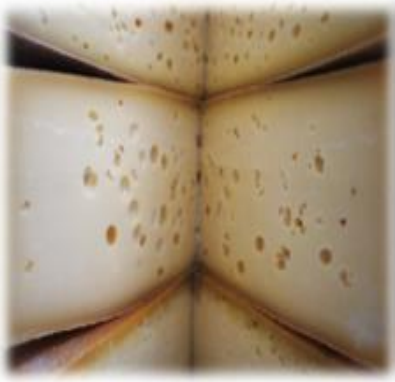
Ilaria Carafa

"*In vitro* and *in vivo* activities of lactic acid bacteria from Italian mountain cheese" and their exploitation in dairy production


PhD School of Sciences

Università Politecnica delle Marche
Fondazione Edmund Mach

XIII PhD School of Sciences

"*In vitro* and *in vivo* activities of lactic acid bacteria from Italian mountain cheese" and their exploitation in dairy production

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To my family

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List of Abbreviations

AP: Amino Peptidase
BA: Biogenic Amines
BSA: Bovine Serum Albumine
BSH: Bile Salts Hydrolysis
Cad: Cadaverine
CFE: Cell-free Extract
CFU: Colony-forming Unit
CLA: Conjugated Linoleic Acid
DGGE: Denaturing Gradient Gel Electrophoresis
GABA: γ-aminobutyric acid
GAD: Glutamate Decarboxylase
GDCA: glycodeoxycholic acid
GI: Gastro-intestinal
His: histamine
KAA: Kanamycin Aesculin Agar
LA: Linoleic Acid
LAB: Lactic Acid Bacteria
MPN: Most Probable Number
MRS: deMan-Rogosa-Sharpe
MSG: Monosodium glutamate
MUG: Methylumbelliferyl-β-D-glucoronide
MV: Mean Value
NSLAB: Non-starter Lactic Acid Bacteria
OD: Optical Density
OPA: o-phtaladehyde
OTUs: Operational Taxonomic Units
PC: Principal Component
PCA: Plate Count Agar
PCR: Polymerase Chain Reaction
PFGE: Pulsed-Field Gel Electrophoresis
Put: Putrescine
RAPD -PCR: Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction
RCM: Reinforced Clostridial Medium
RDP: Ribosomal Database Project
SD: Standard Deviation
SM: Skim Milk
TB: Total bacteria
TBC: Total Bacterial Count
TDCA: taurodeoxycholic acid
TM-cheese: Traditional Mountain cheese
TMM-cheese: Traditional Mountain Malga cheese
Tyr: Tyramine
UHPLC: Ultra High Performance Liquid Cromatography
UPGMA: Unweighted Pair Group Method Arithmetic Averages
VRBA: Violet Red Bile Agar

Abstract

The use of health-promoting lactic acid bacteria (LAB) strains as starter or adjunct cultures for dairy productions could facilitate the *in situ* bio-synthesis of bioactive molecules during the fermentation process, increasing the interest towards dairy products as *multifunctional* foods. Currently, there is much research about genotypic and technological characterization of raw milk cheeses microbiota, which is rich in biodiversity and could be exploited for improving the sensory attributes and add healthy benefits to the cheese. Traditional Mountain (TM) cheese is made from raw cow's milk and spontaneously fermented in small farms called "Malga" located in the alpine areas of Trentino region. For the first time, the microbial population of TM-cheese has been characterized in order to select cocci and non-starter LAB suitable for developing new starter or adjunct cultures, respectively. Samples (n = 120) of milk, curd and cheese at different ripening times (24 hours, 1 month and 7 months) were enumerated in selective culture media. Mesophilic and thermophilic cocci dominated during the first 24 hours following production, and mesophilic lactobacilli were dominant at the end of ripening. Six hundred and forty colonies were isolated from curd and cheese 24 hours following production, and 95 more colonies were isolated from cheese after 7 months of ripening. All isolates were 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. Cocci clustered in 231 biotypes belonging to 16 different species, and non-starter LAB (NSLAB) clustered in 70 biotypes belonging to 13 different species. *Lactococcus lactis*, *Streptococcus thermophilus* and *Enterococcus faecalis* were dominant in curd and 24h-cheese; *Pediococcus pentosaceus* and *Lactobacillus paracasei* were the main species at the end of ripening.

The phenotypic, technological and health-promoting activities of all strains were investigated. In particular, lactococci, streptococci and enterococci were tested for their acidification and proteolytic activity, ability to growth at not optimal temperatures, acetoin production, development of olfactory flavour notes, autolysis

rate and ability to inhibit the growth of coliforms. Forty percent of enterococci showed the ability to inhibit raw milk resident coliforms *in vitro*, but they were excluded as possible starters, owing to the presence of associated risk factors. Among lactococci and streptococci, 4 *Lc. lactis subsp. lactis* and 2 *Sc. thermophilus* were fast acidifiers, produced pleasant flavours, and were subjected to the freeze-drying stability test. *Lc. lactis subsp. lactis* 68 and *Sc. thermophilus* 93 showed the best properties and might be appropriate for cheese production.

NSLAB strains were tested for their growth properties, carbohydrate metabolism, acidifying ability, proteolytic and lipolytic activities, acetoin production, amino-peptidase activity (AP) and biogenic amines production. Concerning the health-promoting properties, the bile salts hydrolysis (BSH) activity was tested qualitatively, the conjugated linoleic acid (CLA) production was measured spectrophotometrically, and the γ-aminobutyric acid (GABA) production was quantified by UHPLC (Ultra High Performance Liquid Cromatography). *Lb. paracasei* isolates resulted to be well adapted to the Malga environment and showed the highest AP activity and acetoin production. Some strains harbored very interesting health-promoting properties and produced bioactive substances. In particular, *Lb. rhamnosus* BT68, *Lb. paracasei* BT18, BT25, BT31, *Pc. pentosaceus* BT3, BT13, BT51 produced between 70 and 130 mg/mL of total CLA *in vitro*. *Lb. brevis* BT66 converted L-glutamate to a high concentration of GABA (129 ± 8.6 mg/L) and showed BSH activity. These first results revealed that TM-cheese is a reservoir of a high microbial diversity, and the resident LAB could be exploited not only for the applicability in dairy production but also for their health-promoting properties.

Lc. lactis subsp. lactis 68 and *Sc. thermophilus* 93, which showed to be the best performing strains, were tested as starter and adjunct cultures, for the production of 9 experimental TM-cheese wheels in a Malga-farm, respectively. Three control (CTRL) cheeses were produced according to the tradition and any starter or adjunct culture was not added; three starter (STR) and three commercial starter (CMS) cheeses were produced inoculating the vat milk with both selected strains and a commercial *Sc. thermophilus* strain, respectively. After 24 hours, 1 month and 7

months of ripening the microbial content of all experimental cheeses was investigated. Mesophilic cocci and lactobacilli dominated in cheese samples after 24 hours and 1 month of ripening, while cocci dominated in full-ripened cheese. The total genomic DNA was extracted, and a fragment of the V1-V3 region was amplified and pyrosequenced. Lactococci and streptococci were the most abundant species in CTRL and STR cheese, and *Lc. lactis subsp. lactis* 68 affected the proliferation of the (raw milk) indigenous *Lc. lactis subsp. cremoris* during the early fermentation. Moreover, the commercial *Sc. thermophilus* showed to be dominant towards *Lc. lactis subsp. lactis* and *cremoris* naturally present in raw milk and to be responsible in decreasing the abundance of *Lactobacillus subp.* and *Enterococcus sp.*

The survival of TM-cheese microbiota *in vitro* was investigated under simulated human gastro-intestinal (GI) conditions. The 9 full ripened experimental TM-cheeses were subjected to a model system that simulates digestive processes in the mouth, stomach and small intestine, comprising sequential incubation in human gastric and duodenal juices. Bacterial counts were performed before and after the simulation: total bacterial count and thermophilic cocci significantly decreased after the simulated digestion. Thirty-six lactobacilli were isolated from cheese after digestion: among them 1 *Lb. paracasei*, 1 *Lb. parabuchneri* and 1 *Lb. fermentum* were tested for their survival after GI transit. *Lc. lactis subsp. lactis* 68 and *Lb. parabuchneri* D34 strains were used to ferment whole milk and digested. The load of *Lb. parabuchneri* D34 decreased by about one log more when grown as pure culture than fermented milk after simulated digestion, suggesting that *Lb. parabuchneri* D34 had in itself the ability to survive digestion, but the fat content and the cheese structure might protect LAB during the GI transit.

Furthermore, our interest towards the GABA producing strains lead us to test the ability of *Lb. brevis* BT66 to produce GABA *in situ* during cheese production, through the decarboxylation of glutamate. Twenty experimental micro-cheeses were produced using a commercial starter strain (10^7 CFU/mL) and *Lb. brevis* BT66 as adjunct culture. Four different concentrations (10^2, 10^3, 10^4, 10^5 CFU/mL) of *Lb. brevis* BT66 were tested in quadruplicate. In order to follow the microbial evolution, samples of milk, curd and cheese after 20 days of ripening were enumerated in

selective media. The control and experimental samples showed a similar trend, suggesting that both milk-resident and starter strains grew during ripening. However, the load of mesophilic lactobacilli in all experimental curd samples was higher than the control ones. The concentration of GABA and glutamic acid in cheese samples after 20 days of ripening was quantified by UHPLC-HQOMS. The amino acidic profiles showed that while the concentration of *Lb. brevis* BT66 in milk increased, the amount of both glutamic acid (from 324 ± 37 to 202 ± 32 mg/kg) and GABA (from 154 ± 31 to 91 ± 20 mg/kg) significantly decreased in cheese. These results suggested that the experimental strain converted glutamic acid to GABA, but that GABA may have subsequently been converted to succinate by GABA transaminases.

The non-protein amino acid GABA has been reported to impact on brain function through the gut:brain axis system, to harbor an anti-obesity and antidiabetogenic effect, to regulate the immune system, the inflammation process and the energy metabolism in mammals including induction of hypotension, diuretic and tranquilizer effects, stimulation of immune cells. Owing to its ability to produce high concentrations of GABA and its BSH activity *in vitro*, *Lb. brevis* BT66 was selected to be tested *in vivo* in mice suffering obesity-associated type-2-diabetes. Another *Lb. brevis* (strain DPC6108), isolated from the human GI tract and harboring the same properties, was simultaneously investigated. The corresponding rifampicin resistant mutants (rif) were generated; their genotypic profile was obtained by RAPD-PCR and PFGE (Pulsed-Field Gel Electrophoresis) and was identical to the native strain. The conversion rates of monosodium glutamate to GABA were investigated by next-generation amino acid analysis: *Lb. brevis* BT66rif produced 840.5 ± 266 µg/mL of GABA with about 73% of bioconversion and *Lb. brevis* DPC6108rif produced 1,218.0 ± 393.2 µg/mL with about 87% of bioconversion. The BSH activity was positive to both qualitative and quantitative assays and the results were similar in both native and mutant strains. The rifampicin resistant strains were freeze-dried and tested for their stability at room temperature, +4 and -20 °C. Both spectrophotometer and plate count methods revealed that freeze-dried strains survived at room temperature during 24 hours after suspending in sterile water. The stability of freeze-dried strains at +4 and -20 °C was investigated enumerating the viable cells in

selective medium during 10 weeks and any significant load reduction was not detected in the first 4 weeks following freeze-drying. Both pharmabiotic-producing *Lb. brevis* BT66rif and DPC6108rif were resistant to freeze-drying, survived transit through mouse GI tract (as proven by a pilot study), and their therapeutic efficiency is being assessed *in vivo* to treat metabolic obesity and type-2-diabetes.

Foreword

Guerrero et al. (2009)[i]defined a traditional food as *"a product frequently consumed or associated with specific celebrations and/or seasons, normally transmitted from one generation to another, made accurately in a specific way according to the gastronomic heritage, with little or no processing/manipulation, distinguished and known because of its sensorial properties and associated with a certain local area, region or country"*.

Trentino is an alpine area located in Northern-East Italy and is characterized by a great variety of environments, local resources and an ancient tradition of food and dairy products. One of the most peculiar products is the Traditional Mountain Malga (TMM), also called Traditional Mountain (TM) or Malga cheese, which perfectly fits with the definition of traditional food. All steps of TM-cheese production are carried out in small farms called "Malga", from June to September, following a traditional procedure passed down from one generation to another for over eight centuries. During any step of production there is no exchange of sources (e.g., milk, water, fresh pasture) with the surrounding areas and silage is never introduced in the cows diet, thus TM-cheese could be defined as traditional and seasonal product made in a closed system. In Trentino there are about 100 Malga (Fig. I), which are located between 1,400 and 2,000 meters above sea level and are strongly related to the mountain tourism.

The organoleptic attributes of TM-cheese originate from the interaction between the Malga environment and the manufacture technology: the altitude, the exposure, and the pasture composition differ among the Malga-farms, and the knowledge about TM-cheese production and the facilities are different depending on the area of production. The autochthonous microbiota of raw milk and cheese plays a fundamental role in determining the uniqueness of TM-cheese: since raw milk is directly processed *in loco* after collection, the fermentation process is spontaneous, the microbial biodiversity and the enzymatic composition remain unchanged, and are consequently related to a richness in sensory attributes. Even though, the final product has a high variability, which is related to the low curd-cooking temperature

and the spontaneous fermentation process. This variability causes a substantial loss of the product, which the cheese-makers have been trying to control by using freeze-dried commercial cultures able to lead the fermentation process. These cultures are adapted to the industrial but not to the traditional cheese productions, thus, they might be antagonistic against the wild TM milk microbiota, ensuring a high level of safety (desired effect) and organoleptic standardization (undesired effect) of the final product.

