starter culture and cheeses were all from the same batch of production. Raw milk was collected directly from the expansion tank in 25 mL sterilized flasks and stored under cooling at 7 °C. Endogenous starter culture was collected from a clean bucket to 25 mL sterilized flasks stored under cooling at 7 °C. Cheeses were wrapped in autoclaved aluminum foil and stored under cooling at 7 °C. All samples under cooling were processed in less than 24 h, in order to prevent major shifts in the microbial community. Sample acquisition, pre-treatment and DNA extraction were carried out at the Instituto de Ciências Biológicas (ICB) of the Universidade Federal de Minas Gerais (Belo Horizonte, Brazil).

2.3. Sample acquisition

Cheese samples were acquired in duplicate (2 cheese wheels) for each treatment. For the rind, sterile razorblades were used to scratch randomly chosen parts of the surface of both cheeses and then pooled to a total of 3 g. For cheese core, new sterile razorblades were used to collect randomly chosen parts of the interior of both cheeses and then pooled to a total of 3 g.

2.4. Physical-chemical analysis and metadata acquisition

Moisture, titratable acidity and pH from samples of milk, starter culture and cheeses were determined according to Castro et al. (2016). Factors such as average regional temperature (°C) and average precipitation (mm) were gathered from the “Instituto Nacional de Meteorologia” (INMET, 2016). Farms' geographic locations such as altitude, longitude and latitude were gathered from the “Intituto Mineiro de Agropecuária” (IMA, 2016, Minas Gerais, Brazil).

2.5. Sample pre-treatment

A total of 3 g of each cheese sample type (rind and core) were diluted (1:10) in 2% sodium citrate solution (w/v) in 15 mL Falcon tubes and homogenized using an Ultra Turrax (24,000 rpm for 1 min). In total, 5 mL of each raw milk sample and endogenous starter culture were diluted in 2% sodium citrate (1:10), transferred to 15 mL Falcon tubes and centrifuged at 4,000 × *g* for 15 min. The supernatant was discarded and the remaining pellet was diluted in 15 mL sodium citrate and homogenized using an Ultra Turrax (24.000 rpm for 1min). The shredder was cleaned with 2 passages in 70% ethanol solution (v/v) and deionized water. Then, 100 μL of iso-amylic acid was added to 1.5 mL of each cheese/milk/starter culture homogenate, incubated in a water bath at 45 °C for 10 min and then centrifuged at 10.000 × *g* for

10 min. The supernatant layer was discarded and the remaining pellet was incubated at 37 °C for 10 min in order to evaporate residual acid. The methodology for pre-treatment and DNA extraction protocol was adapted from Quigley et al. (2011).

2.6. DNA extraction

The pellets were suspended in 500 µL of lysis buffer (Tris HCl 20 nmol/L, EDTA 2 mmol/L, 2% Triton X-100, 10 mg/mL of lysozyme, 200 µg of lysostaphin). Then, 10 µL of proteinase K (25 mg/mL) were added to samples and incubated at 55 °C for 1 h. The sample suspensions were transferred into clean 2.5 µL micro tubes containing 0.3 g of zirconium beads and agitated for 10 min in a vortex. Later, samples were centrifuged at 12.000 × *g* for 10 min. Approximately 500 µL of supernatant was transferred into new clean 2.5 µL micro tubes and equal volume of phenol:chloroform:isoamylic acid (25:24:1) µL was added and then centrifuged at 12.000 × *g* for 10 min. The aqueous phase was transferred to clean 1.5 µL micro tubes and 1/10 of total volume of sodium acetate and 2 vol of cold pure ethanol were added. A no-template control (NTC) was included in the DNA extraction and processed together with all samples to assure contaminating DNA from the reagents could be excluded from the data set. The samples were stored at −20 °C until further processing. Samples were thawed at room temperature, centrifuged at 14.000 RPM/10 min and dried for 5–10 min in a dry incubator (approx. 50 °C). The dried pellets were purified using the Wizard SV Genomics Purification System (Promega, Wisconsin, USA) following the manufacturer's instructions and stored at −20 °C until further processing.

2.7. Library preparation and bacterial 16S rRNA gene amplicon sequencing

First-step PCR amplification and purification were performed at the Institute for Milk Hygiene, Milk Technology and Food Science of the University of Veterinary Medicine Vienna, Vienna, Austria. Standard PCR reaction was performed using *pfu* DNA polymerase (Promega) and V3/4/5 primers with 5‘-tails (357 F_hmp CCTACGGGAGGCAGCAG and 926 R_hmp CCGTCAATTCMTTTRAGT), generating 570 bp amplicons. Final concentrations of reagents were: 10 × Buffer with $MgSO_4$; 200 µM dNTP mix; 0.1 µM of each upstream and downstream primers; 0.5 µg of DNA template and 1.25 U of *pfu* DNA Polymerase. Nuclease-Free Water (Promega) was added to a final volume of 50 µl. Cycling conditions were: 95 °C for 2 min; 25 cycles: 95 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min and a final elongation at 72 °C for 5 min. Amplicon purification was performed using the GeneJET PCR Purification Kit® (Thermo Scientific, Massachusetts, USA) following the manufacturer's instructions.

Second-step PCR and Illumina® MiSeq (Illumina Inc., San Diego, California, USA) sequencing was performed at Microsynth facilities (Balgach, Switzerland). The V3/4/5 hypervariable region of bacterial 16S rRNA genes was amplified using primers with 5′-tails (341 F_CCTACGGGRSGCAGCAG Zakrzewski et al., 2012) and 5′-tails (909 R_TTT CA-GYCTTGCGRCCGTAC Tamaki et al., 2011). Libraries were constructed by ligating sequencing adapters and indexes onto purified PCR products. The Nextera XT Sample Preparation Kit (Illumina Inc.) was used following the manufacturer's recommendations. Amplicons were loaded onto Illumina® MiSeq cartridges with each paired-end sequence attached to its correspondent index. After sequencing, corresponding overlapping paired-end reads were stitched to get an approximate mean read length of 298 bp. Library preparation, sequencing and read-stitching was done by Microsynth.

2.8. Read processing

Paired-end sequences were demultiplexed in FASTQ file formats and downstream analysis was carried out using Qiime v.1.9.1 (Caporaso et al., 2010). Low quality sequences were trimmed using a –phred_quality_threshold of $p > 19$, and chimeras were checked and filtered using Usearch 6.1 by comparing sequences against the reference Gold database (http://drive5.com/uchime/gold.fa). Sequences were clustered into operational taxonomic units (OTU) with a 97% similarity cutoff (0.03 distance), and the SILVA SSU reference database version 119 (Pruesse et al., 2007) was used for alignment. OTUs with less than 10 sequences per OTU were excluded from the data set. An UNIFRAC (Lozupone and Knight, 2008) distance matrix was calculated using both weighted and unweighted measurements and visualization of two-dimensional PCoA plots was generated using Emperor (Vazquez-Baeza et al., 2013).

2.9. Data visualization

Files originated from QIIME pipeline were processed by using the phyloseq software v.1.20.0 (McMurdie and Holmes, 2014) for plot bar graphics using ggplot2 package v.2.2.1, both implemented in RStudio v.1.0.143 (RStudio Team, 2015).

2.10. Sample grouping and variability significance

Analysis of variance (ANOVA) for sample richness and diversity was performed using Graphpad Prism v5.0 software (Graphpad software, San Diego, CA, USA). Statistical significance testing of sample groupings was performed by permutation analysis of variance – adonis using unweighted UNIFRAC distance matrices as input data. In order to verify the variability between the interaction of sample-factors, distance based redundancy analysis (db-RDA) analysis method was applied, also using unweighted UNIFRAC distance matrices as initial input for constrained ordination outputs, both implemented in QIIME (*compare_categories.py* - statistical significance of sample groupings).

2.11. Differentially abundant OTUs

Differential OTU abundance analysis was performed using DeSeq2 package v1.16.1 (Love et al., 2014) in the phyloseq environment. In accordance to McMurdie and Holmes (2014) little to no rarefaction was performed for the differential abundance analysis, while samples that showed only less than 1.000 total counts were removed. Then, variance-mean dependence from sequencing data was calculated and tested for differential expression ($p < 0.05$) based on a model using a negative binomial distribution.

