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Identification and characterization of wild lactobacilli and pediococci from spontaneously fermented Mountain Cheese

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ABSTRACT

The Traditional Mountain Malga (TMM) cheese is made from raw cow's milk by spontaneously fermentation in small farms called "Malga" located in Trentino region. This study was designed to characterize the lactic acid bacteria (LAB) growing on MRS medium, of TMM-cheese at the end of the ripening. Ninety-five LAB were isolated and genotypically characterized by Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) with two primers, species-specific PCR and partial sequencing of 16S rRNA gene. The 95 LAB clustered in 70 biotypes. *Pediococcus pentosaceus* and *Lactobacillus paracasei* were the dominant species. Isolates were tested for their growth properties, carbohydrate metabolism, acidifying ability, proteolytic and lipolytic activities, acetoin production, aminopeptidase (AP) activity, biogenic amines production, bile salts hydrolysis, conjugated linoleic acid and γ-aminobutyric acid production. *Lb. paracasei* isolates resulted to be well adapted to Malga environment and to show the best AP activity and acetoin production. TMM-cheese related LAB showed also interesting health promoting properties and produced bioactive substances. In particular, one *Lb. brevis* biotype produced a GABA mean value of 129 mg/L that is considered a high concentration. The results confirmed that TMM-cheese resident LAB could be exploited for dairy production.

1. Introduction

Traditional Mountain Malga (TMM) cheese is produced following traditional and artisan technologies in small scale on-farm plants called "Malga", which are located between 1400 and 2000 m.a.s.l. in the Trentino alpine region (Northern-east Italy). Each Malga is subjected to a short opening time from the end of May until the beginning of October when cattle from the valley are taken to alpine pastures in the Malga for free grazing. In each Malga the milk collection and the TMM-cheese-making process are carried out in the same place.

TMM-cheese is a semi-cooked cheese made mixing the raw cow's milk from the morning and the overnight skimmed milk collected the previous evening. The milk coagulation is due to a commercial rennet and, after the manual cutting, the curd is cooked at about 45 °C on wood fire. After two days of brine salting, cheeses are ripened for two months in a Malga room at 15–18 °C and for five more months in valley storerooms, where the cheese wheels are delivered after the Malga closure. The microbial fermentation is spontaneous, started and carried on by autochthonous bacteria until the end of the ripening.

It has been reported that raw milk cheeses have more intense flavours than pasteurised milk cheeses (Albenzio et al., 2001; Beuvier et al., 1997; Demarigny et al., 1997) and their organoleptic characteristics are widely correlated with the nutrition of the milk producing cows, the quality and the environmental contamination of the collected milk, the technological procedures and the presence of appropriate lactic acid bacteria (LAB) (Beresford et al., 2001; Corroler et al., 1998; Grappin and Beuvier, 1998). It is well known that mainly LAB and in particular lactobacilli developing during the ripening, influence the typical characteristics of the cheese (Wouters et al., 2002); thus, they represent a fundamental factor for the final attributes and quality of artisan dairy products such as TMM-cheese. Several authors have focused on the genotypic and technological characterization of LAB isolated from spontaneously fermented cheeses (Aquilanti et al., 2007; Fuka et al., 2010; Nieto-Arribas et al., 2010; Turchi et al., 2011) and in many works selected lactobacilli strains were used in order to improve cheese

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organoleptic attributes. Di Cagno et al. (2011) reported that lactobacilli adjunct cultures affected the moisture and texture of the Caciotta-type cheese. Awad et al. (2007) observed that cultures of *Lactobacillus helveticus*, *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus delbrueckii* subsp. *lactis* combined with starter LAB culture, produced free amino acids and free fatty acids which influenced Ras cheese organoleptic attributes. Franciosi et al. (2008) reported that the use of lactobacilli as secondary adjunct cultures may affect the Puzzone di Moena ripening.

During ripening, the cheese environment becomes unfavourable for growth of most microorganisms; there is low water activity, high salt content, low pH and low temperature (Kask et al., 2003). Therefore, LAB are generally analysed for their growth properties, acidifying ability, salt resistance and carbohydrate metabolism (Aquilanti et al., 2007; Kask et al., 2003; Turchi et al., 2011). Others bacterial activities are usually investigated such as caseins proteolysis and aminopeptidase (AP) activity, which contribute to liberation of aminoacids during cheese ripening and play a key role in the hydrolysis of bitter peptides and acetoin production that positively contribute to flavour and aroma of dairy products during the cheese ripening (Bartels et al., 1987; McSweeney, 2004). Dairy LAB are generally assayed also for some undesirable properties, as lipolytic activity which could induce a rancid flavour to cheese during the ripening (Herrero et al., 1996) and biogenic amines (BA) production because they could induce toxicological (palpitations, headaches and flushing) and systemic pathophysiological effects in humans such as allergies and inflammations (Gonzaga et al., 2009; Spano et al., 2010).

In addition to the technological relevance, there is currently much research about raw milk cheeses microbiota, which is rich in biodiversity and could have healthy benefits (Montel et al., 2014). Lactic acid bacteria isolated from dairy environment were found able to produce bioactive compounds, such as conjugated linoleic acid (CLA) and γ-aminobutyric acid (GABA) (Settanni and Moschetti, 2010). CLA have been proposed to possess a number of putative health promoting activities (Pariza et al., 1979, 2001; Ryder et al., 2001; Yang and Cook., 2003; Yu et al., 2002). GABA is produced by LAB through the decarboxylation of glutamate and since the caseins are rich in glutamate which is released by the proteolytic action, the decarboxylation of glutamate to GABA can have an important effect on the formation of eyes in cheese (Zoon and Allersma, 1996). Besides its technological effect in cheese, GABA has several well-characterized physiological functions (Hagiwara et al., 2004; Jakobs et al., 1993; Wong et al., 2003). The bacteria bile salt hydrolysis (BSH) activity has also been reported to have health promoting effects (Begley et al., 2006; De Smet et al., 1994; Jones et al., 2008).

