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Abstract: The present study investigated the dynamic changes in biodiversity population of lactic acid bacteria associated with the natural whey culture (NWC) of Trentingrana PDO cheese. NWC samples were collected from 14 dairies over a period of three years. Culture-independent analysis (pentaplex-PCR) highlighted the continued presence of Lactobacillus helveticus and Streptococcus thermophilus along with a drastic reduction of Lb. delbrueckii subsp. lactis presence. RAPD-PCR performed on 64 LAB strains isolated from 4 dairies pointed out an important decrease in the number of Lb. helveticus and St. thermophilus biotypes over time. This biodiversity loss was reflected also on the volatile organic compounds produced by the NWC isolates. Our results indicate that to preserve the complex pattern of cheese flavour it will be necessary introduce technological innovations to protect the whey culture microbiota preventing adverse effect on typical cheese traits.

Dear Editor Jean-Marc Chobert,

I wish to thank you for your letter of 8 July 2019 referring to our submission to LWT - Food Science and Technology of the manuscript (LWT-D-19-02317) entitled:

"How the biodiversity loss in natural whey culture is affecting ripened cheese quality? The case of Trentingrana cheese".

by: Stefano Morandi, Giovanna Battelli, Tiziana Silvetti, Andrea Goss, Nicola Cologna, Milena Brasca

We have addressed all suggestions and recommendations made by the reviewers, which are detailed below.
I hope that in its present and revised form, the manuscript will be accepted for publication.

With my very best wishes,

Tiziana Silvetti

Reviewer #1:

(X) OVERALL COMMENT
Some minor however important improvements are needed.

(X) INTRODUCTION
A first paragraph about the relevance of cheese as food and production should be added (Journal of Dairy Science, Volume 100, Issue 12, December 2017, Pages 9952-9965). In addition recent studies about cheese too (LWT, Volume 93, July 2018, Pages 287-292; Int J dairy technology, Volume71, Issue2, May 2018, Pages 372-381).
AU: According to the reviewer's suggestion, a brief description of cheese as food and cheese-production was added and the relative references have been included in the text (lines 37 - 43).

line 37-45: it should be decreased.
AU: Sentences has been shortened as suggested (lines 43 - 46).

lines 58-62: add recent reference (Food Research International, Volume 108, June 2018, Pages 18-26)
AU: Reference has been added as suggested (line 62).

(X) MATERIAL AND METHODS
Explain more the 4 dairies chosen. More details and the reason to chose them.
AU: According to the reviewer's suggestion, a description of the 4 dairies chosen has been included in the text (lines 86 - 91).

(X) RESULTS AND DISCUSSION
It lacks a paragraph with the practical significance of the study for the cheese industry.
AU: The request has been addressed in the Conclusion section.

(X) CONCLUSION
Please decrease this part.
AU: The section has been reduced accordingly (lines 334 - 347)

Reviewer #2:
Title: please think about change the word "dairy" to "ripened cheese".
AU: We agree with the reviewer and the title has been modified.

Abstract: exclude "(from 93% of positive samples in April 2014 to 51% in July 2016)".
AU: The sentence has been excluded as suggested.

Line 45: Different studies...
AU: The sentence has been modified as suggested (line 47).

Line 46: first citation for the species please use *Lactobacillus delbrueckii* subsp. *lactis*. Double check all the text.
AU: According to the reviewer's suggestion, the first citation for the species has been written in full (lines 48, 49, 52 and 225).

Please condensate the section 2.1 with 2.2 in the M&M. The introductory sentences are quite similar.
AU: We considered the reviewer's criticism pertinent, consequently the sections 2.1 and 2.2 were reduced.

Line 140: Why 15 days?
AU: LAB are generally known as weak lipolytic and proteolytic microorganisms. To promote these activities, and in consequence VOCs production, the LAB strains were incubated at 42 °C for 15 days as reported by Morandi et al. (International Dairy Journal 16 (2006) 867–875) and in the ISO 27205:2010 (IDF 149:2010; Fermented milk products - Bacterial starter cultures - Standard of identity).

Line 171: what discrepancy?
Line 171: LH-PCR is conducted using DNA from isolated strains. What mean 5 log 10 for limit of detection?
AU: We considered the reviewer's criticism pertinent, consequently the sentence has been removed.

Line 217: Why did you not use whey broth medium?
AU: Whey broth medium is complicated to obtain in laboratory, since the filtration step is very difficult and not easy to standardize. In order to overcome the inability of the isolates to grow in M17 broth, reconstituted skimmed milk was used. This method allowed us to recover the Streptococcus thermophilus *strains considered in this study. A brief description of this procedure has been added in Material and Methods paragraph (lines 103 - 106).*

Conclusion: please dedicate more information regarding how technological innovations can help to mitigate the problematic.
AU: Conclusion has been implemented according to the suggestions

EDITOR'S COMMENTS

Send a revised version with all modifications suggested by Reviewers and Editor (see below) visible.
References indicated in Tables 4 and 5 are not in the references list. Please, add them.
AU: -The references present in Table 4 and 5 have been added in the reference list (lines 476 - 477).

Use et al. (for the first citation) ONLY if the number of authors is >5.
AU: References have been modified as suggested.

l. 434: Huey & Hall not cited
AU: Reference has been eliminated from the reference list.

Highlights

- Novel information is provided about the biodiversity depletion in NWCs.
- RAPD-PCR showed a decrease of *Lb. helveticus* biotypes over time.
- A drastic reduction of *Lb. delbrueckii* subsp. *lactis* in NWCs was observed.
- Production of volatiles compounds in milk is species and strain-dependent.
- Biodiversity loss also affected the VOCs production.

How the biodiversity loss in natural whey culture is affecting ripened cheese quality? The case of Trentingrana cheese

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Abstract.

The present study investigated the dynamic changes in biodiversity population of lactic acid bacteria associated with the natural whey culture (NWC) of Trentingrana PDO cheese. NWC samples were collected from 14 dairies over a period of three years. Culture-independent analysis (pentaplex-PCR) highlighted the continued presence of *Lactobacillus helveticus* and *Streptococcus thermophilus* along with a drastic reduction of *Lb. delbrueckii* subsp. *lactis* presence. RAPD-PCR performed on 64 LAB strains isolated from 4 dairies pointed out an important decrease in the number of *Lb. helveticus* and *St. thermophilus* biotypes over time. This biodiversity loss was reflected also on the volatile organic compounds produced by the NWC isolates. Our results

indicate that to preserve the complex pattern of cheese flavour it will be necessary introduce technological innovations to protect the whey culture microbiota preventing adverse effect on typical cheese traits.

Keywords

Biodiversity loss; Trentingrana cheese; *Lactobacillus helveticus*; *Streptococcus thermophilus*; whey starter

1. Introduction

Cheese and dairy products are considered nutrient-dense foods being a source of proteins, bioactive peptides, lipids, vitamins, and minerals (Silva et al., 2018). Their quality is related not only to the nutritional characteristic but also to the development of desirable texture and flavor (Johnson, (2017). Grana Padano is one of the most important Italian Protected Designation of Origin (PDO) cheese, representing 23% of the Italian milk production (12.079×10^3 ton in 2018) destined for its manufacturing. This gives rise to 4.9 million cheese loaves, about one third of them exported (http://www.clal.it, 2018). Trentingrana PDO cheese, belonging to the consortium of Grana Padano, is produced in a specific alpine area of Northern Italy. It is a hard-textured, cooked, and long-ripened (9-30 months) cheese made using raw cow's milk supplemented with natural whey culture (NWC) and without the addition of lysozyme (Rossetti et al., 2008).

