1 # Transformation of raw ewes' milk applying "Grana" type pressed

2 # cheese technology: development of extra-hard "Gran Ovino" cheese

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4 **Raimondo Gaglio[a], Massimo Todaro[a], Maria L. Scatassa[b], Elena Franciosi[c], Onofrio Corona[a],**

5 **Isabella Mancuso[b], Rosalia Di Gerlando[a], Cinzia Cardamone[b], Luca Settanni[a,]***

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7 *[a] Dipartimento Scienze Agrarie, Alimentari e Forestali, Università degli Studi di Palermo, Viale*

8 *delle Scienze 4, 90128 Palermo, Italy*

9 *[b] Istituto Zooprofilattico Sperimentale della Sicilia "Adelmo Mirri", Via G. Marinuzzi 3, 90129*

10 *Palermo, Italy*

11 *[c] Research and Innovation Centre, Fondazione Edmund Mach (FEM), Via E. Mach 1, San Michele*

12 *all'Adige, Italy*

13

14 *Corresponding author.

15 E-mail address: luca.settanni@unipa.it (L. Settanni).

16 **ABSTRACT**

17 This work was carried out to pursue a double objective: to improve the hygienic safety of cheeses
18 produced from raw ewes' milk; and to produce a new typology of raw ewes' milk through the
19 application of "Grana" technology for which the name "Gran Ovino" was chosen. With this in
20 mind, raw milk from an individual farm was transformed under controlled conditions at a dairy pilot
21 plant. The production technology included the partial skimming of the evening and morning milk
22 mixture by cream surfacing and the addition of a natural whey starter cultures (NWSC) prepared
23 with four selected *Streptococcus thermophilus* strains (PON6, PON244, PON261 e PON413). Ten
24 microbial groups were investigated by plate counts from raw milk until ripened cheeses. Lactic acid
25 bacteria (LAB) were in the range 10^4 – 10^5 CFU/ml before NWSC addition. After curdling, this
26 group increased by 3 log cycles and was counted at 10^6 CFU/g after curd cooking. A rapid pH drop
27 (to 6.05) was registered after almost 3 h from NWSC addition. The levels of members of the
28 *Enterobacteriaceae* family were at about 10^3 CFU/ml in raw milk and decreased after curd cooking
29 to 1 log cycle. A similar behavior was shown by the other undesired microbial groups and a
30 complete disappearance of staphylococci was registered. The microbiological counts of 9-month
31 ripened cheeses showed the dominance of LAB and undetectable levels of the undesired bacteria.
32 MySeq Illumina was applied to better investigate the bacterial composition of ripened cheeses and
33 this technique evidences that the majority of OTUs belonged to *Lactobacillus* and *Streptococcus*
34 genera. The final cheeses were characterized by 67.65% dry matter of which 41.85% of fats and
35 47.02% of proteins. The main cheese fatty acids were palmitic, oleic and myristic acids and the
36 saturated fatty acids/unsaturated fatty acids ratio was 2.17. Forty-one volatile compounds, including
37 acids, esters, ketones, alcohols, aldehydes, phenols and one terpene were emitted from the cheese.
38 Sensory evaluation showed a general appreciation for the new cheese product by judges.

39

40 *Keywords:* hard cheese; Grana type cheese; Illumina technology; lactic acid bacteria; raw ewes'
41 milk; volatile organic compounds

42

43 ## 1. Introduction

44 Olson (1990) affirmed that “there is a cheese for every taste and a taste preference for every
45 cheese”. This statement evidences the high diversity of cheeses produced worldwide. In past,
46 several technologies have been developed to transform a few raw materials, usually bovine, ovine,
47 caprine or buffalo milks (McSweeney et al., 2004) and, nowadays, a great diversity of dairy
48 products, mainly cheeses, are available. Italy boasts a high range of traditional cheeses (Settanni
49 and Moschetti, 2014) and, generally, each cheese possesses unique characteristics that depend on
50 the transformation method applied. Indeed, a given typical cheese is produced following a specific
51 protocol including precise procedures to which a certain milk type undergoes. This is particularly
52 true with regards to the Italian hard and extra-hard cheese varieties, that are subjected to a long
53 ripening period, usually 6 – 24 months (McSweeney et al., 2004). As a matter of fact, in the South
54 part of Italy the main hard cheeses belong to Pecorino cheese typology and are made from raw or
55 pasteurised ewes’ milk. The most important cheeses produced in Northern Italy are Grana Padano
56 and Parmigiano Reggiano cheeses produced from raw cow’s milk, whose productions reached 3,7
57 billions € at production in 2017 (Rapporto Ismea-Qualivita, 2017).
58 Several Italian Pecorino cheeses enjoy a protected designation of origin (PDO) status. Among these
59 cheeses, PDO Pecorino Siciliano cheese is produced throughout Sicily, a large region (25,711 km^2)
60 representing an extended production area with the result that the cheeses produced in distant
61 locations are characterised by different profiles in terms of sensory characteristics and microbial
62 populations (Guarcello et al., 2016). Due to the technology applied, despite the stressing chemico-
63 physical parameters that characterize ripened cheese, PDO Pecorino Siciliano cheese might still
64 host undesired spoilage microorganisms (Settanni et al., 2013; Todaro et al., 2011).
65 Milk is a fragile substance, thus preserving its quality right from milking until it is processed in the
66 dairy industry has always been a challenge and a permanent concern (Vara Martinez et al., 2018).
67 Grana cheeses are generally characterized by a high microbiological quality. Grana and Pecorino

68 cheese productions differ substantially in several points, first of all for the type of milk processed,
69 but, from the hygienic perspective, the most relevant step is represented by the curd cooking
70 (Salvadori del Prato, 1998) carried out during Grana type cheese protocol application. This step
71 represents an effective thermal treatment (55 °C), also because it is applied to the curd after its
72 disruption to rice-size grains, determining a stronger and more rapid temperature penetration than
73 immersion of cheeses in hot deproteinised whey (applied for Pecorino cheeses) with the
74 consequence that temperature sensitive microorganisms decrease in number.
75 Based on the observation that raw ewes' milk is not currently transformed to produce ovine Grana
76 type cheese the main aim of this work was to monitor the main microbiological and chemico-
77 physical parameters of cheese making applying Grana type cheese technology to the raw milk of the
78 Sicilian sheep breed Valle del Belice.
79
80 **2. Materials and methods**
81 *2.1. Natural whey starter culture preparation*
82 In order to carry out the experimentation a natural whey starter culture (NWSC) was developed
83 with four strains of *Streptococcus thermophilus* (PON6, PON244, PON261 and PON413)
84 previously isolated from raw ewes' cheese productions and evaluated for their dairy performances
85 (Gaglio et al., 2014a). All strains were reactivated for 24 h in M17 broth (Oxoid, Milan, Italy)
86 incubated at 44 °C. The cells were subjected to a washing procedure consisting of two consecutive
87 centrifugations at 5000 g × 5 min and resuspension of the pellet in Ringer's solution (Sigma-
88 Aldrich, Milan, Italy). The final resuspension of the cells occurred at an optical density at 600nm
89 (OD_{600}) of ca. 1.00 evaluated by the spectrophotometer Jenway Ltd. model 6400 (Dunmow, UK).
90 Washed cells were then inoculated at about 10^6 CFU/ml in the whey-based medium (WBM)
91 prepared as described by Settanni et al. (2012), using non-acidified ewes' milk whey in place of
92 cows' whey.
93

