International Journal of Food Microbiology 307 (2019) 108277

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Transformation of raw ewes' milk applying “Grana” type pressed cheese technology: Development of extra-hard “Gran Ovino” cheese

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ARTICLE INFO

Keywords:
Hard cheese
Grana type cheese
Illumina technology
Lactic acid bacteria
Raw ewes' milk
Volatile organic compounds

ABSTRACT

This work was carried out to pursue a double objective: to improve the hygienic safety of cheeses produced from raw ewes' milk; and to produce a new typology of raw ewes' milk through the application of “Grana” technology for which the name “Gran Ovino” was chosen. With this in mind, raw milk from an individual farm was transformed under controlled conditions at a dairy pilot plant. The production technology included the partial skimming of the evening and morning milk mixture by cream surfacing and the addition of a natural whey starter cultures (NWSC) prepared with four selected *Streptococcus thermophilus* strains (PON6, PON244, PON261 e PON413). Ten microbial groups were investigated by plate counts from raw milk until ripened cheeses. Lactic acid bacteria (LAB) were in the range 10^4–10^5 CFU/ml before NWSC addition. After curdling, this group increased by 3 log cycles and was counted at 10^6 CFU/g after curd cooking. A rapid pH drop (to 6.05) was registered after almost 3 h from NWSC addition. The levels of members of the *Enterobacteriaceae* family were at about 10^3 CFU/ml in raw milk and decreased after curd cooking to 1 log cycle. A similar behavior was shown by the other undesired microbial groups and a complete disappearance of staphylococci was registered. The microbiological counts of 9-month ripened cheeses showed the dominance of LAB and undetectable levels of the undesired bacteria. MiSeq Illumina was applied to better investigate the bacterial composition of ripened cheeses and this technique evidenced that the majority of OTUs belonged to *Lactobacillus* and *Streptococcus* genera. The final cheeses were characterized by 67.65% dry matter of which 41.85% of fats and 47.02% of proteins. The main cheese fatty acids were palmitic, oleic and myristic acids and the saturated fatty acids/unsaturated fatty acids ratio was 2.17. Forty-one volatile compounds, including acids, esters, ketones, alcohols, aldehydes, phenols and one terpene were emitted from the cheese. Sensory evaluation showed a general appreciation for the new cheese product by judges.

1. Introduction

Olson (1990) affirmed that “there is a cheese for every taste and a taste preference for every cheese”. This statement evidences the high diversity of cheeses produced worldwide. In past, several technologies have been developed to transform a few raw materials, usually bovine, ovine, caprine or buffalo milks (McSweeney et al., 2004) and, nowadays, a great diversity of dairy products, mainly cheeses, are available. Italy boasts a high range of traditional raw milk cheeses (Settanni and Moschetti, 2014) and, generally, each cheese possesses unique characteristics that depend on the transformation method applied.

Milk, due to its richness in nutritional components, represents a growth medium for several microorganisms (Settanni and Moschetti, 2010). For this reason, raw milk may hosts human pathogens, dairy spoilers as well as useful fermentative agents, such as lactic acid bacteria (LAB) (Franciosi et al., 2011). LAB found in raw milk are considered “indigenous milk bacteria” but their presence derives from the contamination of the udder surface, of the milking procedures and milking equipment, stable environment, transport, vat surfaces, and dairy factory environment (Eneroth et al., 1998; Mc Phee and Griffiths, 2002; Scatassa et al., 2015). These bacteria are fundamental to transform milk into cheese (Widyastuti and Febrisiantosa, 2014) because they are responsible for the acidification of the curd (starter LAB) and are implicated in the ripening process (non starter LAB) (Settanni and

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https://doi.org/10.1016/j.ijfoodmicro.2019.108277
Received 14 May 2019; Received in revised form 3 July 2019; Accepted 27 July 2019
Available online 29 July 2019
0168-1605/

Moschetti, 2010). However, when raw milk is transformed by a traditional process, LAB found in cheese do not derive only from milk, because the rennet used for curdling which is generally in form of animal rennet pastes (Cruciata et al., 2014) and the surfaces of the equipment used during cheese making also contribute to LAB transfer (Cruciata et al., 2019).

Italian hard and extra-hard cheese varieties are subjected to a long ripening period, usually 6–24 months (McSweeney et al., 2004), during which non starter LAB are actively responsible for the development of the aroma compounds (Yvon and Rijnen, 2001). From the economic point of view, the most important cheeses produced in Italy are Grana Padano and Parmigiano Reggiano, both belonging to the “Grana” cheese typology, whose productions reached 3,7 billions € at production in 2017 (Rapporto Ismea-Qualivita, 2017). Both cheeses are produced from raw cow's milk and are typical of northern Italy. In the South part of Italy the main traditional hard cheeses belong to “Pecorino” cheese typology and are made from raw ewes' milk. Several Italian Pecorino cheeses enjoy a protected designation of origin (PDO) status. Among these cheeses, PDO Pecorino Siciliano cheese is produced throughout Sicily, a large region (25,711 km^2) representing an extended production area with the result that the cheeses produced in distant locations are characterized by different profiles in terms of sensory characteristics and microbial populations (Guarcello et al., 2016).

Cheese microbial ecology might be particularly affected by the raw milk and the technology applied for transformation. Until the first decade of the 2000s, the microbial ecology of any food sample was basically investigated by a culture dependent approach consisting of the isolation of bacteria and their identification by a combined phenotypic/genotypic methodology. Following this strategy, Grana Padano and Parmigiano Reggiano cheeses were found to host different *Lactobacillus* species, such as *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus*, during ripening (Coppola et al., 1997, 2000; Monfredini et al., 2012; Solieri et al., 2012). LAB collected at dominant levels from ripened Pecorino Siciliano cheeses belong to different genera and those found at consistent levels were identified as *Enterococcus faecium*, *Enterococcus faecalis*, *Lactococcus garvieae*, *Pediococcus acidilactici*, *Streptococcus macedonicus* and *Lactobacillus brevis*, *L. paracasei*, *Lactobacillus plantarum*, *Lactobacillus pentosus*, *L. rhamnosus* and *Lactobacillus curvatus* among non starter lactobacilli (Todaro et al., 2011; Vernile et al., 2008).

We are now living next generation sequencing (NGS) technologies era. The approaches based on these tools have impressively accelerated research in biological science during the last few years by enabling the production of large volumes of sequence data (Knief, 2014). Of course, NGS technologies have been also applied to the study of food microbial ecology (De Filippis et al., 2017), including that of cheeses (De Filippis et al., 2014). The results related to Grana type cheeses can be summarized in the clear dominance of *Lactobacillus* over the LAB community of ripened cheeses (Bassi et al., 2015; Soggiu et al., 2016). So far, no papers are available in literature on the application of NGS tools on the investigation of the microbial community of Pecorino Siciliano cheese, but *Lactobacillus* was the dominant genus throughout ripening of Pecorino Crotonese (De Pasquale et al., 2019) and Pecorino Sardo (O'Sullivan et al., 2015) cheeses.

Due to the technology applied, despite the stressing physicochemical parameters that characterize ripened cheese, Pecorino Siciliano cheese might still host undesired spoilage microorganisms (Settanni et al., 2013; Todaro et al., 2011), while the presence of these microbial groups in Grana type cheeses is much more limited and they are generally characterized by a high microbiological quality. Grana and Pecorino cheese productions differ substantially in several points, first of all for the type of milk processed, but, from the hygienic perspective, the most relevant step is represented by the curd cooking (Salvadori del Prato, 1998) carried out during Grana type cheese protocol application. This step represents an effective thermal treatment (55 °C), also because it is applied to the curd after its disruption to rice-size grains, determining a stronger and more rapid temperature penetration than immersion of cheeses in hot deproteinised whey (applied for Pecorino cheeses) with the consequence that temperature sensitive microorganisms decrease in number during Grana cheese production.

Milk is a fragile substance, thus preserving its quality right from milking until it is processed in the dairy industry has always been a challenge and a permanent concern (Vara Martinez et al., 2018). Based on the observation that raw ewes' milk is not currently transformed to produce ovine Grana type cheese the main objectives of this work were i) to improve the hygienic safety of cheeses processed from raw ewes' milk, and ii) to produce a new typology of raw ewes' milk through the application of Grana type cheese technology. In order to validate the new protocol for processing raw ewes' milk, a natural whey starter culture (NWSC) with selected strains of *Streptococcus thermophilus* was developed and the microbiological populations were monitored from raw milk until ripened cheeses. The final cheeses were subjected to the high-throughput analysis, to the physicochemical characterization, and to the sensory evaluation.