3. Results and discussion

3.1. Overall bacterial community in milk, starter culture, cheese core and cheese rind samples

A total of 4.821,455 sequences were counted resulting in 1.202 OTUs. To eliminate low read counts, a cutoff threshold of 3.066 sequences per sample was applied, achieving a total of 91 samples ($n = 91$ samples, with five being discarded), with a minimum of 164 and a maximum of 209.871 read counts per sample (mean = 50.223). Table 1 shows the total number of sequences per sample sequenced. After rarefaction, a total of 1.009 OTUs were assigned to six phyla: *Firmicutes* (87.0%), *Proteobacteria* (7.5%), *Actinobacteria* (3.7%), *Bacteroidetes* (0.5%), *Deinococcus-Thermus* (0.15%) and *Acidobacteria* (0.15%). As shown in Table 2, the number of observed OTUs was significantly increased ($p < 0.05$) from day one when compared to day fourteen of ripening, showing an increase in total OTU abundance within these cheeses. The total number of observed OTUs was significantly higher in farms B, D and F when compared to farm E ($p < 0.05$; 1.54-fold difference). According to the Simpson's diversity index, farms D and E showed significant higher ($p < 0.05$; 1.23-fold difference) diversity when compared to farm C. No statistical significances of estimated species richness were observed with regards to

Table 1
OTU counts for milk, starter and cheeses samples.

Farm	Source	Ripening time	OTU count
C	Cheese rind	14	164.0
A	Cheese rind	7	414.0
D	Cheese core	60	1557.0
C	Cheese core	28	1746.0
A	Cheese core	14	1890.0
C	Cheese rind	28	3066.0
D	Cheese core	21	3417.0
F	Cheese core	21	4885.0
D	Milk	zero	5072.0
D	Cheese rind	7	6638.0
C	Cheese core	60	9405.0
A	Cheese rind	60	10,779.0
C	Cheese core	45	11,967.0
A	Cheese rind	1	12,723.0
A	Milk	zero	13,290.0
E	Milk	zero	14,884.0
C	Milk	zero	16,116.0
F	Cheese rind	28	17,107.0
C	Chesse rind	14	20,310.0
E	cheese rind	45	21,374.0
B	Milk	zero	22,938.0
B	Cheese rind	60	24,340.0
A	Cheese core	45	24,866.0
F	Milk	zero	28,702.0
B	Cheese core	1	28,964.0
E	Cheese core	60	31,368.0
A	Cheese core	60	31,607.0
C	Cheese core	21	33,477.0
B	Cheese core	60	34,274.0
D	cheese core	14	34,361.0
A	Cheese rind	14	34,546.0
A	Cheese rind	7	37,823.0
C	Cheese rind	45	38,216.0
A	Cheese rind	45	38,421.0
E	Cheese rind	60	38,575.0
F	Cheese rind	60	39,099.0
F	Cheese core	60	39,858.0
B	Starter culture	zero	40,323.0
A	Cheese core	1	42,576.0
F	Starter culture	zero	42,629.0
E	Cheese rind	21	42,979.0
B	Cheese rind	1	43,496.0
B	Cheese rind	14	44,152.0
D	Starter culture	zero	45,328.0
B	Cheese rind	7	45,581.0
C	Cheese rind	7	46,058.0
F	Cheese core	7	46,088.0
D	Cheese core	28	46,114.0
C	Cheese core	7	46,233.0
E	Cheese rind	1	47,057.0
E	Cheese rind	1	47,485.0
F	Cheese rind	60	47,788.0
F	Cheese rind	45	47,865.0
C	Cheese rind	1	48,538.0
F	Cheese core	14	52,598.0
D	Cheese core	45	56,349.0
D	Cheese rind	60	57,122.0
E	Cheese core	14	57,586.0
E	Cheese rind	28	58,753.0
D	Cheese rind	1	59,396.0
A	Starter culture	zero	61,523.0
B	Cheese core	21	62,614.0
E	Starter culture	zero	63,000.0
D	Cheese rind	28	64,437.0
A	Cheese core	28	65,605.0
D	Cheese core	21	66,480.0
A	Cheese core	7	67,938.0
A	Cheese core	21	68,089.0
F	Cheese core	1	68,972.0
D	Cheese rind	45	69,276.0
E	Cheese core	1	69,520.0
E	Cheese core	21	69,784.0
F	Cheese rind	14	70,717.0
B	Cheese rind	45	71,434.0
C	Starter culture	zero	72,685.0
E	Cheese core	45	72,870.0
D	Cheese core	7	72,839.0
B	Cheese core	14	73,935.0
D	Cheese rind	14	47,387.0
F	Cheese core	28	78,465.0
B	Cheese rind	28	79,703.0
B	Cheese core	7	80,522.0
F	Cheese rind	1	81,791.0
F	Cheese core	45	81,997.0
A	Cheese rind	28	85,570.0
E	Cheese core	7	86,267.0
B	Cheese core	45	89,380.0
D	Cheese core	1	90,042.0
F	Cheese rind	21	90,519.0
E	Cheese core	28	90,756.0
D	Cheese rind	21	91,365.0
E	Cheese rind	7	94,543.0
B	Cheese core	28	101,316.0
B	Cheese rind	21	105,655.0
C	Cheese core	1	127,231.0
F	Cheese rind	7	209,871.0

Chao1 and Shannon index estimators. Fig. 2A shows an overall view of the bacterial shifts throughout ripening time regarding its source. Despite low correlation, there was significant difference of group variance (R^2: 0.05, $p < 0.001$) at genus and family levels. Fig. 2C shows that the factors related to ripening periods of 45 and 60 days were linearly significant when compared to other periods. Indeed there is a noticeable increase in relative abundance of the *Leuconostocaceae* and *Lactobacillaceae* families, mostly from day 14th onwards. According to De Pasquale et al. (2016), it is possible to assume that the high prevalence and abundance of the *Streptococcaceae* throughout ripening time may be associated to a secondary proteolysis, providing higher concentrations of essential aminoacids to the growth of non-starter LAB. Starter LAB have in their structure a caseinase associated to their membrane, which facilitates the breakdown of casein in peptides and amino acids that may be used by non-starter LAB. *Lactococcus lactis* is able to produce more than 100 different oligopeptides, due to the presence of the proteinase called PrtP (Steele et al., 2013).

It can be stated that in longer periods of ripening, the relative population of *Lactococcus* and *Streptococcus/Streptococcaceae* may gradually decrease, whereas the population of other families associated with late fermentations could increase significantly. In the present study, however, it is not possible to confirm this fact, although the evidence, especially on the 45th and 60th ripening days, points to this event. According to Gatti et al. (2014), the relationship between SLAB and NSLAB is modulated by the type of culture added to the milk cheese, the cheese production conditions and the maturation duration of the cheese.

Fig. 3A and 3. B show relative abundances at genus and family level from milk, starter, core and rind of different farms when compared amongst each other. OTUs classified as *Streptococcus* (43%), *Lactococcus* (17%)*, Staphylococcus* (11%) *Weissella* (8%) and *Lactobacillus* (6%) were amongst the most prevalent OTUs in all samples, while *Pseudomonas* (2%) was most abundant in raw milk samples. These findings are in accordance with the ones described by Arcurci et al. (2013) who found *Streptococcus salivarius (S. salivarius), Streptococcus thermophilus (S. thermophilus), Lactobacillus plantarum* (*Lb. plantarum*) and *Lactococcus lactis* (*L. lactis)* to be the predominant bacteria from Minas artisanal cheeses collected in four regions (*Serro, Cerrado, Serra da Canastra* and *Araxá*) via DGGE. Lacerda et al. (2011) also isolated and characterized *L. lactis* and *S. salivarius* as the most prevalent in cheeses at seven and sixty days of ripening, respectively. Castro et al. (2016) isolated and identified bacteria from Minas artisanal cheeses from the *Campo das*

Table 2
Statistical analysis of OTU richness of samples regarding ripening time, farm and source.