94 *2.2. Cheese production and sample collection*

95 Cheese productions were carried out in controlled conditions at a dairy pilot plant [Istituto
96 Zooprofilattico Sperimentale (IZS) della Sicilia "Adelmo Mirri", Palermo, Italy] level to avoid
97 environmental contamination by dairy factory LAB. Milk was transformed using the POLYFOOD
98 system (mod. SI-050, INVENTAGRI™, Modena, Italy). Raw ewes' milk from the indigenous
99 Sicilian sheep breed "Valle del Belice" was provided by the artisanal dairy farm (Ovini e Natura,
100 Santa Margherita di Belice, Italy) selected for its high hygienic standards. Bulk milk (100 L) was
101 transformed following the flowsheet reported in Fig. 1 adapted from the classical "Grana" cheese
102 type technology. To this purpose, the entire bulk milk was delivered once daily and, in order to
103 simulate evening and morning milking, 50 l of bulk milk were immediately cooled to 4 °C in a
104 refrigerated vat under low stirring to avoid clustering of fat globules with the consequent floating
105 (Kohnhorst, 2001) as well as microbial proliferation (Franciosi et al., 2011), while the other 50 L
106 were placed into a trapezoidal 60 l-shallow tank to allow the rising of fats (creaming) during the
107 overnight rest at room temperature (Mucchetti and Neviani, 2006). The day after, skimmed milk
108 was transferred into a copper vat, added with the whole milk kept at 4 °C and heated at 38 °C. Bulk
109 milk was inoculated with 1.6 l of NWSC (Gaglio et al., 2016), subjected to vigorous stirring for 20
110 s and added with 25 g of an artisanal lamb rennet paste provided by the Rennet Regional
111 Consortium (Poggioreale, Italy) dissolved in 1 l of tap water. After coagulation, the curd was
112 broken manually with a planetarium stainless steel curd knife until rice-seed grains were obtained.
113 Broken curd was then treated at 55 °C for 8 min (curd cooking step) under agitation and then left to
114 precipitate for 1 h during which the grains welded into a single mass. The rested curd was removed
115 from the vat and left to drip onto a cotton cloth for 30 min and then transferred into a plastic
116 moulder and turned upside down after 3 h for a uniform whey syneresis. Buckets with 15 kg water
117 were put on the top of all drained curds for 12 h in order to facilitate further draining by pressing.
118 Salting was performed by immersion in brine containing NaCl (300 g/l) for 60 h. The ripening

119 occurred at 14 – 16 °C and 85% relative humidity. Cheese production was carried out in
120 quadruplicate in four consecutive weeks (one production per week).
121 The measurement of pH during cheese making (from milk to curd) was carried out with a portable
122 pH-meter (Eutech Instruments, Nijkerk, The Netherlands). The temperature of milk during the
123 skimming process was monitored through the 175-T2 data logger (Testo, Settimo Milanese, Italy)
124 registering data every 30 min. Cheese temperature during ripening (until 9th month) was monitored
125 through Thermo Button 22T 8K data loggers (VWR International Srl, Milano, Italy) inserted in the
126 core of the curds at moulding and registering data every 24 h.
127 The following samples were collected for each cheese production: evening whole milk (EWM),
128 skimmed milk (SM) after overnight separation of fat globules, vat milk (VM) obtained after mixing
129 EWM with SM, inoculated milk (IM) after addition of NWSC, cooked curd (CC) after treatment at
130 55 °C, cooked whey (CW) resulting after curd breaking and 9-month ripened cheese (RC). All
131 samples were kept refrigerated during transport occurred by means of an insulated box containing
132 reusable ice packs to the Agricultural Laboratory of University of Palermo and to the laboratories of
133 Milk Centre and Mastitis Control of Istituto Zooprofilattico Sperimentale della Sicilia (Palermo).
134
135 *2.3. Microbiological analyses*
136 All samples were subjected to the serial decimal dilution procedure. Milk and whey samples (1 ml)
137 were diluted into Ringers' solution. Cheeses were sampled as indicated by Monfredini et al. (2012),
138 in order to analyse the entire cheese profile. To this purpose, three portions (of 10 g each) per
139 cheese were collected, including under rind, middle section and core and mixed together. The first
140 dilution of curds (15 g) and cheeses (30 g) was performed in 2% (w/v) of Na-citrate solution,
141 homogenized by the stomacher BagMixer® 400 (Interscience, Saint Nom, France) at the maximum
142 speed for 2 min. Serial dilutions continued into Ringers' solution.
143 The microbial groups investigated belonged to the dairy desired community as well as to the
144 undesired community including both spoilage and pathogenic populations. Plate count agar (PCA)

145 added with 1 g/l of skimmed milk (SkM) was used for the total mesophilic microorganisms (TMM)
146 when incubated at 30 °C for 72 h or to count total psychrotrophic microorganisms (TPM)
147 performing the incubation at 7 °C per 7 d. LAB community was investigated on five different
148 media/temperature conditions: mesophilic LAB rods were plated on de Man-Rogosa-Sharpe (MRS)
149 agar acidified with 5 M lactic acid to pH 5.4 and incubated at 30 °C for 48 h; thermophilic LAB
150 rods on WBM agar incubated at 44 °C for 48 h; mesophilic and thermophilic LAB cocci on M17
151 agar incubated at 30 and 44 °C, respectively, for 48 h; enterococci on kanamycin aesculin azide
152 (KAA) agar incubated at 37 °C for 24 h. Incubation of all LAB groups except enterococci occurred
153 in anaerobiosis using the AnaeroGen AN25 (Oxoid) in jars closed hermetically. Members of the
154 *Enterobacteriaceae* family were detected on violet red bile glucose agar (VRBGA) after incubation
155 at 37 °C for 24 h. Yeasts were grown on dichloran rose bengal chloramphenicol (DRBC) agar
156 incubated at 28 °C for 48 h. Baird Parker (BP) agar added with rabbit plasma fibrinogen was used
157 to reveal the presence of coagulase-positive staphylococci (CPS) for 48 h at 37 °C. *Escherichia coli*
158 was investigated applying the method AFNOR BIO 12/25-05/09 (2009), *Salmonella* spp. by the
159 method AFNOR BIO 12/32-10/11 (2011), and *Listeria monocytogenes* by the method AFNOR BIO
160 12/11-03/04 (2004). All media and supplements were purchased from Oxoid. All plate counts were
161 carried out in duplicate.
162
163 *2.4. Isolation and identification of cheese LAB*
164 Presumptive LAB, as being Gram-positive (Gregersen KOH method) and catalase-negative (unable
165 to catalyse 3% H_2O_2 to H_2O), were randomly picked up from the highest plated dilutions of cheese
166 suspensions on MRS, M17 and WBM agar considering all different colony types (colour,
167 morphology, edge, surface and elevation). The isolates were purified by successive sub-culturing by
168 streaking on the same media used for plate counts, transferred to the corresponding broth media
169 (isolates from WBM where cultivated in MRS broth) containing 20% glycerol (v/v) and stored at
170 −80 °C until further characterization.

171 The isolates were phenotypically investigated by observing their cell morphology through an optical
172 microscope, by determining their growth at 15 and 45 °C, and their metabolic characteristics such
173 as CO_2 production from glucose, carried out in Durham's tubes with the optimal growth media that
174 did not contain citrate, acid production from different sources (arabinose, ribose, xylose, fructose,
175 galactose, lactose, sucrose and glycerol), NH_3 production from arginine (Abd-el-Malek and Gibson,
176 1948), and aesculine hydrolysis (Qadri et al., 1980). LAB cocci were also evaluated for their ability
177 to grow in presence of 0.65% (w/v) NaCl and at pH 9.2 to directly identify enterococci, showing
178 growth in both conditions.
179 Genomic DNA from cheese LAB was extracted after overnight growth in the optimal media using
180 the Instagene Matrix kit (Bio-Rad, Hercules, CA) following manufacturer's instructions and used
181 for differentiation of the isolates at strain level as well as for their genetic identification.
182 Strain typing was approached by randomly amplified polymorphic DNA (RAPD)-PCR analysis as
183 described by Gaglio et al. (2017) using singly the primers AB111, AB106 and M13 (Stenlid et al.,
184 1994; van den Braak et al., 2000). Electrophoresis on 2% (w/v) agarose gels (Gibco BRL, Cergy
185 Pontoise, France) was performed to separate DNA amplicons which were visualised, after staining
186 with the SYBR® safe DNA gel stain (Molecular probes, Eugene, OR, USA), by an UV trans-
187 illuminator. RAPD profiles were analysed through Gelcompare II software version 6.5 (Applied-
188 Maths, Sint-Marten-Latem, Belgium) and the isolates showing different patterns were considered to
189 represent different strains.
190 All different LAB strains were identified genetically by sequencing of the 16S rRNA gene and
191 comparison of the sequences in public databases (GenBank and EZ-taxon) by BLAST search. PCR
192 reactions were carried out following the protocol described by Weisburg et al. (1991) with the
193 primer pair fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′)/rD1 (5′-AAGGAGGTGATCCAGCC-3′).
194 After confirming the molecular size of the amplicons (about 1600 bp) on agarose gels, the PCR
195 products were purified using the QIAquick purification kit (Quiagen S.p.a., Milan, Italy) and
196 sequenced using the same primers used for PCR amplification at AGRIVET (University of