2. Materials and methods

2.1. Natural whey starter culture preparation

In order to carry out the experimentation a NWSC was developed with four strains of *Streptococcus thermophilus* (PON6, PON244, PON261 and PON413) previously isolated from raw ewes' cheese productions and evaluated for their dairy performances (Gaglio et al., 2014a). All strains were reactivated for 24 h in M17 broth (Oxoid, Milan, Italy) incubated at 44 °C. The cells were subjected to a washing procedure consisting of two consecutive centrifugations at 5000g × 5 min and resuspension of the pellet in Ringer's solution (Sigma-Aldrich, Milan, Italy). The final resuspension of the cells occurred at an optical density at 600 nm (OD_{600}) of ca. 1.00 evaluated by the spectrophotometer Jenway Ltd. model 6400 (Dunmow, UK). Washed cells were then inoculated at about 10^6 CFU/ml in the whey-based medium (WBM) prepared as described by Settanni et al. (2012), using non-acidified ewes' milk whey in place of cows' whey.

2.2. Cheese production and sample collection

Cheese productions were carried out in controlled conditions at a dairy pilot plant [Istituto Zooprofilattico Sperimentale (IZS) della Sicilia “Adelmo Mirri”, Palermo, Italy] level to avoid environmental contamination by dairy factory LAB. Milk was transformed using the POLYFOOD system (mod. SI-050, INVENTAGRI™, Modena, Italy). Raw ewes' milk from the indigenous Sicilian sheep breed “Valle del Belice” was provided by the artisanal dairy farm (Ovini e Natura, Santa Margherita del Belice, Italy) selected for its high hygienic standards. Bulk milk (100 *l*) was transformed following the flowsheet reported in Fig. 1 adapted from the classical “Grana” cheese type technology. To this purpose, the entire bulk milk was delivered once daily and, in order to simulate evening and morning milking, 50 l of bulk milk were immediately cooled to 4 °C in a refrigerated vat under low stirring to avoid clustering of fat globules with the consequent floating (Kohnhorst, 2001) as well as microbial proliferation (Franciosi et al., 2011), while the other 50 l were placed into a trapezoidal 60 l-shallow tank to allow the rising of fats (creaming) during the overnight rest at room temperature (Mucchetti and Neviani, 2006). The day after, skimmed milk was transferred into a copper vat, added with the whole milk kept at 4 °C and heated at 38 °C. Bulk milk was inoculated with 1.6 l of NWSC (Gaglio et al., 2016), subjected to vigorous stirring for 20 s and added with 25 g of an artisanal lamb rennet paste provided by the Rennet Regional Consortium (Poggioreale, Italy) dissolved in 1 l of tap water. After coagulation, the curd was broken manually with a planetarium stainless steel curd knife until rice-seed grains were obtained. Broken

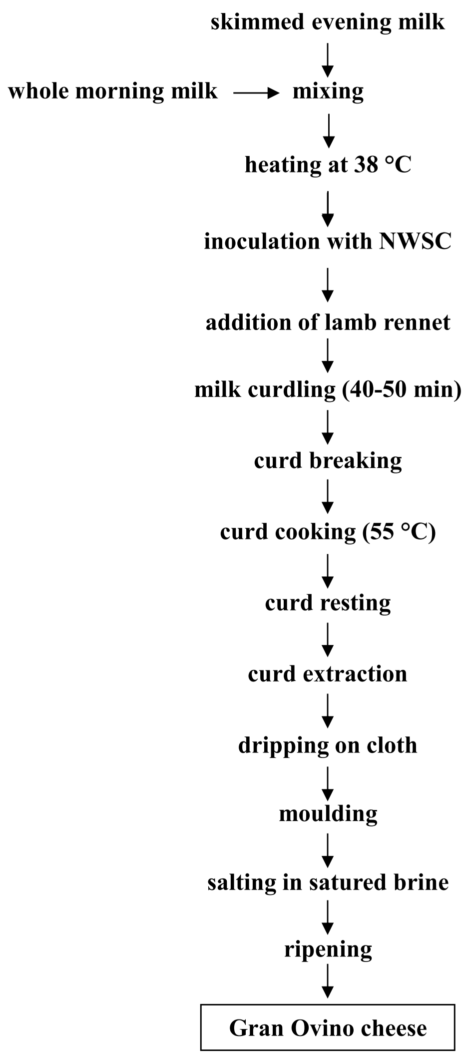


Fig. 1. Flowsheet of Gran Ovino cheese production. Adapted from Grana Padano cheese technology (Mucchetti and Neviani, 2006).

curd was then treated at 55 °C for 8 min (curd cooking step) under agitation and then left to precipitate for 1 h during which the grains welded into a single mass. The rested curd was removed from the vat and left to drip onto a cotton cloth for 30 min and then transferred into a plastic moulder and turned upside down after 3 h for a uniform whey syneresis. Buckets with 15 kg water were put on the top of all drained curds for 12 h in order to facilitate further draining by pressing. Salting was performed by immersion in brine containing NaCl (300 g/l) for 60 h. The ripening occurred at 14–16 °C and 85% relative humidity. Cheese production was carried out in quadruplicate in four consecutive weeks (one production per week).

The measurement of pH during cheese making (from milk to curd) was carried out with a portable pH-meter (Eutech Instruments, Nijkerk, The Netherlands). The temperature of milk during the skimming process was monitored through the 175-T2 data logger (Testo, Settimo Milanese, Italy) registering data every 30 min. Cheese temperature during ripening (until the 9th month) was monitored through Thermo Button 22 T 8 K data loggers (VWR International Srl, Milano, Italy) inserted in the core of the curds at moulding and registering data every 24 h.

The following samples were collected for each cheese production: evening whole milk (EWM), skimmed milk (SM) after overnight separation of fat globules, vat milk (VM) obtained after mixing EWM with SM, inoculated milk (IM) after addition of NWSC, cooked curd (CC) after treatment at 55 °C, cooked whey (CW) resulting after curd breaking and 9-month ripened cheese (RC). All samples were kept refrigerated during transport occurred by means of an insulated box containing reusable ice packs to the Agricultural Laboratory of University of Palermo and to the laboratories of Milk Centre and Mastitis Control of Istituto Zooprofilattico Sperimentale della Sicilia (Palermo).

2.3. Microbiological analyses

All samples were subjected to the serial decimal dilution procedure. Milk and whey samples (1 ml) were diluted into Ringers' solution. Cheeses were sampled as indicated by Monfredini et al. (2012), in order to analyse the entire cheese profile. To this purpose, three portions (of 10 g each) per cheese were collected, including under rind, middle section and core and mixed together. The first dilution of curds (15 g) and cheeses (30 g) was performed in 2% (w/v) of Na-citrate solution, homogenized by the stomacher BagMixer® 400 (Interscience, Saint Nom, France) at the maximum speed for 2 min. Serial dilutions continued into Ringers' solution.

The microbial groups investigated belonged to the dairy desired community as well as to the undesired community including both spoilage and pathogenic populations. Plate count agar (PCA) added with 1 g/l of skimmed milk (SkM) was used for the total mesophilic microorganisms (TMM) when incubated at 30 °C for 72 h or to count total psychrotrophic microorganisms (TPM) performing the incubation at 7 °C per 7 d. LAB community was investigated on five different media/temperature conditions: mesophilic LAB rods were plated on de Man-Rogosa-Sharpe (MRS) agar acidified with 5 M lactic acid to pH 5.4 and incubated at 30 °C for 48 h; thermophilic LAB rods on WBM agar incubated at 44 °C for 48 h; mesophilic and thermophilic LAB cocci on M17 agar incubated at 30 and 44 °C, respectively, for 48 h; enterococci on kanamycin aesculin azide (KAA) agar incubated at 37 °C for 24 h. Incubation of all LAB groups except enterococci occurred in anaerobiosis using the AnaeroGen AN25 (Oxoid) in jars closed hermetically. Members of the *Enterobacteriaceae* family were detected on violet red bile glucose agar (VRBGA) after incubation at 37 °C for 24 h. Yeasts were grown on dichloran rose bengal chloramphenicol (DRBC) agar incubated at 28 °C for 48 h. Baird Parker (BP) agar added with rabbit plasma fibrinogen was used to reveal the presence of coagulase-positive staphylococci (CPS) for 48 h at 37 °C. *Escherichia coli* was investigated applying the method AFNOR BIO 12/25-05/09 (2009), *Salmonella* spp. by the method AFNOR BIO 12/32-10/11 (2011), and *Listeria monocytogenes* by the method AFNOR BIO 12/11-03/04 (2004). All media and supplements were purchased from Oxoid. All plate counts were carried out in duplicate.

2.4. Isolation and identification of cheese LAB

Presumptive LAB, as being Gram-positive (Gregersen KOH method) and catalase-negative (unable to catalyse 3% H_2O_2 to H_2O), were randomly picked up from the highest plated dilutions of cheese suspensions on MRS, M17 and WBM agar considering all different colony types (color, morphology, edge, surface and elevation). The isolates were purified by successive sub-culturing by streaking on the same media used for plate counts, transferred to the corresponding broth media (isolates from WBM where cultivated in MRS broth) containing 20% glycerol (v/v) and stored at −80 °C until further characterization.