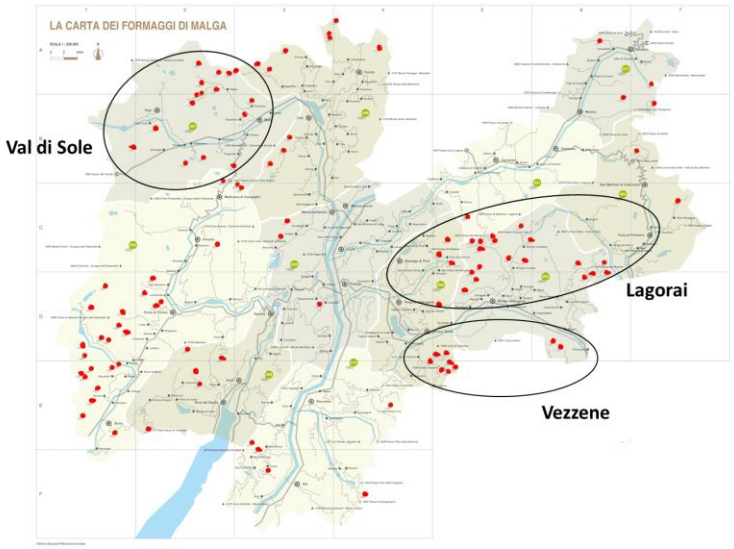

Figure I. TM-cheese map: the red dots indicate the Malga-farms of Trentino (Northern-East Italy); the black ellipse hoop the three areas subjected to investigation during this study.

This research was aimed to characterize the autochthonous LAB established in TM-cheese, and to select strains to be used as starter or adjunct cultures for cheese productions. TM-cheese production was followed in three different areas (Fig. I), and the manufacted cheeses were collected and analysed during the early fermentation process and at the end of ripening. Besides their phenotypical, technological and sensorial properties, the bioactive potential of each strain was investigated in order to develop TM-cheese with desirable organoleptic attributes and added health functionality. The *in vitro* characterization of all strains was followed by the experimental explotation of selected strains *in situ* in cheese and *in vivo* in mice, for a total view of their activities and potential utilization.

[i] Guerrero, L., Guardia, M. D., Xicola, J., Verbeke, W., Vanhonacker, F., Zakowska-Biemans, S., Sajadakowska, M., Sulmont-Rosse, C., Issanchou, S., Contel, M, Scalvedi, M. L., Granli, B. S., & Hersleth, M. (2009). Consumer-driven definition of traditional food products and innovation in traditional foods. A qualitative cross-cultural study. *Appetit, 52*, 345-354.


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1. Microbial evolution of Traditional Mountain cheese and characterization of early fermentation cocci for selection of autochthonous dairy starter strains[a]

Abstract

The microbial population of Traditional Mountain (TM) cheese was investigated and characterized for the selection of cocci suitable for developing new starter cultures. Samples of milk, curd and cheese at different ripening times were enumerated in selective culture media and 640 colonies were isolated from curd and cheese after 24 h of ripening. The Lactic Acid Bacteria (LAB) isolated from M17 were clustered into 231 biotypes by RAPD-PCR analysis and identified as *Lactococcus lactis*, *Streptococcus thermophilus* and *Enterococcus faecalis*. Forty percent of enterococci showed the *in vitro* ability to inhibit raw milk resident coliforms, but they were excluded as possible starters due to the presence of associated risk factors. All lactococci and streptococci were tested for their technological properties; 4 *Lc. lactis subsp. lactis* and 2 *Sc. thermophiles*, which were fast acidifiers and did not produce unpleasant flavours were subjected to the freeze-drying stability test. *Lc. lactis subsp. lactis* biotype 68 and *Sc. thermophilus* biotype 93 showed the best technological properties and may be appropriate for cheese production. This work gave evidence of the high biodiversity of TM-cheese authoctonous biotypes, which could be used as starter cultures for the improvement of TM-cheese technology.

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1.1 Introduction

Trentino alpine area (North Eastern Italy) has an ancient tradition of dairy products. TM-cheese is a raw cow's milk cheese produced in small farms, called *Malga*, which are located between 1,400 and 2,000 meters above sea level. Malga farms are open only during the summer season and are set up to house cows for grazing and to manufacture cheese following a traditional procedure that is not standardized and differs depending on the area of production. TM cheesemakers do not use any commercial starter culture, which is known to standardize the organoleptic properties of the final product (Yann and Pauline, 2014); therefore, the microbial fermentation is totally spontaneous and carried out by the indigenous microbiota, deriving from the raw milk and the Malga-farm environment. The traditional methods used for the production and ripening of raw milk cheeses are still followed to keep the richness and variability of the microbial population in cheese, generating different characteristics in ripened traditional cheeses (Montel et al., 2014). When no starter cultures are added, the autochtonous microbiota contributes to both the fermentation and ripening processes, either directly via its metabolic activity, or indirectly via the release of enzymes into the cheese matrix after autolysis (Pereira et al., 2010). For these reasons, traditional and spontaneously fermented cheeses, like TM-cheese, could be considered as possible sources of microbial diversity and wild LAB harboring good properties for dairy production.

By contrast, starter and/or adjunct cultures as *Streptococcus thermophilus* and *Lactococcus lactis* (Axelsson, 2004; Iyer et al., 2010) are added to the milk in order to obtain cheeses with desired and predictable properties and the cheese-making process is carried out following standardized procedures (Beresford et al., 2001; Høier et al., 2010).

To our knowledge, few studies focused on the technology and microbial population of mountain cheeses (Bonizzi et al., 2009; Franciosi et al., 2008, 2009 and 2015; Giannino et al., 2009; Poznanski et al., 2004), and only one on spontaneously fermented TM-cheese (Carafa et al., 2015). The objectives of this study were i) to follow the microbial evolution, ii) to characterize the autochtonous cocci strains

involved in the early fermentation process of spontaneous fermented TM-cheese, iii) to identify wild strains to be used in mixed starter cultures with non-starter lactobacilli previously isolated (Carafa et al., 2015) for TM-cheese and other dairy productions.

The isolated LAB were genetically characterized and tested for their ability to inhibit the growth of raw milk-resident coliforms. Lactococci and streptococci were screened for their ability to grow at non-optimal temperatures, proteolytic, acidifying and autolytic activities, and production of acetoin and desired flavours notes.

1.2 Materials and methods

1.2.1 Sampling and microbiological counts

The TM-cheese making process was followed in eight different Malga-farms located in the Alpine area of Trentino. For each farm, 3 cheese-making days were followed, and samples of raw milk, curd and cheese at different stages of ripening (24 hours, 1 month and 7 months), were collected for each day of cheese-production, for a total of 120 samples. The milk samples were collected from the vat and the curd samples were collected after the extraction. The pH of cheese 24 hours, 1 month and 7 months following production was measured using the pH meter PT1000 (Knick, Berlin, Germany) equipped with a Hamilton electrode (Hamilton Bonaduz, Bonaduz, Switzerland).

All milk and cheese samples have been analysed within 24 hours for microbiological counts. Cheese samples were homogenized as reported previously by Carafa et al. (2015). Cell suspensions and samples of milk were serially diluted, plated in triplicate and incubated as follows: the total bacterial (TB) were counted onto Plate Count Agar supplemented with 10 g/L of skimmed milk (PCA-SM) and incubated aerobically at 30 °C for 24 h; enterococci onto Kanamycin Aesculin Azide (KAA) agar and incubated aerobically for 24 h at 37 °C, mesophilic cocci-shaped lactic acid bacteria (LAB) onto M17 agar, incubated at 30 °C aerobically for 48 h;

thermophilic cocci-shaped LAB onto M17 agar, incubated at 45 °C anaerobically for 72 h and mesophilic rod-shaped LAB onto MRS agar acidified to pH 5.5 with 5 mol/L lactic acid, incubated at 30 °C anaerobically for 48 h. All culture media were purchased from Oxoid (Milan, Italy).

1.2.2 Pathogen and spoilage bacteria detection

The presence of *Listeria* was detected inoculating the sample in *Listeria* Enrichment Broth (LEB, Merck, Darmstadt, Germany). After 48 hours of incubation at 30 °C, the inoculated broths were streaked on PALCAM *Listeria* selective agar (Merck) and incubated at 35°C in anaerobic conditions for up to 48 hours. Presumptive *Listeria monocytogenes* strains grow as grey-green coloured colonies with a black area.

Coliforms were counted using violet red bile agar (VRBA, Oxoid). Purple-pink colonies were detected after 24 h of incubation at 37 °C, anaerobically. The detection of *Staphylococcus aureus* was carried out by plating on *Staphylococcus* agar No. 110 (Laboratorios Conda S.A., Madrid, Spain). The presence of *Clostridium* spores in milk and 24 h-cheese samples was estimated by using the most probable number (MPN) technique with a 3×3 scheme, as follows: after pasteurization (85 °C for 15 min), the first three decimal dilutions were inoculated in Reinforced Clostridial Medium (RCM) supplemented with 1.4% (v/v) Na-lactate (Merck); the tubes were sealed with sterile melted paraffin:vaseline (1:6) and observed for gas production after incubation at 37 °C for 7 days.

1.2.3 Bacteria isolation and genotypic characterization

Ten or more colonies were randomly picked up from countable M17 agar plates of curd and cheese after 24 hours of ripening for the subsequent starter selection. The selection of putative LAB and their purification and store was done as previously described by Carafa et al. (2015).

The biotype clustering and species identification of the putative LAB isolates was carried out by using Randomly Amplified Polymorphic DNA–PCR (RAPD–PCR) and partial 16S rRNA gene sequencing following the approach described by Carafa et al. (2015). The similarity value for biotypes clustering was set at 85% after testing the RAPD-PCR repeatability as determined by Foschino et al. (2008).

Lc. lactis subspp. lactis and *cremoris, Sc. thermophilus* and *Enterococcus faecium* species were also confirmed by means of species-specific PCR according with Corroler et al. (1998), Lick et al. (1996) and Cheng et al. (1997). All amplifications were performed with a T100^{TM} ThermalCycler (Bio-Rad Laboratories).

1.2.4. Screening for selection of dairy starter strains

One isolate representative of each biotype was analyzed for the detection of prophage, adapting the method of Cochran and Paul (1998) as reported by Carafa et al. (2015).

All biotypes were screened for their ability to inhibit the growth of milk resident coliforms using the Well Diffusion Assay described by Corsetti et al. (2004), with some modifications. One mL of raw cow's milk containing 5.7 Log CFU/mL of coliforms was plated under a bilayer of VRBA with 4-methylumbelliferyl-β-D-glucoronide (MUG) by Oxoid. Thereafter, wells of 10 mm in diameter were generated into the bilayer and 200 µL of cell-free supernatants were inoculated into each well. In order to eliminate the inhibitory effect of lactic acid, supernatants were previously adjusted to pH 7 ± 0.2 with KOH and filtered through a 0.22 µm pore size filter (Minisart®, Sartorius Lab Holding GmbH, Goettingen, Germany). Plates were refrigerated at 4 °C for 4 h to allow the radial diffusion of the compounds contained in the supernatant and finally incubated for 24 h at 37 °C. The inhibitory effect of strains on the growth of coliforms was shown by the presence of a clear halo around each well. A solution of chloramphenicol (1 mg/mL) was used as positive control.

More technological analyses were performed in triplicate on phage-free lactococci and streptococci. The growth ability at different temperatures (30 and 45 °C) was detected as reported previously by Carafa et al. (2015).

The exocellular proteolytic activity was evaluated following the method reported by Franciosi et al. (2009).

The acidifying ability of cell suspensions (1%, v/v) was evaluated in 10 mL of sterile UHT milk (Latte Trento, Trento, Italy) following the method reported by Morandi and Brasca (2012); pH measurements were carried out using a pH meter PT1000 (Knick, Berlin, Germany) equipped with a Hamilton electrode (Hamilton Bonaduz, Bonaduz, Switzerland), every 15 min for 24 h.

The olfactory flavour notes developed by lactococci and streptococci were analyzed following a sniffing test as described by Sánchez et al. (2000), with some modifications. Revitalized cultures were inoculated (1%, v/v) in 15 ml of microfiltered whole milk (Latte Trento, Trento, Italy) for 48 h at optimal conditions. Each sample was evaluated by a panel of eight untrained assessors. Odour attributes (lactic-acid, yoghurt, butter, sulphur, spicy, fruity and fermented herbs) were evaluated and rated in null (0), light (1), medium (2) and strong (3). A statistical interpretation of the results was done by using the principal component analysis (PCA) on the correlation matrix of STATISTICA (data analysis software system), version 9.1. (StatSoft, Inc., 2010) www.statsoft.com.

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

Autolysis ability was determined by inoculating the cell suspensions (2%, v/v) in sterile sodium citrate solution, pH 5.5, and monitoring every 30 min the decrease in OD_{600} using a Spectrophotometer Evolution^{TM} 300 (Thermo Scientific, Rodano, MI, Italy). The monitoring was carried out during 24 h and 48 h of incubation at 37 °C for streptococci and lactococci, respectively. The percentage of dead cells was expressed according to Langsrud et al. (1987).

1.2.5 Screening for enterococci associated risk factors

The hemolytic activity was detected as a clear halo around the colonies after growth of enterococci isolates on Blood Agar Sheep (Biolife Italiana s.r.l., Milan, Italy) and incubation at 37 °C for 24 h.

The presence of virulence genes *asa1*, *gelE*, *cylA*, *esp* and *hyl* was detected by multiplex PCR as described by Vankerckhoven et al. (2004). The *Enterococcus faecalis* strain MMH594 was used as positive control for the detection of *asa1, gelE, cylA*, and *esp* genes; the *Ec. faecium* strains C38 and C68 were used as positive controls for *esp* and both *esp* and *hyl*, respectively.

1.2.6 Stability of starter cultures to freeze-drying process

Four *Lc. lactis subsp. lactis* and 2 *Sc. thermophilus* were selected for freeze-drying. The grown cultures were subcultured twice and inoculated (1%, v/v) in 1 L of M17 broth. The batch cultures were incubated over night at 30 °C (*Lc. lactis subsp. lactis*) and 45 °C (*Sc. thermophilus*), at 100 rpm. Grown cells were harvested by centrifugation at 4,000 rpm for 20 min at 4 °C. The cell pellets were suspended in 100 mL of sterile freeze-drying medium composed of skim milk 10%, (w/v), lactose 3% (w/v), yeast extract 0.1% (w/v) (Oxoid), and frozen in liquid nitrogen, stored over-night at -80 °C and finally dried in a LIO-5P freezedryer (Cinquepascal s.r.l., Trezzano sul Naviglio, MI, Italy) for 48 h (condenser temperature: -45 °C, vacuum: 0.5 mbar). The freeze-dried powder of each strain was weighted and stored in glass flasks at -20 °C.