197 Palermo, Italy). The identities of the sequences were determined by a blastn search against the
198 NCBI nonredundant sequence database and by comparison with the sequences of the sole type
199 strains within the EZTaxon database (https://www.ezbiocloud.net/taxonomy).
200
201 *2.5. Preparation of the MiSeq library*
202 A 464-nucleotide sequence of the bacterial V3-V4 region (Baker et al., 2003) of the 16S rRNA gene
203 (*Escherichia coli* positions 341 to 805) was amplified. Unique barcodes were attached before the
204 forward primers to facilitate the pooling and subsequent differentiation of samples. To prevent
205 preferential sequencing of smallest amplicons, the amplicons were cleaned using the Agencourt
206 AMPure kit (Beckman coulter, Brea, CA, USA) according to manufacturer's instructions. The DNA
207 concentration of amplicons was determined using the Quant-iT PicoGreen dsDNA kit (Invitrogen,
208 Carlsbad, CA, USA) following the manufacturer's instructions. In order to ensure the absence of
209 primer dimers and to assay the purity, the generated amplicon libraries quality was evaluated by a
210 Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the High Sensitivity DNA Kit (Agilent).
211 Following quantitation, the cleaned amplicons were mixed and combined in equimolar ratios. Pair-
212 end sequencing was carried out at Genomic Platform – Fondazione Edmund Mach (San Michele
213 a/Adige, Trento, Italy) using the Illumina MiSeq system (Illumina, USA).
214
215 *2.6. Illumina data analysis and sequences identification by QIIME2*
216 Raw paired-end FASTQ files were demultiplexed using idemp
217 (https://github.com/yhwu/idemp/blob/master/idemp.cpp) and imported into Quantitative Insights
218 Into Microbial Ecology (Qiime2, version 2018.2). Sequences were quality filtered, trimmed, de-
219 noised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences were identified and
220 removed via the consensus method in DADA2. Representative sequences were aligned with
221 MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and
222 phylogeny (Katoh and Standley, 2013; Price et al., 2009). Taxonomic and compositional analyses

223 were conducted by using plugins feature-classifier (https://github.com/qiime2/q2-feature-classifier).
224 A pre-trained Naive Bayes classifier based on the Greengenes 13_8 97% Operational Taxonomic
225 Units (OTUs) database (http://greengenes.secondgenome.com/), which had been previously
226 trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to
227 paired-end sequence reads to generate taxonomy tables.
228 The data generated by Illumina sequencing were deposited in the NCBI Sequence Read Archive
229 (SRA) and are available under Ac. PRJNA542786.
230
231 *2.7. Physico-chemical analyses of cheeses*
232 Cheese samples were analyzed for dry matter (DM), fat, protein (TN×6.38), carbohydrates and ash
233 content according to IDF standards 4A (IDF, 1982), 5B (IDF, 1986), 25 (IDF, 1964a) and 27 (IDF,
234 1964b), respectively. Salt content was determined by Volhard method (AOAC, 2000).
235 Measurements of pH were performed electrometrically by the pH-meter DocuMeter Sartorius (Data
236 Weighing Systems, Inc., Elk Grove, IL, USA). Water activity (a_w) was determined according to the
237 ISO 21807 (2004) using the HygroPalm water activity indicator (Rotronic, Bassersdrof, Germany).
238 Cheese color was analyzed on the top surface by a Minolta tristimulus Chromometer CR-300
239 (Minolta, Osaka, Japan) using CIELAB L*a*b* values (Hunter, 1975). The measure of lightness
240 (L* values, range 0–100) represents black to white, the redness measurement (a* values) describes
241 green to red, and the yellowness measurement (b* values) represents blue to yellow. Beside these
242 attributes, a* and b* values were also used to determine hue angle and chroma: hue angle (a*/b*)
243 gives the predominant wavelength composing the color; chroma or saturation [$\sqrt{(a^2 + b^2)}$] accounts
244 for the vividness or the color purity. The chromometer was standardized using a white standard
245 plate. The results reported are averages of five measurements on the same cheese slice.
246 Fatty acids (FA) were determined on lyophilized cheese samples (100 mg) which were directly
247 methylated with 2 ml of 0.5 M $NaOCH_3$ at 50 °C for 15 min, followed by 1 ml of 5% HCl in
248 methanol at 50 °C for 15 min (Lee and Tweed, 2008). Fatty acid methyl esters (FAME) were

249 recovered in hexane (1.5 ml). One microliter of each sample was injected by auto-sampler into a HP
250 6890 gas chromatography system equipped with a flame-ionization detector (Agilent Technologies
251 Inc., Santa Clara, CA). Fatty acid methyl esters from all samples were separated using a 100-m
252 length, 0.25-mm i.d., 0.25-μm capillary column (cp-sil 88; Chrompack, Middelburg, the
253 Netherlands). The injector temperature was kept at 255 °C and the detector temperature was kept at
254 250 °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.
255 The initial oven temperature was held at 70 °C for 1 min, increased at 5 °C/min to 100 °C, held for
256 2 min, increased at 10 °C/min to 175 °C, held for 40 min, and then finally increased at 5 °C/min to
257 the final temperature of 225 °C and held for 45 min. Helium, with a head pressure of 158.6 kPa and
258 a flow rate of 0.7 ml/min (linear velocity of 14 cm/s) was used as the carrier gas. Fatty acid methyl
259 ester hexane mix solution (Nu-Chek Prep Inc., Elysian, MN, USA) was used to identify each FA.
260 The identification of the conjugated linoleic acid (CLA) isomers was performed using a commercial
261 mixture of cis- and trans-9,11- and 10,12-ocdecadienoic acid methyl esters (Sigma-Aldrich) and
262 published isomeric profiles (Kramer et al., 2004; Luna et al., 2005).
263
264 *2.8. Volatile organic compounds*
265 Volatile organic compound (VOC) were determined using the headspace solid phase
266 microextraction (SPME) technique coupled with gas chromatography with mass spectrometric
267 detection (GC/MS). The cheeses, frozen at −20 °C, were manually grated and 5 g of each cheese
268 were transferred into a vial, added with 10 ml H_2O, 200 μl of internal standard solution (35 mg/l 1-
269 heptanol in 20% ethanol aqueous solution) and 1 g of NaCl. The vials, clear with screw top and hole
270 caps with PTFE/silicone septa 27136 (Supelco, Bellefonte, PA), kept under magnetic stirring, were
271 heated at 60 °C for 25 min (Carlin and Versini, 2005) and the headspace was collected by DBV–
272 carboxen– PDMS fibres (Supelco, Bellefonte, PA) for 30 min at 60 °C. The SPME fibre was
273 inserted directly into a Finnegan TraceMS for GC/MS (Agilent 6890 Series GC system, Agilent
274 5973 NetWork Mass Selective Detector, Milan, Italy) equipped with a DB-WAX capillary column

275 (Agilent Technologies, 30 m, 0.250 mm i.d., film thickness 0.25 μm, part no. 122–7032). The GC-
276 MS system and chromatographic conditions were previously reported by Corona (2010) and
277 Sannino et al. (2013).
278 The detection was carried out by electron impact mass spectrometry in total ion current (TIC) mode
279 using an ionization energy of 70 eV. The mass acquisition range was m/z 30–330. The methodology
280 described by and Alfonzo et al. (2016) and Martorana et al. (2016) was applied for the identification
281 of the compounds. Semiquantitative data (μg/kg of cheese) were obtained by measuring the relative
282 peak area of each identified compound in relation to that of the added internal standard.
283

284 *2.9. Sensory evaluation*

285 After 9-month of ripening, Gran Ovino cheeses were also evaluated for their sensory characteristics.
286 Fifteen descriptive attributes were judged by a panel of 31 assessors members (fifteen men and
287 sixteen woman, from 20 to 57 years old). All panelists were trained at IZS following the ISO 8589
288 (2007) indications. The panelists had available a cubed sample (1 x 1 x 1 cm) in order to evaluate
289 organoleptic attributes and an entire transverse slice of for evaluating appearance attributes. The
290 attributes were organized into: aspect (color and uniformity of structure), smell (strength of odor,
291 milk, butter and unpleasant smell), taste (salty, sweet, acid, spicy and bitter taste), consistency
292 (soft/hard, solubility and grittiness following mastication) and overall acceptability.
293

294 *2.10. Statistical analyses*

295 Statistical analyses of microbiological counts were conducted using STATISTICA software
296 (StatSoft Inc., Tulsa, OK, USA). Microbial, chemical and physical data were analysed using a
297 generalised linear model (GLM procedure, SAS 9.1.2 software). Microbial data were converted to
298 the log scale before statistical elaborations. Differences between means were determined by the
299 post-hoc Tukey's multiple-range test. A $P < 0.05$ was deemed significant.
300

301 **3. Results**

302 *3.1. Monitoring of the acidification process, ripening and microbiological counts*

303 The temperature of milk during skimming increased from 7.7 °C (registered at the time of transfer
304 of the milk into the trapezoidal tank) to 16.3 °C when skimmed milk was tranferred to the copper
305 vat. The average value of pH of vat milk resulting from the mixing of whole and skimmed milk was
306 6.80, while NWSC reached the value of 3.80 thanks to the mixture of *S. thermophilus* PON6,
307 PON244, PON261 and PON413 whose levels (detected on M17 at 44 °C) were 8.5 CFU/ml. After
308 the addition of the NWSC, the milk bulk was characterized by a pH of 6.40 and underwent a rapid
309 acidification; the curds reached 6.05 pH at moulding.