Genomic DNA from cheese LAB was extracted after overnight growth in the optimal media using the Instagene Matrix kit (Bio-Rad, Hercules, CA) following manufacturer's instructions and used for differentiation of the isolates at strain level as well as for their genetic identification.

Strain typing was approached by randomly amplified polymorphic DNA (RAPD)-PCR analysis as described by Gaglio et al. (2017) using singly the primers AB111, AB106 and M13 (Stenlid et al., 1994; van den

Braak et al., 2000). Electrophoresis on 2% (w/v) agarose gels (Gibco BRL, Cergy Pontoise, France) was performed to separate DNA amplicons which were visualised, after staining with the SYBR® safe DNA gel stain (Molecular probes, Eugene, OR, USA), by an UV trans-illuminator. RAPD profiles were analysed through Gelcompare II software version 6.5 (Applied-Maths, Sint-Marten-Latem, Belgium) and the isolates showing different patterns were considered to represent different strains.

The isolates were also investigated for their main phenotypic traits by observing their cell morphology through an optical microscope, by determining their growth at 15 and 45 °C, and their metabolic characteristics such as CO_2 production from glucose, carried out in Durham's tubes with the optimal growth media that did not contain citrate, acid production from different sources (arabinose, ribose, xylose, fructose, galactose, lactose, sucrose and glycerol), NH_3 production from arginine (Abd-el-Malek and Gibson, 1948), and aesculine hydrolysis (Qadri et al., 1980). LAB cocci were also evaluated for their ability to grow in presence of 0.65% (w/v) NaCl and at pH 9.2 to directly identify enterococci, showing growth in both conditions.

All different LAB strains were identified genetically by sequencing of the 16S rRNA gene and comparison of the sequences in public databases (GenBank and EZ-taxon) by BLAST search. PCR reactions were carried out following the protocol described by Weisburg et al. (1991) with the primer pair fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′)/rD1 (5′-AAGGAGGTGATCCAGCC-3′). After confirming the molecular size of the amplicons (about 1600 bp) on agarose gels, the PCR products were purified using the QIAquick purification kit (Quiagen S.p.a., Milan, Italy) and sequenced using the same primers used for PCR amplification at AGRIVET (University of Palermo, Italy). The identities of the sequences were determined by a blastn search against the NCBI non-redundant sequence database and by comparison with the sequences of the sole type strains within the EzTaxon database (https://www.ezbiocloud.net/taxonomy).

2.5. Preparation of the MiSeq library

A 464-nucleotide sequence of the bacterial V3-V4 region (Baker et al., 2003) of the 16S rRNA gene (*Escherichia coli* positions 341 to 805) was amplified. Unique barcodes were attached before the forward primers to facilitate the pooling and subsequent differentiation of samples. To prevent preferential sequencing of smallest amplicons, the amplicons were cleaned using the Agencourt AMPure kit (Beckman coulter, Brea, CA, USA) according to manufacturer's instructions. The DNA concentration of amplicons was determined using the Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. In order to ensure the absence of primer dimers and to assay the purity, the generated amplicon libraries quality was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the High Sensitivity DNA Kit (Agilent). Following quantitation, the cleaned amplicons were mixed and combined in equimolar ratios. Pair-end sequencing was carried out at Genomic Platform – Fondazione Edmund Mach (San Michele a/Adige, Trento, Italy) using the Illumina MiSeq system (Illumina, USA).

2.6. Illumina data analysis and sequences identification by QIIME2

Raw paired-end FASTQ files were demultiplexed using idemp (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights Into Microbial Ecology (Qiime2, version 2018.2). Sequences were quality filtered, trimmed, de-noised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences were identified and removed via the consensus method in DADA2. Representative sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Katoh and Standley, 2013; Price et al., 2009). Taxonomic and compositional analyses were conducted by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier). A pre-trained Naive Bayes classifier based on the Greengenes 13_8 97% Operational Taxonomic Units (OTUs) database (http://greengenes.secondgenome.com/), which had been previously trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads to generate taxonomy tables.

The data generated by Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. PRJNA542786.

2.7. Physico-chemical analyses of cheeses

Cheese samples were analysed for dry matter (DM), fat, protein (TN × 6.38), carbohydrates and ash content according to IDF standards 4A (IDF, 1982), 5B (IDF, 1986), 25 (IDF, 1964a) and 27 (IDF, 1964b), respectively. The maturation index, which indicates the proteolysis in the cheese, was calculated as ratio between N-soluble and total Nitrogen. Salt content was determined by Volhard method (AOAC, 2000). Measurements of pH were performed electrometrically by the pH-meter DocuMeter Sartorius (Data Weighing Systems, Inc., Elk Grove, IL, USA). Water activity (a_w) was determined according to the ISO 21807 (2004) using the HygroPalm water activity indicator (Rotronic, Bassersdrof, Germany).

Cheese color was analysed on the top surface by a Minolta tristimulus Chromometer CR-300 (Minolta, Osaka, Japan) using CIELAB L*a*b* values (Hunter, 1975). The measure of lightness (L* values, range 0–100) represents black to white, the redness measurement (a* values) describes green to red, and the yellowness measurement (b* values) represents blue to yellow. Beside these attributes, a* and b* values were also used to determine hue angle and chroma: hue angle (a*/b*) gives the predominant wavelength composing the color; chroma or saturation [$\sqrt{(a^2 + b^2)}$] accounts for the vividness or the color purity. The chromometer was standardized using a white standard plate. The results reported are averages of five measurements on the same cheese slice.

Fatty acids (FA) were determined on lyophilized cheese samples (100 mg) which were directly methylated with 2 ml of 0.5 M $NaOCH_3$ at 50 °C for 15 min, followed by 1 ml of 5% HCl in methanol at 50 °C for 15 min (Lee and Tweed, 2008). Fatty acid methyl esters (FAME) were recovered in hexane (1.5 ml). One microliter of each sample was injected by auto-sampler into a HP 6890 gas chromatography system equipped with a flame-ionization detector (Agilent Technologies Inc., Santa Clara, CA). Fatty acid methyl esters from all samples were separated using a 100-m length, 0.25-mm i.d., 0.25-μm capillary column (cp-sil 88; Chrompack, Middelburg, the Netherlands). The injector temperature was kept at 255 °C and the detector temperature was kept at 250 °C, with a H_2 flow of 40 ml/min, air flow of 400 ml/min, and a constant He flow of 45 ml/min. The initial oven temperature was held at 70 °C for 1 min, increased at 5 °C/min to 100 °C, held for 2 min, increased at 10 °C/min to 175 °C, held for 40 min, and then finally increased at 5 °C/min to the final temperature of 225 °C and held for 45 min. Helium, with a head pressure of 158.6 kPa and a flow rate of 0.7 ml/min (linear velocity of 14 cm/s) was used as the carrier gas. Fatty acid methyl ester hexane mix solution (Nu-Chek Prep Inc., Elysian, MN, USA) was used to identify each FA. The identification of the conjugated linoleic acid (CLA) isomers was performed using a commercial mixture of cis- and trans-9,11- and 10,12-ocdecadienoic acid methyl esters (Sigma-Aldrich) and published isomeric profiles (Kramer et al., 2004; Luna et al., 2005).

2.8. Volatile organic compounds

Volatile organic compound (VOC) were determined using the headspace solid phase microextraction (SPME) technique coupled with gas chromatography with mass spectrometric detection (GC/MS). The cheeses, frozen at −20 °C, were manually grated and 5 g of each cheese

Table 1
Microbial evolution during experimental Gran Ovino cheese production[a].