Different studies on Grana Padano NWCs showed a constant presence of the dominant species *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *lactis* (10^8-10^9 CFU/mL) along with minor species corresponding to *Streptococcus thermophilus* (10^5-10^6 CFU/mL) and *Lactobacillus fermentum* (10^3-10^4 CFU/mL) (Rossi, Gatto, Sabattini, & Torriani, 2012; Santarelli, Bottari, Lazzi, Neviani & Gatti, 2013). Nevertheless, in the past (Carini, Lodi, Todesco, & Vezzoni, 1977; Bosi et al., 1990) many authors described the presence of *Lactobacillus delbrueckii* subsp. *bulgaricus* in

NWCs, but in this century a difficulty to detect this subspecies was evident (Cremonesi et al., 2011). The prevalence of few dominant species in NWCs suggests that their content, as well as their microbiological richness make a major contribution to the specificity, uniqueness and sensory characteristics of cheese (Montel et al., 2014). The dynamics and balances of volatile organic compounds (VOCs) in the dairy products are associated with differences in the biodiversity present during the cheese-making and ripening period (Buchin, Tessier, Berthier, Salmon, & Duboz Buchin, 2004). In addition, some recent investigations evidenced that the identification of VOCs produced by lactic acid bacteria (LAB) represent a key factor to evaluate microbial consortia and the technological potential of strains involved in cheese production (Bancalari et al., 2017; Gallegos, Arce, Jordano, Arce, & Medina, 2017; Matera et al., 2018).

Up to now, a number of studies are available on the biodiversity of thermophilic lactobacilli (mainly *Lb. helveticus*) isolated from individual whey starter cultures for Grana Padano and Parmigiano Reggiano cheeses (Gatti, Lazzi, Rossetti, Mucchetti, & Neviani, 2003; Andrighetto, Marcazzan, & Lombardi, 2004; Rossetti et al., 2008; Rossi et al., 2012), but nothing is known about biodiversity changes in NWCs over the years. Moreover, few information is available on VOCs generated by strains isolated from NWCs (Sgarbi et al., 2013).

In this study, we aimed i) to study by molecular methods the Trentingrana NWCs biodiversity from 14 dairies over a period of three years ii) to characterize by molecular methods the biodiversity within the prevailing LAB species isolated from whey samples and ii) to investigate the ability of the LAB strains to generate VOCs in milk.

2. Materials and methods

2.1. Microbial composition, enumeration and isolation of LAB in Trentigrana NWCs samples

From April 2014 to July 2016, 98 whey cultures used for Trentigrana production were collected twice a year from 14 dairies located in Trentino-Alto Adige (7 samples per dairy). The NWCs were obtained from the spontaneous fermentation of a part of the whey from the cheese-making

79 performed in the previous day. After collecting, the samples were rapidly cooled to 4 °C and
80 analyzed within 8 h. To mitigate against the potential adverse effects of high acidity, in all whey
81 samples, sterile calcium carbonate (5% v/v) was added. In order to detect the presence of the
82 expected LAB species (*Lb. delbrueckii* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb.*
83 *fermentum*, *Lb. helveticus* and *St. thermophilus*), NWC samples were analyzed by pentaplex
84 polymerase chain reaction (PCR) according to Cremonesi et al. (2011). The DNA was directly
85 extracted from 1 ml of each whey sample according to Cremonesi et al. (2006).
86 At the same time, four dairies (A, B, C and D), located in different zones of the Trento province,
87 were selected in order to study the biodiversity changes over a period of three years. These dairies
88 were chosen based on the following criteria: (1) altitude; (2) ton of milk processed and (3) number
89 of milk producers. All the dairies were located about 900-1.000 m above the sea level and processed
90 from 4.500 to 7.500 ton of milk per year. Dairies A and D were characterized by a high number of
91 milk producers (50 and 67), while B and C collected the milk from 16 and 13 farms respectively.
92 Seven samples per dairy were collected as described above and microbiological analyses were
93 performed. NWC samples were serially diluted in reconstituted (10% w/v) skimmed milk (Sacco
94 System, Cadorago, Italy) and inoculated into the following culture media: de Man Rogosa and
95 Sharpe (MRS) pH: 5.4 agar (Biolife Italiana, Milano, Italy) under anaerobic conditions (Anaerocult
96 A, Merck, Darmstad, Germany) at 42 °C for 72 h for lactobacilli and M17 agar containing lactose
97 (0.5%) (Biolife Italiana) at 42 °C for 48 h for streptococci. Heterofermentative LAB were
98 determined by the most probable number (MPN) method using MRS broth with Durham tubes
99 (MRS+C). Inoculated tubes were incubated at 30 °C for 72 h and later at 37 °C for 48 h and
100 examined daily for gas production. The MPN results were evaluated according to ISO 7218 (ISO,
101 2013). Duplicate analyses were performed on each sample. All colonies with different
102 morphologies were picked from the countable MRS and M17 plates, and sub-cultured using the
103 same isolation media and temperature. If the isolates did not grow in MRS or M17 broth, the
104 colonies were re-cultured in reconstituted (10% w/v) skimmed milk (Sacco System). The purity of

105 the isolates was checked by streaking repeatedly on Homofermentative-Heterofermentative
106 Differential (HHD) agar (Biolife). After purification, the isolates were examined for cell
107 morphology and catalase activity and successively stored in Litmus milk (Biolife Italiana) at -20 °C.
108
109 *2.2. LAB identification*
110 DNA was extracted from overnight bacterial cultures by the Microlysis kit (Aurogene, Rome, Italy)
111 following the manufacturer's instructions. The identification of isolates was performed using the
112 pentaplex PCR as previously described by Cremonesi et al. (2011). Isolates that could not be
113 identified using species-specific PCR were subjected to partial 16S rRNA sequencing as reported
114 by Silvetti et al. (2017).
115
116 *2.3. Randomly Amplified Polymorphic DNA (RAPD) analysis*
117 RAPD-PCR method was applied to explore the biodiversity and genetic relatedness within LAB
118 isolated from NWC samples. RAPD-PCR analysis were performed with 3 primers (M13, D11344
119 and D8635) as described by Morandi, Silvetti, Miranda Lopez & Brasca (2015). Resulting
120 fingerprints were compared with the BioNumeric 5.0 software package (Applied Maths, Sint-
121 Martens-Latem, Belgium), using the UPGMA (unweighted pair group method with arithmetic
122 averages) cluster analysis. The reproducibility value of the RAPD-PCR assay, calculated from two
123 repetitions of independent amplification of type strains, was higher than 90%.
124
125 *2.4. Biodiversity indexes estimation*
126 Simpson's index of diversity (1-*D*) and Shannon-Wiener index (H') were calculated based on
127 RAPD-PCR patterns using the Scripts available in the BioNumerics 5.0 software package (Applied
128 Maths). The value of Simpson's index of diversity ranges between 0 and 1, the greater is this value,
129 the greater is the biodiversity within the species, while the values of Shannon-Wiener index are

generally comprised between 1.5 and 3.5, and when these values increase, the biodiversity richness of the community increases (Magurran, 2004).