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

314 The levels of the different microbial groups investigated in this study are reported in Table 1. The
315 microbiological counts did not included TPM for cooked whey. TPM counts were comparable
316 among milks and the levels registered in vat milk were 5.6 log CFU/ml. The levels of TMM were
317 slightly higher than TPM and were found at 6.1 log CFU/ml before NWSC addition. After starter
318 addition, TMM increased by about 0.5 log cycle. After cooking, the curd was characterized by
319 residual levels of TMM and TPM of 4.9 and <2 log CFU/g, respectively. TMM of cooked whey
320 was particularly low (4.4 log CFU/ml).

321 Regarding LAB, all milk samples (EWM, SM and VM) were dominated by mesophilic cocci with
322 6.0 log CFU/ml detected before NWSC addition. The levels of the other LAB groups registered in
323 VM were 4.3, 5.2 and 2.8 log CFU/ml for thermophilic cocci, mesophilic rods and thermophilic
324 rods, respectively. After NWSC was added, the highest levels (7.2 log CFU/ml) were shown by
325 thermophilic LAB cocci. The cooked curd was characterized by a decrease of about 1 log cycle for

326 the thermophilic cocci, while a slight reduction was observed for the other groups. Enterococci were
327 2.4 log CFU/ml before milk coagulation, but decreased below the detection level in cooked curd.
328 Within the undesired microbial groups, members of *Enterobacteriaceae* family increased during
329 skimming and were detected at 3.3 log CFU/ml in vat milk. Curd cooking determined a decrease of
330 their levels, estimated at 1.1 log CFU/g in CC. CPS were at particularly high levels in EWM,
331 decreased consistently during skimming (until 2.6 log CFU/ml in SM) and completely disappeared
332 after exposure at 55 °C during curd cooking. A similar behaviour was recorder for yeasts, which
333 were at 1.0 log CFU/ml in VM and disappeared in CC. *Salmonella* spp., *E. coli* and *L.*
334 *monocytogenes* were not detected in any milk, whey or curd samples and, for this reason were not
335 object of investigation in ripened cheeses.
336 After 9-month ripening, the cheeses from the four productions were also analysed. TPM levels were
337 a little lower than 5.0 log CFU/g, TMM almost 1 log cycle lower than LAB which were 6.8 log
338 CFU/g in all media considered for the four groups thermophilic and mesophilic rods and cocci. The
339 levels of enterococci were 2 log cycles lower than LAB, while members of *Enterobacteriaceae*
340 family, CPS and yeasts were below the detection levels.
341
342 *3.2. Identification of dominant LAB in ripened cheeses*
343 After enumeration, 172 colonies showing different characteristics and representative of the
344 dominant presumptive LAB (Gram positive and catalase negative) were isolated and purified. The
345 preliminary morphological/physiological/biochemical characterization allowed to distinguish six
346 main LAB groups (Table 2). The most numerous group was Group VI (accounting for more than
347 34% of the isolated cultures) that included 59 isolates of road shape and characterized by a obligate
348 homofermentative metabolism.
349 All isolates were analysed by RAPD-PCR in order to recognise the different strains. Figure 2
350 reports the dendrogram resulting from the combination of the three RAPD patterns of each isolate
351 and shows the presence of 18 strains. The analysis by 16S rRNA gene sequencing indicated that at

352 9-month of ripening the LAB community of Gran Ovino cheese was mainly represented by the
353 species *Lactobacillus fermentum* (Ac. No. MK908201 – MK908205), *Lactobacillus paracasei* (Ac.
354 No. MK908206 – MK908210), *Enterococcus faecium* (Ac. No. MK908197 – MK908200), and
355 *Pediococcus acidilactici* (Ac. No. MK908211 – MK908213). Only one strain was allotted into the
356 species *Lactobacillus delbrueckii* (Ac. No. MK908214).
357

358 *3.3. Characteristics of the Illumina data and taxonomic analysis of the bacterial community*
359 The DNA extracted from the four cheese samples successfully amplified the bacterial V3-V4 16S
360 rRNA and after splitting and quality trimming the raw data, 123,932 reads remained for subsequent
361 analysis. The relative abundance (%) of the different identified bacterial groups is reported in Fig. 3.
362 Only the groups with an incidence of 0.1% were considered. The two most abundant species
363 belonged to the genera *Lactobacillus* and *Streptococcus* that together covered more than 90% of the
364 microbial relative abundance in all cheeses. However, the proportions of the two genera found
365 among the four replicates of Gran Ovino cheese productions differed substantially, e.g. from
366 21.45% of *Lactobacillus* and 68.90% of *Streptococcus* at the second production week until 81.31%
367 and 14.04% of *Lactobacillus* and *Streptococcus*, respectively, at the third week. Furthermore, all
368 cheeses were also characterised by the presence of other unidentified LAB. Regarding the undesired
369 bacterial groups, especially the phylum Gammaproteobacteria to which the members of
370 *Enterobacteriaceae* family belong to, they were at very low levels (at highest 2.28% at the third
371 week).
372

373 *3.4. Physico-chemical characteristics of ripened cheese*
374 Ripened cheeses (Table 3) were characterized by a dry matter of 67.65%. Fat percentage was lower
375 than that of protein. Ripening determined a maturation index (soluble N/total N) closed to 25%. A
376 very low salt percentage was found with an a_w of 0.95 and pH 5.72.

377 Colorimetric parameters (Table 3) showed that Gran Ovino cheese is characterized by a deep
378 yellow paste with a good level of lightness (Chroma and Hue angle values were 20.20 and -0.27,
379 respectively).
380 Cheese fatty acids composition is reported in Table 4. The more represented fatty acids were
381 Palmitic (23.01%), Oleic (12.80%) and Myristic (11.08%) acids, the sum of saturated fatty acids
382 was 68.40% with SFA/UFA ratio of 2.17. Interesting is the content of omega-3 fatty acids (3.05)
383 with a ω-6/ω-3 ratio of 0.67. Fatty acids with healthy interest showed good values: 3.09% for
384 Vaccenic acid, 1.70% for Linoleic acid, 2.39% for Linolenic acid and 1.05% for Rumenic acid.
385
386 *3.5. Volatile organic compound composition of "Gran Ovino" cheese*
387 The volatile organic compounds emitted from Gran Ovino cheese from the four productions are
388 reported in Fig. 4. Forty-one volatile compounds were identified in the headspace of the cheeses: 11
389 acids, 8 esters, 8 ketones, 5 alcohols, 5 aldehydes, 2 phenols and 1 terpene. The VOCs of the cheese
390 samples showed some differences. In particular, high concentrations of hexanoic, octanoic,
391 decanoic and butyric acid among the acids (total acids respectively 64 and 33 μg/kg); ethyl esters
392 (C6, C8, C10), while butyl butyrate, hexanoate, and isoamyl hexanoate among the esters (total
393 esters respectively 12 and 4 μg/kg) were registered. Benzaldehyde was dominant among the
394 aldehydes, 1-hexanol, 2-phenylethanol and 2-nonanol among the alcohols, and 2-decanone, acetoin,
395 3,5-octadien-2-one and 2-nonanone among the ketones. The terpene D-limonene was present at
396 very similar concentrations in the four cheese samples (about 272 μg/kg). Phenol (p-cresol and o-
397 cresol) showed the highest concentration in the cheese produced during the first week.
398
399 *3.6. Sensory evaluation*
400 Figure 5 reports the spider graphic representation of the sensory characteristics evaluated on Gran
401 Ovino cheeses by the judges. The highest scores were registered for color, uniformity, strength of