The viability of strains was evaluated 15 days following freeze-drying, as follows: 1 g of each freeze-dried strain was resuspended in 10 mL of skim milk (10%, w/v) for 30 min, plated in triplicate onto M17 agar and incubated at optimal conditions.

1.3 Results and discussion

1.3.1 pH evolution and microbiological counts

The pH dynamic and microbial counts were followed during all steps of TM-cheese production and maturation and are shown in Fig. 1.1 and 1.2 respectively. The mean pH value of milk samples was 6.7 ± 0.11 and did not significantly change in curd after extraction (6.5 ± 0.22) probably because of the absence of industrial starter culture. The pH decreased significantly ($p < 0.05$) to 5.4 ± 0.27 in cheese after 24 hours of ripening, when mesophilic and thermophilic cocci were the dominant microbial populations (Fig. 1.2). This evidence suggests that the fermentation has been successfully carried out, even if no starter or natural culture had been added, by the indigenous cocci and in particular by *Lc. lactis*, *Sc. thermophilus* and *Ec. faecalis* which were tha main species identified within one day following production.

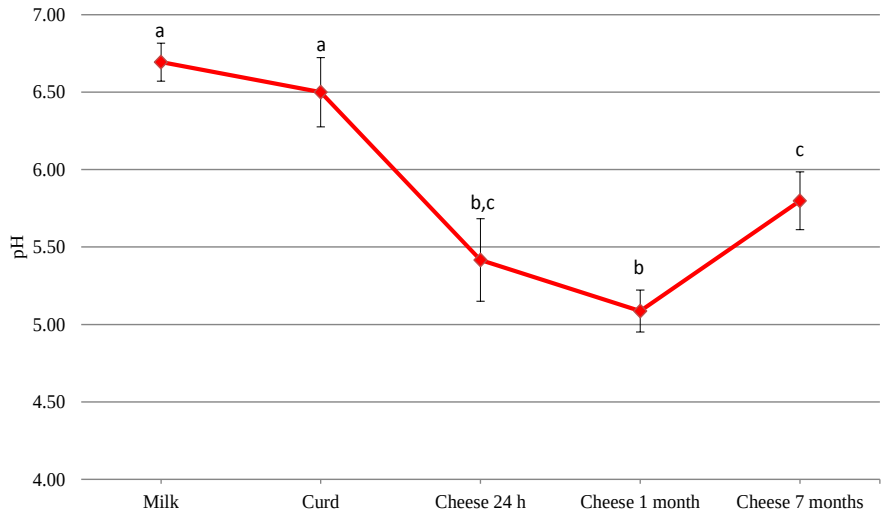


Figure 1.1 pH dynamic of TM-cheese during processing and ripening. Each point is the mean value of 24 samples (milk, curd, and cheese after 24 hours, 1 month and 7 months of ripening). The bars show the standard deviations.

After 1 month of ripening the pH was 5.1 ± 0.14, and rose up to 5.8 ± 0.19 at the end of ripening after 7 months. This change could be related to the degradation of high

molecular weight peptides and consequent release of amines (Sousa et al., 2001). The proteolytic activity may be carried out by both TM-cheese resident lactobacilli proteases (lactobacilli reached the highest concentration after 1 and 7 months of ripening) and the calf rennet used for milk coagulation, which is reported to keep proteolytic activity on cheese caseins during ripening (Harboe et al., 2010).

The microbial development from milk to ripenend cheese was similar to milk and spontaneously fermented cheeses produced in the same region (Franciosi et al. 2009 and 2015) or different area (Alegría et al., 2009; De Pasquale et al., 2014). The high standard deviation in all microbial loads (Supp. Table 1.1) might be due to the variability of each Malga-farm that are isolated, closed systems, without resources exchange (water, milk, cheese-making equipment, etc.) within the sorrounding area. The TB counts of vat milk samples ranged between 4.1 ± 0.3 and 6.5 ± 1.4 Log CFU/mL. The TB count of curd samples was 7.1 ± 1.1 Log CFU/g and significantly increased to 8.6 ± 0.5 Log CFU/g in TM-cheese after 1 month of ripening. Mesophilic rod-shaped LAB were present at low levels in curd (4.8 ± 2.0 Log CFU/g) and significantly increased by about 3.5 orders of magnitude during the first month, to finally stabilize at 7.4 ± 0.5 Log CFU/g at the end of ripening. Both mesophilic and thermophilic cocci-shaped significantly grew during the first 24 hours (the mean counts ranged between 8.1 and 8.4 Log CFU/g), keeping the same load up to 1 month of ripening and significantly decreased by about 1 order of magnitude at the end of ripening. Generally, the decrease of lactococci and streptococci throughout the ripening period could be explained by the reduction in lactose concentration that could slow down the growth of lactococci and streptococci, allowing non-starter LAB to become dominant (McSweeney and Fox, 2004).

We found the highest concentration of enterococci after 1 month of ripening (6.8 ± 0.3 Log CFU/g); they have been associated to the use of raw milk (Marino et al., 2003) and were reported as dominant microbial group in several artisanal cheeses (Gelsomino et al., 2001; Menéndez et al., 2001). Coliforms were detected in almost all samples, and the highest loads (4.7 ± 2.2 Log CFU/g) were recorded 24 hours

following production, probably because the pH reduction by the autochtonous starters required 24 hours.

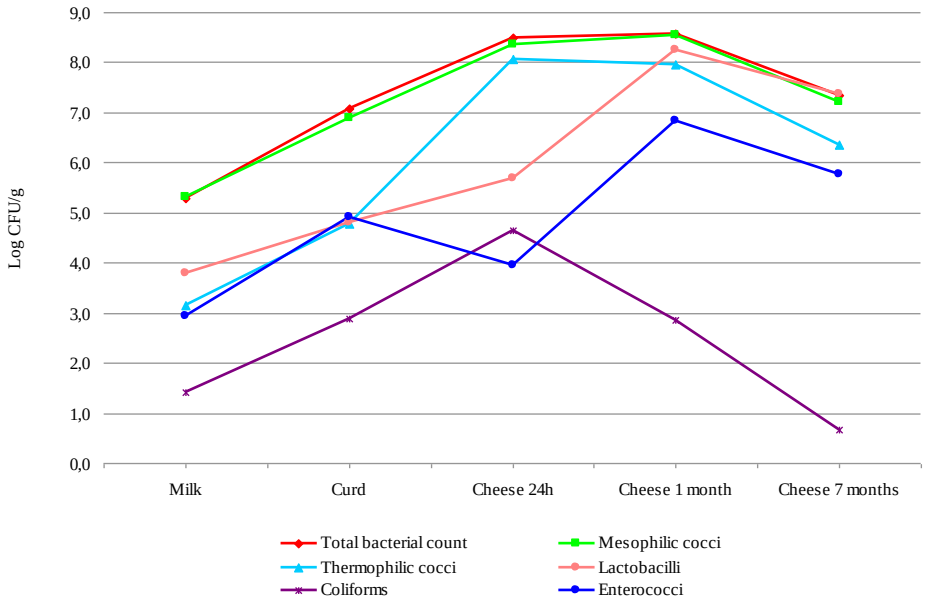


Figure 1.2 Growth on different selective media of the microbial populations resident in samples of milk, curd and TM-cheese at different stages of ripening (24 hours, 1 month, 7 months). Each point is the mean value of 24 samples. All the bacterial counts and standard deviations are shown in Supplementary Table 1.

At the end of ripening coliforms were detected at very low concentrations as subdominant or minor species, similarly to other traditional cheeses (Litopoulou-Tzanetaki and Tzanetakis, 2011; Pangallo et al., 2014).

1.3.2 Pathogen and spoilage bacteria detection

Despite the use of raw milk and the low cooking temperature of cheese, *Listeria spp.*, *Staphylococcus aureus* and clostridial spores were not detected in any sample of milk and 24h-cheese confirming the microbial safety of TM-cheeses. The absence of pathogens has been reported to be related to the presence of wild LAB strains harboring antimicrobial properties (Montel et al. 2014). Clostridial spores were not detected probably because cows are free to graze fresh pasture and never fed with silage, which has been identified as one of the main sources of raw milk

contamination with clostridial spores (Julien et al., 2008; Vissers et al., 2006 and 2007).

1.3.3 Genotypic characterization of isolates

In order to investigate the dominant microbiota in the early 24 hours fermentation, 640 colonies were isolated from M17 agar medium, where the highest microbial loads have been registered in curd and in cheese after 24 hours ripening. A total of 283 isolates were Gram negative and catalase positive and were considered non-LAB bacteria that have previously been detected in cheese by others authors and are known to produce flavour compounds that may modify the final aroma of cheese (Baruzzi et al., 2012; Martìn-Platero et al., 2008). Any influence during the fermentation by these non-LAB strains has been excluded because none of the non-LAB isolates showed acidifying activity in plate.

The RAPD analysis allowed clustering of the other 357 LAB into 231 biotypes with 85% of similarity index (Fig. 1.3a, b and c); amongt these biotypes, 57 clustered together two or more strains, and 174 were single isolate whose similarity index was lower than 85% with the closeste related strain. These great presence of "singletons" confirmed that TM-cheese is a reservoir of high LAB biodiversity from the first day of ripening as well as in the full-ripened TM-cheese, as it has been previously reported by Carafa et al. (2015). One hundred and sixteen isolates were identified as latococci and clustered into 82 biotypes (Fig. 1.3a), 115 isolates were identified as streptococci and clustered into 65 biotypes (Fig. 1.3b), and 126 isolates were identified as enterococci and clustered into 84 biotypes (Fig. 1.3c). *Sc. thermophilus* (97 isolates, 51 biotypes), *Lc. lactis subsp. lactis* (70 isolates, 53 biotypes) and *Ec. faecalis* (72 isolates, 51 biotypes) were the dominant species both in curd and 24-h cheese. *Lc. lactis* and *Sc. thermophilus* are considered the most important species of industrial LAB (Iyer et al., 2010); they were widely found in raw milk artisanal cheeses (Alegría et al., 2009; Fuka et al., 2010; Hati et al., 2013) and produce lactic acid, reducing the cheese pH at the beginning of fermentation.

Furthermore, lactococci are known to be also involved in the first step of casein breakdown and in the aroma formation during cheese maturation because of their proteinases and amino acid convertases (Wouters et al., 2002).

Ec. faecalis, *Ec. faecium*, *Enterococcus italicus* and *Enterococcus durans* have been frequently isolated from raw-milk cheeses (Aquilanti et al., 2006 and 2007; Franciosi et al., 2008). Enterococci belong to a highly controversial bacterial group: depending on the strain, they are considered as pro-technological, probiotic, spoilage or pathogenic organisms (Conde-Estevez et al., 2011; Franz et al., 2011). Undoubtedly this microbial group plays a relevant role for the development of sensory characteristics (Franz et al., 1999; Giraffa, 2003) and, consequently, enterococcal strains have been successfully used as starter or adjunct cultures (Franz et al., 2003; Izquierdo et al., 2009).

1.3.4 Screening for selection of dairy starter strains

The mitomycin C induced a sharp decrease in plate counts of 30 streptococci and lactococci biotypes (Fig. 1.3), which were excluded from further analysis.

223 LAB isolates were positive to the qualitative acidifying activity test; 8 non-LAB and no LAB isolate showed exoproteolytic activity.

The ability to inhibit the growth of raw milk resident coliforms was considered as an important parameter for choosing novel starter strains. Presence and development of coliforms during the first few days of ripening could cause gassy and unpleasant flavour formation and is sign of a poor hygiene (Farkye, 2000). Thirty-four enterococci (31 *Ec. faecalis*, 2 *Ec. faecium* and 1 *Ec. durans*), 4 *Sc. thermophilus*, 2 *Lactococcus garviae*, 2 *Lc. lactis subsp. lactis* and 1 *Lc. lactis subsp. cremoris* were positive to the coliforms inhibition well diffusion assay. The radius of the inhibition halos generated by the positive control was 8 mm; TM-cheese related enterococci generated halos ranging from 4 to 6 mm, while the streptococci and lactococci positive biotypes generated always halos with a radius smaller than 2 mm.