	Ripening time							60	*p*-value
	0	1	7	14	21	28	45		
observed OTUs	70 ± 28[a,b]	56.1 ± 18.67[a]	76.73 ± 26.27[a,b]	108.8 ± 40[b]	87 ± 32.09[a,b]	91.82 ± 46.61[a,b]	98.73 ± 38.25[a,b]	92.09 ± 29.07[a,b]	< 0.05[a]
Chao 1	158 ± 21	626 ± 41	290 ± 30	289 ± 33	310 ± 34	290 ± 29	167 ± 86	258 ± 136	NS
Shannon	221 ± 111	229 ± 128	251 ± 97	266 ± 139	248 ± 126	275 ± 90	257 ± 131	142 ± 126	NS
Simpson	0.54 ± 0.24	0.57 ± 0.32	0.68 ± 0.13	0.74 ± 0.14	0.61 ± 0.24	0.62 ± 0.16	0.65 ± 0.20	0.55 ± 0.21	NS
Farm									
	A	B	C	D	E	F	*p*-value		
observed OTUs	95.08 ± 39.63[a,b]	74.5 ± 25[b]	85.2 ± 37.85[a,b]	74.3 ± 22.23[b]	117.5 ± 45[a]	79.9 ± 24.26[b]	< 0.05[b]		
Chao 1	120 ± 24	102 ± 22	155 ± 20	145 ± 58	175 ± 61	231 ± 12	NS		
Shannon	209 ± 110	215 ± 109	123 ± 103	304 ± 100	305 ± 113	247 ± 087	NS		
Simpson	0.55 ± 0.18[a,b]	0.6 ± 0.21[a,b]	0.45 ± 0.27[a]	0.78 ± 0.23[b]	0.73 ± 0.21[b]	0.6 ± 0.22[a,b]	< 0.05[b]		
Source									
	Milk	Starter culture	Core	Rind	*p*-value				
observed OTUs	84 ± 30	58 ± 17	81 ± 26	94 ± 43	NS				
Chao 1	123.89 ± 33.4	175.46 ± 27.4	169.729 ± 2.69	146.596 ± 2.39	NS				
Shannon	2.28 ± 1.33	2.18 ± 0.7	2.33 ± 1.18	2.36 ± 1.32	NS				
Simpson	0.64 ±	0.48	0.62 ± 0.21	0.64 ± 0.20	NS				

[a] Ripening time contrasts were tested between all groups and non-significant differences were marked with same letters.
[b] Farm contrasts were tested between all groups and non-significant differences were marked with same letters.

Vertentes region and found a large dominance of *Enterococcus* species in these products. *Lactobacillus* species were amongst the most frequently detected by length-heterogeneity PCR in cheeses from the *Serra do Salitre* region, as described by Perin et al. (2017).

3.2. - Bacterial communities in milk

Milk samples showed a high relative abundance of *Pseudomonas* and *Streptococcus*-OTUs followed by OTUs classified as *Lactococcus,* and *Acinetobacter*. *Streptococacceae*, *Pseudomonadaceae*, *Moraxellaceae*, *Staphylococcaceae* and *Planococcaceae* were amongst the most abundant families. Differences between farms were observed at genus and family level, while farms A and B being the ones with highest relative abundance of *Pseudomonas* OTUs and *Pseudomonadaceae* family. Farms C and D showed highest relative abundance of *Streptococcus* and *Lactococcus* OTUs and *Streptococcaceae* family, whereas farm E showed highest relative abundance of *Acinetobacter*-OTUs, as well as *Streptococacceae* and *Staphylococcaceae* families. Farm F showed highest relative abundance of *Acinetobacter*-OTUs with major abundance of *Moraxellaceae* and *Planococcaceae* families. These findings are in accordance with Desmasures and Gueguén (1997) and Michel et al. (2001) who described high inter-farm variabilities. In the present study, this inter-farm variability is possibly due to different milking practices amongst the cheese producers such as teat cleaning, milk machine and milk line; indirect sources such as feed, litter, drinking and washing water, stable and milking parlour; air and environmental surfaces such as shelves, racks, wall and floors (Montel et al., 2014; Quijada et al., 2018). According to Gobbettii (2018) and Frétin (2018), after milking the first microbial contaminations are via the channel and the surface of the teat. Teat skin is a major source of microbial contamination for milk and is responsible for inoculating *Staphylococcus*, coryneforms, *Enterobacteriaceae*, *Clostridium* and *Pseudomonas* in the milk.

3.3. - Bacterial communities in starter cultures

Regarding the endogenous starter culture, the clear majority of OTUs were composed of *Streptococcus* and *Lactococcus* with a strong dominance of the *Streptococcaceae* family, endorsing the idea that starter cultures may greatly contribute to the high abundance of *Streptococcus*-OTUs in the cheese environment, while milk might rather introduce other genera such as *Lactococcus*, *Lactobacillus, Weissella, Brevibacterium,* etc. According to Irlinger et al. (2015), *Lactococcus lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* are the major components of the mesophilic lactic starter cultures widely used in cheese making and are frequently combined with *Streptococcus* genera. To the best of our knowledge, this is the first study describing the association between starter cultures and the *Streptococcaceae* family. This fact points towards the importance of maintaining cheese making traditions, especially regarding the Minas artisanal cheeses, where milk and starter cultures may contribute to a unique endogenous microbiota of cheeses (Montel et al., 2014). In the present study, all starter culture samples showed similar results regarding relative abundance, as shown in Fig. 2A, 2B, 3A and 3B. The clear majority of OTUs were associated to the *Streptococcaceae* family and *Lactococcus* genus, and no statistical significance was found regarding group variance. Most OTUs associated with *Streptococcaceae* and *Lactococcus* showed no significant difference in relation to inter-farm variability. According to Gatti et al. (2014), curd cooking temperature, the maintenance of a temperature gradient during the fermentation of starter culture and the increase of the acidity can lead to the selection of a characteristic microbiota, consisting mainly of thermophilic, aciduric and moderately heat resistant LAB. Starter culture is collected at the moment of cheese pressing and stored at room temperature overnight. This should be one of the main factors associated with the predominance of these microorganisms in the starter cultures of the *Serra do Salitre* region. Pogačić et al. (2013) showed that some *Lactobacillus* spp. strains were associated to natural whey cultures, while other strains such as *Lb. plantarum* were not detected in the starter culture used to make P.D.O Grana Padano cheeses. These findings support the idea that starter culture may be inoculating different strains of *Streptococcus* spp. in the cheese environment, which could explain, alongside specific clusters found in the PCoA (Fig. 3E) why the *Streptococcaceae* family is prevalent in all sources from the *Serra do Salitre* region. Other studies are necessary to establish a comparison between the microbiota of *Serra do Salitre* and other regions, in