402 odor, chewiness and solubility while the lower score was evidenced by unpleasant odor. The overall
403 assessment, intended as an overall rating of the cheeses expressed considering all parameters with
404 their levels of evaluation, indicated a certain appreciation of this novel cheese expressed by the
405 judges.
406
407 **4. Discussion**
408 In past, cheese making represented a means for the preservation of raw milk through the
409 fermentation process. During its first production step, cheese can be described as an aggregate of
410 casein micelles forming a gel containing all solid components of milk (Dalgleish and Corredig,
411 2012) in which all microorganisms present in the raw milk are trapped. Due to the technology
412 applied during processing, each cheese variety will dictate the potential for the growth of desired
413 LAB as well as for the survival of undesired (spoilage and pathogenic) microorganisms (Donnelly,
414 2004).
415 This work was aimed to evaluate technological alternatives for processing raw ewes' milk into
416 cheeses with high hygienic quality. To this purpose, the technology of Grana type cheeses,
417 generally applied to transform raw cows' milk (Mucchetti and Neviani, 2006), was tested on raw
418 ewes' milk. This technology is mainly characterised by a curd cooking step.
419 Recently, an approach based on the use of selected starter and non starter LAB was applied to
420 ameliorate the production of raw milk cheeses, such as PDO Pecorino Siciliano with the
421 modification of the production protocol from a raw milk production without bacterial culture
422 addition to a protocol including the addition of selected strains (Settanni et al., 2013). Based on the
423 positive results registered in terms of reduction of undesired bacterial groups (pseudomonads and
424 *Enterobacteriaceae*) the innovation respectful of the traditional production technology was applied
425 at large scale level on the entire Sicilian area improving the hygienic characteristics of all final
426 cheeses (Guarcello et al., 2016). However, although consistently reduced in number, these bacteria

427 were still found during ripening and some defects in cheese structure, due to the presence of eyes,
428 was noticed.
429 The main hypothesis of this study was that Grana type technology applied to raw ewes' milk
430 contained the development of the undesired microbial groups. This strategy is not completely new
431 in Sicily, because Maiorchino cheese is produced by curd cooking after coagulation of a mixed
432 cows', ewes' and goats' bulk milk (Conte and Panebianco, 2001). However, a NWSC was prepared
433 *ad hoc* in order to perform a driven fermentation for Gran Ovino cheese. The inclusion of adjunct
434 cultures might influence the ripening profiles of hard cheeses (Cuffia et al., 2019), for this reason
435 non starter LAB were not added to the milk in order to evaluate the natural evolution of indigenous
436 raw ewes' cheese strains.
437 In the present work, the microbiological parameters were first evaluated by plate count. TMM and
438 TPM of milk samples (EWM, SM and VM) did not show great variations after skimming and
439 mixing in vat. Generally, typical ewes' milk cheese productions performed in Sicily do not include
440 a curd cooking step and, after coagulation, an increase of the microbial counts is registered as a
441 consequence of whey draining (Gaglio et al., 2014b; Settanni et al., 2013). On the contrary, in the
442 present study lower values of TMM and TPM were found in curds showing a strong effect of the
443 treatment of curd grains at 55 °C for 8 min; the values of these microbial groups were 2 and 5 log
444 cycles lower than bulk milk used for transformation.
445 Mesophilic LAB cocci dominated the microbial community of milk before NWSC addition, but no
446 differences among the levels of LAB cocci and LAB rods were registered in ripened cheeses.
447 Similar results are generally reported for Grana type cheeses produced from raw cows' milk (De
448 Dea Lindner et al., 2008; Monfredini et al., 2012) The levels of enterococci were 2 log cycles lower
449 than other LAB, similarly to what reported for Parmigiano Reggiano cheese (Coppola et al., 2000).
450 The most interesting results were displayed by the members of *Enterobacteriaceae* family which
451 increased during skimming but strongly decreased during curd cooking until disappearance in

452 ripened cheese as observed for cows' Grana cheeses (Coppola et al., 2000; Monfredini et al., 2012).
453 The cooking step determined also the complete disappearance of CPS and yeasts.
454 LAB communities were firstly studied by a culture-dependent approach which recognised six
455 phenotypic groups including rods and cocci. The LAB most frequently isolated were *Lb. paracasei*
456 and *Lb. fermentum*. In particular, *Lb. paracasei* is often isolated during the ripening of different
457 Grana type cheeses (Gala et al., 2008; Monfredini et al., 2012; Solieri et al., 2012; Zago et al.,
458 2007), while *Lb. fermentum* is less frequent, but found in Parmigiano Reggiano cheese during the
459 first production stages (Neviani et al., 2009). *Pediococcus acidilactici* was also isolated from Gran
460 Ovino cheese and this species is associated to ripened Grana cheeses (Gala et al., 2008; Neviani et
461 al., 2009). Enterococci of Gran Ovino were represented by *E. faecium* which is commonly found in
462 raw ewes' milk cheeses (Gaglio et al., 2014a; Pino et al., 2017; Todaro et al., 2011), but for other
463 Grana cheeses, such as Parmigiano Reggiano cheese, it presence is only reported at the beginning of
464 production (Pogačić et al., 2013). Regarding the presence of a viable strain of the thermophilic *Lb.*
465 *delbrueckii* after nine months of ripening, this finding is not surprising since Di Grigoli et al. (2015)
466 also isolated viable colonies belonging to this species from ripened Caciacavallo Palermitano
467 cheeses.
468 The bacterial community of Gran Ovino cheese was also approached by a culture-independent
469 perspective, analysing total DNAs extracted from the four replicate cheeses. This tools showed data
470 almost completely in agreement with the culture-based study concerning lactobacilli, but also
471 showed a consistent presence of streptococci. Since no *Streptococcus* was isolated, at least at the
472 dominant levels, from 9-month ripened cheeses, the high percentages of OTUs identified as
473 *Streptococcus* might probably derive from residual DNAs of death cells. This statement is also
474 supported by the temperatures monitored during ripening which were below 15 °C and the
475 thermophilic starter *Streptococcus thermophilus* cannot grow at this temperatures (Hardie and
476 Whiley, 1995). Similar findings were reported by Bassi et al. (2015) who found *Lactobacillus*

477 (65.3%) and *Streptococcus* (14.4%) in Grana Padano cheeses applying a next generation sequence
478 approach performed with MySeq Illumina.
479 Ripened cheeses presented a dry matter percentage similar to analogous Sicilian cheeses with long
480 ripening periods (Guarcello et al., 2016). Maiorchino cheese is very similar to ours cheeses with the
481 difference that it is made from entire milk, dry matter percentage of Maiorchino with 8-month of
482 ripening is around 70% (Conte et al., 2015). Fat and protein percentages found in our cheeses
483 displayed values similar to those of PDO Pecorino Siciliano (Guarcello et al., 2016) and Grana
484 Padano cheese (Consorzio Tutela Grana Padano, 2002), while Maiorchino cheese present the same
485 fat content, but lower protein percentages (Conte et al., 2015). Maturation index showed a good
486 proteolysis activity and resulted slightly higher than PDO Pecorino Siciliano at 5-month of ripening
487 (Guarcello et al., 2016).
488 Salt content was very low for a ripened cheeses made with sheep milk and lower than others Italian
489 Pecorino cheeses at the same ripening period (Di Cagno et al., 2003; Guarcello et al., 2016); this
490 fact is to be considered as positive to increase the consumer satisfaction and reduce the human
491 pathologies due to high consumption of salt; but the low salt content in cheese is permitted only
492 when its microbiological quality is high. a_w values were higher than those of other cheeses with
493 analogous ripening period; e.g. a_w for PDO Pecorino Siciliano is on average 0.92 (Guarcello et al.,
494 2016), the same values were reported for Maiorchino cheese (Conte et al., 2015). The value of pH
495 resulted similar to those of PDO Pecorino Siciliano (Guarcello et al., 2016), but higher than
496 Maiorchno cheese (Conte et al., 2015). Color parameters showed that our cheeses were
497 characterized by a deep yellow and high lightness, values clearly higher than PDO Pecorino
498 Siciliano (Todaro et al., 2011), making Gran Ovino cheese more attractive to consumers.
499 Fatty acids composition of these cheeses were similar to those of hard cheeses made from sheep's
500 milk (Prandini et al., 2011), but our cheeses showed a higher percent of PUFA (8.60 vs 4.93%) and
501 lower SFA/UFA ratio, probably due to the high level of pasture in the diet of the sheep (Bonanno et
502 al., 2016) that produced the milk used in this study. Regarding healthy fatty acids, our cheeses