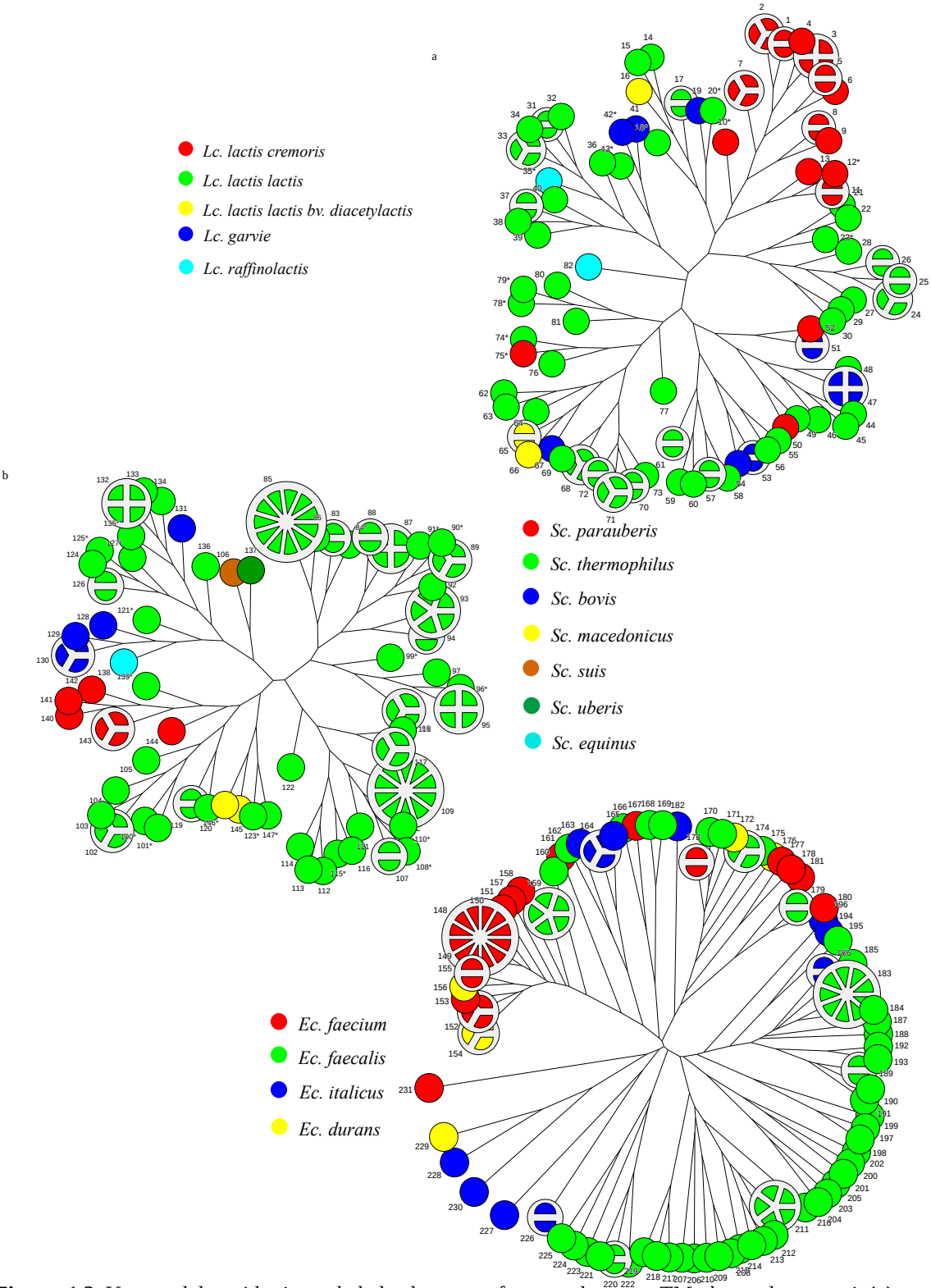


Figure 1.3 Unrooted logarithmic scaled dendrograms for autochtonous TM-cheese lactococci **(a)** streptococci **(b)** and enterococci **(c)** obtained analyzing the RAPD-PCR patterns from primers M13 and OPA-09. The enterococci dendogram is not logarithmic scaled. The similarity level was calculated using the Pearson product-moment correlation coefficient: isolates having at least 85% of similarity were grouped into the same biotype and were represented as slices in the same circle. The star (*) indicates the phage infected biotypes.

The increasing report of enterococci associated with disease (Arias and Murray, 2012; Fisher and Phillips, 2009) led us to investigate their hemolytic activity and the presence of virulence genes associated with adhesion, translocation, and immune evasion (Johnson, 1994). Despite undetected hemolytic activity (except in biotype 20), enterococci were excluded from further analysis because 58% of them harbored one or more virulence genes (data not shown).

Only streptococci and lactococci were exploited in order to find wild and novel starter strains. Forty-one of the 47 thermophilic phage-free biotypes were able to grow at 30 °C, corresponding to the curd cooking temperature; 39 of the 70 mesophilic phage-free biotypes were able to grow at 45 °C (Table 1.1), which was the temperature of cheese wheels during the first 8 hours following curd moulding (data not shown). These results suggested that isolates could be wild strains adapted to the dairy environment; a similar environmental adaptation by cheese resident LAB was observed by Monfredini et al. (2012). None of the tested biotypes showed exoproteolytic activity when assayed on SM (data not shown).

The ability and speed of the isolates in reducing the pH was investigated because a fast pH decrease in curd is essential for the coagulation and prevents or reduces the development of adventitious bacterial populations (Ayad et al. 2004; Johnson and Law, 2010). According to the ranking described by Huggins and Sandine (1984), biotypes were classified in fast, fair and slow acidifiers (Fig. 1.4 and Supp. Table 1.2). Few biotypes (4 *Lc. lactis subsp. lactis* and 2 *Sc. thermophilus*) reduced the milk pH below 5.0 in 6 hours and were considered as fast acidifiers. A similar result was reported by Ayad et al. (2004). Thirty-three biotypes (25 *Lc. lactis subsp. lactis*, 5 *Sc. thermophilus*, 2 *Lc. lactis subsp. lactis bv. diacetylactis*, 1 *Sc. suis*) reduced the milk pH below 6.0 in 6 hours and were considered as fair acidifiers; the remaining 78 tested biotypes were slow acidifiers because reduced the milk pH below 6.5 in more than 6 hours.

We investigated for the ability of strains to produce desired flavour, which is one of the most important acceptance attribute for consumers (Heap, 1998). Many different flavours were detected in milk fermented by the tested isolates suggesting that the sensory characteristics of TM-cheese might be complex and heterogeneous.

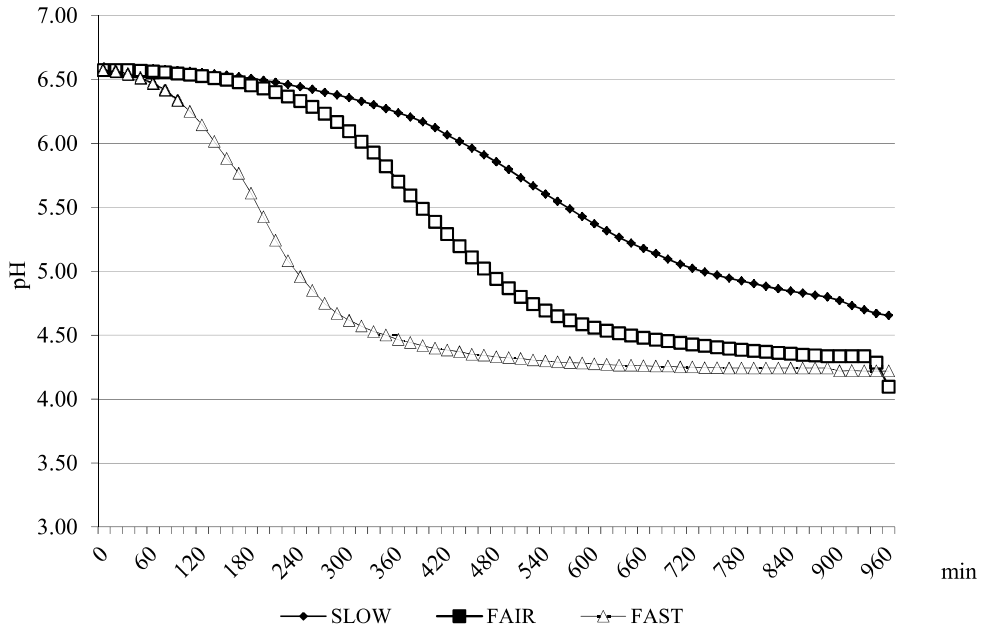


Figure 1.4. Acidification ability of *Lactococcus* and *Streptococcus* biotypes. Mean values of slow (n = 78), fair (n = 33) and fast (n = 6) acidifiers are shown. Standard deviations are indicated in the Supplementary Table 1.

All panelists noticed a good visual appearance of the fermented milk samples and detected lactic acid, yoghurt, butter, fruity, sulphur and fermented herbs flavours (see Table 1.1), which were used as variables to perform a Principal Component Analysis (PCA). Two data matrices were made with the milk samples fermented by the tested strains: the curd-PCA (Supp. Fig. 1.1a) included the olfactory characteristics generated by the biotypes isolated from curd, and the 24h-PCA (Supp. Fig. 1.1b) included those produced by the biotypes isolated from 24h-cheese. In the curd-PCA, the variables having high loadings with PC1 ($\left|\text{loadings values}\right| \geq 0.70$) were related to yoghurt (positive values) and fermented herbs notes (negative values). The variables having high loadings with PC2 ($\left|\text{loadings values}\right| \geq 0.50$) were flower, butter, sulphur (positive values) and lactic acid notes (negative values). In the 24h-PCA, the variables having high loadings with PC1 ($\left|\text{loadings values}\right| \geq 0.50$) were mainly related to fermented herbs, lactic acid (positive values), yoghurt and flowers (negative value) notes. The variables having high loadings with PC2 ($\left|\text{loadings values}\right| \geq 0.50$) were butter and sulphur notes (negative values). More than 50% of the biotypes

produced desired and pleasant flavour notes like yoghurt, flowers and butter, and were located on the left side of the graph. The yoghurt note may be attributed to high proportions of lactic acid and acetaldehyde, and the butter note to the production of diacetyl; the spicy note likely derived from lipolytic activity; sulphur and fermented herb notes, similar to fermented cabbage and sauerkraut, could come from the metabolism of L-methionine (Garabal et al., 2008; McSweeney, 2004). Some of the detected flavour notes, such as fermented herb, sulphur and fruity, are unusual if compared with some industrial strains tested by Ayad et al. (1999), but were also described by other authors (Ayad et al., 2004; Garabal et al., 2008).

Table 1.1 Temperature of growth, acetoin production, inhibition of coliforms, autolysis activity and flavours produced by lactococci (a) and streptococci (b) biotypes isolated from curd and 24h-cheese.

a)

Biotype	Sample of isolation	Growth at 45 °C[a]	Acetoin production	Inhibition of coliforms	Autolysis[b]	Flavour notes
1	24h-cheese	+	+	+	+	Butter
2	Curd	+	-	-	+	Fermented herbs and sulphur
3	24h-cheese	+	-	-	+	Fermented herbs and sulphur
4	24h-cheese	+	-	-	+	Butter
5	24h-cheese	-	-	-	-	Fermented herbs and sulphur
6	Curd	-	-	-	+	Fermented herbs and sulphur
7	24h-cheese	-	-	-	-	Butter
8	24h-cheese	-	-	-	++	Lactic acid
9	24h-cheese	+	-	-	++	Lactic acid
11	Curd	-	-	-	+	Fermented herbs and sulphur
13	Curd	-	-	-	+	Yoghurt
14	24h-cheese	+	+	-	-	Yoghurt and flowers
15	24h-cheese	+	+	-	-	Butter
16	24h-cheese	-	-	-	-	Yoghurt and flowers
17	24h-cheese	-	+	-	++	Fermented herbs and sulphur
19	Curd	+	+	-	-	Fermented herbs and sulphur
21	Curd	-	+	-	++	Fermented herbs and sulphur
22	Curd	-	+	-	-	Lactic acid
24	24h-cheese	-	+	-	+	Lactic acid
25	24h-cheese	-	-	-	-	Yoghurt and flowers
26	Curd	+	+	-	-	Fermented herbs and sulphur
27	Curd	+	-	-	-	Lactic acid
28	24h-cheese	-	+	-	-	Fermented herbs and sulphur
29	24h-cheese	+	+	-	-	Butter
30	Curd	-	+	-	+	Flowers and butter
31	Curd	+	-	-	+	Yoghurt
32	24h-cheese	-	+	-	+	Yoghurt and flowers

Biotype	Sample of isolation	Growth at 45 °C[a]	Acetoin production	Inhibition of coliforms	Autolysis[b]	Flavour notes
33	24h-cheese	-	-	-	+	Yoghurt and flowers
34	24h-cheese	+	+	-	-	Yoghurt and flowers
36	24h-cheese	-	+	-	-	Fermented herbs and sulphur
37	Curd	-	-	-	-	Fermented herbs and sulphur
38	Curd	-	+	-	+	Flowers and butter
39	24h-cheese	-	+	-	+	Yoghurt and flowers
40	Curd	-	+	-	+	Fermented herbs and sulphur
41	Curd	+	+	+	-	Lactic acid
44	24h-cheese	+	-	-	+	Fermented herbs and sulphur
45	24h-cheese	+	-	-	+	Yoghurt and flowers
46	Curd	-	+	-	-	Fermented herbs and sulphur
47	Curd	+	-	-	+	Fermented herbs and sulphur
48	24h-cheese	-	+	-	+	Lactic acid
49	24h-cheese	+	-	-	+	Yoghurt and flowers
50	24h-cheese	+	-	-	+	Lactic acid
51	Curd	+	-	-	-	Flowers and butter
52	Curd	+	-	-	+	Lactic acid
53	Curd	+	+	+	-	Flowers and butter
54	Curd	+	-	-	-	Yoghurt
55	24h-cheese	-	+	-	+	Yoghurt and flowers
56	Curd	+	+	+	-	Fermented herbs and sulphur
57	24h-cheese	+	+	-	-	Fermented herbs and sulphur
58	Curd	-	-	-	-	Lactic acid
59	24h-cheese	+	-	-	-	Yoghurt and flowers
60	24h-cheese	+	-	-	++	Yoghurt and flowers
61	24h-cheese	+	-	-	+	Yoghurt and flowers
62	24h-cheese	+	+	-	+	Fermented herbs, sulphur and lactic acid
63	24h-cheese	+	-	-	+	Fermented herbs and sulphur
64	24h-cheese	+	+	-	+	Butter
65	24h-cheese	+	+	-	-	Lactic acid
66	Curd	+	-	-	+	Lactic acid
67	Curd	-	-	-	++	Yoghurt
68*	24h-cheese	+	+	-	++	Butter
69	24h-cheese	+	+	-	+	Fermented herbs and sulphur
70	24h-cheese	-	-	-	+	Yoghurt and flowers
71*	Curd	+	-	+	-	Lactic acid
72*	24h-cheese	+	-	-	-	Lactic acid
73	24h-cheese	+	+	-	-	Fermented herbs and sulphur
76*	24h-cheese	+	-	-	-	Lactic acid
77	24h-cheese	-	-	-	-	Fermented herbs and sulphur
80	Curd	-	+	-	-	Lactic acid
81	24h-cheese	-	-	-	-	Lactic acid
82	Curd	-	-	-	+	Fermented herbs, sulphur and lactic acid

b)