2.5. VOCs analysis

The strains (19 biotypes) subjected to the VOCs analysis were selected based on the results of RAPD analysis. VOCs analysis was performed by Solid Phase Micro Extraction-Gas Chromatography-Mass Spectrometry (SPME-GC-MS) technique after the inoculation of the single strain in UHT whole milk (Parmalat, Parma, Italy). LAB biotypes were previously cultured overnight at 42 °C in UHT milk and then re-inoculated in 5 mL of UHT milk directly in vials for SPME GC-MS analysis. In detail, 5 mL of UHT whole milk were added to a 20 mL sterile head-space glass vial sealed with PTFE/silicone septa (Sigma-Aldrich S.r.l., Milan, Italy) and inoculated with the overnight-grown bacterial culture, obtaining a final concentration of approximately 10^6 CFU/mL of milk. After incubation at 42 °C for 15 d, the vials were immediately stored at -20 °C. In order to check for background compounds derived from the matrix, several vials containing 5 mL of UHT milk were incubated under the same conditions employed for the LAB strains. Prior to the SPME-GC-MS analysis, the vials were thawed at room temperature, opened, 3.5 g NaCl were added, and then immediately resealed. The analysis was performed by means of a Combi-Pal automated sampler CTC Analytics, Zwingen, Switzerland equipped with DVB/CAR/PDMS 50/30 μm fiber (Supelco, Bellefonte, USA) and coupled to a 6890N/ 5973N Agilent gas chromatograph-mass spectrometer (Technologies, Inc., Wilmington, DE). Extraction and chromatographic conditions were previously described (Masotti, Battelli, & De Noni, 2012). Data were expressed as $\log_{10}$ of arbitrary unit (AU) and refer to the peak area of the quant ion of each compound. Two independent replicates (inoculated vials) were analyzed for each biotype considered.

2.6. Statistical analysis

155 Statistical analysis was performed with the software package MINITAB ver. 14.13 (Minitab Inc.,
156 State College, PA, USA).
157
158 **3. Results and discussion**
159 During the last decades the improvement of the hygienic conditions in the dairy chain led to a
160 decrease in pathogen contamination, but at the same time to a reduction of bacterial count, LAB
161 content and consequently the microbial biodiversity. The contribution of the dairy microbiota to
162 flavour development and the overall quality of the cheese is of critical significance, as many of the
163 final characteristics of a cheese are due to the complex dynamics and interactions among the
164 different LAB species present during the cheese-making (Afshari, Pillidge, Dias, Osborn & Gill,
165 2018). For this reasons, in the present study we investigated the dynamic changes in biodiversity of
166 LAB associated with the NWC of Trentingrana PDO cheese.
167 Pentaplex-PCR revealed that NWCs were mainly composed by *Lb. helveticus* (present in all
168 samples analyzed) and by a variable presence of *St. thermophilus* (from 60 to 100%) and *Lb.*
169 *delbrueckii* subsp. *lactis* (from 50 to 93%) (Table 1).
170 The LAB species detected in the whey starters were the same as those found by other authors using
171 length heterogeneity-PCR (LH-PCR) (Rossetti et al., 2008; Santarelli et al., 2013), evidencing a
172 higher presence percentages of *St. thermophilus* and *Lb. delbrueckii* subsp. *lactis*.
173 It is interesting to notice that the presence of *Lb. delbrueckii* subsp. *lactis* dramatically decreased
174 during the time frame considered (from 93% of positive samples in April 2014 to 51% in July
175 2016). These data are not easy to explain and require further investigations considering biotic and
176 abiotic drives along the entire dairy chain (Gobbetti et al., 2018; Morandi et al, 2018). Recently,
177 Morandi et al. (2018) demonstrated that the sodium hypochlorite used for the disinfection of
178 milking machines can influence the composition of milk microbiota and, in consequence, the
179 structure of the whey microbial populations. In this research, the authors showed that in the NWCs
180 obtained from milk collected from milking machines cleaned using detergent containing sodium

hypochlorite *Lb. delbrueckii* subsp. *lactis* was present with a relative abundance of 28.1%, while in the whey starter obtained when a nonchlorine detergent was applied, the concentration of this species increased more than two times (from 28.1% in chlorine period to 58.6% in nonchlorine period) (Morandi et al., 2018).

Lactobacillus fermentum was found at low percentage only in samples collected in April 2014 (20%), in February and July 2016 (13 and 14%), while *Lb. delbrueckii* subsp. *bulgaricus* was never detected (Table 1). *Lb. delbrueckii* subsp. *bulgaricus* was abundant in the past (Carini et al., 1977; Bosi et al., 1990) but, in the subsequent years, there was a dramatic reduction in its content (Andrighetto et al., 2004; Rossetti et al., 2008; Cremonesi et al., 2011) probably linked to change in technology or other interventions that affected the NWC biodiversity (Gobbetti et al., 2018). Different authors attributed the abundance of this subspecies in whey starters to an imprecise identification based on phenotypic traits. For the same reason, 13 strains isolated in Grana Padano NWCs during the '80s (previously identified phenotypically as *Lb. bulgaricus*) were re-identified by molecular method confirming the phenotypic identification (data not shown).

In order to know the evolution of Trentingrana NWCs biodiversity over a period of three years, four dairies (A, B, C and D), out of 14 Trentingrana producers, were chosen. The microbial counts of NWC samples collected over the years are shown in Table 2.

Thermophilic lactobacilli were dominant in all samples and their content resulted to be similar in the four dairies considered, showing average values of 8.15±0.39, 8.20±0.42, 8.06±0.62 and 8.19±0.30 $\log_{10}$ CFU/mL respectively. The highest values were recorded from February to June 2015. It is interesting to note that the content of thermophilic lactobacilli observed in this research was 1-2 log higher than previously found in Trentigrana whey samples (Rossi et al., 2012). This discrepancy could be ascribed to the different medium used for the lactobacilli count (MRS *vs* Whey Agar Medium (WAM)).

Thermophilic cocci were present at various levels (from $<10^5$ to 10^8 CFU/mL) and, differently from lactobacilli, the highest values were recorded from June 2015 to February 2016. The content of

thermophilic cocci of Trentingrana NWCs were comparable to those reported in whey starters used for Grana Padano production by Rossetti et al. (2008). Heterofermentative LAB were found at low levels (not exceeding 10^3 MPN/mL), confirming the available data of Neviani & Carini (1994).

As previously reported by Rossi et al. (2012) a little monthly variation in the Trentingrana whey culture counts was observed. This suggested that changes occurring during the year in artisanal cheeses quality are due to the variations not only in milk composition but also in the microbiota involved in fermentation.

A total of 96 catalase-negative isolates with different morphologies were collected from M17 (39 isolates) and MRS (57 isolates) agar plates. Twenty-four isolates from M17 agar (1 in 2014, 13 in 2015, 8 in 2016) did not growth after plate isolation. This is not surprising as the NWC microbiota is well adapted to the whey conditions (e.g. low pH) and the dependence on this substrate is so strict that these bacteria multiply with difficulty outside of whey environment (Fornasari, Rossetti, Carminati, & Giraffa, 2006). Nevertheless, it is worth to note that the prevalence of strains not cultivable in M17 broth increased over the time, highlighting a progressive loss of strains biodiversity.

The pentaplex-PCR revealed that 71 strains belonged to four different species: *Lb. helveticus* (49 strains), *Lb. fermentum* (4), *Lb. delbrueckii* subsp. *lactis* (3) and *St. thermophilus* (15). Only one strain was not identified by pentaplex-PCR and the 16S rRNA gene sequence analysis recognised it as *Lactobacillus frumenti* sharing an identity of 99% (NCBI accession number KU851164).

RAPD-PCR fingerprinting was performed to investigate the biodiversity within the two most abundant species: *St. thermophilus* and *Lb. helveticus*.