503 showed triple levels of Linolenic acids, also this result is due to sheep feeding, than those registered
504 for the sheep grazed on Sulla meadows. In fact, one factor known to increase the concentration of n-
505 3 FA in sheep milk (Cabiddu et al., 2005) is the presence of legumes in the feed ration. It is likely
506 that this is the result of plant secondary compounds which are often higher concentrated in legumes,
507 as Sulla forage. Important representative of plant secondary compounds are tannins (Cabiddu et al.,
508 2009), which may partially inhibit ruminal biohydrogenation and, thus, reduce the loss of native
509 plant FA like C18:3 n-3 during digestion. However, it has to be taken into account that the lipolysis
510 is influenced by the temperature during ripening of Grana cheeses (Sihufe et al., 2007).
511 Furthermore, FFA profiles also depend on the starter strains (Perotti et al., 2005).
512 The biochemical processes which lead to the synthesis of volatile compounds in cheese are very
513 complex (Kilcawley, 2017; Thierry et al., 2017). It is known that the volatile compounds identified
514 in cheese are mainly the products of lipolysis, proteolysis, metabolism of residual lactose, lactate,
515 and citrate. Lipolysis of the triglycerides by microbial and indigenous milk enzymes, and also
516 enzymes from added rennet pastes, result in the development of medium-chain (carbon chain
517 lengths $\leq$ 10) and long-chain (carbon chain lengths >10) FFAs (Free Fatty Acids) (Collins et al.,
518 2003; Thierry et al., 2017). The flavor contribution of FFAs in cheese is mainly influenced by the
519 pH. FFAs at high pH levels are less flavor active and are often perceived as "soapy" as they are
520 converted to nonvolatile salts. At low pH FFAs exist in free form and are perceived as rancid at
521 high concentrations (Singh et al., 2003). The main components of the volatile fraction of Gran
522 Ovino cheeses analyzed in this study were free fatty acids mostly represented by hexanoic,
523 octanoid, decanoic and butyric acid. The four productions of Gran Ovino cheese had significantly
524 different FFAs, acids from C5 to C10. FFAs contribute to the formation of cheese flavor not only
525 directly, but also indirectly as they are precursors of methyl ketones, secondary alcohols, straight-
526 chain aldehydes, lactones and esters (Collins et al., 2003; Smit et al., 2005; Thierry et al., 2017).
527 Also the content is high of the ethyl esters of medium chain fatty acids (from C6 to C10), alcohols
528 and aldehydes of Gran Ovino cheeses. Benzaldehyde is very high in Gran Ovino cheese, especially

529 at the second production week; this compound can be formed by enzymatic activities (proteolysis
530 and peptidolysis) or by chemical conversion by phenyl-pyruvic acid (Smit et al. 2005). The
531 differences in the VOCs emitted from the cheeses produced in the four weeks are a direct
532 consequence of the differences revealed in the bacterial communities. This is a common observation
533 when cheeses are analysed, since cheeses produced in a given cheese factory in different days or
534 even in different vats the same days might be different (Fitzsimons et al., 1999; Williams et al.,
535 2002).
536 Sensory evaluation indicated that the ripened Gran Ovino cheese was characterized by a general
537 appreciation by judges and, in particular, the level of unpleasant odors, which represents one of the
538 main parameters for tasters' acceptance of a new product (Herz, 2006), was very low.
539 In conclusion, in this work a post-milking approach was applied to ameliorate the hygienic
540 characteristics of raw ewes' milk cheeses by introducing a curd cooking step during milk
541 transformation. Even though TMM of bulk milk was 6.1 log CFU/ml that is above the limit of
542 500.000 CFU/ml established for the raw ewes' milk for cheese production (CE, 2004), the strategy
543 tested in this work allowed to obtain an extra-hard cheese, namely Gran Ovino, characterised by the
544 absence of undesired microorganisms. Furthermore, sensory evaluation determined the appreciation
545 by judges with high values of overall acceptance indicating the possible positive response by
546 consumers enlarging the offer of raw ewes' milk processed products.
547

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554

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765

766 **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]	***

767 768 769 770 771 772

773 [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).
774 [b] Data within a line followed by the same letter are not significantly different according to Tukey's test.
775 P value: *, P≤0.05; **, P≤0.01; ***, P≤0.001.
776 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
777 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,
778 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
779 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
780 glucose agar for *Enterobacteriaceae*; CPS, coagulase-positive staphylococci; DRBC, dichloran rose bengal chloramphenicol agar for yeasts; n.d., not determined.

Table 2. Phenotypic grouping of the LAB isolated from Gran Ovino cheeses.

Characters	Clusters					
	1 (n=39)	2 (n=16)	3 (n=5)	4 (n=44)	5 (n=9)	6 (n=59)
Morphology[a]	C	C	C	R	R	R
Cell disposition[b]	sc	tr	tr	sc	sc	sc
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	-	-	-	+	-	-

[a] R, rod; C, coccus.
[b] sc, short chain; tr, tetrads.
Abbreviation: n.d., not determined.

797 **Table 3.** Physicochemical parameters of experimental Gran Ovino cheeses cheeses.

Parameters	Mean	SD[1]
Dry matter (%)	67.65	0.53
Fat (% on DM[2])	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[3]	20.20	0.58
Hue angle[4]	-0.27	0.02

798 [1]SD = standard deviation
799 [2]DM = Dry matter
800 [3]Croma=$\sqrt{(a^2+b^2)}$;
801 [4]Hue angle=a/b.

802 **Table 4.** Cheese fatty acid composition (g/100 g FAME)

Fatty acids	Mean	SD[1]
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[2]	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[3]	1.70	0.07
C18:3 n-3 *c9 c12 c15*	2.39	0.01
CLA C18:2 *c*9 *t*11, RA[4]	1.05	0.02
C20:0	0.39	0.09
C20:5 n-3, EPA[5]	0.12	0.00
C22:5 n-3, DPA[6]	0.22	0.02
OBCFA[7]	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[8]	22.97	0.48
PUFA[9]	8.62	0.14
Unsaturated FA	31.60	0.60
Saturated/unsaturated	2.17	0.06
HPI[10]	0.44	0.02

803 [1]SD = standard deviation
804 [2]VA = *trans* vaccenic acid. [3]LA = linoleic acid. [4]RA = rumenic acid. [5]EPA = eicosapentaenoic acid. [6]DPA = docosapentaenoic acid. [7]OBCFA = odd
805 and branched chain fatty acids. [8]MUFA = Monounsaturated fatty acids; [9]PUFA = Polyunsaturated fatty acids; [10]HPI = Health Promoting Index =
806 unsaturated fatty acids/[C12:0 + (4 × C14:0) + C16:0] (Chen et al., 2004).
807

808 **Legend to figures**

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

811 **Fig. 2.** Dendrogram obtained with combined RAPD-PCR patterns of the LAB strains isolated from
812 Gran Ovino cheese. Abbreviations: *E.*, *Enterococcus*; *Lb.*, *Lactobacillus*; *P.*, *Pediococcus*.