Growth media	Samples							Statistical significance[b]
	EWM	SM	VM	IM	CC	CW	RC	
PCA-SkM 7 °C	5.9 ± 0.2^{a}	5.5 ± 0.3^{ab}	5.6 ± 0.2^{a}	5.3 ± 0.3^{ab}	$< 2^{c}$	n.d.	4.9 ± 0.2^{b}	***
PCA-SkM 30 °C	6.6 ± 0.2^{a}	6.0 ± 0.3^{a}	6.1 ± 0.2^{a}	6.6 ± 0.2^{a}	4.9 ± 0.4^{b}	4.4 ± 0.2^{b}	6.0 ± 0.1^{a}	***
MRS	3.5 ± 0.1^{c}	5.3 ± 0.4^{b}	5.2 ± 0.4^{b}	7.0 ± 0.2^{a}	6.3 ± 0.4^{a}	5.1 ± 0.1^{b}	6.7 ± 0.2^{a}	***
WBAM	2.3 ± 0.1^{d}	3.8 ± 0.4^{c}	2.8 ± 0.2^{d}	6.9 ± 0.3^{a}	6.3 ± 0.4^{a}	5.2 ± 0.3^{b}	6.8 ± 0.2^{a}	***
M17 30 °C	6.6 ± 0.2^{a}	6.1 ± 0.3^{a}	6.0 ± 0.4^{a}	6.3 ± 0.4^{a}	6.0 ± 0.3^{a}	5.1 ± 0.2^{b}	6.8 ± 0.2^{a}	***
M17 44 °C	4.1 ± 0.1^{d}	4.7 ± 0.3^{cd}	4.3 ± 0.2^{cd}	7.2 ± 0.4^{a}	6.0 ± 0.4^{b}	4.9 ± 0.1^{c}	6.8 ± 0.2^{b}	***
KAA	2.7 ± 0.2^{ab}	3.0 ± 0.3^{a}	2.5 ± 0.3^{ab}	2.4 ± 0.1^{b}	$< 2^{c}$	$< 1^{c}$	2.7 ± 0.1^{ab}	***
VRBGA	3.0 ± 0.3^{b}	3.6 ± 0.2^{a}	3.3 ± 0.2^{ab}	3.0 ± 0.1^{b}	1.1 ± 0.1^{c}	$< 1^{d}$	$< 1^{d}$	***
CPS	4.1 ± 0.3^{a}	2.6 ± 0.2^{b}	2.5 ± 0.4^{b}	2.3 ± 0.2^{b}	$< 2^{c}$	$< 1^{c}$	$< 2^{c}$	***
DRBC	1.7 ± 0.2^{a}	1.1 ± 0.2^{b}	1.0 ± 0.2^{b}	1.0 ± 0.1^{b}	$< 2^{c}$	$< 1^{c}$	$< 2^{c}$	***

P value: *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.
Abbreviation: EWM, evening whole milk; SM, skimmed milk after overnight separation of fat globules; VM, vat milk obtained after mixing EWM with SM; IM, inoculated milk after addition of NWSC; CC, cooked curd after treatment at 55 °C; CW, cooked whey resulting after curd breaking; RC, ripened cheese; PCA-SkM 7 °C, plate count agar added with skimmed milk incubated at 7 °C for total psychrotrophic microorganisms; PCA-SkM 30 °C, plate count agar added with skimmed milk incubated at 30 °C for total mesophilic microorganisms; MRS, de Man-Rogosa-Sharpe agar for mesophilic rod LAB; WBAM, whey-based agar medium for thermophilic rod LAB; M17 30 °C, medium 17 agar incubated at 3 0 °C for mesophilic coccus LAB; M17 44 °C, medium 17 agar incubated at 44 °C for thermophilic coccus LAB; KAA, kanamycin aesculin azide agar for enterococci; VRBGA, violet red bile glucose agar for *Enterobacteriaceae*; CPS, coagulase-positive staphylococci; DRBC, dichloran rose bengal chloramphenicol agar for yeasts; n.d., not determined.
[a] Units are log CFU/ml for liquid samples and log CFU/g for solid samples. Results indicate mean values ± standard deviation (SD) of eight plate counts (carried out in duplicate for four independent productions).
[b] Data within a line followed by the same letter are not significantly different according to Tukey's test.

were transferred into a vial, added with 10 ml H_2O, 200 µl of internal standard solution (35 mg/l 1-heptanol in 20% ethanol aqueous solution) and 1 g of NaCl. The vials, clear with screw top and hole caps with PTFE/silicone septa 27136 (Supelco, Bellefonte, PA), kept under magnetic stirring, were heated at 60 °C for 25 min (Carlin and Versini, 2005) and the headspace was collected by DBV–carboxen– PDMS fibres (Supelco, Bellefonte, PA) for 30 min at 60 °C. The SPME fibre was inserted directly into a Finnegan TraceMS for GC/MS (Agilent 6890 Series GC system, Agilent 5973 NetWork Mass Selective Detector, Milan, Italy) equipped with a DB-WAX capillary column (Agilent Technologies, 30 m, 0.250 mm i.d., film thickness 0.25 µm, part no. 122–7032). The GC-MS system and chromatographic conditions were previously reported by Corona (2010) and Sannino et al. (2013).

The detection was carried out by electron impact mass spectrometry in total ion current (TIC) mode using an ionization energy of 70 eV. The mass acquisition range was m/z 30–330. The methodology described by and Alfonzo et al. (2016) and Martorana et al. (2016) was applied for the identification of the compounds. Semiquantitative data (µg/kg of cheese) were obtained by measuring the relative peak area of each identified compound in relation to that of the added internal standard.

2.9. Sensory evaluation

After 9-month of ripening, Gran Ovino (GO) cheeses were also evaluated for their sensory characteristics. Fifteen descriptive attributes were judged by a panel of 31 assessors members (fifteen men and sixteen woman, from 20 to 57 years old). All panelists were trained at IZS following the ISO 8589 (2007) indications. The panelists had available a cubed sample (1 × 1 × 1 cm) in order to evaluate organoleptic attributes and an entire transverse slice for evaluating appearance attributes. The attributes were organized into: aspect (color and uniformity of structure), smell (strength of odor, milk, butter and unpleasant smell), taste (salty, sweet, acid, spicy and bitter taste), consistency (chewiness, solubility and grittiness following mastication) and overall acceptability. The sensory evaluation of GO cheese was compared to that of 10-month (minimum ripening time for commercialization) ripened Grana Padano (GP) and 9-month Pecorino Siciliano (PS) cheeses. GP cheese was purchased in a retail market, while PS cheese was provided by the consortium for the protection of this traditional cheese and was produced in the same factory that provided the raw milk for GO cheese production. Both cheeses were presented to the judges in the same way of GO cheese. In order to avoid the influence of GP and PS on the acceptability of GO cheese, the three cheeses were tasted in the following order: 1. GO cheese; 2. GP cheese; 3. PS cheese.

2.10. Statistical analyses

Statistical analyses of microbiological counts were conducted using STATISTICA software (StatSoft Inc., Tulsa, OK, USA). Microbial, chemical and physical data were analysed using a generalised linear model (GLM procedure, SAS 9.1.2 software). Microbial data were converted to the log scale before statistical elaborations. Differences between means were determined by the post-hoc Tukey's multiple-range test. A $P < 0.05$ was deemed significant.

3. Results

3.1. Monitoring of the acidification process, ripening and microbiological counts

The temperature of milk during skimming increased from 7.7 °C (registered at the time of transfer of the milk into the trapezoidal tank) to 16.3 °C when skimmed milk was tranferred to the copper vat. The average value of pH of vat milk resulting from the mixing of whole and skimmed milk was 6.80, while NWSC reached the value of 3.80 thanks to the mixture of *S. thermophilus* PON6, PON244, PON261 and PON413 whose levels (detected on M17 at 44 °C) were 8.5 CFU/ml. After the addition of the NWSC, the milk bulk was characterized by a pH of 6.40 and underwent a rapid acidification; the curds reached 6.05 pH at moulding.

The average temperature of the cheeses soon after moulding was 45.8 °C. After 24 h from production, the temperature at cheese core dropped to 21.3 °C. The temperature continued to drop until 14.4 after 5 d and remained almost constant (ranging between 14.1 and 14.8 °C) during the nine months of ripening.

The levels of the different microbial groups investigated in this study are reported in Table 1. The microbiological counts did not included TPM for cooked whey. TPM counts were comparable among milks and the levels registered in vat milk were 5.6 log CFU/ml. The levels of TMM were slightly higher than TPM and were found at 6.1 log

CFU/ml before NWSC addition. After starter addition, TMM increased by about 0.5 log cycle. After cooking, the curd was characterized by residual levels of TMM and TPM of 4.9 and < 2 log CFU/g, respectively. TMM of cooked whey was particularly low (4.4 log CFU/ml).

Regarding LAB, all milk samples (EWM, SM and VM) were dominated by mesophilic cocci with 6.0 log CFU/ml detected before NWSC addition. The levels of the other LAB groups registered in VM were 4.3, 5.2 and 2.8 log CFU/ml for thermophilic cocci, mesophilic rods and thermophilic rods, respectively. After NWSC was added, the highest levels (7.2 log CFU/ml) were shown by thermophilic LAB cocci. The cooked curd was characterized by a decrease of about 1 log cycle for the thermophilic cocci, while a slight reduction was observed for the other groups. Enterococci were 2.4 log CFU/ml before milk coagulation, but decreased below the detection level in cooked curd.

Within the undesired microbial groups, members of *Enterobacteriaceae* family increased during skimming and were detected at 3.3 log CFU/ml in vat milk. Curd cooking determined a decrease of their levels, estimated at 1.1 log CFU/g in CC. CPS were at particularly high levels in EWM, decreased consistently during skimming (until 2.6 log CFU/ml in SM) and completely disappeared after exposure at 55 °C during curd cooking. A similar behaviour was recorder for yeasts, which were at 1.0 log CFU/ml in VM and disappeared in CC. *Salmonella* spp., *E. coli* and *L. monocytogenes* were not detected in any milk, whey or curd samples and, for this reason were not object of investigation in ripened cheeses.