Fifteen *St. thermophilus* were isolated from whey cultures of three out of the four dairy factories (A, B and C) and among these strains 10 different biotypes were observed (Fig. 1). Some of these biotypes were recovered in samples from different dairies, in particular: biotype 1 was detected in dairies A and B, biotypes 3 and 8 in B and C, while the others (2, 4, 5, 6, 7, 9 and 10) were specific

for C cheese factory (Fig. 1). These results confirmed the variability of *St. thermophilus* biotypes in Trentingrana whey cultures (Rossi et al., 2012).

Persistent *St. thermophilus* (biotype 3) was found from farm C, during the years 2014 and 2016 (Fig. 1). Twelve out of 15 *St. thermophilus* biotypes were isolated during the year 2014 (Table 3). This finding highlights that a lot of biotypes of *St. thermophilus* were present and metabolically active in the cultures analyzed in the successive years (Table 2), but only a low number was able to develop in M17 broth, confirming the difficulty of these strains to grow in synthetic medium.

RAPD fingerprints analysis revealed a high heterogeneity of *Lb. helveticus* in the Trentingrana whey fermentation process (23 biotypes) (Fig. 2).

As shown for *St. thermophilus*, also some *Lb. helveticus* biotypes were detected in different dairies, in particular: biotype 3 was present in dairies A and B, biotypes 5, 8 and 14 in C and D, and biotypes 10 and 16 were found in three cheese factories A, B and C (Fig. 2). The other 17 biotypes resulted to be peculiar for each dairy and no persistent strains were recovered.

These results were in contrast with those reported by Rossi et al. (2012) that found a single genotype common in the Grana Trentino production zone.

Considering the different *Lb. helveticus* biotypes in relation to the year of isolation, it is possible to note a decrease in biodiversity (Table 3). In all the four dairies in the 2014 were identified 13 different biotypes among the 15 strains, while in the 2015 and 2016, with an equal number of strains isolated, the biotypes detected were 6 and 4 respectively.

The change of biodiversity within the *Lb. helveticus* species was measured by Simpson's index of diversity (1-*D*) and Shannon-Wiener index (H'). The values of these indexes decreased from the 2014 to 2016 (1-*D*: 0.98 (2014), 0.74 (2015), 0.68 (2016) and H': 2.52 (2014), 1.43 (2015), 1.23 (2016)), confirming the reduction of biodiversity richness in the dominant species of Trentigrana whey samples.

The presence of different biotypes in NWCs is strictly related to the cheese microbial biodiversity, in turn linked to the quality and sensory characteristics of the cheese. In fact, the simultaneous

presence of various *Lb. helveticus* strains is also important at technological level, for example in preventing possible phage attacks.

Considering the "kill the winner" hypothesis, which allows to the bacteriophages infect the dominant bacterial strains (Ellegaard & Engel, 2016), the whey cultures composed by 1 or 2 biotypes resulted to be more susceptible to phage infections; on the contrary, a high degree of biodiversity makes the NWCs highly resistant to bacteriophage attacks (Spus et al., 2015).

Production of flavour compounds by the NWCs strains grown in UHT whole milk after 15 days of incubation was detected by means of SPME GC–MS analysis. The strains were chosen according to their genetic relatedness and, to better evaluate the differences in VOCs production, a RAPD-PCR similarity level of 80% was considered. This threshold allowed the selection of 5 and 14 different biotypes between *St. thermophilus* and *Lb. helveticus* species (Fig. 1 and 2).

Twenty-seven compounds, belonging to 5 chemical classes, were recognized as specific products of each biotype metabolism (Table 4 and 5). Ketones (ranged between 4.27 and 6.97 $\log_{10}$AU) and fatty acids (comprised between 4.24 and 6.87 $\log_{10}$AU) were the most frequent classes detected in this study. These compounds constitute the key flavour in a wide range of dairy products, and are generated from the β-oxidation of fatty acids (McSweeney & Sousa, 2000) and from the amino acids catabolism (Smit, Smit, & Engels, 2005).

Considering the ketones, in all fermented milk a high amount of acetone (from 5.35 to 6.21 $\log_{10}$AU) and butane-2,3-dione (diacetyl) (from 5.52 to 6.51 $\log_{10}$AU) was detected, while a high level of 3-hydroxybutan-2-one (acetoin) (from 6.37 to 6.94 $\log_{10}$AU) and heptan-2-one (from 5.92 to 6.97 $\log_{10}$AU) was produced by *St. thermophilus* and *Lb. helveticus*, respectively. Diacetyl and acetoin deriving from citrate metabolism give an aromatic note related to a pleasant buttery/creamy odour to dairy products, while 2-heptanone is characterized by a cheesy, fruity, herbaceous flavour that is perceived as the fruity flavour of the cheese (Qian & Burbank, 2007; Bancalari et al., 2017).

Among fatty acids, butanoic (also known as butyric), 3-methylbutanoic (isovaleric) and hexanoic (caproic) acids were the most abundant (Table 4 and 5). Straight-chain fatty acids (butanoic and

hexanoic) are short-chain fatty acids that derive from the lipolysis of milk triglycerides, while the branched-chain 3-methylbutanoic acid derives from the amino acid leucine. These compounds have a considerable impact on sensory quality of dairy products. Due to their strong aroma and low sensory threshold, they can develop an undesired flavour in cheese like fermented milk or rancid (Marilley & Casey, 2004). The highest production of acetic acid was registered among the *Lb. helveticus* biotypes (from 6.59 to 7.03 $\log_{10}$AU). This short-chain fatty acid contributed to a strong, pungent, vinegary note and was usually detected in Grana Padano cheese (Lazzi et al., 2016).

Aldehydes are generated from the autoxidation of unsatured fatty acids and are usually synthesized by thermophilic LAB. In accordance with previous studies (Lazzi et al., 2016), *St. thermophilus* (with the exception of TR11 strain) and *Lb. helveticus* were able to produce benzaldehyde from the amino acid phenylalanine, hexanal and furfural responsible for the green aroma and baked note in Grana type cheese (Table 4 and 5). 3-Methybutanal was detected only in milk fermented by *Lb. helveticus* and derives, like the corresponding acid, from the catabolism of leucine. This finding confirms how the proteolytic activity of *Lb. helveticus* significantly contributes to the production of aroma compounds through the amino acid catabolism (Klein, Maillard, Thierry, & Lortal, 2001). Highly variability in alcohols abundance was observed among the *St. thermophilus* and *Lb. helveticus* biotypes. These compounds are formed by the reduction of the aldehydes and ketones and their production has been associated with LAB that grow during cheese ripening. Butan-1-ol and 3-methyl-3-buten-1-ol were detected in all *St. thermophilus* and *Lb. helveticus* even if to a different extent, while 18 out 19 strains were able to synthesize 3-methylbutan-1-ol (Table 4 and 5). This last compound, like the corresponding acid and aldehyde,- is produced by leucine degradation and is recognised as a key flavour of handcrafted cheeses produced by wild strains of LAB (Gallegos et al., 2017). Ethanol was revealed in 5 different biotypes (*St. thermophilis* I and *Lb. helveticus* VI, VII, VIII, XII), while 2-methylpropan-1-ol was produced respectively by 3 *St. thermophilus* (I, IV, V) and 3 *Lb. helveticus* (VI, VIII, XII). Variability in alcohol production by thermophilic LAB species was previously observed by Imhof, Glättli, & Bosset (1995) and could be

ascribed to the different metabolic behaviour of the biotypes during fermentation. Esters are important contributors to the flavour of many dairy products since they give fruity notes to the cheese. They are formed during the esterification of alcohols and fatty acids either by microorganisms or by chemical reactions (Marilley & Casey, 2004). In our study, ethyl acetate was produced by all the tested strains except for *Lb. helveticus* biotype XIII (Table 4 and 5).