There are many studies analyzing the chemical composition of semi-hard alpine cheeses made by raw cows' milk, e.g., Toma piemontese, Bitto and Asiago cheeses (Chion et al., 2010; De Noni and Battelli, 2008; Favaro et al., 2005), but to our knowledge, nothing is known about the microbiota of alpine cheeses and in particular of malga cheeses. The aim of this study was to characterize the species and biotypes of LAB growing on MRS medium present at the end of ripening of TMM-cheese produced in eight different area in Trentino province. The microbial biodiversity investigation was added with information about some technological properties and the ability to produce bioactive substances.

2. Materials and methods

2.1. Cheese sampling and bacterial isolation

Eight TMM-cheeses produced in summer season following the traditional cheese-making techniques were sampled from eight different Malga dairies at the end of ripening (7 months). Ten grams of each sample were homogenized in 90 mL of sterile 2% of Na-citrate solution pH 5.5 by a ULTRA-TURRAX® (IKA® – Werke GmbH & Co.KG, Staufen, Germany) for 5 min at speed 3 and five serial decimal dilutions were done. All the dilutions were plated on MRS agar (Oxoid, Milan, Italy) acidified to pH 5.5 with 5 mol/L lactic acid, for selection of putative lactobacilli. Plates were incubated for 48 h at 30 and 45 °C, anaerobically. Ten or more colonies were randomly picked up from countable MRS agar plates for bacterial isolation and purified by subsequent culturing. Pure cultures were stored at −80 °C in glycerol (20% v/v) stocks. Cell morphology was determined by microscopic observation, Gram characterization was performed applying the KOH method (Gregersen, 1978) and catalase activity was tested after addition of 5% H_2O_2 on the colonies.

2.2. Biotypes clustering and identification

All isolates were subjected to Randomly Amplified Polymorphic DNA–PCR (RAPD–PCR). DNAs were prepared from MRS plates colonies after 48 h of incubation at the isolation temperature, by Instagene Matrix (Bio-Rad, Hercules, CA, USA), following the manufacturer's instruction.

RAPD–PCRs were carried out using the primers M13 (Huey and Hall, 1989) and OPA09 (5′-GGGTAACGCC-3′). Amplification reactions were performed according to the protocol described by Giraffa et al. (2000). PCR products were separated by electrophoresis on 2.5% (w/v) agarose gel (Gibco BRL, Cergy Pontoise, France) and stained with ethidium bromide (0.5 μg/L). DNA patterns were analysed through the Unweighted Pair Group Method Arithmetic averages (UPGMA) using the GelCompar II-BioNumerics software (package version 6.0; Applied Maths, Belgium). Calculation of similarity of the PCR fingerprinting profiles was based on the Pearson product–moment correlation coefficient. Isolates with a similarity coefficient higher than 85% were considered belonging to the same biotype, as described by Gatti et al. (2008).

Genotypic identification of different LAB biotypes was carried out by partial 16S rRNA gene sequencing and species-specific PCRs. The 16S rRNA gene sequence analysis was performed using 27f (5′-GAGAGTTTGATCCTGGCTCAG) and 1495r (5′-CTACGGCTACCTTGTTACGA) primers, designed by Grifoni et al. (1995). Each obtained PCR product (ca. 30 ng) was purified with Exo-SAP-IT™ kit (USB Co., Cleveland, OH) and sequenced through the BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in a ABI PRISM 3100 sequencer (Applied Biosystems, Italy). For species assignment, sequences were compared using the BLAST algorithm made available by the National Center for Biotechnology Information (NCBI, USA). *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus* species were also confirmed by means of species-specific PCR with the primers Y2, Casei, Para and Rham described by Ward and Timmins (1999). All amplifications were performed with a T100™ ThermalCycler (Bio-Rad Laboratories, Hercules, CA, USA).

2.3. Determination of lysogenic state

The lysogenic state of LAB isolates was determined by measuring the induction of prophage adapting the method of Cochran and Paul (1998). Broth cultures grown for 48 h were splitted into two 1 mL aliquots and one was added with mitomycin C (1 μg/mL; Sigma Chemical Co., USA). After 24 h of incubation at room temperature, in the dark, the presence of phage was detected by the plaque assay modified as follows: 50 μL of each cell suspension was inoculated into 7 mL of soft agar (0.7%, w/v) medium, plated on a layer of 1.8% (w/v) agar and incubated for 24 h at conditions allowing the optimal growth. The presence of phage was

visible comparing the cell growth onto plates with/without mitomycin C.

2.4. Screening for technological potentialities

Cells grown anaerobically in MRS broth medium for 48 h at 30 °C, were harvested by centrifugation at 5000 rpm for 5 min, washed, suspended in peptone water and subjected to following tests, all performed in triplicate.

The growth at different temperatures (15, 30, and 45 °C) and in presence of different NaCl concentrations (2, 4, 6 and 8%, w/v) was evaluated by qualitative tests onto MRS plates incubated in anaerobic conditions. The period of incubation was 48 h for all tests, except for growth at 15 °C (96 h).

Exocellular proteolytic and lipolytic activities were evaluated by qualitative test as reported by Franciosi et al. (2009) and Buffa et al. (2005), respectively.

Acetoin production was determined by a qualitative test according to Benjaminson et al. (1964).