After 9-month ripening, the cheeses from the four productions were also analysed. TPM levels were a little lower than 5.0 log CFU/g, TMM almost 1 log cycle lower than LAB which were 6.8 log CFU/g in all media considered for the four groups thermophilic and mesophilic rods and cocci. The levels of enterococci were 2 log cycles lower than LAB, while members of *Enterobacteriaceae* family, CPS and yeasts were below the detection levels.

3.2. Identification of dominant LAB in ripened cheeses

After enumeration, 172 colonies showing different characteristics and representative of the dominant presumptive LAB (Gram positive and catalase negative) were isolated and purified. All isolates were analysed by RAPD-PCR in order to recognise the different strains. To this purpose, when 2 or more isolates share the same RAPD pattern are considered to represent the same strain (Charlton et al., 1999). Fig. 2 reports the dendrogram resulting from the combination of the three RAPD patterns of each isolate and shows the presence of 18 strains (Fig. 2A) from the total of 172 collected LAB isolates grouped, after their preliminary morphological/physiological/biochemical characterization, into six main groups (Fig. 2B). The most numerous LAB were characterized by an obligate homofermentative metabolism (59 rod isolates). The analysis by 16S rRNA gene sequencing indicated that at 9-month of ripening the LAB community of GO cheese was mainly represented by the species *Lactobacillus fermentum* (Ac. No. MK908201 – MK908205), *Lactobacillus paracasei* (Ac. No. MK908206 – MK908210), *Enterococcus faecium* (Ac. No. MK908197 – MK908200), and *Pediococcus acidilactici* (Ac. No. MK908211 – MK908213). Only one strain was allotted into the species *Lactobacillus delbrueckii* (Ac. No. MK908214).

3.3. Characteristics of the Illumina data and taxonomic analysis of the bacterial community

The DNA extracted from the four cheese samples successfully amplified the bacterial V3-V4 16S rRNA and after splitting and quality trimming the raw data, 123,932 reads remained for subsequent analysis. The relative abundance (%) of the different identified bacterial groups is reported in Fig. 3. Only the groups with an incidence of 0.1% were considered. The two most abundant species belonged to the genera *Lactobacillus* and *Streptococcus* that together covered > 90% of the microbial relative abundance in all cheeses. However, the proportions of the two genera found among the four replicates of GO cheese productions differed substantially, e.g. from 21.45% of *Lactobacillus* and 68.90% of *Streptococcus* at the second production week until 81.31% and 14.04% of *Lactobacillus* and *Streptococcus*, respectively, at the third week. Furthermore, all cheeses were also characterized by the presence of other unidentified LAB. Regarding the undesired bacterial groups, especially the phylum Gammaproteobacteria to which the members of *Enterobacteriaceae* family belong to, they were at very low levels (at highest 2.28% at the third week).

3.4. Physico-chemical characteristics of ripened cheese

Ripened cheeses (Table 2) were characterized by a dry matter of 67.65%. Fat percentage was lower than that of protein. Ripening determined a maturation index (soluble N/total N) closed to 25%. A very low salt percentage was found with an a_w of 0.95 and pH 5.72.

Colorimetric parameters (Table 2) showed that GO cheese is characterized by a deep yellow paste with a good level of lightness (Chroma and Hue angle values were 20.20 and − 0.27, respectively).

Cheese fatty acids composition is reported in Table 3. The more represented fatty acids were Palmitic (23.01%), Oleic (12.80%) and Myristic (11.08%) acids, the sum of saturated fatty acids was 68.40% with SFA/UFA ratio of 2.17. Interesting is the content of omega-3 fatty acids (3.05) with a ω-6/ω-3 ratio of 0.67. Fatty acids with healthy interest showed good values: 3.09% for Vaccenic acid, 1.70% for Linoleic acid, 2.39% for Linolenic acid and 1.05% for Rumenic acid.

3.5. Volatile organic compound composition of “Gran Ovino” cheese

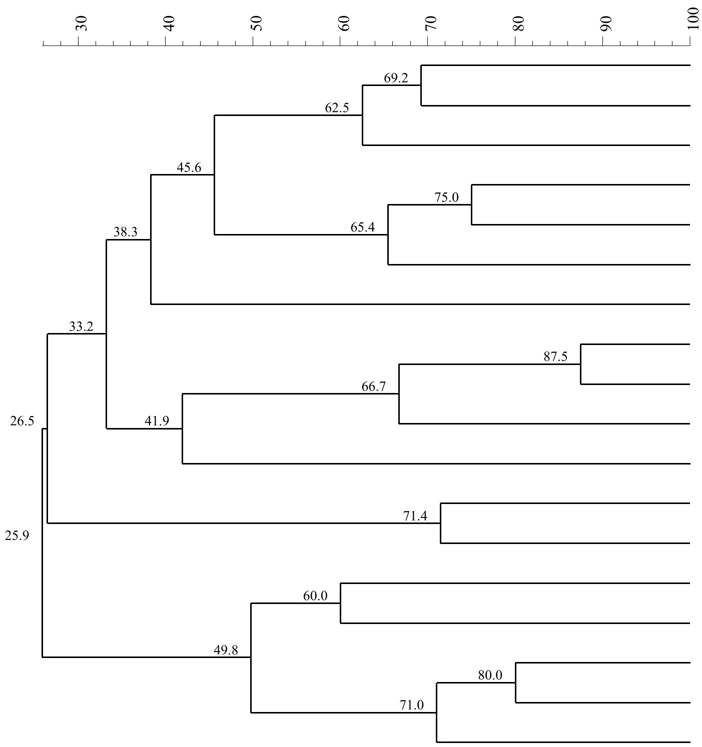
The volatile organic compounds emitted from GO cheese from the four productions are reported in Fig. 4. Forty-one volatile compounds were identified in the headspace of the cheeses: 11 acids, 8 esters, 8 ketones, 5 alcohols, 5 aldehydes, 2 phenols and 1 terpene. The VOCs of the cheese samples showed some differences. In particular, high concentrations of hexanoic, octanoic, decanoic and butyric acid among the acids (total acids respectively 64 and 33 μg/kg); ethyl esters (C6, C8, C10), while butyl butyrate, hexanoate, and isoamyl hexanoate among the esters (total esters respectively 12 and 4 μg/kg) were registered. Benzaldehyde was dominant among the aldehydes, 1-hexanol, 2-phenylethanol and 2-nonanol among the alcohols, and 2-decanone, acetoin, 3,5-octadien-2-one and 2-nonanone among the ketones. The terpene D-limonene was present at very similar concentrations in the four cheese samples (about 272 μg/kg). Phenol (p-cresol and o-cresol) showed the highest concentration in the cheese produced during the first week.

3.6. Sensory evaluation

Fig. 5 reports the spider graphic representation of the sensory characteristics evaluated on GO cheeses by the judges. The highest scores were registered for color, uniformity, strength of odor, chewiness and solubility while the lower score was evidenced by unpleasant odor. The overall assessment, intended as an overall rating of the cheeses expressed considering all parameters with their levels of evaluation, indicated a certain appreciation of this novel cheese expressed by the judges.

The sensory characteristics of GO cheese were also compared to those of GP and PS cheeses in order to retrieve the differences among the novel raw ewes' milk cheeses and two conventional highly consumer's appreciated products. GO cheese was characterized by a more intense color than PS cheese, but quite lower than GP cheese. The three cheeses showed almost superimposable scores for butter and milk odors. GO and GP cheeses were similar for the perception of unpleasant odors, salt and grittiness (lower than PS cheese), uniformity of structure, solubility and overall satisfaction (higher than PS cheese). GO cheese was more similar to PS cheese rather than GP cheese regarding

A



Strain	Phenotypic group	Number of isolates	Species
PGC161	II	7	*P. acidilactici*
PGC164	II	9	*P. acidilactici*
PGC128	III	5	*P. acidilactici*
PGC101	I	4	*E. faecium*
PGC103	I	17	*E. faecium*
PGC143	I	11	*E. faecium*
PGC157	I	7	*E. faecium*
PGC5	VI	9	*L. paracasei*
PGC6	VI	13	*L. paracasei*
PGC39	VI	14	*L. paracasei*
PGC106	V	9	*L. delbrueckii*
PGC19	VI	16	*L. paracasei*
PGC99	VI	7	*L. paracasei*
PGC145	IV	15	*L. fermentum*
PGC3	IV	5	*L. fermentum*
PGC45	IV	8	*L. fermentum*
PGC90	IV	12	*L. fermentum*
PGC148	IV	4	*L. fermentum*

B

Characters	Phenotypic clusters					
	I	II	III	IV	V	VI
Morphology	C	C	C	R	R	R
Cell disposition	s.c.	t.	t.	s.c.	s.c.	s.c.
Growth:						
15 °C	+	+	+	-	-	+
45 °C	+	+	+	+	+	-
pH 9.6	+	+	+	n.d.	n.d.	n.d.
6.5% NaCl	+	+	+	n.d.	n.d.	n.d.
Resistance to 60 °C	-	+	+	+	-	-
Hydrolysis of:						
arginine	+	+	+	-	-	-
aesculin	+	+	-	-	-	-
Acid production from:	+	+	+			
arabinose	+	+	+	+	-	+
ribose	+	+	+	+	-	+
xylose	+	+	+	+	-	+
fructose	+	+	+	+	+	-
galactose	+	+	+	+	+	+
lactose	+	+	+	+	+	+
sucrose	+	+	+	+	+	+
glycerol	+	+	+	+	+	+
CO_2 from glucose	-	-	-	+	-	-

Fig. 2. Differentiation of LAB isolates from ripened Gran Ovino cheese. A, dendrogram obtained with combined RAPD-PCR patterns of the LAB strains identified. B, phenotypic grouping of the LAB isolates based of morphological, physiological and biochemical traits. Abbreviations: *E.*, *Enterococcus*; *Lb.*, *Lactobacillus*; *P.*, *Pediococcus*; R, rod; C, coccus; s.c., short chain; t., tetrads; n.d., not determined.