Other organic compounds such as dimethyl sulfide and dimethyl sulfone were produced by the biotypes tested. These sulfur compounds are considered important flavour contributors to the Cheddar cheeses and were synthesized during the ripening process, thanks to decomposition of sulfur containing amino acids, such as methionine (Burbank & Qian, 2005).

To highlight the variability among the LAB biotypes, a PCA analysis was carried out taking into account all the VOCs data set (Fig. 3). The first two components of the PCA account for 64.9% of the total variability of the original data set. The first principal component, that explains the 44.9% of the total variability, clearly classified horizontally the NWCs biotypes into two groups according the taxonomically characteristics. All *St. thermophilus* strains were gathered in the left part of the plot, except for the biotype I (strain TR11). A higher intraspecies variability was observed for *Lb. helveticus*. The majority of the *Lb. helveticus* were grouped on the right part of the PCA plot. Three biotypes (VI, VIII and XII) fell in the high part of the plot and were characterised by production of alcohols, such as butan-1-ol and 2-methylpropan-1-ol. Interesting to note that the LAB biotypes positively scored on PC2 (*St. thermophilus* biotype I and *Lb. helveticus* biotypes VI, VII, VIII and XII) were good producers of alcohols (butan-1-ol, 2-methylpropan-1-ol, 3-methylbutan-1-ol), esters (ethyl acetate) and organic acid (3-methylbutanoic acid) that contribute to the fruity aroma in cheeses.

4. Conclusions

The characterization of NWCs is essential to avoid loss of sensory quality of cheese. The results of this study provide a first evidence regarding the biodiversity depletion in the dairy chain that could influence the peculiar characteristics strictly connected with PDO and traditional cheeses.

In the period considered, we observed a progressive reduction of biodiversity, both at species levels and within the LAB species. A worrying trend was the decrease of *Lb. delbrueckii* subsp. *lactis* and *Lb. helveticus* biotypes number in NWCs. This biodiversity loss can affect VOCs production during ripening, and as VOCs are strongly correlated to cheese flavour, it can lead to grim consequences on the quality and sensory characteristics of the cheese. For these reasons, to preserve the cheese quality it will be necessary to recognize the microbial whey depletion drivers, to identify and introduce technological innovations to protect the whey culture microbiota preventing adverse effect on typical cheese traits. The addition of autochthonous LAB both in milk pre-maturation and in NWC represents a possible way of optimizing the fermentative process during cheesemaking, maintaining the full complexity of flavour profile preserving the typicality of the cheese minimizing texture defects

Figure 1: Unweighted Pair Group Method with Arithmetic Averages-Based Dendrogram Derived from the Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction Patterns of the *St. thermophilus* isolated from natural whey cultures collected in 4 different dairies during the years 2014-2016.

Figure 2: Unweighted Pair Group Method with Arithmetic Averages-Based Dendrogram Derived from the Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction Patterns of the *Lb. helveticus* isolated from natural whey cultures collected in 4 different dairies during the years 2014-2016.

Figure 3: Plots of loading (A) and score (B) obtained by PCA analysis of the volatile organic compounds produced by lactic acid bacteria incubated at 42 °C in UHT whole milk for 15 days. Squares and circles correspond to the different *St. thermophilus* and *Lb. helveticus* biotypes, respectively.

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Table 1: Presence percentages of *Lb. delbrueckii* subsp. *lactis*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. fermentum*, *Lb. helveticus* and *St. thermophilus* in Trentingrana natural whey cultures samples, collected from April 2014 to July 2016 and analyzed by pentaplex PCR assay.

Year	Month	Percentage of presence in NWC samples (%) collected from 14 dairies				
		St. thermophilus	***Lb. bulgaricus***	***Lb. lactis***	***Lb. helveticus***	***Lb. fermentum***
2014	Apr.	60	0	93	100	20
	July	100	0	89	100	0
	Nov.	93	0	86	100	0
2015	Feb.	86	0	79	100	0
	June	93	0	50	100	0
2016	Feb.	64	0	57	100	13
	July	72	0	51	100	14

Table 2: Microbial counts of Trentingrana natural whey cultures collected from four dairies (A, B, C and D) twice a year from April 2014 to July 2016. The data were expressed as means and standard deviation of bacterial number of CFU/mL or MPN/mL transformed to $\log_{10}$.

	Year	Month	Dairies							
			A		B		C		D	
			Mean	SD	Mean	SD	Mean	SD	Mean	SD
Lactobacilli in MRS ($\log_{10}$ CFU/mL)	2014	Apr.	8.50	0.08	8.47	0.04	8.48	0.02	8.26	0.11
		July	7.47	0.03	7.46	0.23	7.82	0.10	8.11	0.04
		Nov.	8.28	0.06	8.47	0.05	8.27	0.12	8.47	0.02
	2015	Feb.	8.47	0.04	8.63	0.05	8.79	0.04	8.29	0.07
		June	8.56	0.08	8.52	0.07	8.50	0.04	8.58	0.08
	2016	Feb.	7.94	0.04	7.89	0.02	6.93	0.05	7.89	0.03
		July	7.85	0.03	7.96	0.03	7.61	0.05	7.70	0.07
Streptococci in M17 ($\log_{10}$ CFU/mL)	2014	Apr.	7.16	0.06	6.75	0.04	7.64	0.10	<5.00	0.00
		July	7.07	0.02	6.08	0.06	7.33	0.04	<5.00	0.00
		Nov.	6.75	0.09	6.48	0.03	<5.00	0.00	<5.00	0.00
	2015	Feb.	7.89	0.05	7.36	0.23	7.29	0.05	7.27	0.33
		June	7.93	0.05	7.41	0.03	7.55	0.05	7.69	0.10
	2016	Feb.	7.71	0.04	6.58	0.66	7.94	0.03	8.44	0.07
		July	5.24	0.08	<5.00	0.00	<5.00	0.00	<5.00	0.00
Heterofermentative LAB in MRS+C ($\log_{10}$ MPN/mL)	2014	Apr.	3.47	0.15	<2.48	0.00	2.52	0.06	2.71	0.21
		July	<2.48	0.00	<2.48	0.00	<2.48	0.00	<2.48	0.00
		Nov.	<2.48	0.00	<2.48	0.00	<2.48	0.00	2.96	0.12
	2015	Feb.	<2.48	0.00	<2.48	0.00	<2.48	0.00	<2.48	0.00
		June	2.71	0.21	<2.48	0.00	3.07	0.15	1.96	0.12
	2016	Feb.	<2.48	0.00	<2.48	0.00	<2.48	0.00	2.52	0.06
		July	<2.48	0.00	<2.48	0.00	<2.48	0.00	<2.48	0.00

Table 3: *Streptococcus thermophilus* and *Lactobacillus helveticus* strains isolated from natural whey cultures collected in 4 different dairies during the years 2014-2016. Values in parentheses represent the number of biotypes determined by RAPD-PCR analysis.