The aminopeptidase (AP) activity was evaluated by a quantitative test, as described by Requena et al. (1993) with some modifications: L-lysine *p*-nitroanilide and L-leucine *p*-nitroanilide (Sigma) were the tested substrates. After 16 h of growth, cells were resuspended in 50 mM of sodium–phosphate buffer pH 7. The cell suspensions were lysed by sonication in an ice-water bath to prevent significant heating in the sample for 10 min. The supernatants (cell-free extracts, CFEs) were separated by centrifugation (12,000 × *g*, 30 min, 4 °C) and protein concentration in CFEs was measured using the Bradford reagent (Sigma). Two different 15.5 mM substrate solutions (L-lysine and L-leucine *p*-nitroanilide) were prepared in 50 mM of sodium–phosphate buffer pH 7 (1.9 mL) and mixed to 100 μL of CFE. AP activity was measured spectrophotometrically at 410 nm (Spectrophotometer Evolution™ 300, Thermo Scientific, Rodano, MI, Italy) at 2 min intervals, for at least 30 min and the measure unit was defined as the change of 0.01 of absorbance in one minute for each mg of protein.

The acidifying capacity of cell suspensions (1%, v/v) was evaluated by a quantitative test in 10 mL of sterile UHT skim milk (Latte Trento Sca, Trento, Italy) and incubated at the optimal growth temperature. pH measurements were carried out using a pH meter PT1000 (Knick, Berlin, Germany) equipped with a Hamilton electrode (Hamilton Bonaduz, Bonaduz, Switzerland), after 8, 16 and 24 h from inoculation.

Two different qualitative tests were carried out in order to understand the carbohydrate metabolism of isolates from TMM-cheese. The facultative heterofermentative lactobacilli, intrinsically resistant to vancomycin (Ammor et al., 2007), were detected by spotting each culture onto MRS agar with vancomycin (8 μg/mL).

The presence of obligate heterofermentative lactobacilli was detected by using Durham tubes filled with Hugh Leifson broth medium, in presence of lactose, glucose or galactose, as carbohydrate sources, as reported by Gerhardt et al. (1984).

The ability to produce cadaverine, tyramine, histamine and putrescine respectively from L-lysine, tyrosine disodium salt, L-histidine monohydrochloride and L-ornithine monohydrochloride was investigated by a qualitative test, according to the method proposed by Bover-Cid and Holzapfel (1999). All amino acids were purchased from Sigma.

2.5. Screening for bioactive properties

2.5.1. Bile salt hydrolysis (BSH) assay

The capacity of isolated strains to hydrolyze bile salts was tested by using a plate assay according to the method previously described by Ren et al. (2011), with some modifications. Each culture was spotted (10 μL) onto MRS agar plates supplemented with 0.5% (w/v) taurodeoxycholic acid (TDCA; Sigma) and 0.5% (w/v) glycodeoxycholic acid (GDCA; Sigma). The white precipitates around colonies and the clearing of the medium are indicative of BSH activity.

2.5.2. CLA production and quantification

Cells grown for 24 h anaerobically at 30 °C in MRS broth supplemented with 0.5 mg/mL of linoleic acid (LA, Sigma) and 0.1 mg/mL of bovine serum albumin (BSA, Sigma), were incubated for 24 h in skim milk (5% v/v) added with 2.5 mg/mL LA and 0.5 mg/mL BSA. Total CLA extraction was carried out according to the method described by Alonso et al. (2003), with some modifications: 1 mL of culture was centrifuged at 7500 rpm for 5 min, at 4 °C. After the addition of 2 mL of isopropanol to the supernatants and vortexing for 3 min, 1.5 mL of hexane was added and further vortexed for 3 min. A last centrifugation step at 2000 rpm for 5 min at 4 °C allowed to collect the supernatants containing the total CLA and to quantify it by UV spectroscopy as proposed by Barrett et al. (2007). Absorbance values of the tested samples were obtained through a spectrophotometer Evolution™ 300 (Thermo Scientific) at 233 nm, with a scan program (190–350 nm). For each isolate, 2 mL of lipid extract were placed into quartz cuvettes and analysed in triplicate in order to measure the ability to convert free LA to total CLA. In order to verify the suitability of this method, a standard curve was constructed for the absorbance at 233 nm versus the pure C18:2 *cis-9,trans-11* CLA isomer (Oxoid) concentration (0–280 μg/mL), which is the most representative isomer in milk fat (Stanton et al., 2003). This isomer is reported as one of the most bioactive and is produced by intestinal bacteria in higher amount than the other 27 isomers (O'Shea et al., 2012).

2.5.3. γ-Aminobutyric acid (GABA) production and quantification

Glutamate decarboxylase (GAD) activity of LAB strains and the resulting production of GABA were checked through the method described by Nomura et al. (1999), with some modifications: cultures were centrifuged (9000 rpm for 15 min at 4 °C), washed twice with sterile PBS and resuspended in sterile 0.85% NaCl solution in order to achieve the A_{620nm} value of 2.5. Afterward, 100 μL of cell suspension were mixed with 900 μL of 50 mM sodium acetate buffer (pH 4.7) containing 7.0 mM L-glutamate and 0.1 mM pyridoxal phosphate. The reaction mixture was incubated at 30 °C for 24 h and filtered through a 0.22 μm pore size filter (Minisart, Sartorius Stedim Biotech, Goettingen, Germany). The sample, diluted 10 times with sodium tetraborate 0.1 M (Carlo Erba; pH adjusted to 10.5) and added of glycine (Sigma; as internal standard to a final concentration of 10 mg/L), was stored at −20 °C before the analysis. L-glutamic acid (Sigma–Aldrich), glycine and GABA (Merck) were quantified as o-phthalaldehyde (OPA) adducts modifying the method proposed by Lehtonen (1996): separation was carried out with sodium acetate 0.05 M (adjusted pH 7.5; eluent A; Sigma) and methanol (eluent B; Sigma) using a column Chromolith Performance RP-18e (100 × 4.6 mm; Merk, Germany) with Guard Cartridge Chromolith RP-18e (10 × 4.6 mm; Merk) at 40 °C. The flow rate was set at 2 mL/min. The analytical gradient for eluent B was: 0.5 min at 40%, back to 25% in 1.5 min, to 100% in 0.2 min, held at 100% for 0.3 min for cleaning, and to 60% for reconditioning in 0.2 min. The sample (10 μL), kept at 10 °C by the autosampler, was automatically introduced in the loop, added of 10 μL derivatising solution, mixed for 1 min, and injected. The derivatising mix was prepared with 4.5 g/L of OPA (Fluka) in sodium tetraborate 0.1 M, corrected to pH 10.5, 10% methanol and 2% 2-mercaptoethanol (Fluka). The measures were performed using an UHPLC Ultimate 3000 (Thermo Scientific) equipped with a fluorescence detector (Ex = 336 nm, Em = 445 nm). The detection limit for GABA was estimated at 0.025 mg/L (3 times the standard