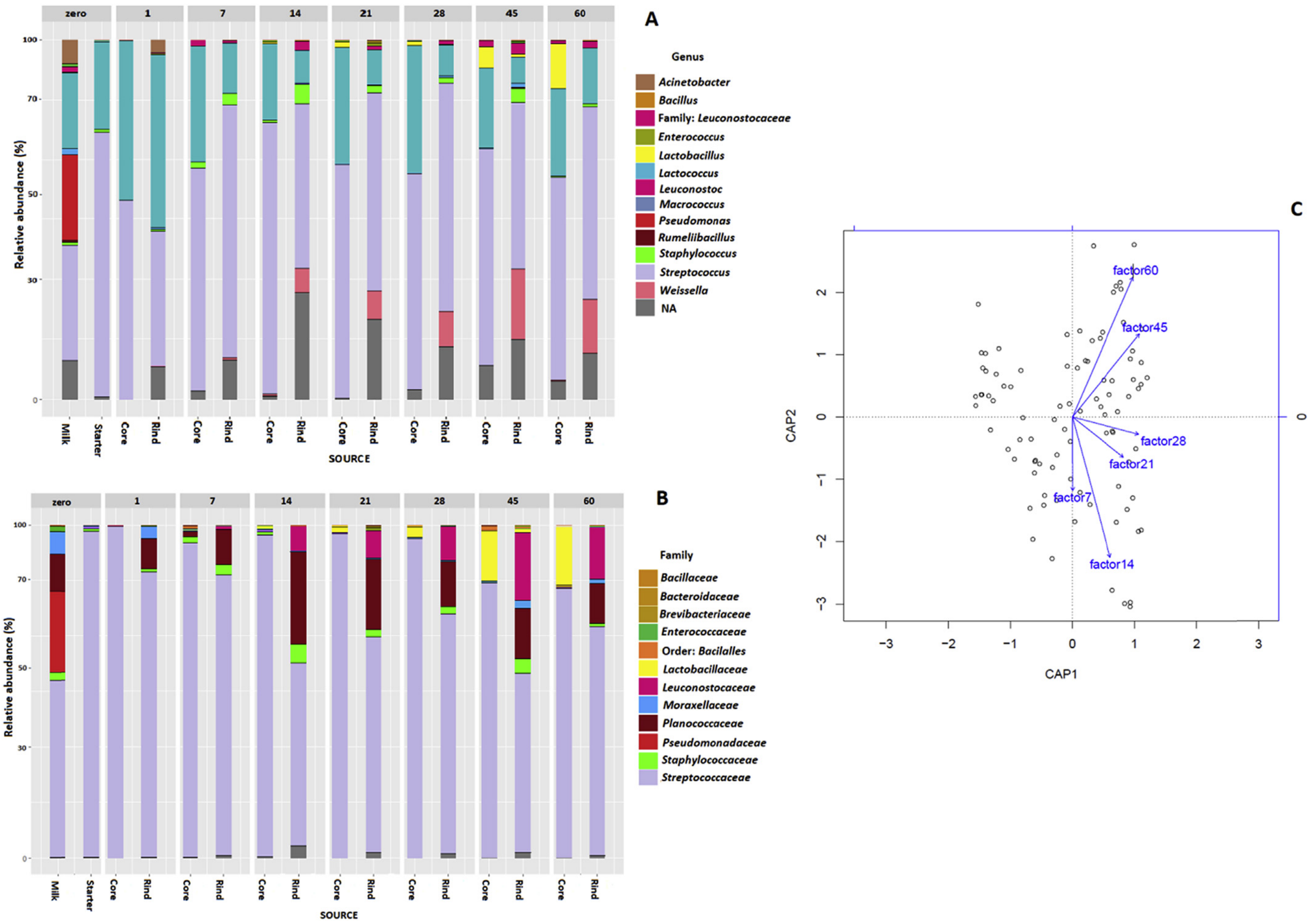


Fig. 2. Microbial shifts from milk, starter culture and cheeses (rind and core) throughout ripening time. A – Relative abundances (%) of microbial population from milk, starter and cheeses from 1 to 60th day of ripening at genus level. Horizontal gray boxes from zero to 60 represent ripening time, respectively (adonis: R^2: 0.05, $p < 0.001$). B - Relative abundances (%) of microbial population from milk, starter and cheeses from 1 to 60th day of ripening at family level. Horizontal gray boxes from zero to 60 represent ripening time, respectively (adonis: R^2: 0.05, $p < 0.001$). C - Distance-based redundancy analysis for ripening factor (db-RDA: $p < 0.001$, pseudo-f 1.932). Each factor represents a ripening time.

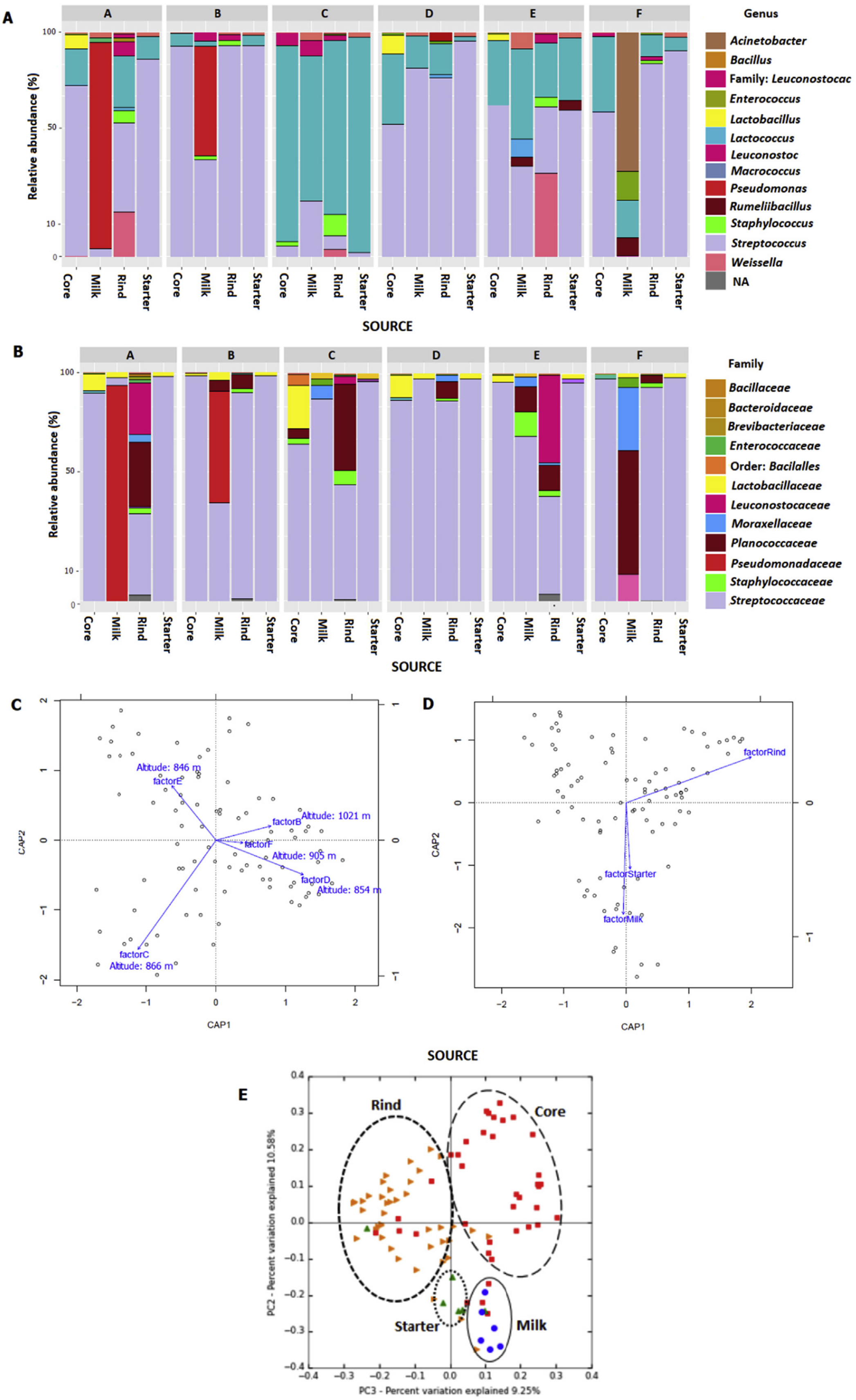
order to verify major intrinsic or extrinsic factors responsible for directing the microbial shifts in the starter culture.

3.4. Bacterial communities in cheese cores

The cheese core microbiota was mainly comprised of starter culture derived *Streptococcaceae* and *Lactobacillaceae* families in all farms. The most abundant genera were *Streptococcus, Lactococcus* and *Lactobacillus*. Farm C showed higher relative abundance of *Lactococcus* OTUs in comparison to the other farms. Interestingly, no OTUs for the *Pseudomonadaceae/Pseudomonas*; *Enterococcaceae/Enterococcus* and *Moraxellaceae/Acinetobacter* were detected in cheese cores at day 1 originated from farms that showed relative abundance of these microorganisms in the milk. During cheese making, the main shifts in microbial composition occurred during curd production and ripening, which is endorsed by the detection of *Lactobacillus* OTUs in the core of these cheeses (O'Sullivan et al., 2015; Ogier et al., 2004). According to Bove et al. (2011), the detection of biotypes that correlate with specific moments in cheese ripening or differential development throughout this process suggests that these strains may have specific roles closely linked to their peculiar technological properties. Due to physical–-chemical shifts, selective pressure on the microbiota is intense, consequently favoring microbial species that are specifically adapted to these environmental constraints (Irlinger and Mournier, 2009; Goerges et al., 2008), and possibly influenced by the "Jameson effect" - a term used to describe a once fast increase in a certain microbial population, rapidly ending after a short growth period because of limitation in some nutrients, or competition with some specific microbial populations (Jameson, 1962). In accordance to this study, Luiz et al., 2017 isolated and identified bacteria from cheeses of the *Araxá* region throughout ripening time (from days one to 57) in dry and rainy seasons and found that *Lactococcus* develop an important role at the start of the ripening process, while *Lactobacillus* species slowly grow to establish dominance in the cheese environment. Bouton et al. (2009) described that in the cores of ripened cheeses, volatile profiles associated with cheese flavor were affected only by *Lactobacilli*. Further studies are necessary to verify the influence of the *Lactobacillus* species over the sensorial aspects of the Minas artisanal cheese.