Species	Years	Dairies				
		A	**B**	**C**	**D**	**total**
St. thermophilus	2014	2 (2)	4 (3)	6 (6)	-	12 (8)
	2015	-	1 (1)	1 (1)	-	2 (2)
	2016	-	-	1 (1)	-	1 (1)
	total	2 (2)	5 (4)	8 (8)	-	15 (10)
Lb. helveticus	2014	4 (4)	3 (3)	3(3)	5 (5)	15 (13)
	2015	4 (1)	2 (1)	3 (2)	6 (4)	15 (6)
	2016	5 (1)	4 (2)	5 (2)	5 (3)	19 (4)
	total	13 (6)	9 (6)	11 (7)	16 (12)	49 (23)

Table 4: Volatile organic compounds (VOC) produced by the *St. thermophilus* strains in UHT whole milk in 15 days at 42 °C. Data expressed as $\log_{10}$ of arbitrary units (AU) of the peak area of the characteristic ion. Each value is the mean of 2 determinations.

Classes	**Compounds**	**Odour description***	***St. thermophilus* biotypes/strains**				
			I TR11	**II TR17**	**III TR55**	**IV TR60**	**V TR37**
Alcohols	Ethanol	Sweet, alcoholic	6.55	**	**	**	**
	Butan-1-ol	Green, fruity	5.34^{c}	3.88^{a}	4.05^{b}	3.33^{a}	3.56^{a}
	2-Methylpropan-1-ol	Fruity	5.92^{b}	**	**	3.81^{a}	3.73^{a}
	3-Methylbutan-1-ol	Green, fruity	6.16^{c}	4.02^{b}	4.20^{b}	3.65^{a}	3.99^{b}
	3-Methyl-3-buten-1-ol	Fresch cheese	4.27	4.73	4.66	4.24	4.70
	Heptan-2-ol	Green	4.47	**	**	**	**
Aldehydes	Hexanal	Green	4.67	5.11	4.97	4.88	5.26
	3-Methylbutanal	Green, fruity	**	**	**	**	**
	Benzaldehyde	Bitter, almond	4.95	4.12	4.16	4.68	4.56
	Furfural	Sweet, almond	**	2.88^{a}	3.14^{b}	4.42^{c}	3.86^{b}
Ketones	Acetone	Fruity	5.35	6.01	6.02	5.96	6.04
	Butan-2-one	Fruity	5.02	5.64	5.64	5.58	5.65
	Pentan-2-one	Ether	4.21	4.45	4.45	4.27	4.44
	Heptan-2-one	Fruity	5.94	5.76	5.72	5.75	5.80
	Nonan-2-one	Fruity	4.73	4.66	4.65	4.48	4.60
	Butane-2,3-dione	Buttery, cream	6.31	6.36	6.36	6.39	6.34
	3-Hydroxybutan-2-one	Buttery, cream	6.41	6.47	6.41	6.55	6.38
Esters	Ethyl acetate	Pineapple	5.24	4.34	4.39	4.23	4.30
Acid	Acetic acid	Sour	5.20	5.61	5.64	5.79	5.41
	Butanoic acid	Cheesy, rancid	5.79	6.25	6.26	6.43	6.22
	Propionic acid	Sour	**	3.84	3.79	4.10	3.90
	3-Methylbutanoic acid	Rancid	6.73	4.42	4.35	4.08	4.09
	Pentanoic acid	Sweat	4.14	4.24	4.27	4.40	4.24
	Hexanoic acid	Cheesy, fatty	6.31	6.65	6.67	6.76	6.68
	Benzoic acis	Urine	4.39	4.40	4.41	5.23	4.28
Others	Dimethyl sulfone	Burnt	3.94	4.63	4.70	3.99	3.89
	Dimethyl sulfide	Cabbage	4.12	4.32	4.21	4.18	4.48

* Odour description reported derived mainly from Flavornet database (http://www.flavornet.org) and from Qian and Reineccius ,2002

**: VOC below detection limit (< 2.00 $\log_{10}$ AU).

Table 5: Volatile organic compounds (VOCs) produced by the *Lb. helveticus* strains in UHT whole milk in 15 days at 42 °C. Data expressed as $\log_{10}$ of arbitrary units (AU) of the peak area of the characteristic ion. Each value is the mean of 2 determinations.

Classes	Compound	Odour description*	*Lb. helveticus* biotypes/strains												
			I TR34	II TR42	III TR33	IV TR120	V TR139	VI TR25	VII TR62	VIII TR51	IX TR20	X TR28	XI TR22	XII TR24	XIII TR71	XIV TR73
Alcohols	Ethanol	Sweet, alcoholic	**	**	**	**	**	6.60	4.95	5.95	**	**	**	6.50	**	**
	Butan-1-ol	Green, fruity	3.82^{a}	3.62^{a}	3.81^{a}	3.71^{a}	3.84^{a}	5.69^{c}	3.76^{a}	4.76^{b}	4.00^{a}	3.64^{a}	3.95^{a}	5.73^{c}	3.89^{a}	3.94^{a}
	2-Methylpropan-1-ol	Fruity	**	**	**	**	**	6.12^{b}	**	3.94^{a}	**	**	**	5.42	**	**
	3-Methylbutan-1-ol	Green, fruity	3.17^{a}	7.12^{d}	3.52^{a}	4.04^{b}	3.32^{a}	5.85^{c}	3.60^{a}	**	3.81^{b}	3.57^{a}	3.80^{b}	5.85^{c}	3.59^{a}	3.88^{b}
	3-Methyl-3-buten-1-ol	Fresch cheese	5.49	4.79	5.31	5.22	4.75	4.46	5.25	5.15	5.44	5.24	4.79	4.74	5.30	4.95
	Heptan-2-ol	Green	**	**	**	**	**	**	**	**	**	**	**	**	**	4.20
Aldehydes	Hexanal	Green	4.55	4.75	4.80	4.88	4.57	4.17	4.58	4.04	5.37	4.63	5.32	4.20	4.59	4.84
	3-Methylbutanal	Green, fruity	5.43^{c}	4.68^{b}	5.67^{c}	5.46^{c}	4.65^{b}	3.63^{a}	5.29^{c}	4.99^{c}	5.30^{c}	5.43^{c}	4.60^{b}	4.54^{b}	5.05^{c}	4.85^{b}
	Benzaldehyde	Bitter, almond	6.08^{c}	6.27^{c}	6.47^{c}	6.20^{c}	6.31^{c}	5.00^{b}	6.24^{c}	6.25^{c}	6.08^{c}	6.23^{c}	6.02^{c}	3.98^{a}	6.01^{c}	6.33^{c}
	Furfural	Sweet, almond	6.04^{c}	6.25^{c}	6.01^{c}	6.04^{c}	6.08^{c}	5.21^{b}	6.05^{c}	6.28^{c}	6.07^{c}	6.11^{c}	6.05^{c}	3.03^{a}	5.97^{c}	6.52^{c}
Ketones	Acetone	Fruity	6.02	5.99	6.00	5.99	5.91	5.81	5.98	5.95	5.99	5.96	6.04	6.21	6.01	6.04
	Butan-2-one	Fruity	6.05	5.81	6.03	6.02	5.68	5.48	5.96	5.88	6.07	5.99	6.13	5.87	6.08	5.88
	Pentan-2-one	Ether	4.71	4.48	4.68	4.78	4.49	4.67	4.68	4.58	4.70	4.74	4.67	4.38	4.75	4.47
	Heptan-2-one	Fruity	6.72	6.14	6.74	6.83	6.38	6.87	6.80	6.97	6.76	6.80	6.51	5.92	6.70	6.17
	Nonan-2-one	Fruity	5.57	4.92	5.55	5.75	5.19	5.93	5.70	6.08	5.69	5.64	5.33	4.72	5.51	4.91
	Butane-2,3-dione	Buttery, cream	5.95	6.30	5.79	6.20	6.41	6.16	6.36	6.34	5.69	6.13	5.67	5.81	5.81	5.52
	3-Hydroxybutan-2-one	Buttery, cream	5.69^{b}	6.20^{c}	5.67^{b}	5.94^{b}	6.67^{c}	6.65^{c}	6.49^{c}	6.60^{c}	4.61^{a}	6.86^{c}	4.29^{a}	6.51^{c}	5.52^{b}	**
Esters	Ethyl acetate	Pineapple	4.48^{a}	4.39^{a}	4.38^{a}	4.40^{a}	4.35^{a}	5.30^{b}	4.54^{a}	5.19^{b}	4.47^{a}	4.43^{a}	4.61^{a}	6.02^{c}	**	4.53^{a}
Acid	Acetic acid	Sour	6.76	6.89	6.81	6.81	6.97	6.62	6.84	6.89	6.85	6.81	6.84	6.59	6.76	7.03
	Butanoic acid	Cheesy, rancid	6.53	6.44	6.56	6.53	6.49	6.44	6.47	6.45	6.52	6.53	6.49	6.35	6.47	6.43
	Propionic acid	Sour	5.01	5.20	5.37	5.12	5.22	4.30	5.20	5.37	5.04	5.17	4.94	4.78	4.99	5.24
	3-Methylbutyric acid	Rancid	5.69^{a}	5.29^{a}	5.58^{a}	5.18^{a}	5.88^{a}	7.02^{c}	6.45^{b}	7.49^{c}	5.39^{a}	5.34^{a}	5.46^{a}	7.27^{c}	5.88^{a}	5.60^{a}
	Pentanoic acid	Sweat	4.60	4.50	4.67	4.59	4.60	4.71	4.59	4.76	4.58	4.63	4.58	4.81	4.52	4.49
	Hexanoic acid	Cheesy, fatty	6.76	6.72	6.87	6.77	6.82	6.84	6.76	6.81	6.73	6.84	6.72	6.59	6.72	6.67
	Benzoic acis	Urine	5.01	5.35	4.79	4.73	5.83	4.99	4.75	4.69	4.73	5.43	4.59	**	4.75	4.77
Others	Dimethyl sulfone	Burnt	4.45	4.33	4.30	4.36	4.40	4.18	4.38	4.31	4.37	4.50	4.42	4.33	4.55	4.29
	Dimethyl sulfide	Cabbage	5.09	4.74	4.78	4.89	4.76	4.62	4.91	4.78	5.03	4.76	4.87	4.07	4.79	4.75