Biotype	Sample of isolation	Growth at 30 °C[a]	Acetoin production	Inhibition of coliforms	Autolysis[b]	Flavour notes
83	Curd	-	-	-	-	Lactic acid
85	24h-cheese	+	-	+	-	Lactic acid
86	Curd	-	+	-	-	Lactic acid
87*	Curd	+	+	-	-	Flowers and butter
88	Curd	+	+	-	+	Fermented herbs and sulphur
89	Curd	+	+	-	-	Flowers and butter
92	24h-cheese	+	+	-	-	Butter
93*	Curd	+	+	-	-	Yoghurt
94	24h-cheese	+	-	+	-	Fermented herbs and sulphur
95	24h-cheese	+	-	-	-	Yoghurt and flowers
97	24h-cheese	+	-	-	-	Yoghurt
98	24h-cheese	+	+	-	-	Yoghurt and flowers
102	24h-cheese	+	+	-	++	Yoghurt and flowers
103	24h-cheese	+	-	-	-	Yoghurt and flowers
104	24h-cheese	+	-	-	-	Fermented herbs and sulphur
105	24h-cheese	-	+	-	-	Yoghurt and flowers
106	24h-cheese	+	+	-	-	Butter
107	Curd	+	+	-	+	Yoghurt
109	24h-cheese	+	+	-	-	Yoghurt, flowers and butter
111	24h-cheese	+	-	-	+	Yoghurt and flowers
112	24h-cheese	+	+	-	-	Butter
113	Curd	+	+	-	-	Flowers and butter
114	Curd	+	+	-	-	Yoghurt
116	24h-cheese	+	+	-	-	Lactic acid
117	24h-cheese	+	+	-	-	Butter
118	Curd	+	-	-	-	Lactic acid
119	Curd	+	+	-	-	Lactic acid
120	Curd	+	+	+	+	Flowers and butter
122	Curd	+	+	+	-	Flowers and butter
124	24h-cheese	+	-	-	-	Fermented herbs and sulphur
126	Curd	+	-	-	-	Yoghurt
128	Curd	+	-	-	-	Yoghurt
129	24h-cheese	-	-	-	-	Yoghurt and flowers
130	24h-cheese	+	-	-	-	Butter
131	24h-cheese	+	-	-	+	Butter
132	24h-cheese	+	+	-	-	Butter
133	24h-cheese	+	-	-	-	Lactic acid
134	24h-cheese	+	+	-	-	Butter
136	Curd	+	-	-	-	Yoghurt and lactic acid
137	Curd	+	-	-	-	Yoghurt and lactic acid
138	Curd	-	-	-	-	Yoghurt
140	24h-cheese	+	+	-	-	Butter
141	24h-cheese	+	-	-	-	Fermented herbs and sulphur
142	24h-cheese	+	+	-	+	Flowers and butter
143	Curd	+	+	-	-	Fermented herbs and sulphur
144	Curd	-	-	-	-	Fermented herbs and sulphur
145	24h-cheese	+	-	-	-	Yoghurt and flowers

[a] Mesophilic strains were tested for the ability to grow at 45 °C and thermophilic ones for the ability to grow at 30 °C.

[b] The autolysis rates were indicated as – (poor; 5–29%), + (fair; 30–59%) and ++ (good ; 60–94%).

* Biotypes showing fast acidifying activity and selected for freeze-frying test.

Fifty-seven of the 117 tested biotypes produced acetoin (Table 1.1) and the majority of them belonged to *Lc. lactis subsp. lactis* (28 biotypes) and *Sc. thermophilus* (21 biotypes), followed by few others biotypes: 3 *Sc. parauberis*, 3 *Lc. garviae*, 1 *Lc. lactis subsp. cremoris* and 1 *Sc. suis.* Furthermore, most of the *Lc. lactis* and *Sc. thermophilus* strains that generated butter flavour in milk produced also acetoin which is a product of the citrate metabolism and is related to the detection of butter flavour in cheese (McSweeney, 2004).

The ability of strains to lyse and release their intracellular enzymes could positively affect the cheese ripening (El-Soda et al., 2000; Wilkinson et al., 1994); in TM-cheese resident strains it was between 5 and 94%, and enabled us to classify strains into three groups: poor (5–29%), fair (30–59%) and good (60–94%) (Table 1.1). Nineteen *Lc. lactis subsp. lactis*, 9 *Lc. lactis subsp. cremoris*, 1 *Lc. raffinolactis*, 1 *Lc. lactis subsp. lactis bv. diacetylactis*, 1 *Lc. garviae*, 4 *Sc. thermophilus*, 1 *Sc. bovis* and 1 *Sc. parauberis* showed a fair autolysis activity and only 8 biotypes (4 *Lc. lactis subsp. lactis*, 2 *Lc. lactis subsp. cremoris*, 1 *Lc. garviae* and 1 *Sc. thermophilus*) had a good autolysis rate. *Lc. lactis subsp. lactis* was the best autolytic species amongst lactococci, in agreement with a previous work by Ayad et al. (2004).

1.3.5 Stability of starter cultures to freeze-drying process

Six biotypes (*Lc. lactis subsp. lactis* 68, 71, 72, 76 and *Sc. thermophilus* 87, 93) were selected as putative starter because fast acidifiers, able to grow at both 30 and 45 °C and not producing unpleasant flavour notes. The resistance and stability of these strains to freeze-drying process were tested. In this test we included also four non-starter biotypes isolated from the same cheese samples: one *Lactobacillus rhamnosus* (biotype 68), that was selected because able to produce CLA from linoleic acid, and three *Lactobacillus paracasei* (biotypes 18, 37 and 38) selected from the previous work (Carafa et al., 2015) for their aminopeptydolytic and acydifiant properties. These strains could be mixed to the starters for cheese making process. The microbial loads, the amount of powder and the yield after freeze-drying

are shown in Table 1.2. The freeze-dried material obtained varied depending on the strain and generally ranged between ca. 9 and 12 g; the yield of *Sc. thermophilus* and lactobacilli biotypes was higher than *Lc. lactis* biotypes.

After freeze-drying, cocci were tested for their ability to grow at non-optimal temperature and acidifying property, and lactobacilli for keeping their aminopeptydolytic and acydifiant activities: *Lc. lactis subsp. lactis* 68, *Sc. thermophilus* 93, *Lb. rhamnosus* BT68 and *Lb. paracasei* BT18 kept the properties shown before freeze-drying.

Table 1.2 The microbial counts of the six biotypes selected as starter candidates are reported as mean value (MV) and standard deviations (SD) before (1 mL from the 1 L batch) and after the freeze-drying process (1 g from the total final powder). The yield is expressed as ratio between the bacterial load after and before the freeze-drying process.

Biotype	**Species**	**Before freeze-drying (CFU/mL)**		**After freeze-drying (CFU/g)**		**Powder weight (g)**		**Yield (%)**
		MV	SD	MV	SD	MV	SD	
68	*Lc. lactis*	10.1	0.5	11.3	0.2	9.9	0.1	15.7
71	*Lc. lactis*	9.6	0.4	9.9	0.8	12	0.2	4.7
72*	*Lc. lactis*	9.6	0.1	10.2	0.3	11.7	0.1	4.7
76*	*Lc. lactis*	9.7	0.2	10	0.6	11.6	0.1	2.3
87*	*Sc. thermophilus*	7.7	0.3	9.5	0.1	10	0.1	63
93*	*Sc. thermophilus*	7.5	0.1	9.3	0.3	8.9	0.1	56

* The enumeration of these strains is referenced as in Carafa et al. paper (2015).

1.4 Conclusions

This study confirmed that the Malga environment is a reservoir of different wild LAB which may be used as starter cultures for dairy production. *Lc. lactis subsp. lactis* 68 and *Sc. thermophilus* 93 showed the best performance among our isolates and are currently in use in mixed starters with the two non-starter strains *Lb. rhamnosus* BT68 and *Lb. paracasei* BT18 in experimental TM-cheeses.

Supplementary Table 1.1 Mean Value (MV) and related standard deviations (SD) of microbial counts of milk, curd and TM-cheese at different stages of ripening (24 hours, 1 month and 7 months) collected in 8 different Malga (A-H), for a total of 120 samples. MV is expressed in Log CFU/mL for milk and Log CFU/g for cheese samples.

	Total bacterial count		**Mesophilic cocci**		**Thermophilic cocci**		**Lactobacilli**		**Coliforms**		**Enterococci**	
	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD
Milk[a]												
Malga A	4.5	0.5	4.6	0.4	3.2	0.1	3.7	0.2	4.2	0.3	0.9	1.0
Malga B	5.5	1.2	5.7	0.8	4.5	0.3	3.4	0.6	0.6	1.1	5.4	1.3
Malga C	4.9	0.9	4.7	1.5	1.3	2.3	4.2	1.3	0.0	0.0	2.5	0.7
Malga D	5.6	1.5	6.0	0.7	4.5	1.6	4.7	1.3	1.3	1.1	2.3	2.0
Malga E	6.5	1.4	6.6	0.3	2.9	0.3	3.9	3.4	0.0	0.0	2.6	0.8
Malga F	4.7	1.2	4.9	1.4	2.0	2.0	3.2	1.8	1.6	1.6	2.9	0.4
Malga G	4.1	0.3	4.4	0.1	1.5	2.6	3.4	1.2	0.0	0.0	2.1	0.6
Malga H	6.1	0.9	5.6	1.0	4.6	1.1	4.2	0.8	2.1	1.5	4.2	0.9
Malga A-H	**5.3a**	**1.2**	**5.3a**	**1.2**	**3.1a**	**1.9**	**3.8a**	**1.4**	**1.4a**	**1.6**	**2.9a**	**1.4**
Curd												
Malga A	8.8	0.0	4.2	3.6	7.3	0.3	1.2	2.1	3.5	0.9	2.9	0.6
Malga B	7.8	0.1	7.7	0.3	5.7	0.9	7.5	0.6	3.0	2.8	6.1	1.0
Malga C	5.7	0.9	6.8	0.4	1.6	2.7	6.4	1.0	2.3	0.7	4.8	0.4
Malga D	7.8	1.2	8.0	0.7	7.1	0.2	6.9	1.2	3.5	0.6	4.1	1.1
Malga E	7.7	0.0	7.5	0.2	3.7	3.2	6.6	1.1	3.0	0.5	4.8	1.0
Malga F	6.2	0.5	6.0	0.4	2.7	2.5	4.9	2.1	3.8	2.2	4.9	1.1
Malga G	7.7	1.1	8.1	1.0	6.2	0.4	6.2	0.3	0.7	1.2	5.1	0.9
Malga H	7.3	1.0	7.4	1.0	6.0	2.4	5.9	2.5	3.1	2.0	4.8	1.1
Malga A-H	**7.1b,c**	**1.1**	**6.9a,b**	**1.5**	**4.8b**	**2.8**	**4.8b,c**	**2.0**	**2.9a**	**1.0**	**4.7a**	**0.9**

	Total bacterial count		**Mesophilic cocci**		**Thermophilic cocci**		**Lactobacilli**		**Coliforms**		**Enterococci**	
	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD
Cheese 24 h												
Malga A	7.2	1.3	4.3	3.8	8.5	0.3	2.7	2.4	0.0	0.0	3.2	0.8
Malga B	8.8	0.3	8.7	0.1	8.7	0.6	8.3	0.0	5.6	0.4	6.3	0.8
Malga C	8.9	0.6	9.1	0.5	8.5	0.5	8.4	0.1	4.4	1.5	5.3	1.0
Malga D	7.8	0.9	8.8	0.4	7.2	1.9	6.5	1.3	4.7	0.4	4.5	0.9
Malga E	8.7	0.1	8.8	0.1	8.4	0.2	8.0	0.9	5.3	0.6	5.0	0.6
Malga F	8.4	1.3	8.5	1.3	7.5	0.9	6.3	2.9	6.4	1.4	4.0	0.5
Malga G	9.1	0.1	9.1	1.1	8.2	0.5	8.7	0.5	5.2	0.6	5.0	1.1
Malga H	8.7	0.5	8.7	0.7	8.2	0.7	7.4	1.7	3.8	2.3	3.8	0.2
Malga A-H	**8.5d**	**0.9**	**8.4b**	**1.7**	**8.1a,c**	**0.9**	**5.7c**	**1.9**	**4.7a,b**	**2.2**	**4.6a**	**1.0**
Cheese 1 month												
Malga A	9.3	0.6	4.3	0.1	8.1	0.3	9.0	0.3	0.0	0.0	6.6	0.9
Malga B	8.3	0.3	8.7	0.3	8.6	0.2	8.2	0.1	5.7	0.4	7.2	0.4
Malga C	7.9	0.3	9.1	0.3	7.5	0.5	7.7	0.3	0.0	0.0	6.4	1.1
Malga D	7.9	0.4	8.8	0.5	6.9	0.9	8.2	0.4	0.9	1.6	6.6	0.8
Malga E	8.7	0.2	8.8	0.2	7.9	0.3	8.3	0.4	2.5	0.8	7.0	0.4
Malga F	8.5	0.3	8.5	0.5	7.8	0.6	8.1	0.6	3.9	2.3	6.6	0.7
Malga G	8.3	0.1	9.1	0.3	7.8	0.7	8.3	0.0	3.5	3.1	7.4	1.5
Malga H	9.1	0.3	8.7	0.4	8.4	0.7	8.5	0.3	3.0	2.6	7.0	1.1
Malga A-H	**8.6b,d**	**0.5**	**8.5b,c**	**0.5**	**7.9a,c**	**0.7**	**8.3a,d**	**0.5**	**2.9a,b**	**2.5**	**6.8b**	**0.3**

	Total bacterial count		**Mesophilic cocci**		**Thermophilic cocci**		**Lactobacilli**		**Coliforms**		**Enterococci**	
	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD
Cheese 7 months												
Malga A	7.7	0.1	7.7	0.1	6.9	0.1	7.4	0.2	0.4	0.5	6.2	0.5
Malga B	7.2	0.3	7.3	0.2	6.8	0.1	7.7	0.1	0.0	0.0	6.4	0.6
Malga C	7.4	0.5	5.3	2.1	2.8	2.7	6.5	0.7	1.2	1.2	5.0	2.0
Malga D	6.5	0.1	6.6	0.1	5.5	0.8	7.1	0.2	2.0	0.5	4.0	1.3
Malga E	7.4	0.2	7.6	0.1	7.1	0.0	8.0	0.0	2.0	1.8	6.7	0.3
Malga F	7.3	0.3	7.4	0.2	7.0	0.3	7.4	0.3	0.1	0.1	6.1	0.4
Malga G	7.3	1.8	7.9	1.0	5.3	0.2	7.4	0.1	0.0	0.0	5.1	1.6
Malga H	7.5	0.7	7.6	0.6	7.3	0.8	7.5	0.4	0.3	0.6	6.0	1.0
Malga A-H	**7.3c,e**	**0.6**	**7.2a,d**	**1.0**	**6.4a,d**	**1.6**	**7.4a,d**	**0.5**	**0.7a,c**	**1.0**	**5.7a**	**0.9**

[a] Microbial concentration of milk is expressed in Log CFU/mL.