3.5. Bacterial communities in cheese rinds

Rind samples showed linear differences when compared to other sources. Still, a *Streptococcus*-OTU was dominant within all farms, followed by a *Lactococcus*-OTU. Farm A showed a higher relative abundance of *Brevibacterium*, *Staphylococcus* and *Weissella*-OTUs, while *Acinetobacter*-OTU was detected in high abundance in samples from farm D. Farm E showed a high abundance of a *Weissella*-OTU, whilst a *Leuconostoc*-OTU was enriched in cheese rinds from farm F. The most prevalent OTUs in cheese rinds were classified as *Streptococcaceae*, *Planococcaceae* and *Staphylococcaceae*. Cheese ecosystems may be



(caption on next page)

Fig. 3. Microbial population from milk, starter culture and cheese (rind and core) according to farms. A – Relative abundances (%) of microbial population from farms at genus level. Horizontal gray boxes from A to F represent farms, respectively (adonis: R^2: 0.17, $p < 0.001$). B - Relative abundances (%) of microbial population from farms at family level. Horizontal gray boxes from A to F represent farms, respectively (adonis: R^2: 0.12, $p < 0.001$). C - Distance-based redundancy analysis among farms (db-RDA: $p < 0.001$, pseudo-f 3.57). Each letter represents a farm with their respective altitude values. D - Distance-based redundancy analysis for source (db-RDA: $p < 0.001$, pseudo-f 3.98). Each factor represents a source. E − Principal Coordinates (2D-PCoA) of UNIFRAC unweighted matrices from milk (blue circles), starter (green triangles), core and rind sources (red squares and orange triangles, respectively), represented by different circled lines. NA – not assigned samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

associated with the presence of unique microbes and thus leading to unique microbial interactions that can develop remarkable flavors, such as a cheese signature. Moreover, these microbial interactions are most frequently related to cheese rinds and their association with human intervention. (Cheeseman et al., 2014; Passerini et al., 2010). Cheese-specific signatures may be affected by a greater diversity of rind samples, relative to the presence of a set of different microbial species, rather than a particular species. This might explain why *Staphylococcus* is a commonly prevalent genus in Minas artisanal cheeses, mainly because the wheels are routinely manipulated by hand to turn the cheese wheels throughout the ripening period (Castro et al., 2016), also by using raw milk, in which *Staphylococcus* is a commonly found species as one of the main causes of subclinical mastitis in cows. Many phylotypes are common to most cheese varieties but have varying relative abundances and levels of complexity within the microbial community. The microbiota of cheese rinds consists in complex assemblages, harboring *Firmicutes* (*Lactobacillus* ssp., *Lactococcus* ssp., *Enterococcus* ssp., *Staphylococcus aureus, Staphylococcus equorum, Staphylococcus sciuri*), *Actinobacteria (Arthrobacter* ssp., *Brevibacterium linens, Brevibacterium aurantiacum, Brevibacterium jeotgali, Corynebacterium variabile), Proteobacteria (Acinetobacter* ssp., *Escherichia coli, Enterobacter* ssp., *Halomonas* ssp., *Pseudomonas gessardii, Pseudomonas formosensis, Pseudoalteromonas haloplanktis*, *Proteus* ssp.), *Bacteroidetes* (*Sphingobacterium lactis*), yeasts (*Candida* ssp., *Debaromyces* ssp., *Geotrichum* ssp.) and moulds (*Cladosporium* ssp., *Penicillium* ssp., *Scopulariopsis* ssp.) (Irlinger et al., 2015; Schornsteiner et al., 2014). The *Planococcaceae* family was prevalent in all cheese rind samples from all farms, which could be specific within these cheeses and indicate that this family may play a major role in the ripening processes, although studies describing the role of this family in foods are sparse. According to Wang et al. (2015), it is hypothesized that the *Planococcaceae* family may affect the quality and flavor of fermented beverages by producing secondary metabolites, which could also be possible to occur in these cheeses. Interestingly, the *Rummeliibacillus* species - a *Planococcaceae* member - was previously isolated from soils of the Cerrado region, where the farms are located (Coba et al., 2012). To the best of our knowledge, this is the first study describing a prevalence of this family associated with cheese rinds. According to this study, Wolfe et al. (2014) described the genera *Jeotgalibacillus*, a *Planococcaceae* member, as sub-dominant in cheese rind communities. The role of the *Weissella* genus and *Leuconostocaceae* family have not yet been fully elucidated regarding cheese environments (Chombo-Morales et al., 2016; Fuka et al., 2013; Masoud et al., 2012). According to McSweeney and Sousa (2000) *Leuconostoc* spp. are used as secondary starters in Dutch type cheeses, primarily to produce diacetyl, acetoin and CO_2, added intentionally to grow by environmental conditions. Escobar-Zepeda et al. (2016) described that the presence of these microorganisms could reveal an authentic signature in the Cotija cheese. Litoupoulou-Tzanetaki and Tzanetakis (2014) isolated both genera from a Greek cheese facility, while Settanni et al. (2012) and Masoud et al. (2012) described the presence of these strains isolated from wood surface of the containers used in cheese manufacture and raw milk, respectively. The presence of *Planococacceae* and *Leuconostocaceae* families in the rind suggests that the Minas artisanal cheese is strongly associated with the environment (cheese wheel manipulation, ripening shelves, ripening chamber/cellars), highlighting the concept of geographical location and regional microbiota, once many of the microbes that co-occur within rind communities also co-occur in their source environment (Quijada et al., 2018; Engel et al., 2010; Verdier-Metz et al., 2012). Bokulich and Mills (2013), Goerges et al. (2008) and Mounier et al. (2005) also described that the processing environment forms distinct functional niches, selecting for the species that perform the best in that environment regardless of inoculation.

Fig. 3C and 3 D show distance-based redundancy analysis among farms (db-RDA: $p < 0.001$, pseudo-f 3.57/adonis: R^2: 0.17, $p < 0.001$) and source (db-RDA: $p < 0.001$, pseudo-f 3.98/adonis R^2: 0.12, $p < 0.001$), indicating that these factors contribute to variability amongst microbial diversity. Interestingly, farms E (altitude 846 m) and C (altitude 866 m) were more discriminant in contrast to other values of altitude, with both showing greater diversity at genus and family OTU-levels, which could indicate an interaction of altitude and other abiotic factors such as pasture, cow feeding, breed composition, quality of water, etc. Fig. 3E shows principal coordinates (PCoA) of UNIFRAC unweighted matrices from milk, starter, core and rind sources, indicating that phylogeny is source-dependent. De Filippis et al. (2016) detected differences in gene expression within microbial communities of cheese surfaces and cores, possibly contributing to this natural source-dependence. According to current results, further studies are necessary to confirm the same findings from De Filippis and collaborators. De Pasquale et al. (2016) also identified differentiated metabolic profiles in samples from the core of the cheese and samples from the border region between core and rind, detecting high levels of proteolysis and synthesis of volatile compounds associated to the rind and core border. According to the results of this work, more in-depth studies are needed to confirm that these processes are also occurring in the Minas artisanal cheese of the *Serra do Salitre* region. Wolfe et al. (2014) showed that divergence in community composition is best explained by the rind type of the cheese (washed, bloomy, and natural), whereas country of origin, milk treatment or milk source are weakly associated with community divergence. In the present study, despite low correlation values, farm and sources are discriminant factors for the bacterial diversity.

3.6. *Abiotic factors play a major role in cheese bacterial community shifts*

Fig. 3A and 4. A shows bacterial diversity and relative abundance at family level of samples throughout ripening time correlated with the acidity content of milk, starter, cheese rind and core, as well as moisture content for each sample, given in percentage. Table 6 shows values of acidity and moisture for each individual sample. *Planococcaceae* family was prevalent throughout ripening process and not affected by moisture, which supports our previous assumption that it is indeed a prevalent family in the environment and consequently in cheese rinds. *Leuconostocaceae* and *Lactobacillaceae* families showed increased abundance associated with lower moisture values throughout ripening times, which is supported by their non-starter lactic acid bacteria (NSLAB) characteristics (Gobbetti et al., 2018 and Wouters et al., 2002). Wolfe et al. (2014) found that moisture is the best predictor of rind community composition, being significantly associated with the gradient in surface moisture measured across natural, washed, and bloomy rind cheeses. In the present study, moisture content from all sources was measured throughout ripening time and microbial communities are strongly correlated with this factor (adonis $R^2 = 0.65$, $p < 0.01$). PCoA depicts distinct microbial community clusters (Fig. 4D) as well as linear relationships of moisture (Fig. 3B) (db-RDA