* Odour description reported derived mainly from Flavornet database (http://www.flavornet.org) and from Qian and Reineccius ,2002

**: VOCs not detected or present in traces (< 2.00 $\log_{10}$ AU).

Figure

Figure 1

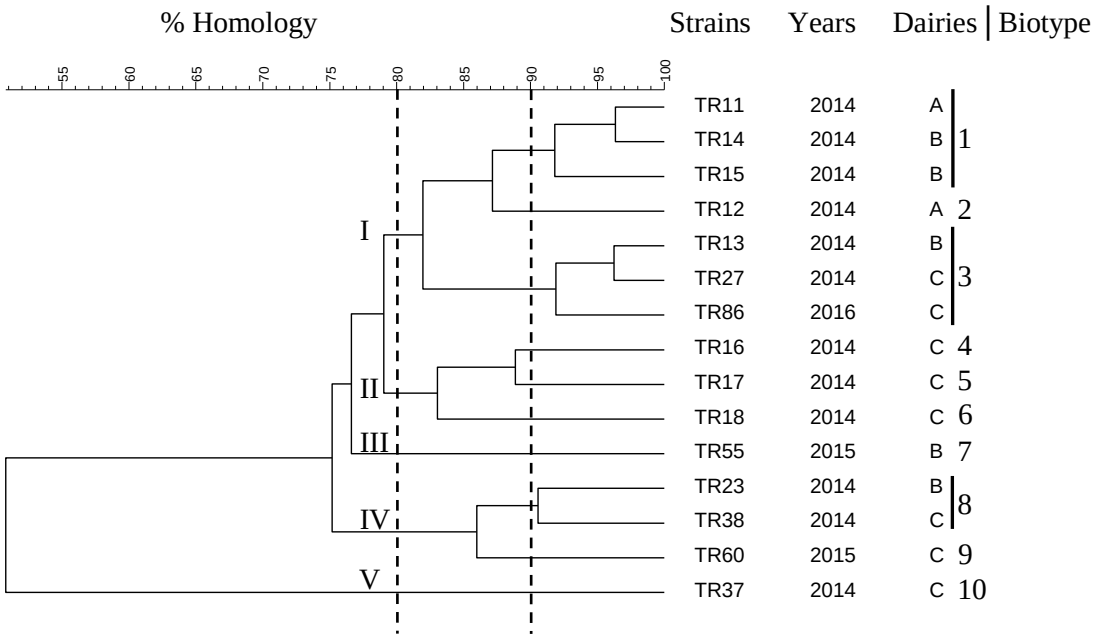


Figure 2

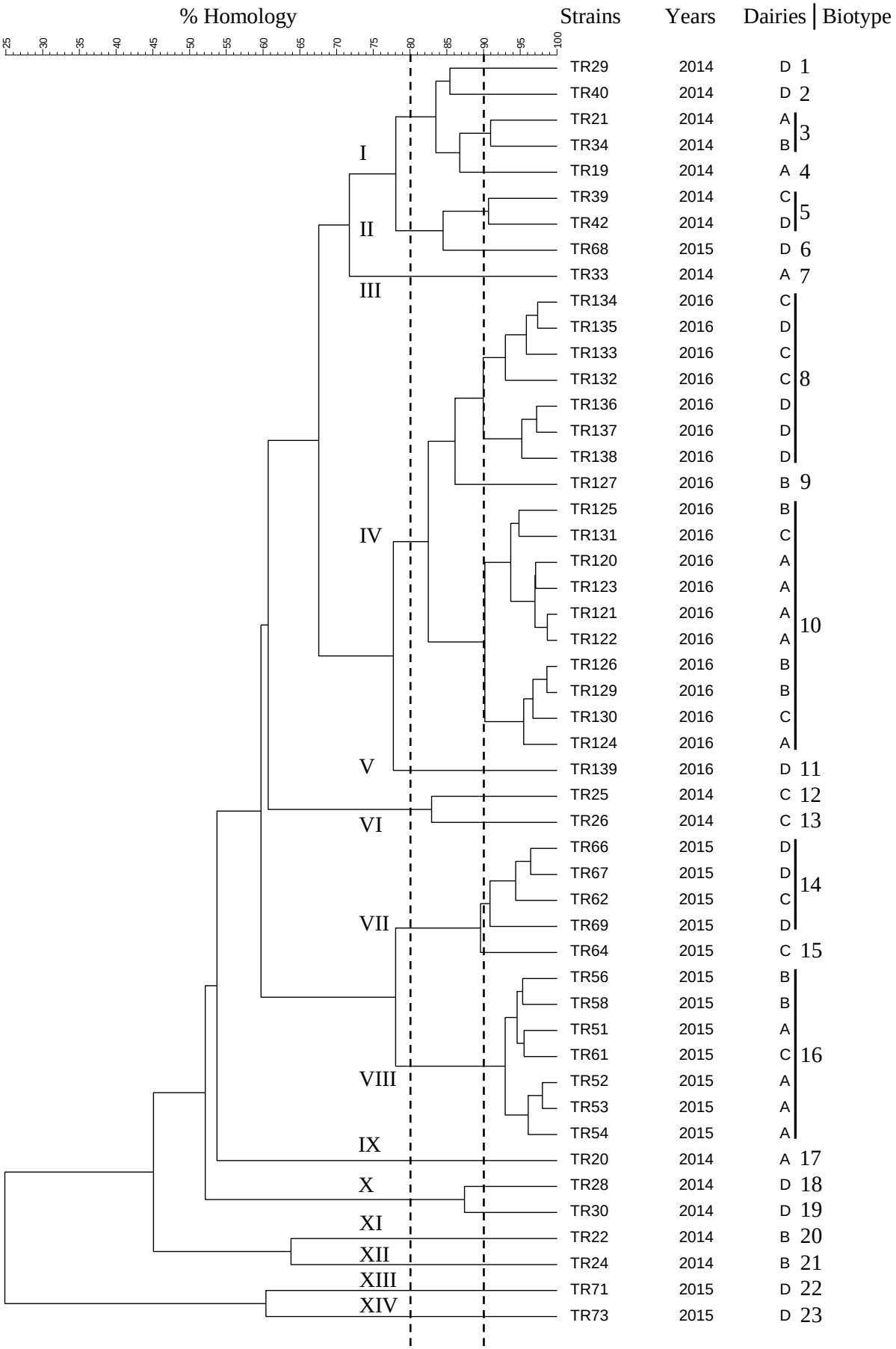
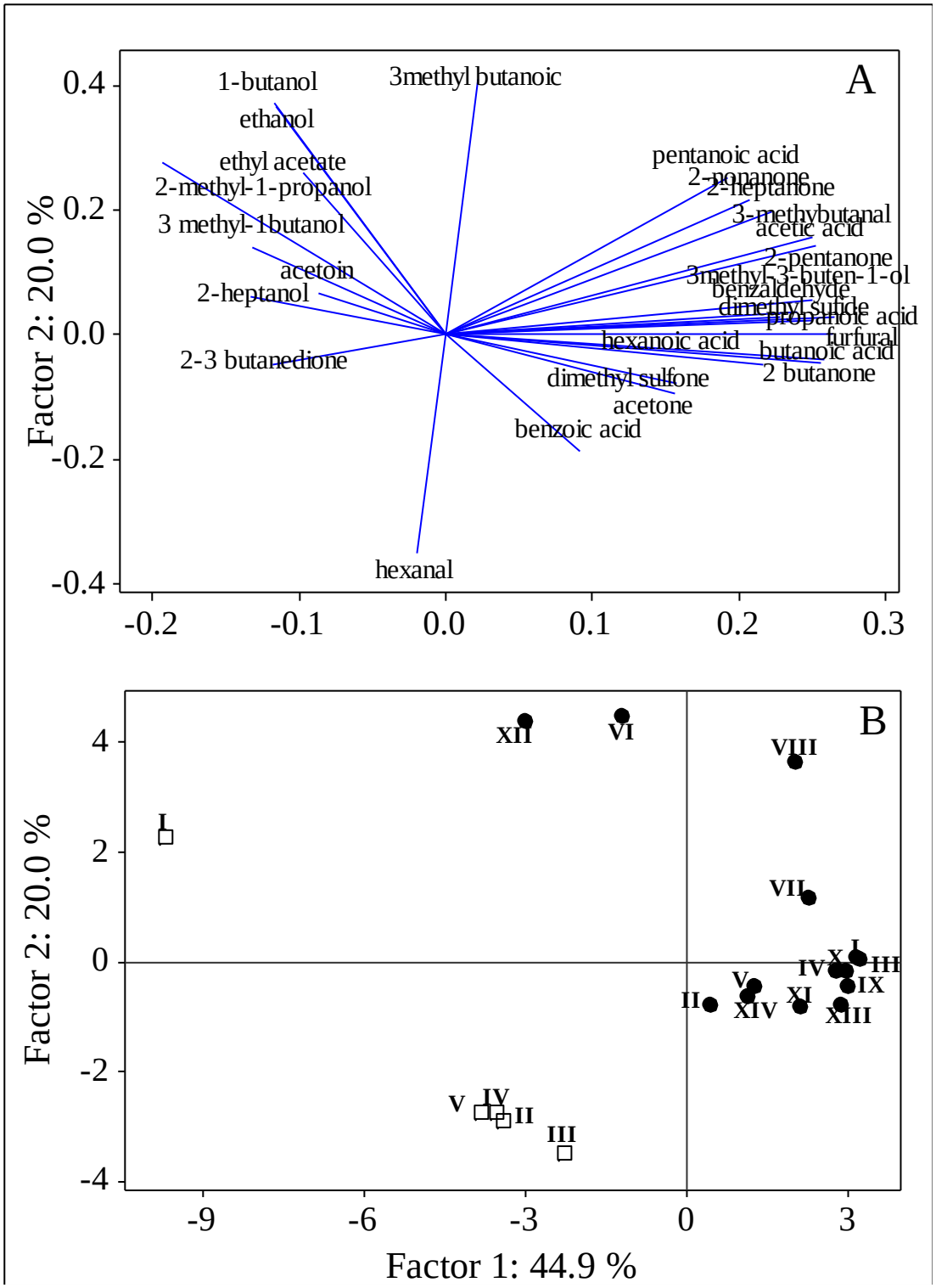

% Homology
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
Strains
Years
Dairies
Biotype
TR29 2014 D 1
TR40 2014 D 2
TR21 2014 A 3
TR34 2014 B
TR19 2014 A 4
TR39 2014 C 5
TR42 2014 D
TR68 2015 D 6
TR33 2014 A 7
TR134 2016 C 8
TR135 2016 D
TR133 2016 C
TR132 2016 C
TR136 2016 D
TR137 2016 D
TR138 2016 D
TR127 2016 B 9
TR125 2016 B 10
TR131 2016 C
TR120 2016 A
TR123 2016 A
TR121 2016 A
TR122 2016 A
TR126 2016 B
TR129 2016 B
TR130 2016 C
TR124 2016 A
TR139 2016 D 11
TR25 2014 C 12
TR26 2014 C 13
TR66 2015 D 14
TR67 2015 D
TR62 2015 C
TR69 2015 D