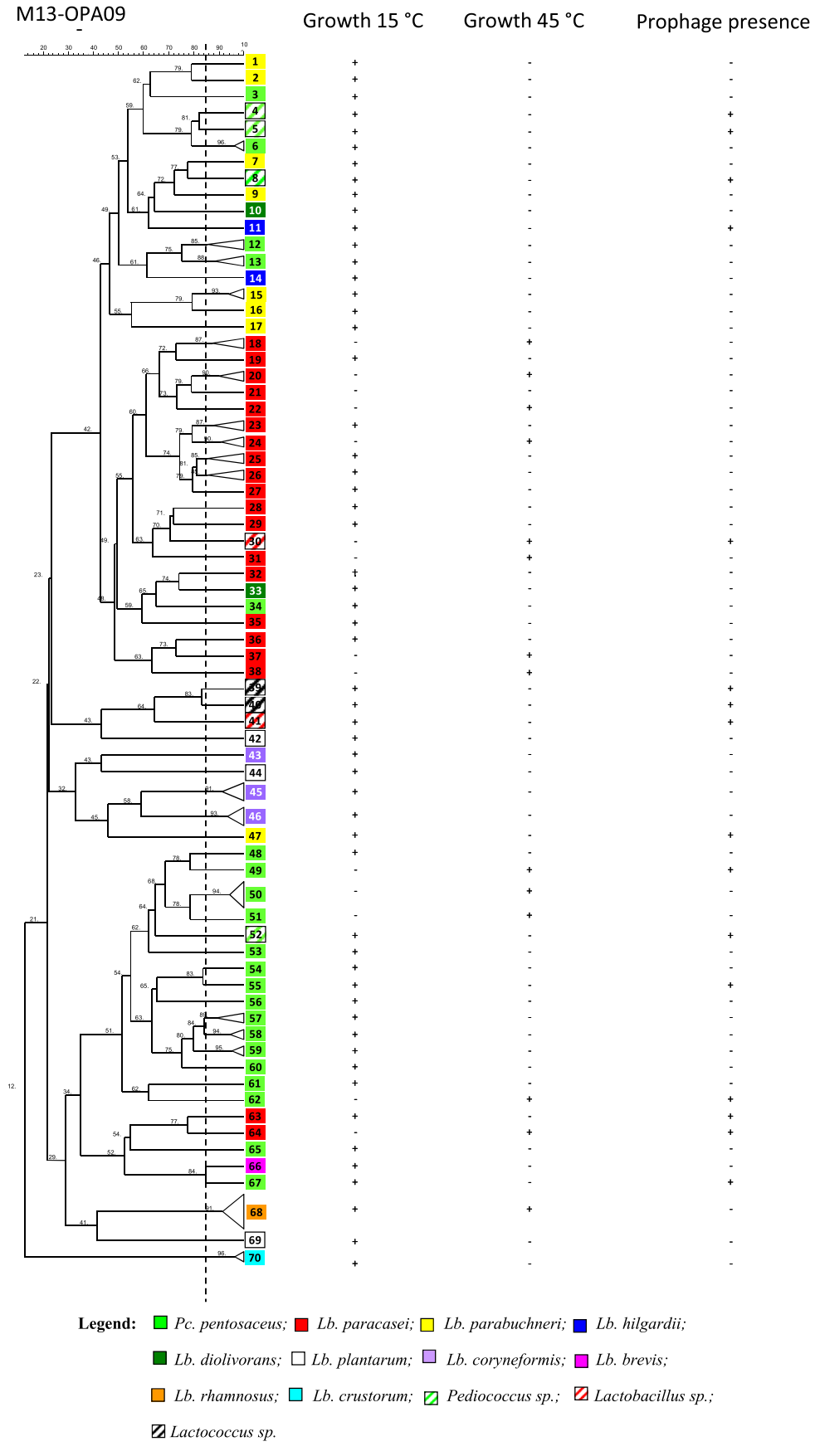

Fig. 1. Dendrogram obtained from M13 and OPA-09 RAPD-PCR patterns of 95 isolates. The upper scale indicates the similarity level calculated by using the Pearson product–moment correlation coefficient: isolates having 85% (black line) of similarity were collapsed into the same biotype. Temperature growth ability and determination of the lysogenic state are shown in the beside columns.

deviation of the GABA contents measured repeating 10 times the analysis of a sample at unquantifiable content).

3. Results and discussion

This research was aimed to characterize the autochthonous LAB growing onto MRS and establishing in TMM-cheese at the end of ripening and to screen their technological and health promoting properties. It has been reported that raw cows' milk from Trentino alpine region has a high biodiversity and some wild LAB strains showed interesting technological properties (Franciosi et al., 2009). Since there is no detailed study about the TMM-cheese microbiota, a polyphasic approach was chosen for the investigation of the LAB isolated from the TMM-cheeses at the end of ripening.