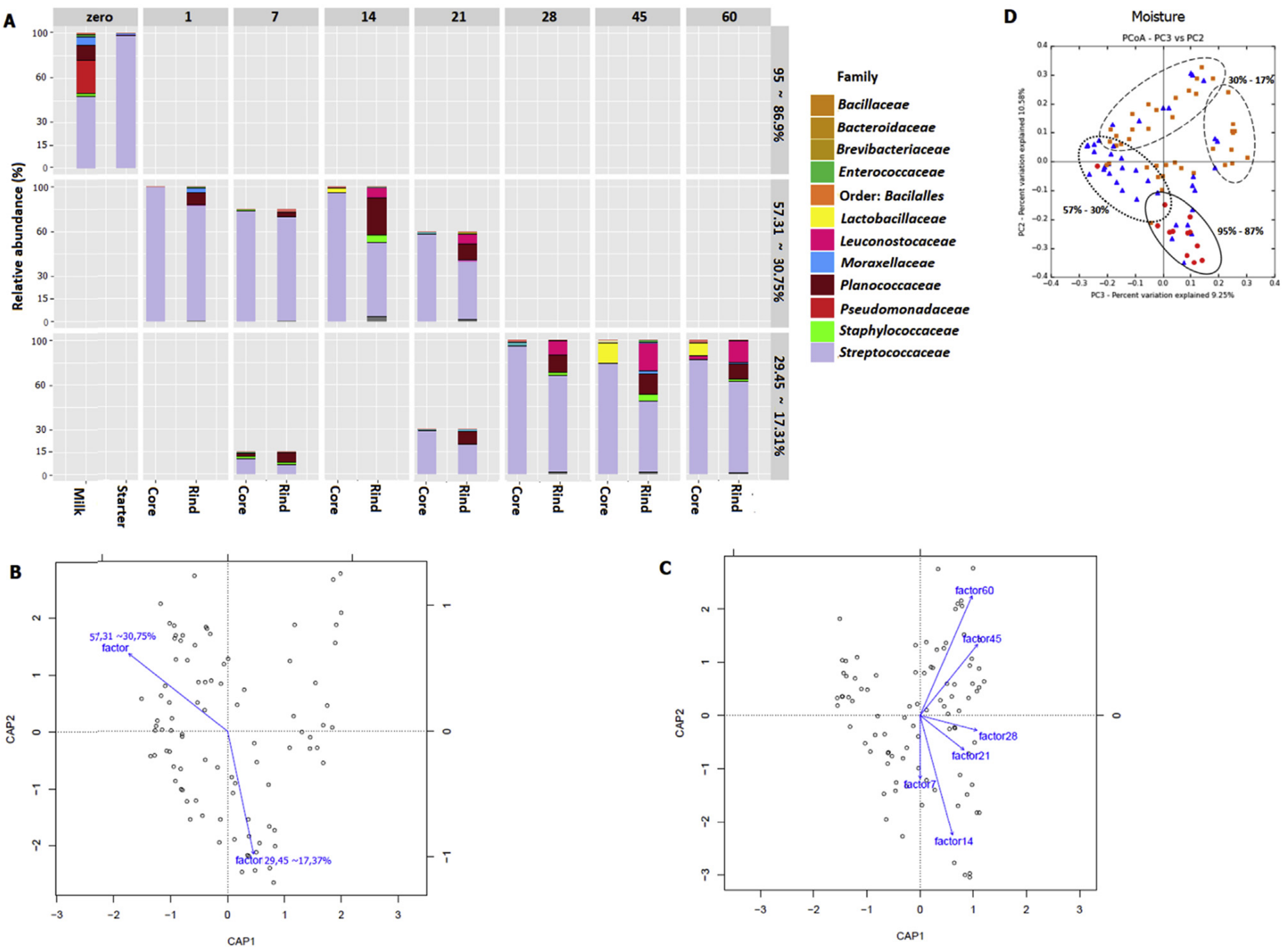


Fig. 4. Microbial shifts from milk, starter culture and cheese (rind and core) according to moisture content. A – Relative abundances (%) of microbial shifts in a combination of source, ripening time and moisture content percentage (adonis $R^2 = 0.65$, $p < 0.01$). Horizontal gray boxes from 1 to 60 represent ripening period. Vertical gray boxes represent moisture content. Moisture content percentage was obtained by gravidity method. B - Distance-based redundancy analysis for moisture content ($p < 0.01$, pseudo-F: 3.42). Each factor represents a moisture content percentage. C - Distance-based redundancy analysis for ripening time ($p < 0.01$, pseudo-F: 1.93). Each factor represents a ripening period. D - Principal Coordinates (2D-PCoA) of UNIFRAC unweighted matrices from distinct moisture content percentage, represented by different circled lines. Orange squares represents a 17–30% of moisture content; blue triangles represent 30–57% of moisture content and red circles represents 87–95% of moisture content. NA – not assigned samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

($p < 0.01$, pseudo-F: 3.42). Ripening time also presented a linear relationship for this model ($p < 0.01$, pseudo-F: 1.93) (Fig. 4C), with factors 45 and 60 days as ripening periods being the most discriminant when compared with other ripening time days. Due to lower activity of water and higher O_2 concentrations, an adverse environment on the cheese surfaces is set, delaying NSLAB development until further periods (De Filippis et al., 2016). In the present study, these abiotic factors are discriminately different by the increasing abundance of *Leuconostocaceae* and *Lactobacillaceae* families.

As expected in lower acidity, *Pseudomonadaceae*, *Moraxellaceae* and *Streptococcaceae* families were more abundant when compared with higher acidity parameters (Fig. 5A) (Bachman and Spahr, 1995). This could be explained by the fact that *Streptococcaceae* members are acting as starter lactic acid bacteria, possibly by the interaction of both *Lactococcus* and *Streptococcus* genera (De Filippis et al., 2014; Ercolini et al., 2008 Fox et al., 2004). Results show that acidity was also highly correlated (adonis $R^2 = 0.43$, $p < 0.01$) and linearly significant (db-rda $p < 0.01$, pseudo-F: 3.00) with the microbial shifts (Fig. 5B). PCoA analysis (Fig. 5C) shows distinct source and acidity clusters, possibly by the establishment of microbial interactions related with the fermentation process, once the *Streptococcacea* family associated with milk consists of different OTUs when compared to the ones found in starter, which has a higher acidity value when compared to milk, due to natural fermentation process. *Planococcaceae* family was surprisingly prevalent in both acidity values (0.17–0.31 and 0.45 to 0.78), indicating that these bacteria are quite pH resistant and play a major role in both situations by probably establishing strong interactions with the *Leuconostocaceae* family in the surface of these cheeses, leading to a natural separation of phylotypes. However, further studies are necessary in order to unravel these assumptions. In the present study, pH from all sources was measured and also showed strong correlation with microbial shifts during ripening time (adonis R^2: 0.43, $p < 0.001$), presenting strikingly similar results in the microbial shifts when compared to acidity values, probably due to a higher concentration of lactic acid in these cheeses. According to Sadler and Murphy (2010) titratable acidity is not related to pH because the acidity detection method is based only on the total concentration of lactic acid in a sample whereas pH is a combined function of titratable acidity and conjugated bases, supporting the idea that nonstarter LABs are in fact contributing to pH reduction via lactic acid metabolism.

Average temperature in the region also influenced the microbial shifts throughout ripening as shown in Fig. 6A, as *Lactobacillaceae* and *Leuconostocaceae* families achieved higher values of relative abundance at an average temperature of 23 °C (adonis R^2: 0.04, $p < 0.001$). In the

Table 3
DESeq2 analysis shows differentially abundant OTUs by source comparison.

Source	OTU abundance	log2FoldChange
Starter x core	↑*Macrococcus caseolyticus*	9.50
	↑*Planococcaceae*	9.02
	↑*Streptococcus agalactiae*	7.00
	↑*Staphylococcus*	5.80
	↑*Lactococcus*	4.45
	↓*Streptococcus*	−2.20
Starter x milk	↑*Planococcaceae*	14.00
	↑*Weissella*	13.90
	↓*Streptococcus agalactiae*	−9.60
	↓*Lactococcus garvieae*	−10.30
	↓*Streptococcus*	−10.32
	↓*Enterobacteriaceae*	−10.50
Starter x rind	↓*Streptococcus*	−3.60
	↓*Planococcaceae*	−5.45
	↓*Lactococcus*	−6.16
	↓*Streptococcus dispersa*	−6.45
	↓*Staphylococcus*	−7.43
	↓*Weissella*	−9.77
Milk x rind	↑*Pseudomonas*	13.27
	↑*Lactococcus garviae*	12.83
	↑*Streptococcus agalactiae*	8.62
	↓*Streptococcus*	−4.32
Milk x core	↑Enterobacteriaceae	9.75
	↑*Macrococcus caseolyticus*	9.50
	↑*Lactococcus garviae*	9.50
	↑Planococcaceae	6.80
	↑*Streptococcus agalactiae*	8.76
	↑*Staphylococcus*	5.87
	↑*Lactococcus*	4.45
Rind x core	↑*Weissella*	8.60
	↑*Staphylococcus*	8.20
	↑*Brevibacterium*	8.10
	↑*Corynebacterium variable*	8.70
	↑*Staphylococcus sciuri*	8.10
	↑*Staphylococcus aureus*	8.10
	↑*Staphylococcus equorum*	7.90
	↑*Macrococcus caseolyticus*	7.65
	↑*Brevibacterium aureum*	7.70
	↑*Pseudomonas*	4.30
	↑Micrococcaceae	4.20
	↓*Streptococcus*	−2.90
	↓*Lactococcus*	−2.80
	↓*Lactobacillus brevis*	−2.40
	↓*Lactobacillus zeae*	−2.70

Log2FoldChange negative values indicates that the OTU is more abundant in the second factor when compared to the first one, as shown by the UP or DOWN arrows. Table is showing only significant values for Log2FoldChange ($p < 0.05$).