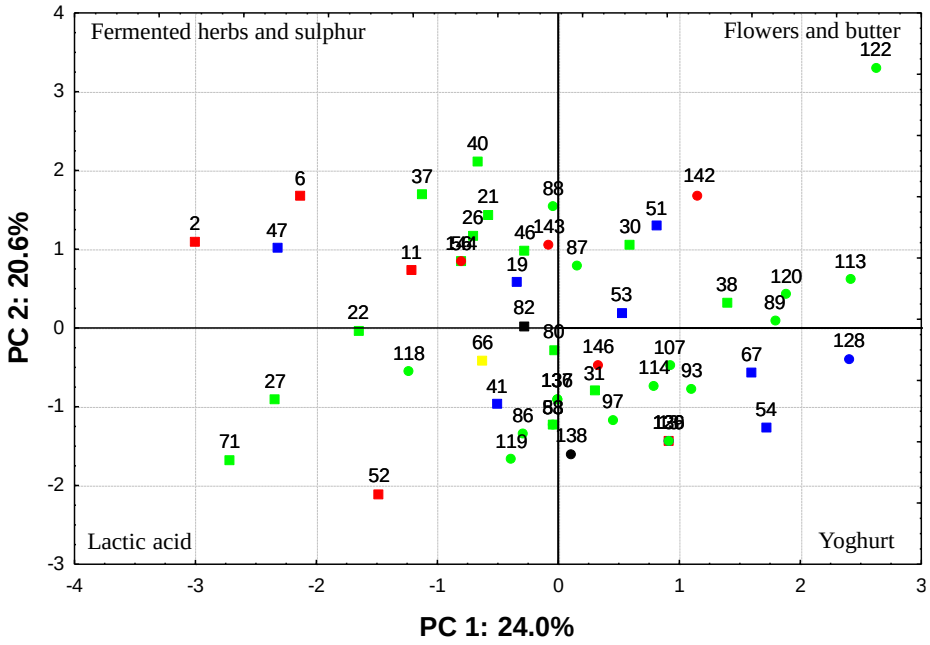
Different letters (a, b, c, d, e) on the same column indicate significant differences ($p < 0.05$).

Supplementary Table 1.2 Mean acidification ability and standard deviations (SD) of LAB from TM-cheese grouped as slow (n=82), fair (n=33) and fast (n=6) acidifiers.

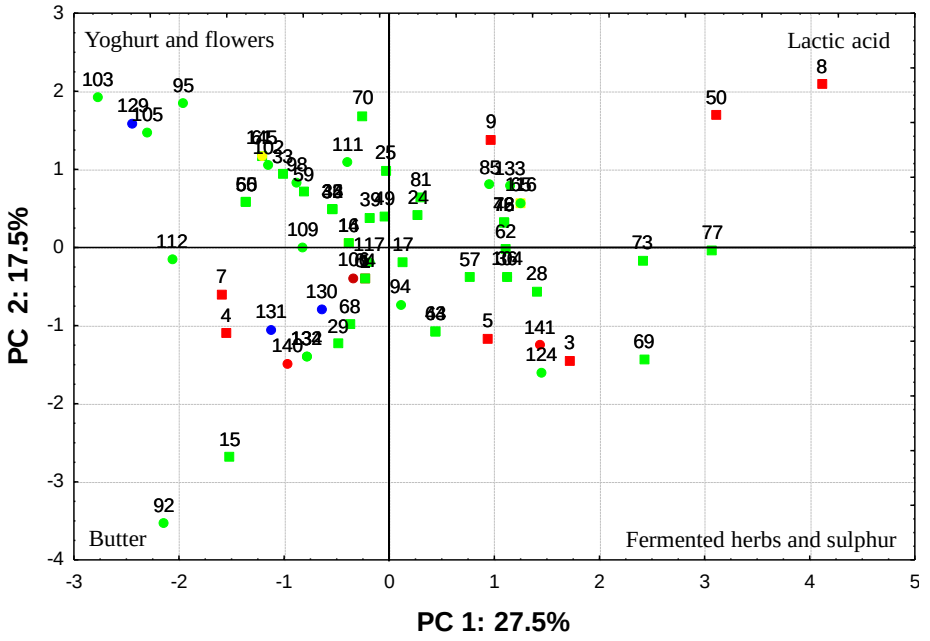
Minutes	***Slow acidifiers***		***Fair acidifiers***		***Fast acidifiers***	
	pH	**SD**	**pH**	**SD**	**pH**	**SD**
0	6.60	0.075	6.57	0.099	6.57	0.028
15	6.59	0.075	6.57	0.091	6.56	0.042
30	6.59	0.072	6.57	0.091	6.54	0.071
45	6.59	0.071	6.57	0.089	6.5	0.12
60	6.58	0.070	6.56	0.087	6.5	0.16
75	6.58	0.070	6.55	0.086	6.4	0.23
90	6.57	0.070	6.55	0.087	6.3	0.31
105	6.56	0.072	6.54	0.091	6.3	0.42
120	6.55	0.074	6.53	0.088	6.1	0.53
135	6.54	0.076	6.51	0.087	6.0	0.64
150	6.53	0.079	6.50	0.086	5.9	0.73
165	6.52	0.082	6.48	0.082	5.8	0.73
180	6.51	0.081	6.45	0.080	5.6	0.64
195	6.49	0.083	6.43	0.079	5.4	0.50
210	6.48	0.084	6.40	0.080	5.2	0.39
225	6.46	0.083	6.37	0.078	5.1	0.34
240	6.44	0.082	6.33	0.079	5.0	0.36
255	6.42	0.083	6.29	0.084	4.9	0.37
270	6.40	0.082	6.23	0.088	4.7	0.36
285	6.38	0.085	6.17	0.091	4.7	0.34
300	6.36	0.086	6.09	0.096	4.6	0.32
315	6.33	0.090	6.01	0.099	4.6	0.30
330	6.3	0.10	5.9	0.10	4.5	0.27
345	6.3	0.10	5.8	0.11	4.5	0.25
360	6.2	0.11	5.7	0.12	4.5	0.23
375	6.2	0.12	5.6	0.16	4.4	0.22
390	6.2	0.13	5.5	0.18	4.4	0.20
405	6.1	0.15	5.4	0.19	4.4	0.19
420	6.1	0.17	5.3	0.20	4.4	0.18
435	6.0	0.18	5.2	0.21	4.4	0.17
450	6.0	0.19	5.1	0.21	4.4	0.16
465	5.9	0.20	5.0	0.21	4.3	0.15
480	5.9	0.22	4.9	0.20	4.3	0.14
495	5.8	0.24	4.9	0.19	4.3	0.14
510	5.7	0.27	4.8	0.17	4.3	0.13
525	5.7	0.31	4.7	0.16	4.3	0.12
540	5.6	0.34	4.7	0.14	4.3	0.12
555	5.5	0.37	4.6	0.12	4.3	0.11
570	5.5	0.40	4.6	0.11	4.3	0.11
585	5.4	0.42	4.6	0.11	4.3	0.11
600	5.4	0.45	4.6	0.10	4.28	0.106
615	5.3	0.48	4.53	0.097	4.27	0.099

Minutes	***Slow acidifiers***		***Fair acidifiers***		***Fast acidifiers***	
	pH	**SD**	**pH**	**SD**	**pH**	**SD**
630	5.3	0.50	4.51	0.092	4.27	0.092
645	5.2	0.51	4.50	0.092	4.27	0.092
660	5.2	0.52	4.48	0.090	4.26	0.085
675	5.1	0.53	4.46	0.085	4.26	0.088
690	5.1	0.54	4.45	0.085	4.26	0.088
705	5.1	0.55	4.44	0.085	4.25	0.085
720	5.0	0.55	4.43	0.084	4.25	0.085
735	5.0	0.56	4.42	0.085	4.25	0.078
750	5.0	0.56	4.41	0.086	4.25	0.078
765	4.9	0.56	4.39	0.086	4.24	0.081
780	4.9	0.55	4.38	0.085	4.24	0.081
795	4.9	0.55	4.38	0.082	4.24	0.081
810	4.9	0.54	4.37	0.085	4.24	0.081
825	4.9	0.54	4.36	0.085	4.24	0.081
840	4.8	0.54	4.35	0.086	4.24	0.081
855	4.8	0.53	4.35	0.085	4.24	0.081
870	4.8	0.53	4.34	0.086	4.24	0.081
885	4.8	0.52	4.33	0.086	4.24	0.081
900	4.8	0.50	4.33	0.086	4.22	0.057
915	4.7	0.46	4.33	0.089	4.22	0.053
930	4.7	0.44	4.33	0.088	4.22	0.057
945	4.7	0.42	4.28	0.089	4.22	0.057
960	4.7	0.48	4.10	0.085	4.22	0.057

a



b



Supplementary Figure 1.1 Score plot of the first and second Principal Component (PC) according to PC analysis of the flavour notes generated by the 121 LAB isolated from curd (a) and cheese after 24 hours of production (b) Squares represent lactococci (green: *Lactococcus lactis subsp. lactis*; red: *Lactococcus lactis subsp. cremoris*; yellow: *Lactococcus lactis subsp. lactis bv. dyacetilactis*; blue: *Lactococcus garviae*; black: *Lactococcus raffinolactis*); dots represent streptococci (green: *Streptoccus thermophilus*; yellow: *Streptococcus macedonicus*; red: *Streptococcus uberis/parauberis*; blue: *Streptococcus bovis*; orange: *Streptococcus suis*; black: *Streptococcus equinus*). The flavour notes having high loadings with PC1 and PC2 are shown in both score plots.

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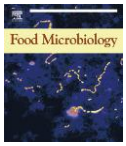

2. Identification and characterization of wild lactobacilli and pediococci from spontaneously fermented Mountain Cheese[b]

Abstract

The Traditional Mountain Malga (TMM) cheese is made from raw cow's milk by spontaneous 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, amino-peptidase (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.

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2.1 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 1,400 and 2,000 mteres above sea level in the Trentino alpine region (Northern-east Italy). Each Malga is open for a short 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 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 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 *Lb. helveticus*, *Lb. paracasei* subsp. *paracasei* and *Lb. delbrueckii* subsp. *lactis* combined with starter LAB culture, produced free amino acids and free fatty acids which influenced the organoleptic attributes of Ras cheese. 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). Other bacterial activities are usually investigated such as caseins proteolysis and aminopeptidase (AP) activity, which contribute to liberation of amino acids 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; Pariza et al., 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 areas in Trentino province. The microbial biodiversity investigation was followed by some experiments on technological properties and the ability to produce bioactive substances.

2.2 Materials and methods

2.2.1 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.

2.2.2 Biotype 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 using a T100^{TM} ThermalCycler (Bio-Rad Laboratories, Hercules, CA, USA).

2.2.3 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.

2.2.4 Screening for technological potentialities

Cells grown anaerobically in MRS broth medium for 48 h at 30 °C, were harvested by centrifugation at 5,000 rpm for 5 min, washed and resuspended 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 hours).

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 Benjanminson 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 EvolutionTM 300, Thermo Scientific, Rodano, MI, Italy) at 2 min intervals, for at least 30 minutes 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.

2.2.5 Screening for bioactive properties

2.2.5.1 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.

2.2.5.2 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 7,500 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 2,000 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).

2.2.5.3 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 (9,000 rpm for 15 min at 4 °C), washed twice with sterile PBS and resuspended in sterile 0.85% (w/v) 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 was diluted 10 times with sodium tetraborate 0.1 M (Carlo Erba; pH adjusted to 10.5) containing added 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 x 4.6 mm; Merk, Germany) with Guard Cartridge Chromolith RP-18e (10 x 4.6 mm; Merk) thermostated 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, adjusted to pH 10.5, 10% methanol and 2% 2-mercaptoethanol (Fluka). The measurements

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

2.3 Results and discussion

This research was aimed to characterize the autochthonous LAB growing onto MRS and established 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.

2.3.1 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 they were 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. 2.1); 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 percentage of pediococci (26.4%).