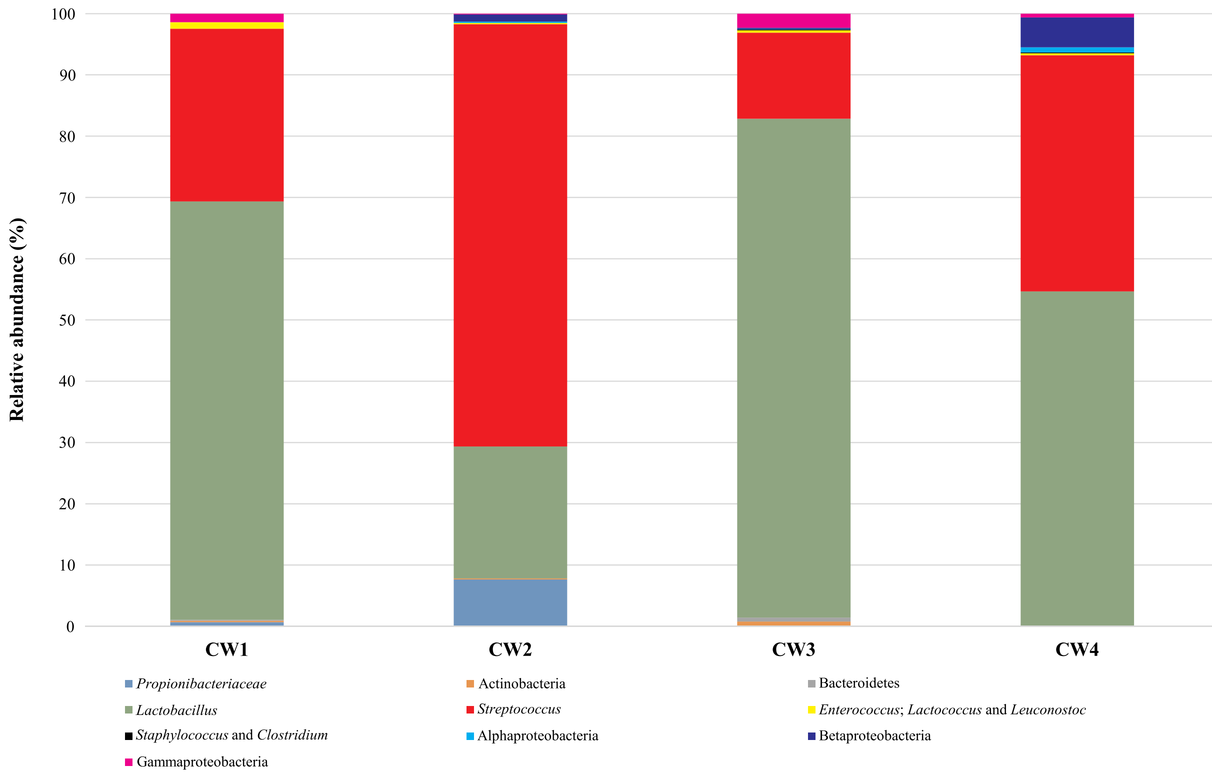


Fig. 3. Relative abundances (%) of bacteria identified by MiSeq Illumina in Gran Ovino cheeses after 9-month of ripening. Abbreviation: CW1, cheese week one; CW2, cheese week two; CW3, cheese week three; CW4, cheese week four.

Table 2
Physicochemical parameters of experimental Gran Ovino cheeses cheeses.

Parameters	Mean	SD[a]
Dry matter (%)	67.65	0.53
Fat (% on DM[b])	41.85	1.06
Protein (% on DM)	47.02	0.58
N-soluble (% on DM)	1.83	0.27
Maturation index (%)	24.87	3.66
Carbohydrates (% on DM)	4.49	1.13
Ash (% on DM)	6.64	0.20
Salt (% on DM)	1.16	0.03
a_w	0.95	0.01
pH	5.72	0.05
Lightness (L*)	78.56	1.12
Redness (a*)	−5.31	0.21
Yellowness (b*)	19.49	0.62
Croma[c]	20.20	0.58
Hue angle[d]	−0.27	0.02

[a] SD = standard deviation.
[b] DM = Dry matter.
[c] Croma = $\sqrt{(a^2+b^2)}$.
[d] Hue angle = a/b.

sweet, acid and chewiness. Regarding strength of odor, bitter and spicy the three cheeses were scored different with scores for GO cheese higher than those registered for GP cheese but lower than those resulting from PS cheese.

4. Discussion

In past, cheese making represented a means for the preservation of raw milk through the fermentation process. During its first production step, cheese can be described as an aggregate of casein micelles forming a gel containing all solid components of milk (Dalgleish and Corredig, 2012) in which all microorganisms present in the raw milk are trapped. Due to the technology applied during processing, each cheese variety will dictate the potential for the growth of desired LAB as well as for the survival of undesired (spoilage and pathogenic) microorganisms (Donnelly, 2004).

This work was aimed to evaluate technological alternatives for processing raw ewes' milk into cheeses with high hygienic quality. To this purpose, the technology of Grana type cheeses, almost exclusively applied to transform raw cows' milk (Mucchetti and Neviani, 2006), was tested on raw ewes' milk. This technology is mainly characterized by a curd cooking step.

Recently, an approach based on the use of selected starter and non starter LAB was applied to ameliorate the production of raw milk cheeses, such as PDO Pecorino Siciliano with the modification of the production protocol from a raw milk production without bacterial culture addition to a protocol including the addition of selected strains (Settanni et al., 2013). Based on the positive results registered in terms of reduction of undesired bacterial groups (pseudomonads and *Enterobacteriaceae*) the innovation respectful of the traditional production technology was applied at large scale level on the entire Sicilian area improving the hygienic characteristics of all final cheeses (Guarcello et al., 2016). However, although consistently reduced in number, these bacteria were still found during ripening and some defects in cheese structure, due to the presence of eyes, was noticed.

The main hypothesis of this study was that Grana type technology applied to raw ewes' milk contained the development of the undesired microbial groups. This strategy is not completely new in Sicily, because Maiorchino cheese is produced by curd cooking after coagulation of a mixed cows', ewes' and goats' bulk milk (Conte and Panebianco, 2001). However, a NWSC was prepared *ad hoc* in order to perform a driven fermentation for GO cheese using "autochthonous" *S. thermophilus* strains adapted to ewes' milk transformation at high temperatures. For this reason, the strains PON6, PON244, PON261 and PON413 were isolated from ewes' cheeses subjected to thermal treatments (stretching) during manufacturing (Gaglio et al., 2014a) and some of them were

Table 3
Cheese fatty acid composition (g/100 g FAME).

Fatty acids	Mean	SD[a]
C4:0	3.04	0.25
C6:0	2.72	0.14
C8:0	2.57	0.10
C10:0	7.04	0.21
C12:0	3.84	0.05
C14:0	11.08	0.33
C16:0	23.01	0.23
C16:1 *c9*	0.92	0.06
C17:0	0.77	0.03
C18:0	9.92	0.34
C18:1 *t*11, VA[b]	3.09	0.12
C18:1 *c*6	2.05	0.11
C18:1 *c*9	12.80	0.20
C18:2 n-6 *c9 c12,* LA[c]	1.70	0.07
C18:3 n-3 *c9 c12 c15*	2.39	0.01
CLA C18:2 *c*9 *t*11, RA[d]	1.05	0.02
C20:0	0.39	0.09
C20:5 n-3, EPA[e]	0.12	0.00
C22:5 n-3, DPA[f]	0.22	0.02
OBCFA[g]	2.23	0.08
Σ omega-6	2.05	0.12
Σ omega-3	3.05	0.02
omega-6/omega-3	0.67	0.04
Saturated FA	68.40	0.60
MUFA[h]	22.97	0.48
PUFA[i]	8.62	0.14
Unsaturated FA	31.60	0.60
Saturated/unsaturated	2.17	0.06
HPI[j]	0.44	0.02

[a] SD = standard deviation.
[b] VA = *trans* vaccenic acid.
[c] LA = linoleic acid.
[d] RA = rumenic acid.
[e] EPA = eicosapentaenoic acid.
[f] DPA = docosapentaenoic acid.
[g] OBCFA = odd and branched chain fatty acids.
[h] MUFA = Monounsaturated fatty acids.
[i] PUFA = Polyunsaturated fatty acids.
[j] HPI = Health Promoting Index = unsaturated fatty acids/[C12:0 + (4 × C14:0) + C16:0] (Chen et al., 2004).

already tested during ewes' milk cheese productions (Gaglio et al., 2014b). *Streptococcus thermophilus* is one of the main bacterium of natural whey starter cultures used for the production of Grana type cheeses (Bottari et al., 2010; Giraffa et al., 1997). The inclusion of adjunct cultures might influence the ripening profiles of hard cheeses (Cuffia et al., 2019), for this reason non starter LAB were not added to the milk in order to evaluate the natural evolution of indigenous raw ewes' cheese strains.