Table 4
DESeq2 analysis shows differentially abundant OTUs by titratable acidity comparison.

Acidity (°D)	OTU abundance	Log2FoldChange
0,17–0,31 × 0,45–0,78	↓*Brevibacterium aureum*	−7.42
	↓*Weissella*	−5.85
	↓*Lactobacillus brevis*	−5.44
	↓*Lactobacillus zeae*	−5.33
	↓Micrococcaceae	−3.94
	↑*Lactococcus*	2.80

Log2FoldChange negative values indicates that the OTU is more abundant in the second factor when compared to the first one, as shown by the UP or DOWN arrows. Table is showing only significant values for Log2FoldChange ($p < 0.05$). Acidity values shown consists of titratable acidity using °D (Dornic).

Table 5
DESeq2 analysis shows differentially abundant OTUs by moisture content comparison.

Moisture	OTU abundance	Log2FoldChange
29.45–17.37%	↓*Streptococcus*	−1.266
	↓*Lactobacillus brevis*	−3.96
	↓*Leuconostoc mesenteroides*	−4.43
	↓*S.agalactiae*	−4.45
	↓*Weissella*	−5.38

Log2FoldChange negative values indicates that the OTU is more abundant in the second factor when compared to the first one, as shown by the UP or DOWN arrows. Table is showing only significant values for Log2FoldChange ($p < 0.05$). Moisture values are shown in percentage by the gravidity method.

Table 6
Moisture (%) and acidity values (°D) from samples of milk, starter culture and Minas artisanal cheese throughout ripening time.

Source	Ripening time	Farm	Moisture (%)	Acidity (°D)
Milk	zero	A	87,3	0,15
		B	86,9	0,16
		C	88,1	0,14
		D	87,6	0,18
		E	86,9	0,17
		F	87,9	0,15
Starter	zero	A	92,3	0,45
		B	95	0,60
		C	93,7	0,57
		D	90	0,71
		E	91,2	0,50
		F	93,3	0,78
Cheese (rind and core)	1	A	55,33	0,17
		B	54,55	0,14
		C	49,44	0,11
		D	57,31	0,11
		E	48,48	0,27
		F	49,22	0,21
Cheese (rind and core)	7	A	43,4	0,19
		B	39,89	0,17
		C	29,71	0,34
		D	36,08	0,26
		E	41,83	0,18
		F	30,75	0,23
Cheese (rind and core)	14	A	39,65	0,27
		B	34,84	0,31
		C	31,16	0,4
		D	35,18	0,41
		E	38,79	0,42
		F	32,28	0,42
Cheese (rind and core)	21	A	31,88	0,4
		B	28,65	0,53
		C	26,94	0,51
		D	32,38	0,55
		E	31,87	0,60
		F	31,75	0,40
Cheese (rind and core)	28	A	28,38	0,45
		B	24,21	0,59
		C	22,6	0,87
		C	22,6	0,87
		D	26,22	0,62
		E	28,42	0,63
		F	23,24	0,72
Cheese (rind and core)	45	A	29,45	0,45
		B	27,75	0,59
		C	18,45	0,87
		D	25,42	0,62
		E	23,37	0,63
		F	29	0,72
Cheese (rind and core)	60	A	21,16	0,53
		B	19,54	0,58
		C	17,37	0,68
		D	19,82	0,64
		E	21,97	0,65
		F	26,04	0,36

present study, daily and regular temperature measurement in the ripening room was not possible to cover due to some difficulties the cheese makers had with the thermostat handling given to them. The measured data for average temperature was acquired from a local

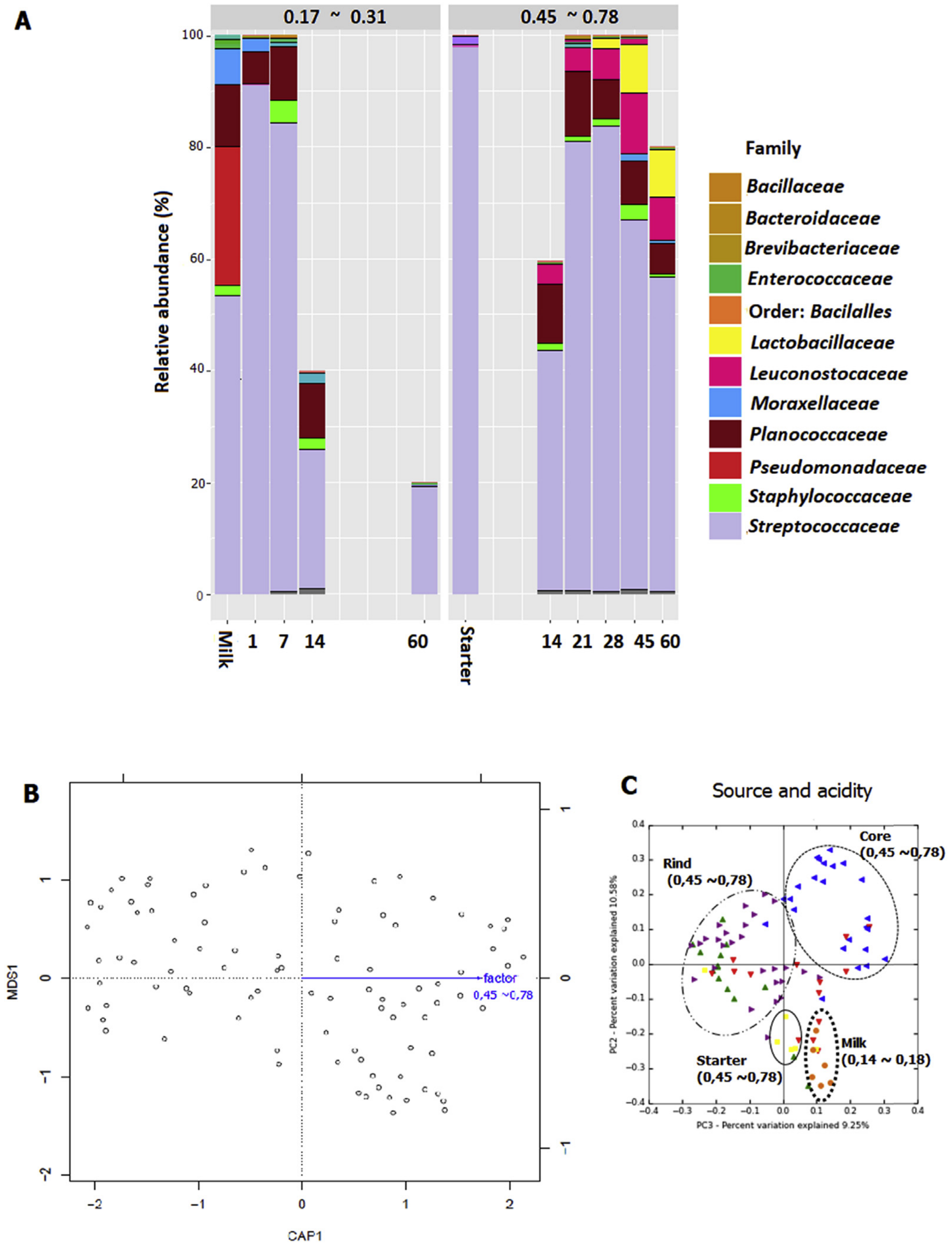


Fig. 5. Microbial shifts of milk, starter culture and cheeses in all ripening periods, according to acidity values. A – Relative abundances (%) of microbial shifts in a combination of ripening time and acidity values (adonis $R^2 = 0.43$, $p < 0.01$). Horizontal gray boxes represent acidity intervals (titratable acidity in °D). B - Distance-based redundancy analysis for moisture content ($p < 0.01$, pseudo-F: 3.2). Each factor represent a moisture content percentage. C - Principal Coordinates (2D-PCoA) of UNIFRAC unweighted matrices from combined source and acidity intervals, represented by different circled lines. Orange circles represents milk acidity values from 0.14 to 0.18 ^{0}D; yellow squares represent starter culture acidity values from 0.45 to 0.78 ^{0}D; purple triangles represents rind acidity values from 0.45 to 0.78 ^{0}D and blue triangles represents core acidity values from 0.45 to 0.78 ^{0}D. NA – not assigned samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

station located near all farms. Despite the fact that these values were not daily based, it can be assumed that average temperature drives microbial shifts especially at temperatures higher than 22 °C, as depicted in Fig. 6B showing factors 22 °C and 23 °C being linearly different (db-rda: $p < 0.001$, pseudo-F: 2.09). This fact also endorses the importance of geographical locations, as samples from this study were collected at the dry season (Brazil's winter) and the average temperature is distinctly higher than the mean temperature for other cheese producing regions in the state.