3.1. Biotyping and identification of isolates

One hundred and ten colonies were isolated from spontaneously fermented TMM-cheese samples after seven month of ripening. Ninety-five isolates were considered LAB because Gram-positive and catalase negative and 31 of them were identified as cocci-shaped by microscope observation. The RAPD-PCR analysis clustered the isolates into 70 biotypes having 85% similarity index (Fig. 1); 18 clustered together two or more strains, and 52 were "singletons" (isolates whose similarity index with other isolates was lower than 85%). Colonies isolated from cheese sampled in different Malga-farms had always a similarity level lower than 85%, thus, they never clustered into the same biotype. The partial 16S rRNA gene sequencing revealed that TMM-cheese at the end of ripening was mainly composed of lactobacilli (73.6%), followed by lower amounts of pediococci (26.4%). *Lb. paracasei* (26 isolates, 20 biotypes) was the most represented species amongst lactobacilli and *Pediococcus pentosaceus* (29 isolates, 21 biotypes) was the only species found among the cocci-shaped isolates. Both species were previously isolated from raw cow milk cheeses. *Lb. paracasei* resulted to be the main species in traditional cheeses as Spanish farmhouse and Manchego cheeses (Martín-Platero et al., 2008; Sánchez et al., 2006) and *Pc. pentosaceus* was isolated from Domiati and Cheddar cheeses at the end of ripening (El-Baradei et al., 2006; Thomas et al., 1985) but was not a dominant species as in TMM-cheese. The other isolates were clustered into 8 biotypes of *Lb. parabuchneri*, 3 biotypes of *Lb. plantarum* and *Lb. coryniformis*, 2 biotypes of *Lb. diolivorans* and *Lb. hilgardii*, 1 biotype of *Lb. brevis*, *Lb. rhamnosus* and *Lb. crustorum* (Fig. 1). All these species were previously found in raw cow's milk cheeses made following traditional cheese-making processes (Didienne et al., 2012; Dolci et al., 2008, 2010; Sánchez et al., 2006; Yu et al., 2011).

The high number of bacilli and pediococci species and biotypes recorded confirmed the higher LAB biodiversity of TMM-cheese when compared to other Italian cheeses produced from cow milk: in Grana Trentino 345 bacterial isolates belonged to only 4 microbial species (Monfredini et al., 2012); 352 isolates from Puzzone di Moena clustered into about 100 biotypes belonging to 6 different species (Franciosi et al., 2009) and 320 LAB, isolated from Caciocavallo Pugliese, clustered in 40 biotypes belonging to 10 species (Aquilanti et al., 2007). The TMM-cheese high LAB biodiversity could be related to: i) the standing-alone character of Malga-farms; in facts there is no exchange of sources with surrounding areas because both milk collection and the cheese-making process are carried out in the same place; ii) the spontaneous fermentation which allows the growth of different species during the cheese-ripening; iii) the low cooking temperature (never higher than 45 °C) which is not bacteria-selective.

3.2. Screening for technological properties

All 70 tested clusters were facultative heterofermentative, able to grow at 30 °C and in presence of 8% of NaCl (data not shown). None of them showed exoproteolytic activity when assayed on SM, nor lipolytic activity in PCA added with milk cream (data not shown). Growth at 15 and 45 °C was observed in 56 and 14 of the 70 tested biotypes, respectively. Only *Lb. rhamnosus* (cluster 68) was able to grow at both temperatures (Fig. 1). The psychrotrophic attitude and the salt tolerance may be determined by the cheese-ripening environment: in fact, TMM-cheese wheels are brine salted for two days and ripened for seven months in a store-room with the temperature range of 15–18 °C. The role of the dairy environment (ripening store temperature) and the cheese-making procedures (cheese cooking temperature) on the selection of dairy adapted LAB were previously reported by Monfredini et al. (2012).

The presence of resident prophage in the genome of all biotypes was determined by induction with sub-lethal concentrations of mitomycin C. In 16 out of the 70 tested biotypes, mitomycin C induced a sharp decrease (more than 5 magnitude orders) in plate counts (Fig. 1). The 16 biotypes infected by lysogenic phage were discarded for further analysis and 54 isolates representative of the 54 prophage-free clusters were selected for further analysis.

All 54 identified prophage-free clusters were tested for their acidifying ability. All isolates lowered the milk pH below 5.2 after 24 h (data not shown), and only 6 biotypes (5 *Lb. paracasei* and 1 *Pc. Pentosaceus*) showed a rapid acidification to pH values lower than 5.2 within 8 h (Table 2). Similar results were expected because the *Lactobacillus* genus is known to have a slow ability to metabolize lactose (Gonzales et al., 2010; Herreros et al., 2003).

In order to evaluate the flavor formation ability of strains from TMM-cheese, the acetoin production and the AP activity were tested on all 54 phage-free biotypes. Sixteen biotypes produced acetoin which is responsible for the buttery flavor of dairy products: all acetoin producers were *Lb. paracasei* with the exception of two biotypes n° 16 (*Lb. parabuchneri*) and n° 60 (*Pc. pentosaceus*) (Table 1).

All 54 phage-free biotypes showed AP activity (Table 2) and results were broadly different amongst the clusters. Only two *Lb. paracasei* biotypes (18 and 31) showed a good AP activity in particular toward leucine (more than 750 U/mg). Ten of them (4 *Pc. pentosaceus*, 3 *Lb. paracasei*, 1 *Lb. plantarum*, 1 *Lb. parabuchneri* and 1 *Lb. coryniformis*) showed a fair AP activity (values in the range of 300–750 U/mg) and the remaining 42 clusters showed a poor activity (Table 2).