TR64 2015 C 15
TR56 2015 B 16
TR58 2015 B
TR51 2015 A
TR61 2015 C
TR52 2015 A
TR53 2015 A
TR54 2015 A
TR20 2014 A 17
TR28 2014 D 18
TR30 2014 D 19
TR22 2014 B 20
TR24 2014 B 21
TR71 2015 D 22
TR73 2015 D 23
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV

Figure 3


A
1-butanol
3methyl butanoic
ethanol
ethyl acetate
2-methyl-1-propanol
3 methyl-1butanol
acetoin
2-heptanol
2-3 butanedione
hexanal
pentanoic acid
2-nonanone
2-heptanone
3-methybutanal
acetic acid
2-pentanone
3methyl-3-buten-1-ol
benzaldehyde
dimethyl sufide
propanoic acid
furfural
hexanoic acid
butanoic acid
2 butanone
dimethyl sulfone
acetone
benzoic acid
Factor 2: 20.0 %
0.4
0.2
0.0
-0.2
-0.4
-0.2
-0.1
0.0
0.1
0.2
0.3
B
XII
VI
VIII
I
VII
X
IV
III
V
IX
II
XI
XIV
XIII
V
IV
II
III
Factor 2: 20.0 %
4
2
0
-2
-4
-9
-6
-3
0
3
Factor 1: 44.9 %

Conflict of interest

All the authors declare no conflict of interest in this paper