3.7. Differentially abundant OTUs

In order to investigate specific shifts in relative abundance of OTUs, DESeq2 analysis was performed to detect which OTUs were over or under represented, associated with sources and abiotic factors. Negative values for log2FoldChange implies that this OTU is more abundant in the second factor when compared to the first one. Table 3 shows differentially abundant OTUs with regards to the sample sources. *Planococcaceae*, *Staphylococcus, Macrococcus caseolyticus, Streptococcus agalactiae* and *Lactococcus* OTUs were significantly more abundant in the endogenous starter culture and milk samples when compared to core samples (average of 6.78 fold-enrichment), which could be explained by the fact that both milk and starter cultures are not very distant regarding phylogenetic distances in microbial diversity, as shown in multivariate analysis (Fig. 3E). *Macrococcus caseolyticus* is considered an adventitious bacterium and is reported to disappear when milk and/or cheese curd is submitted to high temperatures, e.g. *pasta filata* cheeses. The presence of this species at lower abundances in the milk could be linked to longer storage time under irregular temperature (Gianinno et al., 2009; Randazzo et al., 2002). *Streptococcus agalactiae* is

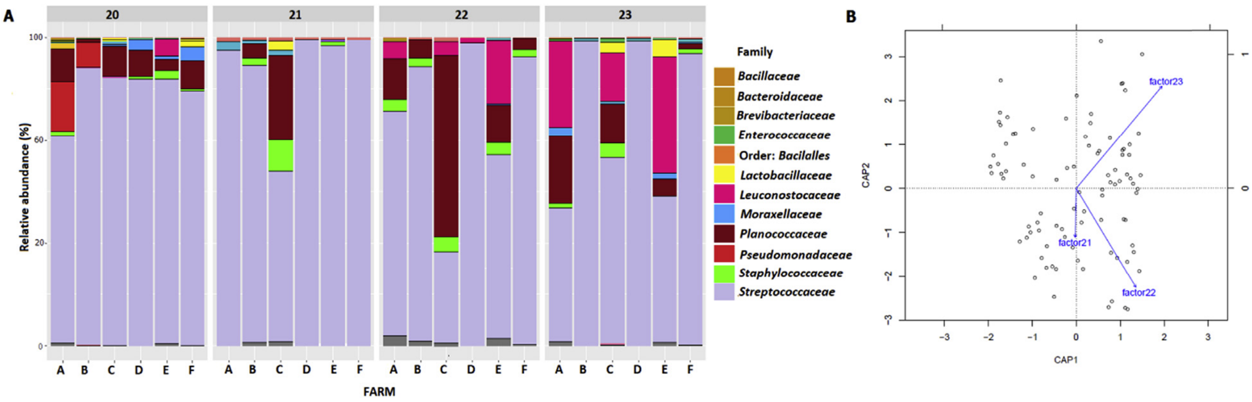


Fig. 6. Shifts in microbial population from all farm sources according to average temperature in the region. A – Relative abundances (%) of microbial shifts in a combination of farms (averaged values of milk, starter culture, rind and core) and average temperature values (°C) (adonis $R^2 = 0.04$ $p < 0.01$). Horizontal gray boxes represent average temperature values. B - Distance-based redundancy analysis of average temperature content ($p < 0.001$, pseudo-F: 2.09). Each factor represents an average temperature of the region. NA – not assigned samples.

a very well-described bacterium associated with mastitis (Soggiu et al., 2018). *Planococcaceae* and *Weissella* OTU were more abundant in starter samples when compared to milk, indicating that the latter might be more responsible for inoculating starter LAB in the cheese, while starter culture could provide environmental and non-starter lactic acid bacteria. Cheese rinds showed higher numbers of differentially abundant OTUs when compared to the core, while only *Lactobacillus brevis* (*Lb. brevis)* and *Lactobacillus zeae* (*Lb. zeae*) were linked to the core. De Filippis et al. (2016) also described that cheese ripening was driven by few non-starter lactobacilli, e.g. *Lb. casei* and *Lb. buchneri*, groups whose abundance was greater in the cheese core compared to the rind. The presence of *Brevibacterium* and *Corynebacterium* OTUs are important for cheese ripening due to their proteolytic activity and because they produce volatile sulfur compounds or ammonia (Eliskases-Lechner and Ginzinger, 1995). Carnio et al. (2000) described that *S. equorum* is known for producing aromatic characteristics and orange pigments by proteolytic and lipolytic enzymes. Indeed, some cheese wheels showed a yellow to orange color, which could be explained by the presence of this particular species in the rind. As expected, NSLAB OTUs classified as *Brevibacterium, Weissella* and *Lactobacillus* were more abundant in lower acidity values, as shown in Table 4. This reinforces the idea that the ripening time must be adequate in order to let the establishment of these bacteria settle down as temperature rises. Table 5 shows that only in lower moisture content the abundance of *Streptococcus, Lb. brevis, Leuconostoc mesenteroides, S. agalactiae*- and *Weissella*-OTUs was higher. In accordance to current results, De Filippis et al. (2016) stated that NSLAB at 10 days of ripening were significantly more abundant when reduction in relative moisture and increase in the ripening temperature were observed, when compared to the control conditions. In the present study, NSLAB started to increase in relative abundance at the 14th day of ripening with highest abundances at the 45th and 60th days of ripening. Interestingly, not only NSLAB were differentially abundant, but also *Streptococcus*-OTUs which could be related to a specific environmental adaptation by these genera.

4. Conclusions

The bacterial community in the Minas artisanal chees is greatly affected by the bacterial community of the milk and starter culture used as raw material. Shifts in the bacterial community structure throughout the ripening time are strongly correlated with abiotic factors such as geographical location, moisture and acidity. Given the artisanal nature of the cheese, natural variation in the microbiota may occur regarding seasonal aspects, environmental aspects and human intervention. Further studies are needed in order to establish the true core microbiota from these cheeses. However, this study points to a trend in the core microbiota being highly associated to the *Streptococcaceae* in rind and core, as well as the *Planococcaceae* mostly associated with the rind of these cheeses. There seems to be an environmental adaptation by the bacteria, as shown in distinct clusters by PCoA from source, moisture and acidity. *Streptococcaceae* and *Planococcaceae* were prevalent in cheeses throughout ripening time, while *Lactobacillaceae* and *Leuconostocaceae* appeared in later stages of the ripening process. The *Planococcaceae* family is linked to the environmental aspect of the region and possibly plays a major role in the ripening process, especially on cheese rinds. It is possible that the microbial signature of these cheeses, as well as their interactions with other microbial communities rely on the presence of this *Planococcaceae* family. Further studies will be necessary to uncover the putative benefits of this family for the cheese environment regarding physical-chemical parameters and the functional analysis of the microbial community.

Conflicts of interest

The authors declares that there is no conflict of interest regarding the publication of this article.

Acknowledgments

This work was conducted during a scholarship supported by the International Cooperation Program CAPES/COFECUB at the University of Veterinary Medicine in Vienna.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.02.016.

Funding

This work was financed by CAPES – Brazilian Federal Agency for Support and Evaluation of Graduate Education within the Ministry of Education of Brazil; the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) – Brazilian Federal Agency of the Ministry of Technology, Innovations and Communication and the University of Veterinary Medicine in Vienna. Wetzels, S.U. and Wagner, M. are affiliated to the FFoQSI, funded by the Austrian ministries BMVIT, BMDW and the Austrian provinces Niederoesterreich, Upper Austria and Vienna within the scope of COMET -Competence Centers for Excellent Technologies. The Program COMET is handled by the Austrian Research Promotion Agency FFG.

We would also like to thanks Andreas Zaiser, Benjamin Zwirzitz, Canan Keskinöz, Isabel Rabanser, Viktoria Neubauer, Sandra and Viviane from EMATER – Empresa de Assistência Técnica e Extensão Rural do Estado de Minas Gerais and the IMA – Instituto Mineiro de Agropecuária for their support and also the cheese-making community for their cooperation throughout this entire experiment. The support of Monika Dzieciol during raw data submission is also highly acknowledged.

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