3.3. In vitro production of biogenic amines

Biogenic amines (BA) are generated through amino acid decarboxylation, which is generally considered an undesirable trait for food grade microorganisms. Flushing, headache, dilatation of peripheral blood vessels and hypertension could be the risks associated with a high intake of BA (Shalaby, 1996; Valsamaki et al., 2000). The 54 biotypes were tested *in vitro* for the production of tyramine, histamine, cadaverine and putrescine. The test was performed in triplicate and colonies growth was always observed but there is the possibility of false negative results due to the acidifying ability of the isolates that could inhibit the shift of the pH indicator contained in the decarboxylase agar medium. Only eight biotypes (*Pc. pentosaceus* biotypes 48, 51, 57, 60, 61; *Lb. paracasei* biotypes 24, 27 and *Lb. hilgardii* biotypes 14) synthesized tyramine from tyrosine and three *Lb. parabuchneri* biotypes (1, 7, 17) converted the L-histidine monohydrochloride to histamine. None produced cadaverine or putrescine (Table 1). The production of both tyramine and histamine was previously detected in dairy LAB belonging to *Lb.*

Table 1
Identification at species level and screening of acetoin and biogenic amines production by the 70 biotypes found after RAPD-PCR clustering of the 95 LAB isolated from TMM cheese.

Biotype	Closest genus and species	Maximum identity in NCBI database (%)	Number of isolates	Acetoin production	Biogenic amines production[a]			
					His	Tyr	Put	Cad
1	*Lactobacillus parabuchneri*	98.8	1	−	+	−	−	−
2	*Lactobacillus parabuchneri*	98.9	1	−	−	−	−	−
3	*Pediococcus pentosaceus*	97.0	1	−	−	−	−	−
4	*Pediococcus* sp.	92.9	1	n.d.	n.d.	n.d.	n.d.	n.d.
5	*Pediococcus* sp.	91.0	1	n.d.	n.d.	n.d.	n.d.	n.d.
6	*Pediococcus pentosaceus*	98.9	2	−	−	−	−	−
7	*Lactobacillus parabuchneri*	99.2	1	−	+	−	−	−
8	*Lactobacillus* sp.	96.9	1	n.d.	n.d.	n.d.	n.d.	n.d.
9	*Lactobacillus parabuchneri*	98.6	1	−	−	−	−	−
10	*Lactobacillus diolivorans*	99.7	1	−	−	−	−	−
11	*Lactobacillus hilgardii*	99.6	1	n.d.	n.d.	n.d.	n.d.	n.d.
12	*Pediococcus pentosaceus*	100	2	−	−	−	−	−
13	*Pediococcus pentosaceus*	99.5	2	−	−	−	−	−
14	*Lactobacillus hilgardii*	99.6	1	−	−	+	−	−
15	*Lactobacillus parabuchneri*	98.6	2	−	−	−	−	−
16	*Lactobacillus parabuchneri*	99.4	1	+	−	−	−	−
17	*Lactobacillus parabuchneri*	97.2	1	−	+	−	−	−
18	*Lactobacillus paracasei*	98.6	2	−	−	−	−	−
19	*Lactobacillus paracasei*	99.4	1	+	−	−	−	−
20	*Lactobacillus paracasei*	99.6	2	+	−	−	−	−
21	*Lactobacillus paracasei*	99.9	1	+	−	−	−	−
22	*Lactobacillus paracasei*	99.7	1	+	−	−	−	−
23	*Lactobacillus paracasei*	99.8	2	+	−	−	−	−
24	*Lactobacillus paracasei*	99.9	2	+	−	+	−	−
25	*Lactobacillus paracasei*	99.9	2	+	−	−	−	−
26	*Lactobacillus paracasei*	99.4	2	+	−	−	−	−
27	*Lactobacillus paracasei*	99.9	1	−	−	+	−	−
28	*Lactobacillus paracasei*	99.0	1	+	−	−	−	−
29	*Lactobacillus paracasei*	100	1	+	−	−	−	−
30	*Lactobacillus* sp.	90.5	1	n.d.	n.d.	n.d.	n.d.	n.d.
31	*Lactobacillus paracasei*	99.2	1	+	−	−	−	−
32	*Lactobacillus paracasei*	99.5	1	−	−	−	−	−
33	*Lactobacillus diolivorans*	99.6	1	−	−	−	−	−
34	*Pediococcus pentosaceus*	99.8	1	−	−	−	−	−
35	*Lactobacillus paracasei*	99.7	1	−	−	−	−	−
36	*Lactobacillus paracasei*	99.7	1	+	−	−	−	−
37	*Lactobacillus paracasei*	99.1	1	+	−	−	−	−
38	*Lactobacillus paracasei*	99.5	1	+	−	−	−	−
39	*Lactococcus* sp.	85.5	1	n.d.	n.d.	n.d.	n.d.	n.d.
40	*Lactococcus* sp.	84.4	1	n.d.	n.d.	n.d.	n.d.	n.d.
41	*Lactobacillus* sp.	91.6	1	n.d.	n.d.	n.d.	n.d.	n.d.
42	*Lactobacillus plantarum*	100	1	−	−	−	−	−
43	*Lactobacillus coryniformis*	99.5	1	−	−	−	−	−
44	*Lactobacillus plantarum*	99.6	1	−	−	−	−	−
45	*Lactobacillus coryniformis*	99.4	3	−	−	−	−	−
46	*Lactobacillus coryniformis*	99.7	3	−	−	−	−	−
47	*Lactobacillus parabuchneri*	99.5	1	n.d.	n.d.	n.d.	n.d.	n.d.
48	*Pediococcus pentosaceus*	99.9	1	−	−	+	−	−
49	*Pediococcus pentosaceus*	99.6	1	n.d.	n.d.	n.d.	n.d.	n.d.
50	*Pediococcus pentosaceus*	99.7	4	−	−	−	−	−
51	*Pediococcus pentosaceus*	99.2	1	−	−	+	−	−
52	*Pediococcus* sp.	95.2	1	n.d.	n.d.	n.d.	n.d.	n.d.
53	*Pediococcus pentosaceus*	99.7	1	−	−	−	−	−
54	*Pediococcus pentosaceus*	99.9	1	−	−	−	−	−
55	*Pediococcus pentosaceus*	99.9	1	n.d.	n.d.	n.d.	n.d.	n.d.
56	*Pediococcus pentosaceus*	99.9	1	−	−	−	−	−
57	*Pediococcus pentosaceus*	100	2	−	−	+	−	−
58	*Pediococcus pentosaceus*	100	2	−	−	−	−	−
59	*Pediococcus pentosaceus*	99.5	2	−	−	−	−	−
60	*Pediococcus pentosaceus*	99.9	1	+	−	+	−	−
61	*Pediococcus pentosaceus*	99.8	1	−	−	+	−	−
62	*Pediococcus pentosaceus*	99.9	1	n.d.	n.d.	n.d.	n.d.	n.d.
63	*Lactobacillus paracasei*	99.9	1	n.d.	n.d.	n.d.	n.d.	n.d.
64	*Lactobacillus paracasei*	99.5	1	n.d.	n.d.	n.d.	n.d.	n.d.
65	*Pediococcus pentosaceus*	99.7	1	−	−	−	−	−
66	*Lactobacillus brevis*	99.8	1	−	−	−	−	−
67	*Pediococcus pentosaceus*	99.6	1	n.d.	n.d.	n.d.	n.d.	n.d.
68	*Lactobacillus rhamnosus*	99.9	5	−	−	−	−	−
69	*Lactobacillus plantarum*	99.6	1	−	−	−	−	−
70	*Lactobacillus crustorum*	97.6	2	−	−	−	−	−