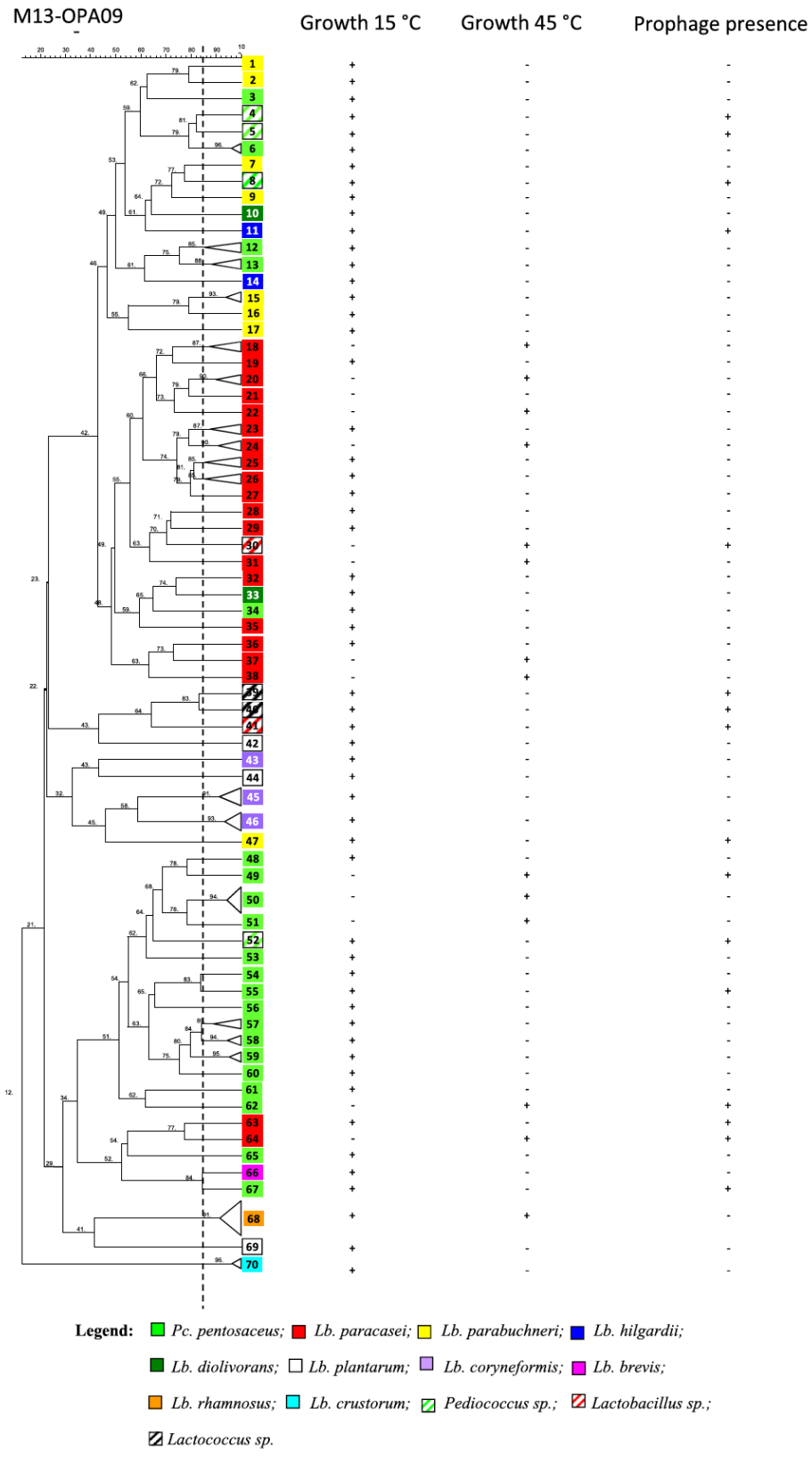

Fig. 2.1 Dendrogram obtained from M13 and OPA-09 RAPD-PCR patterns of 95 isolates. The upper scale indicates the similarity level calculated by using the Pearson productemoment correlation coefficient: isolates having 85% (black line) of similarity were collapsed into the same biotype. Temperature growth ability and determination of the lysogenic state are shown beside.

Lb. paracasei (26 isolates, 20 biotypes) was the most represented species amongst lactobacilli and *Pc. 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. 2.1). 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; Dolci et al., 2010; Sanchez 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's 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 stand-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.

2.3.2 Screening for technological properties

All 70 tested clusters were facultatively 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. 2.1). The psychrotrophic habit 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 were temperature ranges between 15 and 18 °C, but can be lower than 10 °C by night. 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 orders of magnitude) in plate counts (Fig. 2.1). The 16 biotypes infected by lysogenic phage were not considered 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 2.2). 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 2.1).

All 54 phage-free biotypes showed AP activity (Table 2.2) 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 2.2).

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 colony 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 2.1). The production of both tyramine and histamine was previously detected in dairy LAB belonging to *Lb. 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*.

Table 2.1 Identification at species level and screening of acetoin and biogenic amines production by the 70 biotypes found after RAPD-PCR clustering of the 95 isolated LAB 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	+	-	-	-	-

Biotype	Closest genus and species	Maximum identity in NCBI database (%)	Number of isolates	Acetoin production	Biogenic amines production[a]			
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 = Histydine. Tyr = Tyramine. Put = putrescine; Cad = Cadaverine

2.3.4 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 2.2). 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 2.2). 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 1,410 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 2.2). 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).

Table 2.2 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
45c	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
51c	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
66c	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.

One *Lb. brevis* (biotype 66), showed the highest glutamate decarboxylase activity generating 129 mg/L of GABA (Table 2.2), which is considered a good amount. In fact, 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 investigations are in progress to test the ability of this isolate to produce GABA not only *in vitro* but also in fermented milk

2.4 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 habit; 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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3. Antimicrobial activity and risk factors of wild enterococci isolated from spontaneously fermented Mountain cheese

Abstract

The object of this study was to screen the bile salt hydrolysis (BSH), the bile salt resistance (BSR) and the virulence factors harboured by the 84 enterococcal strains previously isolated from Traditional Malga (TM) cheese samples (curd and cheese 24 hours following production). Two *Ec. faecalis* and one *Ec. durans* biotypes showed the bile salt hydrolysis (BSH) activity. The production of histamine, tyramine, cadaverine, and putrescine was demonstrated, the last two being produced with the highest frequency by different strains belonging to *Ec. faecalis, Ec. faecium, Ec. italicus* and *Ec. durans* species. Only one *Ec. faecalis* strain showed hemolytic activity on sheep blood. *Ec. faecalis* isolates were more frequently resistant to vancomycin, ampicillin, tetracycline and erythromycin than other species. All tested strains were sensitive to chloramphenicol, with some exceptions. Seventy percent of tested isolates was sensitive to vancomycin and only 10 strains harbored *vanA* and *vanB* genes. The presence of some virulence determinants was investigated, revealing that biotypes belonging to *Ec. faecalis* and *Ec. faecium* species harbored *gelE*, *asa1*, and *cylA*. The incidence of *esp* and *hyl* was low. Many strains harbored some CRISP locus associated genes but no correlation with antibiotic resistance genotype could be established.

3.1 Introduction

Enterococci belong to a highly controversial bacterial group: depending on the strain, they are considered as pro-technological, probiotic, spoilage or pathogenic organisms (Conde-Estevez et al., 2011; Franz et al., 2011; Khan et al., 2010; Peel et al., 2011;). Due to their tolerance to salts and acids, *Enterococcus spp.* are highly

adapted to several food systems and commonly occur in many fermented foods of meat, dairy and vegetable origins (Ben Omar et al., 2004). In particular, they have been frequently found in traditional cheeses produced from raw or pasteurized goat's, ewe's, water-buffalo or bovine milk (Cabezas et al., 2007; Cogan et al., 1997; Giraffa, 2003; Psoni et al., 2006; Tornadijo et al., 1995). In some cases enterococci represented the predominant microbiota of cheese, as in Manchego, Canestrato Pugliese, Pecorino, Semicotto Caprino, Comté and Kashar (Aquilanti et al., 2006, 2007; Aran, 1998; Ballesteros et al., 2006; Bouton et al., 1998; Cabezas et al., 2007). Undoubtedly, this microbial group plays a relevant role in the development of sensorial characteristics (Franz et al., 1999; Giraffa, 2003) and, consequently, enterococcal strains have been successfully used as starter (Franz et al., 2003; Giraffa et al., 1997) or adjunct cultures (Centeno et al., 1999; Izquierdo, et al., 2009; Manolopoulou et al., 2003) for the production of different cheeses in various European countries. Some distinctive strains of enterococci producing hydrogen peroxide or enterocins were also exploited as biopreservatives or shelf life extender (Franz et al., 2007; Morandi et al., 2012).

Ec. faecalis and *Ec. faecium* are the species most commonly isolated from cheese (Foulquié Moreno et al., 2006); unfortunately, the same species are also related to human infections (Bourgogne et al., 2008). Accordingly, a careful and exhaustive analysis of the potential risk factors of isolates is necessary in order to differentiate safe versus unsafe enterococci. Several virulence factors, e.g., aggregation substance, gelatinase, cytolysin, enterococcal surface protein and hyaluronidase have been described in enterococci (Jett et al., 1994; Vankerckhoven et al., 2004), in addition to the occurrence of antibiotic-resistances. However, De Vuyst et al. (2003) concluded that enterococcal strains lacking hemolytic activity and not carrying cytolysin nor vancomycin resistance genes may be regarded as safe and can be used as starter cultures, cocultures, or probiotics. CRISPR loci, encoded by some bacterial strains, were recently described as a defence system that recognizes, cleaves and silences exogenous genetic elements (Barrangou et al., 2007; Deveau et al., 2008); it has been found exclusively in bacteria that are vertebrate pathogens or

commensals. *Ec. faecalis* strains harboring the CRISPR loci have the possibility to avoid prophage infection (Bourgogne et al., 2008).

As previously described (Carafa et al., 2016), 126 isolates were genotypically identified as *Ec. faecalis* (57%), *Ec. faecium* (25%), *Ec. italicus* (12%), and *Ec. durans* (6%). Randomly Amplified Polymorphic DNA (RAPD) analysis clustered the isolates into 84 biotypes with 85% similarity index. Thirty-six biotypes belonging to *Ec. faecalis*, *Ec. faecium* and *Ec. durans* species, representing 43% of tested enterococcal cultures, showed the *in vitro* ability to inhibit the growth of coliforms.

The objective of this study was to investigate the safety of enterococci resident in TM-cheese and evaluate their potential use as adjunct cultures; in particular, the presence of antibiotic-resistance, virulence factors and CRISP locus associated genes were genotypically evaluated. In addition, the production of biogenic amines, the bile salt resistance and the haemolytic activity were investigated.

3.2 Materials and methods

3.2.1 Bile Salt Resistance (BSR) and Hydrolysis (BSH)

Eighty-four enterococcal isolates were selected as representative of each biotype identified through the RAPD cluster analysis (from strain Carafa et al., 2016).

BSR was determined according to the method of Vinderola and Reinheimer (2003) with some modifications: each biotype cultivated in M17 was inoculated (2%, v/v) into M17 broth supplemented with 0.5% (w/v) taurodeoxycholic acid (TDCA; Sigma Chemical Co., USA). After 24 h of incubation at 30 °C, the absorbance at 560 nm (A_{560nm}) was measured and compared to that of the control culture (M17 broth without TDCA). Results were expressed as the percentage of growth in presence of bile salts with respect to that of the control culture (A_{560nm}bile salts/A_{560nm}control*100).

The BSH activity was tested using the plate assay, as described by Carafa et al. (2015).

3.2.2 Enterococci associated risk factors

3.2.2.1 Production of biogenic amines (BA)

The ability to produce cadaverine, tyramine, histamine and putrescine from L-lysine, tyrosine disodium salt, L-histidine monohydrochloride and L-ohrnytine monohydrochloride, respectively, was investigated according to the method proposed by Bover-Cid and Holzapfel (1999). All amino acids were purchased from Sigma. Enterococci, previously subcultured for three weeks in MRS broth supplemented with 0.1% (w/v) of each amino acid, were streaked on decarboxylase agar medium plates supplemented with the single amino acids. Decarboxylase agar medium plates without the amino acid supplement were used as negative controls. After 4 days of aerobic incubation at 37 °C the amino acid decarboxilation to the respective BA was detected as presence of purple halos surrounding the colonies. Tyramine was detected as a transparent halo in the medium.

3.2.2.2 Hemolysis

Overnight enterococcal cultures (M17 at 30 or 45 °C) were streaked on Blood Agar Sheep (Biolife Italiana s.r.l., Milan, Italy) and aerobically incubated at 37 °C for 24 h. The hemolytic activity was detected through the formation of a clear halo around the colonies.

3.2.2.3 Antibiotic resistance: microbiological and genotypic screening

The sensitivity of TM-cheese resident enterococci to some of the most commonly used antimicrobials was tested according to the cut-off concentration suggested by European Food Safety Authority (EFSA, 2012). Pure enterococcal cultures were streaked onto M17 agar plates supplemented with ampicillin (2 mg/L), erythromycin (4 mg/L), vancomycin (4 mg/L), chloramphenicol (16 mg/L) and tetracycline (4 mg/L). After 48 h of aerobic incubation at 37 °C, the antibiotic

resistant biotypes showed positive growth. The presence of vancomycin resistance genes was detected, by using the specific PCR primer pairs VanAf, VanAr and VanBf, VanBr (Table 3.1), following the method described by Dutka-Malen et al. (1995).

Table 3.1 PCR primers used in this work and PCR products.

PCR	Primer	Lenght (bp)	Sequence 5'-3'	Size of PCR products (bp)	Reference
Vancomycin resistance	VanA f	17	GGGAAAACGACAATTGC	732	Dutka-Malen et al., 1995
	VanA r	17	GTACAATGCGGCCGTTA		
	VanB f	17	ATGGGAAGCCGATAGTC	635	
	VanB r	17	GATTTCGTTCCTCGACC		
Multiplex PCR for virulence factors	ASA 11	21	GCACGCTATTACGAACTATGA	375	Vankerckhoven et al., 2004
	ASA 12	21	TAAGAAAGAACATCACCACGA		
	GEL11	21	TATGACAATGCTTTTTGGGAT	213	
	GEL12	21	AGATGCACCCGAAATAATATA		
	CYT I	18	ACTCGGGGATTGATAGGC	688	
	CYT IIb	18	GCTGCTAAAGCTGCGCTT		
	Esp 14F	22	AGATTTCATCTTTGATTCTTGG	510	
	Esp 12R	21	AATTGATTCTTTAGCATCTGG		
	HYL n1	21	ACAGAAGAGCTGCAGGAAATG	276	
	HYL n2	21	GACTGACGTCCAAGTTTCCAA		
CRISP cas locus	cas_csn2 F	20	CTACCACTTGTTGCACTTCA	486	Bourgogne et al., 2008
	cas_csn2 R	20	CGATTAAAGACGTTCAAACC		
	cas1 F	20	TTTCATTATCGGAAATGCTT	614	
	cas1 R	20	TTGATGTTTTGTTGTTGGAA		
	cas_csn1 F	20	GCAAAGTCAACCACTCTCTC	877	
	cas_csn1 R	20	AAGTCCAAATCATCACCTTG		

3.2.2.4 Multiplex PCR for detection of virulence factors

The total genomic DNA of the selected biotypes was amplified as described by Vankerckhoven et al. (2004), using the primer pairs ASA 11, ASA 12, GEL 11, GEL 12, CYT I, CYT IIb, ESP 14F, ESP 12R, HYL n1, HYL n2 (Table 3.1) that amplify the virulence genes *asa1, gelE*, *cylA, esp* and *hyl*, respectively. *Ec. faecalis* MMH594 was used as positive control for the detection of *asa1*, *gelE, cylA*, and *esp* genes. The *Ec. faecium* biotypes C38 and C68 were used as positive controls for *esp* and both *esp* and *hyl*, respectively.