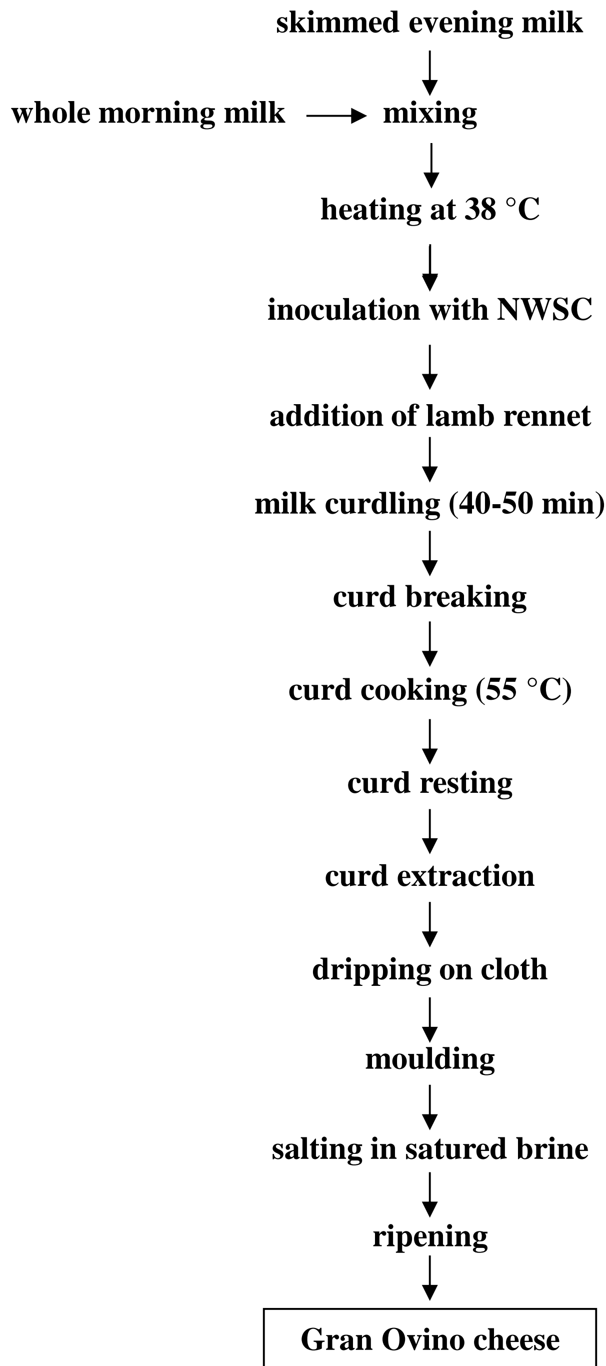
813 **Fig. 3.** Relative abundances (%) of bacteria identified by MySeq Illumina in Gran Ovino cheeses
814 after 9-month of ripening. Abbreviation: CW1, cheese week one; CW2, cheese week two; CW3,
815 cheese week three; CW4, cheese week four.

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

818 **Fig. 5.** Spider diagrams of descriptive sensory analysis of Gran Ovino cheeses. Abbreviation: RC,
819 9-month ripened cheese.

820

821 **Fig. 1.**



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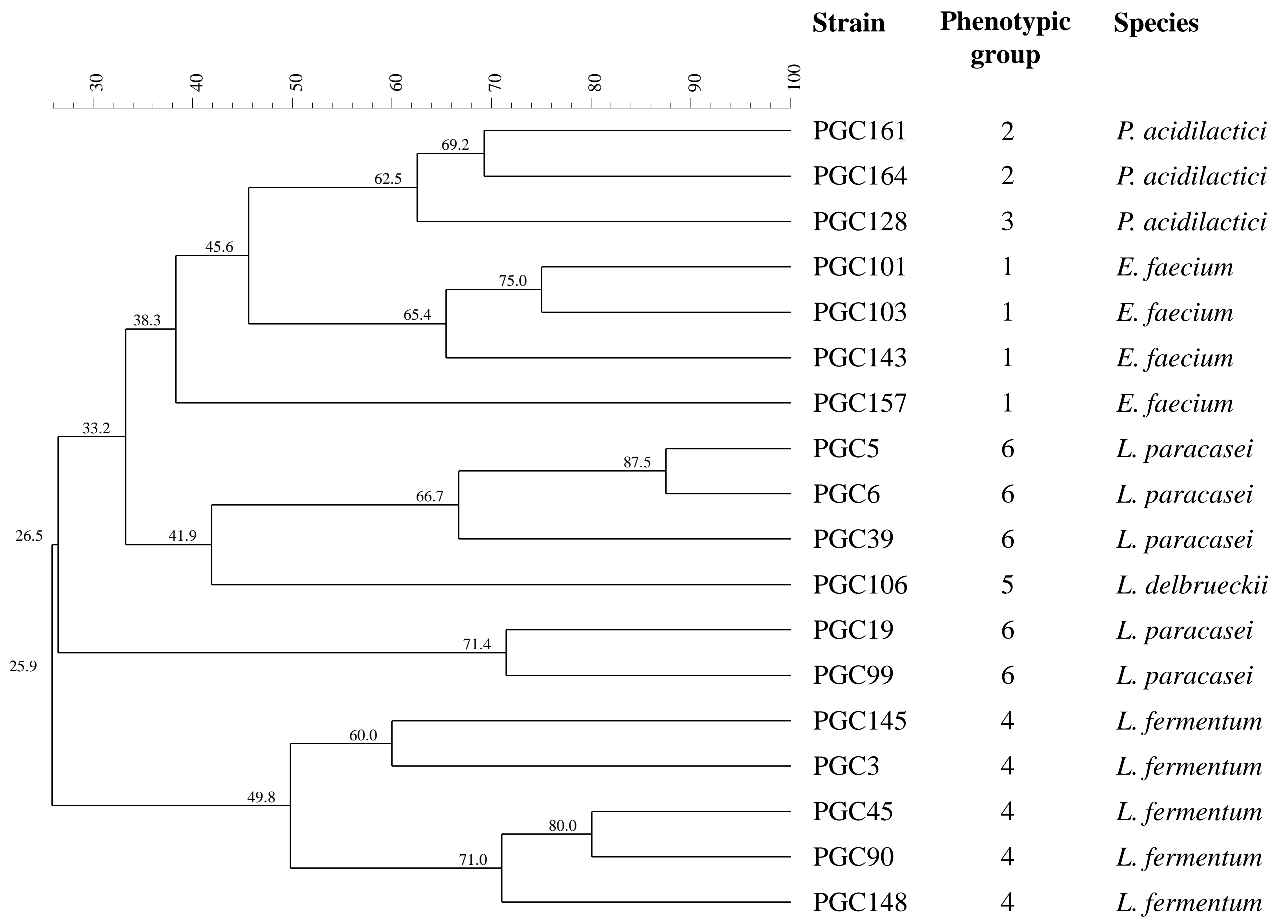
824 **Fig. 2.**



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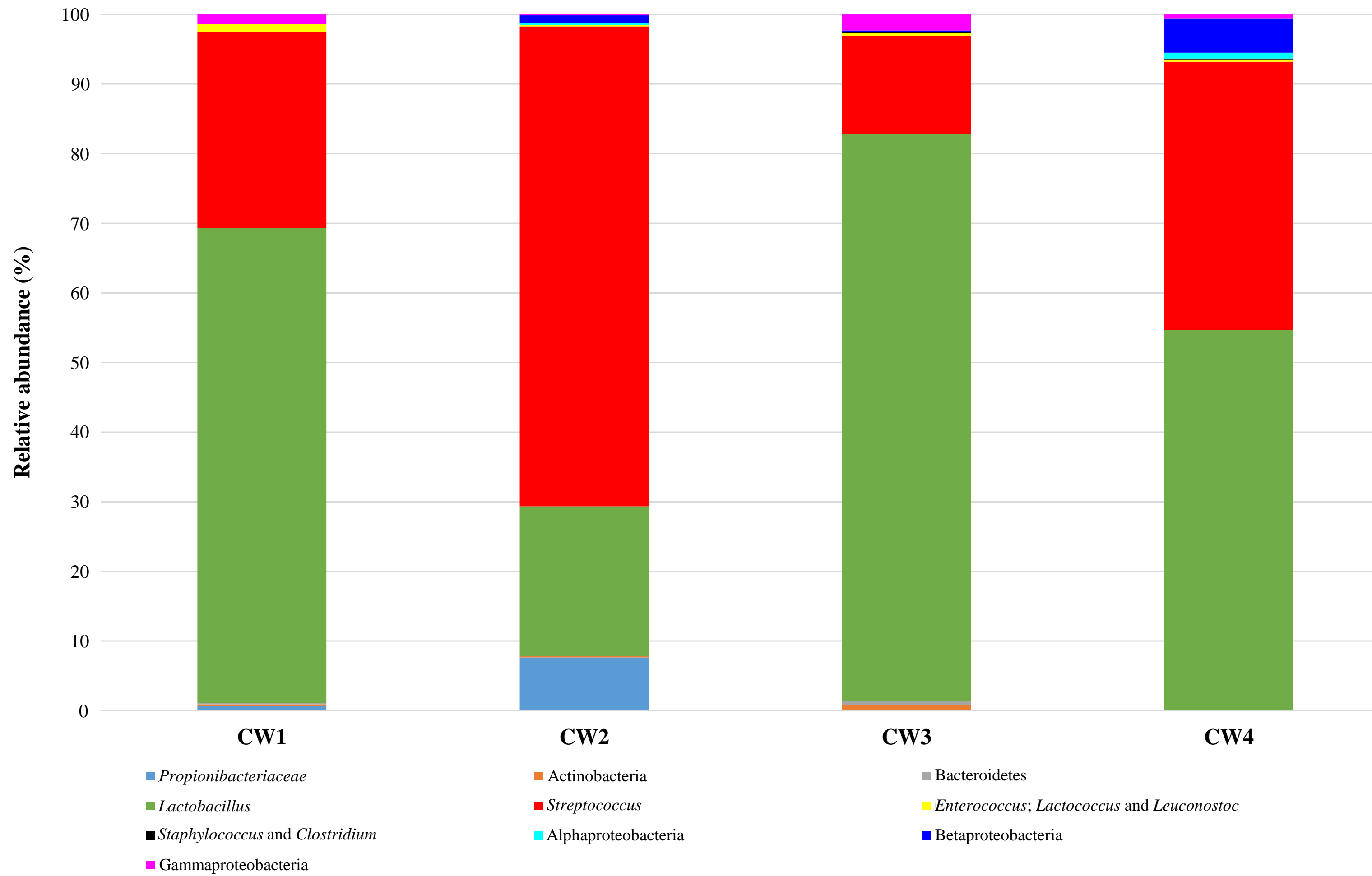
827 **Fig. 3.**