n.d.: not determined; isolates with resident prophage were not tested.
[a] His = Histidine, Tyr = Tyramine, Put = putrescine; Cad = Cadaverine.

Table 2
Screening of the acidifying, AP, BSH activities, CLA and GABA production by the 54 prophage-free biotypes found after RAPD-PCR clustering of the 95 LAB isolated from TMM-cheese.

Biotype	Acidifying activity[a] (pH after 8 h)	Aminopeptidase activity[b] (U/mg)		CLA 24 h (μg/mL)	GABA production (mg/L)
		Leucine	Lysine		
1	6.37	129	102	<0.05	12 ± 0.5
2	5.94	121	118	<0.05	<0.5
3	6.40	<10	<10	103 ± 11	0.9 ± 0.7
6	5.52	141	115	<0.05	1.6 ± 0.2
7	5.91	81	86	1 ± 0.2	<0.5
9	6.02	12	13	24 ± 1.5	<0.5
10	6.15	39	45	22 ± 2.1	<0.5
12	5.86	276	233	<0.05	1.6 ± 0.1
13	6.35	<10	<10	81 ± 9	1.5 ± 0.2
14	6.09	76	68	33 ± 7	<0.5
15	6.18	20	23	5.9 ± 0.9	<0.5
16	5.38	328	278	22 ± 3	5.4 ± 0.1
17	6.21	78	58	23 ± 3.3	<0.5
18	5.64	798	389	130 ± 10	4.4 ± 0.3
19	5.92	31	26	52 ± 7	<0.5
20	5.49	50	42	<0.05	3.2 ± 0.6
21	6.23	33	20	10 ± 1.9	3.8 ± 0.2
22	5.59	254	17	47 ± 3.1	3.0 ± 0.3
23	4.92	230	183	14 ± 3	3.6 ± 0.2
24	5.02	307	641	<0.05	5.5 ± 0.2
25	6.64	115	93	90 ± 11	5.4 ± 0.6
26	5.39	148	119	22 ± 2.9	3.8 ± 0.2
27	5.26	37	30	13 ± 1.7	5.8 ± 0.2
28	5.08	215	146	18 ± 2.7	4.0 ± 0.5
29	5.42	41	30	41 ± 6.9	7.0 ± 0.9
31	4.47	877	561	96 ± 8.3	4.7 ± 0.2
32	6.35	172	142	<0.05	7.0 ± 0.6
33	5.87	119	96	29 ± 4.6	<0.5
34	5.94	166	135	30 ± 5	1.3 ± 0.1
35	5.10	12	11	12 ± 2.2	5.4 ± 0.2
36	5.23	503	392	<0.05	2.9 ± 0.4
37	5.46	282	229	32 ± 5	4.4 ± 0.4
38	5.51	383	202	33 ± 3.8	7 ± 1.2
42	6.06	36	26	<0.05	13 ± 1.6
43	6.57	<10	<10	<0.05	<0.5
44	5.35	461	392	24 ± 3	14 ± 6.1
45[c]	6.45	540	471	5.9 ± 0.7	0.8 ± 0.7
46	6.03	184	154	60 ± 5	2.3 ± 0.2
48	5.59	356	117	21 ± 4.1	1.3 ± 0.1
50	5.69	280	256	31 ± 3.4	1.0 ± 0.5
51[c]	6.41	492	417	73 ± 6.1	1.5 ± 0.5
53	6.17	31	27	22 ± 3	1.5 ± 0.3
54	5.94	62	42	60 ± 7	1.7 ± 0.3
56	4.92	218	190	14 ± 2.8	9.6 ± 0.9
57	6.20	535	239	<0.05	1.2 ± 0.2
58	5.89	277	107	<0.05	<0.5
59	5.85	138	106	<0.05	2.0 ± 0.2
60	5.69	34	28	30 ± 6	1.3 ± 0.1
61	5.71	132	124	24 ± 3.2	1.1 ± 0.1
65	5.67	133	101	30 ± 3	1.7 ± 0.2
66[c]	6.56	<10	<10	22 ± 3.1	129 ± 8.6
68	6.57	22	19	94 ± 10.6	8.3 ± 0.1
69	5.96	364	296	<0.05	10 ± 4.9
70	6.64	<10	<10	<0.05	1.9 ± 0.1