In the present work, the microbiological parameters were first evaluated by plate count. TMM and TPM of milk samples (EWM, SM and VM) did not show great variations after skimming and mixing in vat. Generally, typical ewes' milk cheese productions performed in Sicily do not include a curd cooking step and, after coagulation, an increase of the microbial counts is registered as a consequence of whey draining (Gaglio et al., 2014b; Settanni et al., 2013). On the contrary, in the present study lower values of TMM and TPM were found in curds showing a strong effect of the treatment of curd grains at 55 °C for 8 min; the values of these microbial groups were 2 and 5 log cycles lower than bulk milk used for transformation.

Mesophilic LAB cocci dominated the microbial community of milk before NWSC addition, but no differences among the levels of LAB cocci and LAB rods were registered in ripened cheeses. Similar results are generally reported for Grana type cheeses produced from raw cows' milk (De Dea Lindner et al., 2008; Monfredini et al., 2012). The levels of enterococci were 2 log cycles lower than other LAB, similarly to what reported for Parmigiano Reggiano cheese (Coppola et al., 2000). The most interesting results were displayed by the members of *Enterobacteriaceae* family which increased during skimming but strongly decreased during curd cooking until disappearance in ripened cheese as observed for cows' Grana cheeses (Coppola et al., 2000; Monfredini et al., 2012). The cooking step determined also the complete disappearance of CPS and yeasts.

LAB communities were firstly studied by a culture-dependent approach which identified 18 strains belonging to six phenotypic groups. This approach showed that strains allotted into diverse species were characterized by several traits in common, indicating that the combination of genotypic information with those from morphology, physiology and biochemistry is important to understand the role of the isolates collected. The LAB most frequently isolated were *Lb. paracasei* and *Lb. fermentum*. In particular, *Lb. paracasei* is often isolated during the ripening of different Grana type cheeses (Gala et al., 2008; Monfredini et al., 2012; Solieri et al., 2012; Zago et al., 2007), while *Lb. fermentum* is less frequent, but found in Parmigiano Reggiano cheese during the first production stages (Neviani et al., 2009) and at very low isolation levels after at least 12-month of ripening (Solieri et al., 2012). *Pediococcus acidilactici* was also isolated from GO cheese and this species is associated to ripened Grana cheeses (Gala et al., 2008; Neviani et al., 2009). Enterococci of GO cheese were represented by *E. faecium* which is commonly found in raw ewes' milk cheeses (Gaglio et al., 2014a; Pino et al., 2017; Todaro et al., 2011), but for other Grana cheeses, such as Parmigiano Reggiano cheese, it presence is only reported at the beginning of production (Pogačić et al., 2013). Regarding the presence of a viable strain of the thermophilic *Lb. delbrueckii* after nine months of ripening, this finding is not surprising since Di Grigoli et al. (2015) also isolated viable colonies belonging to this species from ripened Caciacavallo Palermitano cheeses.

The bacterial community of GO cheese was also approached by a culture-independent perspective, analysing total DNAs extracted from the four replicate cheeses. This tools showed data almost completely in agreement with the culture-based study concerning lactobacilli, but also showed a consistent presence of streptococci. Since no *Streptococcus* was isolated, at least at the dominant levels, from 9-month ripened cheeses, the high percentages of OTUs identified as *Streptococcus* might probably derive from residual DNAs of death cells. This statement is also supported by the temperatures monitored during ripening which were below 15 °C and the thermophilic starter *Streptococcus thermophilus* cannot grow at this temperatures (Hardie and Whiley, 1995). Similar findings were reported by Bassi et al. (2015) who found *Lactobacillus* (65.3%) and *Streptococcus* (14.4%) in GP cheeses applying a next generation sequence approach performed with MiSeq Illumina. However, in the last work, all cheese samples were affected by blowing defects and the results, in terms of abundance %, could have been influenced by *Clostridium* spp. contamination.

Ripened cheeses presented a dry matter percentage similar to analogous Sicilian cheeses with long ripening periods (Guarcello et al., 2016). Maiorchino cheese is very similar to ours cheeses with the difference that it is made from entire milk, dry matter percentage of Maiorchino with 8-month of ripening is around 70% (Conte et al., 2015). Fat and protein percentages found in our cheeses displayed values similar to those of PDO Pecorino Siciliano (Guarcello et al., 2016) and GP cheese (Consorzio Tutela Grana Padano, 2002), while Maiorchino cheese present the same fat content, but lower protein percentages (Conte et al., 2015). Maturation index showed a good proteolysis activity and resulted slightly higher than PDO Pecorino Siciliano at 5-month of ripening (Guarcello et al., 2016).

Salt content was very low for a ripened cheeses made with sheep milk and lower than others Italian Pecorino cheeses at the same ripening period (Di Cagno et al., 2003; Guarcello et al., 2016); this fact is to be considered as positive to increase the consumer satisfaction and reduce the human pathologies due to high consumption of salt; but the low salt content in cheese is permitted only when its microbiological quality is high. a_w values were higher than those of other cheeses with

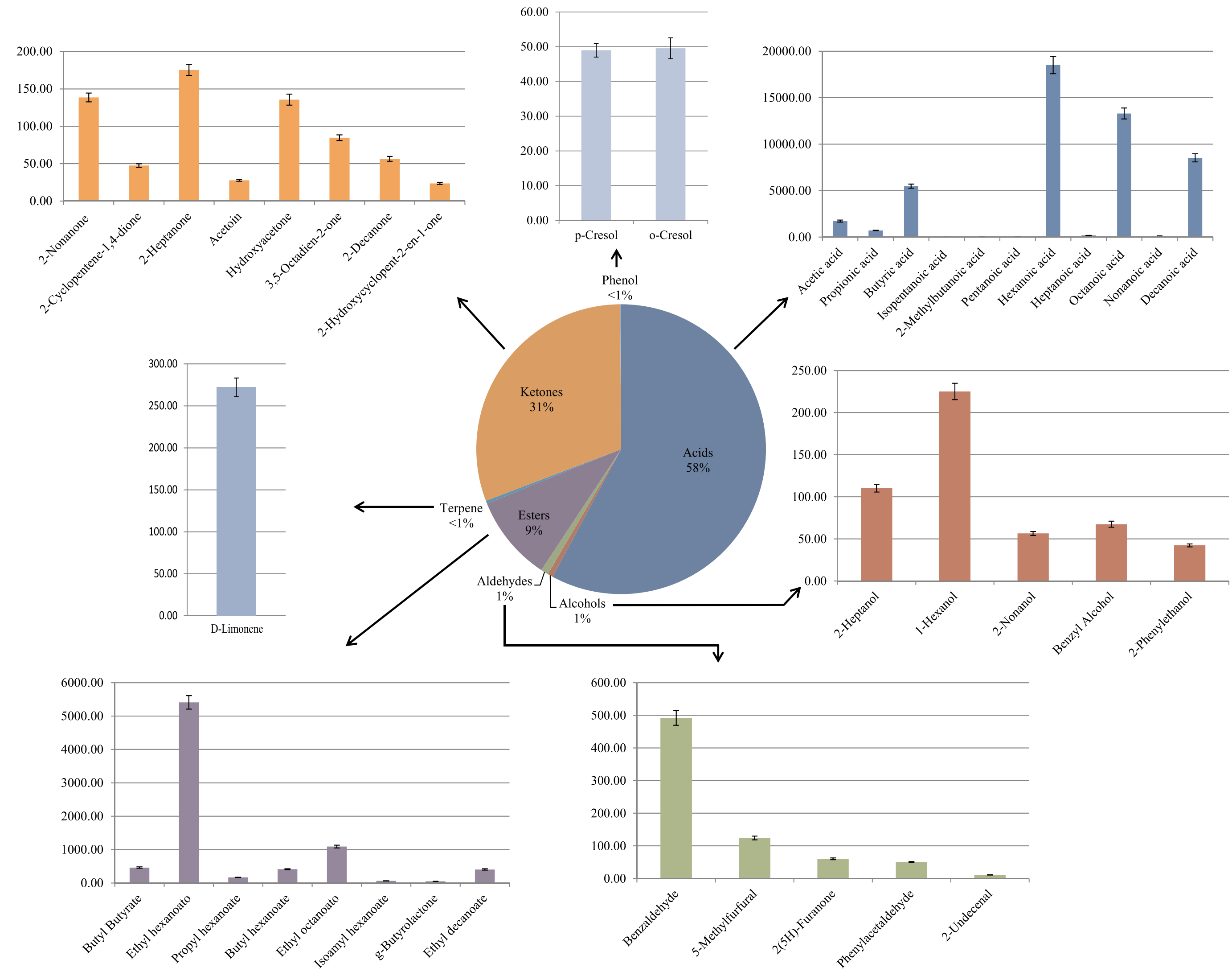


Fig. 4. Analysis of volatile organic compounds emitted from 9-month ripened Gran Ovino cheeses. Results are expressed in mg/kg.