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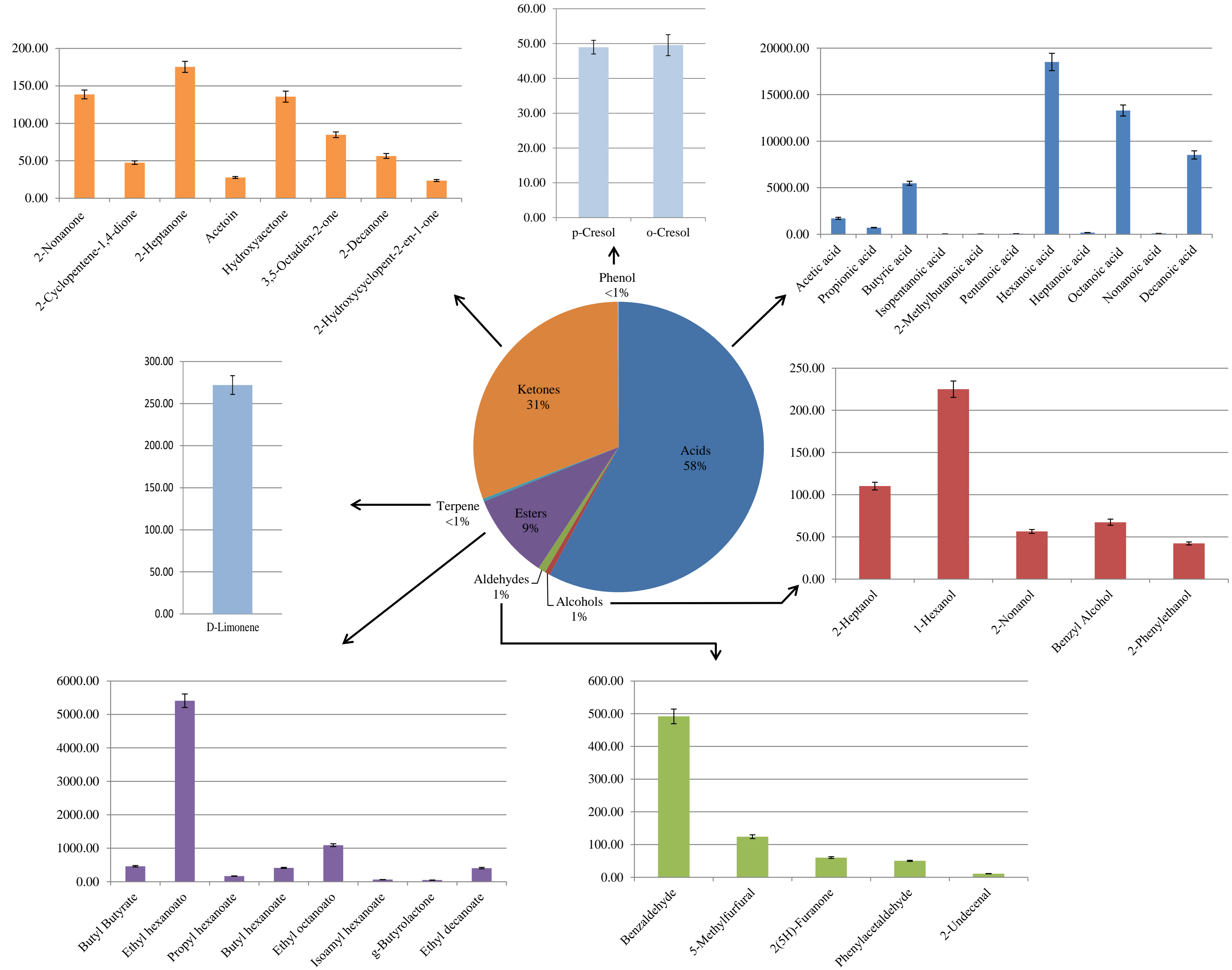
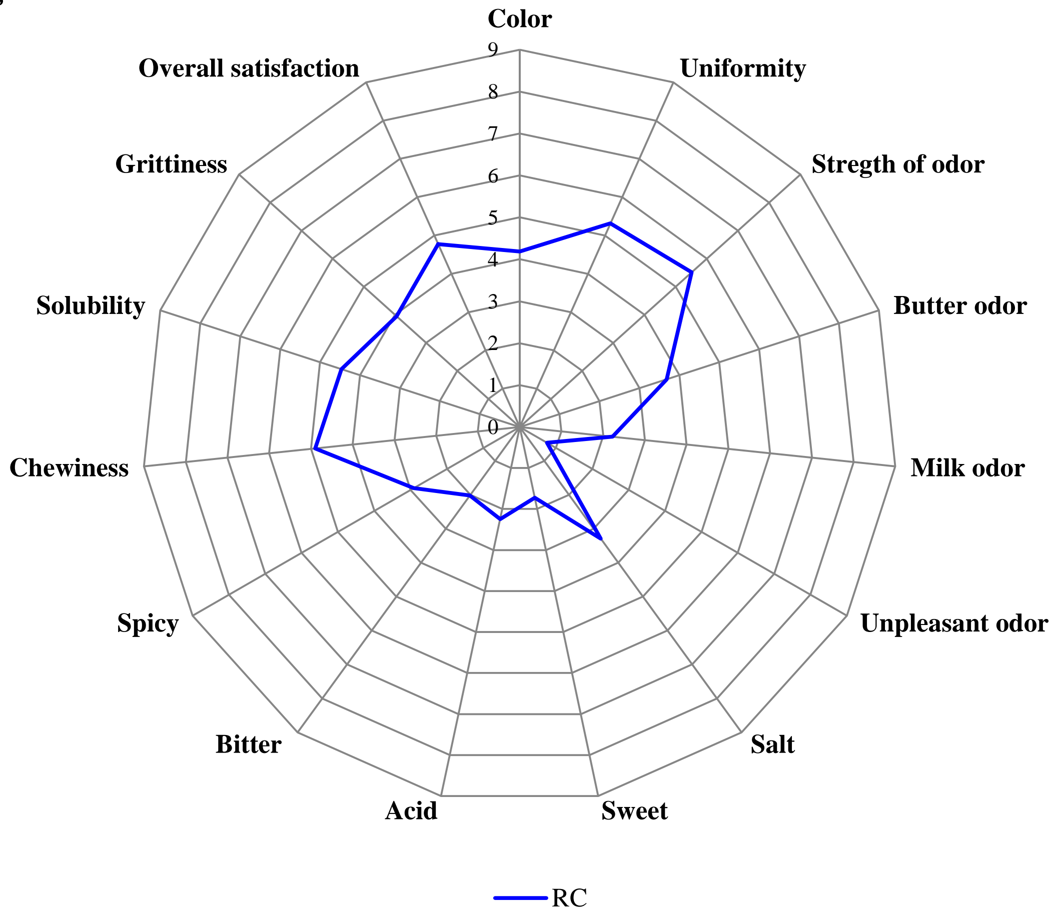
830 **Fig. 4.**



831

832 **Fig. 5.**



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