[a] The standard deviation was never higher than 0.04.
[b] AP activity was measured in AP unit which corresponds to an increase of 0.01 units of absorbance in 1 min for each mg of protein. [AP unit = $(A_{410sample} - A_{410blank})/(0.01*30)$]. The standard deviation was never higher than 10.
[c] Biotypes positive for BSH activity; the test was performed on MRS agar plates supplemented with 0.5% (w/v) taurodeoxycholic acid and 0.5% (w/v) glycodeoxycholic acid.

paracasei (Nieto-Arribas et al., 2009) and *Lb. parabuchneri* (Fröhlich-Wyder et al., 2013) species. BA production by *Pc. pentosaceus* and *Lb. hilgardii* was found in wine (Alberto et al., 2007; García-Ruiz et al., 2011) but never in cheese isolates. We have not verified nor quantified the presence of BA in TMM-cheese. Some physico-chemical factors could affect the amount of BA in cheese, e.g., pH, temperature, NaCl concentration, water activity and redox potential (Gardini et al., 2001; Pinho et al., 2001; Santos et al., 2003), therefore the BA profile in the cheese could differ from results obtained analysing the isolates *in vitro*.

3.4. Health promoting effects

The 54 phage-free biotypes were further tested for *in vitro* production of bioactive molecules. Seven biotypes (*Lb. paracasei* biotypes 24, 31; *Pc. pentosaceus* biotypes 12, 51; *Lb. coryniformis*

biotypes 45 and *Lb. brevis* biotypes 66) were able to grow onto 1% of bile salts but only three biotypes (45, 51 and 66) showed the BSH activity on solid media containing 1% of bile salts (Table 2). The capacity to hydrolyze bile salts has been detected in several members of *Lactobacillus* genus, such as *Lb. acidophilus* (McAuliffe et al., 2005), *Lb. johnsonii* (Elkins et al., 2001), *Lb. reuteri* (De Boever et al., 2000), *Lb. plantarum* (Christiaens et al., 1992) and *Lb. casei* (Zhang et al., 2009). Recently, some *Pc. pentosaceus* strains isolated from *Idly* batter (Vidhyasagar and Jeevaratnam, 2013) have been shown to exhibit the BSH activity, but never *Lb. brevis* and *Lb. coryniformis* species.

The CLA and GABA producing abilities were screened *in vitro* because considered a source of health promoting effects (Pariza et al., 1979; 2001; Ryder et al., 2001; Yang and Cook, 2003; Yu et al., 2002). The total CLA concentration present in culture supernatants was measured spectrophotometrically comparing the measured values in a calibration curve (linear increase $R^2 = 0.9994$) built at 233 nm with the C18:2 *cis-9,trans-11* CLA isomer from 0 to 10 ppm. Most of tested strains produced less than 60 μg/mL of CLA; strains belonging to biotypes 68 (*Lb. rhamnosus*), 25, 18, 31 (*Lb. paracasei*), 3, 13 and 51 (*Pc. pentosaceus*) produced between 70 and 130 μg/mL of total CLA, respectively (Table 2). Few studies investigated the CLA production by strains isolated from dairy products (Jiang et al., 1998; Rodríguez-Alcalá et al., 2011); Kishino et al. (2002) found some *Lb. paracasei* strains, belonging to a Culture Collection, able to produce between 70 and 90 μg/mL of CLA and one *Lb. rhamnosus* producing 1410 μg/mL.

Forty-three out of 54 biotypes synthesized GABA after 24 h of incubation at 30 °C in presence of glutamic acid. In particular, ten (1 *Lb. parabuchneri*, 7 *Lb. paracasei*, 1 *Lb. rhamnosus* and 1 *Pc. pentosaceus*) were able to produce GABA concentrations between 5 and 10 mg/L and 5 (1 *Lb. parabuchneri*, 3 *Lb. plantarum* and 1 *Lb. brevis*) produced 10 mg/L or more (Table 2). The production of GABA by various LAB isolated from traditional fermented food has been reported, in particular *Lactobacillus* sp. isolated from kimchi (Li and Cao, 2010) and from several Italian cheese varieties (Siragusa et al., 2007). Our results are different from those obtained by Siragusa et al. (2007) who found that only 14% of strains isolated from different Italian cheeses were GABA producers. *Lb. brevis* and *plantarum* species are commonly found as high GABA producers (Fröhlich-Wyder et al., 2013; Li and Cao, 2010). One *Lb. brevis* (biotype 66), showed the highest glutamate decarboxylase activity generating 129 mg/L of GABA (Table 2), which is considered a good amount. In facts, some studies reported that a daily intake of only 10 mg of GABA decreased blood pressure in hypertensive patients (Inoue et al., 2003; Kajimoto et al., 2004). Further investigation are in progress to test the ability of this isolate to produce GABA not only *in vitro* but also in fermented milk.

4. Conclusions

This is a preliminary study confirming that TMM-cheese has a high microbial biodiversity and it may represent a model of raw cow milk cheese, spontaneously fermented, according to the dairy mountain tradition in the Italian Alpine area. *Lb. paracasei* and *Pc. pentosaceus* were the dominant species. Most of the biotypes isolated from TMM-cheese were subjected to a selection by the dairy environment during the dairy production: all species were resistant to high salt concentration and had psychrotrophic attitude; most of them showed AP activity, acetoin production and did not generate BA. Many other different species were found, showing technological and bioactive properties, in particular one *Lb. brevis* biotype showed a high GABA production. All these results confirmed the Malga environment as a good reservoir of wild lactobacilli with technological and bioactive potentials that could be exploited for the improvement of cheese quality. It is our intention to evaluate if the tested LAB are able to produce compounds such as CLA or GABA also in experimental cheeses and consequently to perform detailed genotypic and biotechnological analysis.

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