analogous ripening period; e.g. a_w for PDO Pecorino Siciliano is on average 0.92 (Guarcello et al., 2016), the same values were reported for Maiorchino cheese (Conte et al., 2015). The value of pH resulted similar to those of PDO Pecorino Siciliano (Guarcello et al., 2016), but higher than Maiorchno cheese (Conte et al., 2015). Color parameters showed that our cheeses were characterized by a deep yellow and high lightness, values clearly higher than PDO Pecorino Siciliano (Todaro et al., 2011), making GO cheese more attractive to consumers.

Fatty acids composition of these cheeses were similar to those of hard cheeses made from sheep's milk (Prandini et al., 2011), but our cheeses showed a higher percent of PUFA (8.60 vs 4.93%) and lower SFA/UFA ratio, probably due to the high level of pasture in the diet of the sheep (Bonanno et al., 2016) that produced the milk used in this study. Regarding healthy fatty acids, our cheeses showed triple levels of Linolenic acids, also this result is due to sheep feeding, than those registered for the sheep grazed on Sulla meadows. In fact, one factor known to increase the concentration of n-3 FA in sheep milk (Cabiddu et al., 2005) is the presence of legumes in the feed ration. It is likely that this is the result of plant secondary compounds which are often higher concentrated in legumes, as Sulla forage. Important representative of plant secondary compounds are tannins (Cabiddu et al., 2009), which may partially inhibit ruminal biohydrogenation and, thus, reduce the loss of native plant FA like C18:3 n-3 during digestion. However, it has to be taken into account that the lipolysis is influenced by the temperature during ripening of Grana cheeses (Sihufe et al., 2007). Furthermore, FFA profiles also depend on the starter strains (Perotti et al., 2005).

The biochemical processes which lead to the synthesis of volatile compounds in cheese are very complex (Kilcawley, 2017; Thierry et al., 2017). It is known that the volatile compounds identified in cheese are mainly the products of lipolysis, proteolysis, metabolism of residual lactose, lactate, and citrate. Lipolysis of the triglycerides by microbial and indigenous milk enzymes, and also enzymes from added rennet pastes, result in the development of medium-chain (carbon chain lengths ≤ 10) and long-chain (carbon chain lengths > 10) FFAs (Free Fatty Acids) (Collins et al., 2003; Thierry et al., 2017). The flavor contribution of FFAs in cheese is mainly influenced by the pH. FFAs at high pH levels are less flavor active and are often perceived as “soapy” as they are converted to nonvolatile salts. At low pH FFAs exist in free form and are perceived as rancid at high concentrations (Singh et al., 2003). The main components of the volatile fraction of GO cheeses analysed in this study were free fatty acids mostly represented by hexanoic, octanoid, decanoic and butyric acid. The four productions of GO cheese had significantly different FFAs, acids from C5 to C10. FFAs contribute to the formation of cheese flavor not only directly, but also indirectly as they are precursors of methyl ketones, secondary alcohols, straight-chain aldehydes, lactones and esters (Collins et al., 2003; Smit et al., 2005; Thierry et al., 2017). Also the content is high of the ethyl

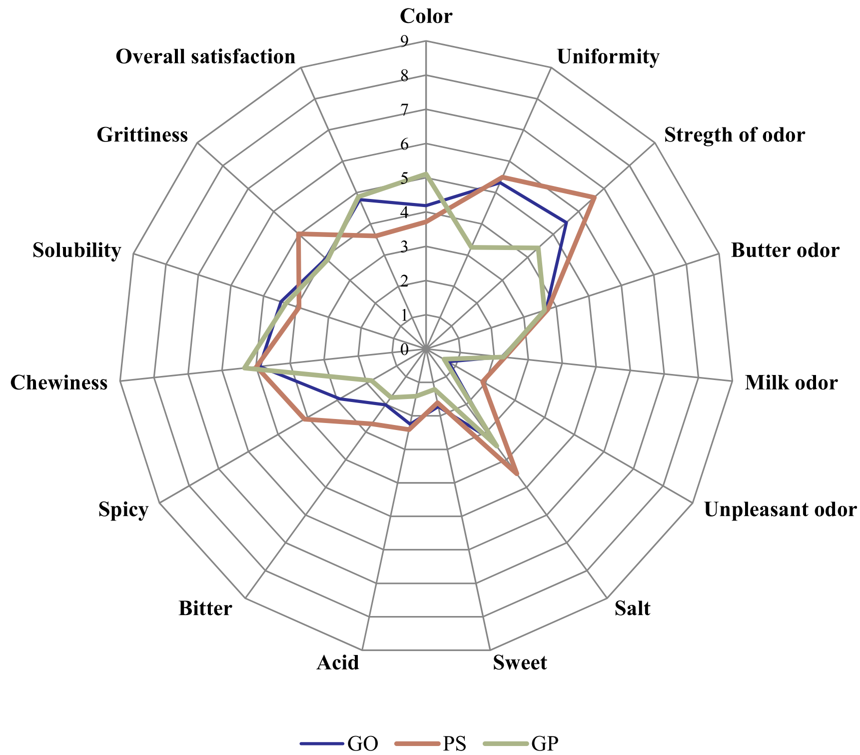


Fig. 5. Spider diagrams of descriptive sensory analysis of Gran Ovino (GO), Grana Padano (GP) and Pecorino Siciliano (PS) cheeses ripened for 9–10 months.

esters of medium chain fatty acids (from C6 to C10), alcohols and aldehydes of GO cheeses. Benzaldehyde is very high in GO cheese, especially at the second production week; this compound can be formed by enzymatic activities (proteolysis and peptidolysis) or by chemical conversion by phenyl-pyruvic acid (Smit et al., 2005). The differences in the VOCs emitted from the cheeses produced in the four weeks are a direct consequence of the differences revealed in the bacterial communities. This is a common observation when cheeses are analysed, since cheeses produced in a given cheese factory in different days or even in different vats the same days might be different (Fitzsimons et al., 1999; Williams et al., 2002).

Sensory evaluation indicated that the ripened GO cheese was characterized by a general appreciation by judges and, in particular, the level of unpleasant odors, which represents one of the main parameters for tasters' acceptance of a new product (Herz, 2006), was very low. The sensory comparison of GO cheese with GP and PS cheeses clearly showed that the three cheeses where characterized by several differences and that the highest differences were found with PS rather than GP, even though the last cheese is obtained from cows' milk.

In conclusion, in this work a post-milking approach was applied to ameliorate the hygienic characteristics of raw ewes' milk cheeses by introducing a curd cooking step during milk transformation. Even though TMM of bulk milk was 6.1 log CFU/ml that is above the limit of 500.000 CFU/ml established for the raw ewes' milk for cheese production (CE, 2004), the strategy tested in this work allowed to obtain an extra-hard cheese, namely Gran Ovino, characterized by the absence of undesired microorganisms. Furthermore, the sensory evaluation determined the appreciation by judges with high values of overall acceptance indicating the possible positive response by consumers enlarging the offer of raw ewes' milk processed products. Regarding the main hypothesis of the work, it was demonstrated that the novel cheese “Gran Ovino” was characterized by better hygienic features than Pecorino Siciliano cheese and that Grana cheese type technology can be applied on raw ewes' milk determining the production of a well appreciated cheese.

Conflict of interest

The authors have declared no conflicts of interest for this article.

Acknowledgments

This work was financially supported by the project for industrial research Project F/050267/03/X32, Asse 1, azione 1.1.3, Programma Operativo Nazionale Imprese e Competitività 2014-2020 “Approccio integrato per lo sviluppo di prodotti innovativi nei settori trainanti del comparto agroalimentare siciliano” (CUP: B78I17000260008), from the Italian Ministry of Economic Development. The authors wish to thank “Caseificio Passalacqua” (Castronovo di Sicilia, PA) for the ripening of Gran Ovino cheese.

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