3.2.2.5 CRISP locus associated genes

The selected biotypes were analyzed for the presence of CRISP locus associated genes (*cas* genes), using three PCR primer pairs (Table 3.1): cas1 F, cas1 R, cas_csn1 F, cas_csn1 R, cas_csn2 F, cas_csn2 R) according to the method described by Bourgogne et al. (2008),

Vancomicyn resistance genes, virulence factors and CRISP locus amplicons were analyzed by electrophoresis on 2.0% (w/v) agarose el (Gibco, BRL) at 100 V for 90 minutes in 1X TAE buffer and were revealed by staining with ethidium bromide (0.5 μg/L). Amplifications were performed with a Piko96 ThermalCycler (Thermo Fisher Scientific Inc., Vantaa, Finland).

3.3 Results and discussion

3.3.1 Enterococci harboring technologically interesting properties

In a previous work, 231 LAB strains were isolated from samples of curd and 24h-cheese in order to study the microbial population that initiate the fermentation process in TM-cheese during the first 24 hours of production (Carafa et al., 2016). Enterococci represented 20% of the total microbial population 24 hours following

production, with a high biodiversity amongst the 126 isolates that grouped into 84 biotypes. Forty percent of tested enterococcal biotypes showed a great *in vitro* ability to inhibit the growth of coliforms resident in raw milk (Table 3.2). More bioactive properties and the safety level of TM-cheese resident enterococci were investigated in order to evaluate their possible exploitation for dairy productions. Forty-seven biotypes showed a BSR higher than 95%, suggesting that they could resist to human gut digestion; 1 *Ec. faecium* and 2 *Ec. italicus* biotypes showed a BSR range between 50 and 90%, 6 biotypes lower than 50% and the remaining 28 biotypes did not show any ability to grow in presence of bile salts (Table 3.2).

Only two *Ec. faecalis* and one *Ec. durans* biotypes showed BSH activity (Table 3.2), which has been reported to have health promoting effects (Begley et al., 2006; De Smet et al., 1994; Jones et al., 2008): this result is in agreement with other authors who found food-resident isolates belonging to *Ec. faecalis* and *Ec. durans* species able to hydrolyze bile salts (Franz et al., 2001; Gomes et al., 2008).

3.3.2 Risk factors associated with enterococci

In order to exploit their technological and bioactive potential, the risk factors potentially associated with enterococci were evaluated. The production of biogenic amines, the hemolytic activity and the antibiotic-resistances were analyzed by plate assays, and the presence of some virulence factors and CRISP locus associated genes were genotypically detected.

Histamine, tyramine, putrescine, and cadaverine are considered the most important BA in cheese (Linares et al., 2011) and are associated with the presence of the genera *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Lactococcus*, and *Streptococcus* (Martín et al., 2005; Bonetta et al., 2008; Calles-Enríquez et al., 2010; Ladero et al., 2011). The decarboxylase activity by cheese-related enterococci has been detected by several authors (Bonetta et al., 2008; Fernández et al., 2007; Ladero et al., 2012a) and *Ec. faecalis* strains have been identified as putrescine producers (Ladero et al., 2012b; Llácer et al., 2007). In TM-cheese, forty-seven biotypes

decarboxilated one or more amino acids. Cadaverine and putrescine were produced with the highest frequency: 18 *Ec. faecalis*, 9 *Ec. faecium*, 7 *Ec. italicus* and 1 *Ec. durans* biotypes produced both these BA; 16 *Ec. faecalis*, 3 *Ec. faecium*, 1 *Ec. durans* and 2 *Ec. italicus* biotypes generated tyramine and only 9 biotypes produced histamine (Table 3.2). Only one *Ec. faecalis* strain (biotype 166) showed the hemolytic activity on sheep blood (Table 3.2). This result is in accordance with the study of Semedo et al. (2003), who detected the hemolytic activity in two *Ec. faecalis* strains isolated from Portuguese ewes' cheese.

Concerning the sensitivity to antimicrobial substances, food chain has been considered a reservoir of animal and environment related bacteria carrying antibiotic resistances, which could move either to pathogenic bacteria or to other commensal bacteria in the gut (Clementi and Aquilanti, 2011). Amongst LAB isolated from spontaneously fermented foods, the antibiotic resistance has been commonly detected in the genus *Enterococcus* (Teuber et al., 1999). According to the definition by EFSA (2013), *enterococci are considered good indicators of the selective pressure exerted by the use of antimicrobials on intestinal populations of Gram-positive bacteria*. In particular, the occurrence of *Ec. faecium* and *Ec. faecalis* in the intestinal tract of animals or in food may constitute a reservoir of resistance genes. Nevertheless, De Vuyst et al. (2003) suggested that enterococci lacking hemolytic activity, cytolysin and vancomycin resistance genes might be considered safe. In this study we screened the antimicrobial resistance of TM-cheese resident enterococci, testing the cut-off values of five antibiotics proposed in EFSA (2012). Five biotypes (2 *Ec. italicus*, 1 *Ec. faecalis*, 1 *Ec. faecium* and 1 *Ec. durans*) were resistant to none of the tested antibiotics, 18 biotypes (11 *Ec. faecalis*, 4 *Ec. italicus*, 2 *Ec. durans* and 1 *Ec. faecium*) had a single antibiotic resistance and the remaining 61 biotypes had a multiple resistance (Table 3.2). Seventy percent of the tested strains showed resistance to vancomycin (42 *Ec. faecalis* biotypes, 14 *Ec. faecium*, 3 *Ec. italicus* and 1 *Ec. durans*), 58% to ampicillin (24 *Ec. faecalis* biotypes, 14 *Ec. facium*, 6 *Ec. italicus* and 3 *Ec. durans*), 58% to tetracycline (28 *Ec. faecalis* biotypes, 13 *Ec. faecium*, 6 *Ec. italicus* and 2 *Ec. durans*) and 48% to erythromycin (28 *Ec. faecalis* biotypes, 10 *Ec. faecium* and 3 *Ec. italicus*), whereas only few strains were not

sensitive to chloramphenicol (5 *Ec. faecalis* biotypes and 1 *Ec. faecium*) (Fig. 3.1). These results are in agreement with other authors who detected enterococci from food with resistances to vancomycin, tetracycline, erythromycin, chloramphenicol and ampicillin (Franz et al., 2003; Teuber et al., 1999).

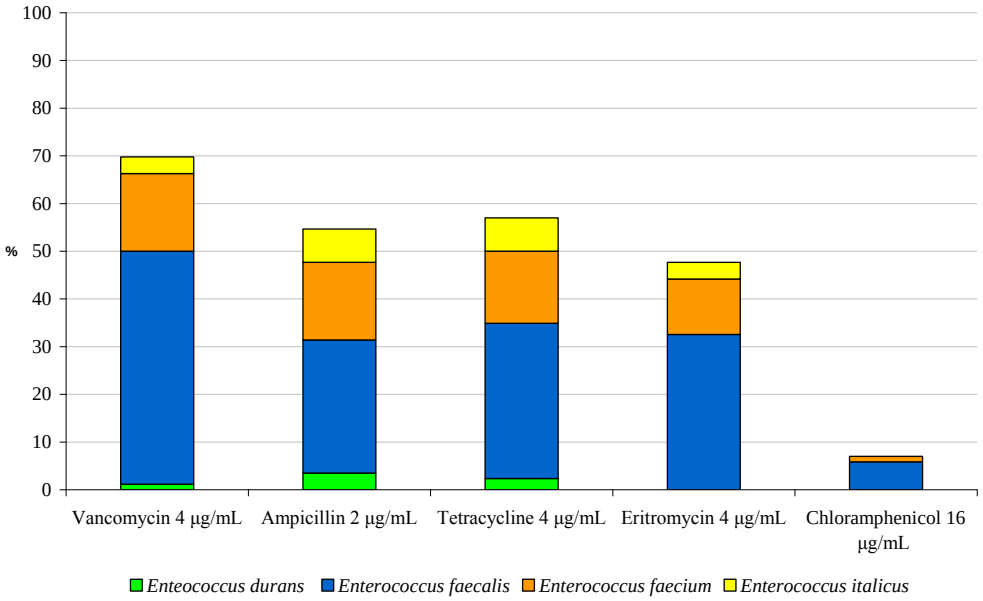

Fig. 3.1 Distribution of antibiotic resistance among the species of TM-cheese resident enterococci.

All biotypes were genotypically analyzed for vancomycin resistance, which has been reported to be conserved at species level in enterococci (Descheemaeker et al., 1999; Jensen et al., 1998; Willems et al., 1999): 5 *Ec. faecalis* biotypes, 4 *Ec. faecium* and 1 *Ec. durans* had *VanA* gene, and only 2 *Ec. italicus VanB* gene (Table 3.2). The presence of *vanA* in *Ec. faecium* and *Ec. durans* species was also detected by De Vuyst et al. (2003), who studied enterococci from food and non-food origins.

The antibiotic resistance represents a risk only if is spread to other bacteria through horizontal promiscuous transfer (Clementi and Aquilanti, 2011) and is not directly correlated with the virulence of enterococci; in fact, their pathogenicity is rather developed through the expression of virulence genes associated with adhesion, translocation, and evasion of immune responses (all factors which cause pathology) (Johnson et al., 1994). Some virulence determinants have been well determined including the proteolytic enzyme gelatinase (gel) which acts on a variety of

substrates such as insulin-β chain and collagenous material in tissues (Waters et al., 2003); cytolysin (Cyl) which was shown to enhance the virulence of pathogenic strains in animal models (Chow et al., 1993; Gilmore et al., 1993; Jett et al., 1992, 1994); and the aggregation substance (AS) which is an adhesion protein encoded by a pheromone responsive plasmid. It was shown *in vitro* that AS presence enhanced the adherence of *Ec. faecalis* to cultured renal epithelial cells and the invasion of eukaryotic mucosal cells (Kreft et al., 1992; Olmsted et al., 1994). Our results showed that TM-cheese related enterococci harbored virulence factors such as gelatinase, AS, cytolysin, enterococcal surface protein and hyaluronidase. *gelE* and *asa1* were the most frequently detected genes, *esp* and *hyl* were present only in 3 *Ec. faecalis* biotypes and cylA was detected in 10 *Ec. faecalis* and 2 *Ec. faecium*. Thirty-six biotypes (15 *Ec. faecalis*, 12 *Ec. faecium*, 6 *Ec. italicus* and 3 *Ec. durans*) showed no presence of any tested virulence genes. Figure 3.2 shows the percentage of biotypes harboring the five virulence genes. Biotypes belonging to *Ec. faecalis* species showed more virulence determinants than those belonging to *Ec. faecium*. Both *Ec. faecalis* and *Ec. faecium* biotypes harbored *gelE, asa1* and *cylA*, conversely the incidence of *esp* and *hyl* was very low. These results are in contrast with the study of Shankar et al. (1999) who detected *gelE* exclusively in *Ec. faecalis* strains and not in any tested *Ec. faecium* strain (Coque et al., 1995).

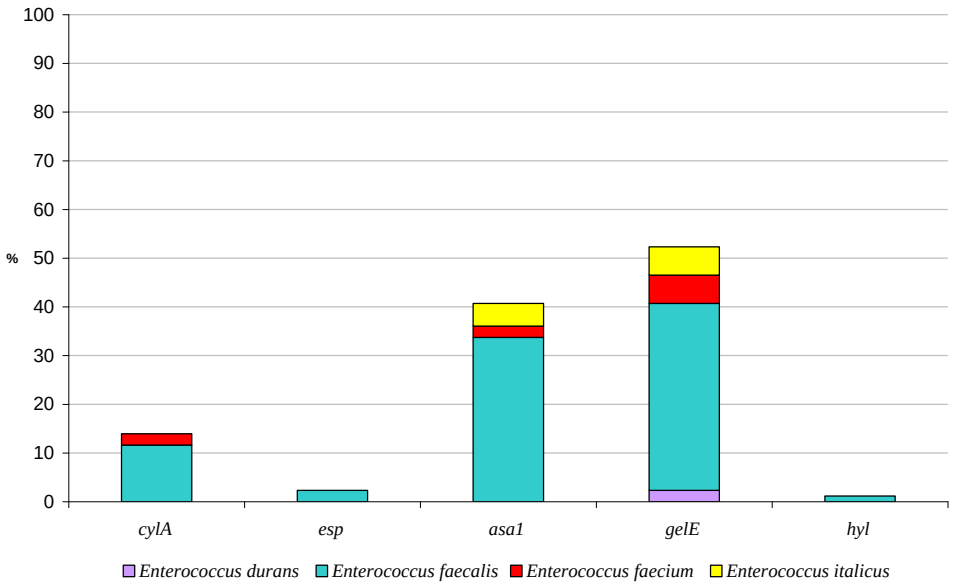

Fig. 3.2 Distribution of virulence genes among the species of TM-cheese resident enterococci.

Since the study of Palmer and Gilmore (2010) generated the evidence of an inverse correlation between multiple antibiotic resistance and detection of CRISPR1 loci in *Ec. faecalis*, we determined by specific PCR the presence of the following CRISPR locus associated genes: Cas_csn1 (possible endonuclease), Cas1 (novel nuclease) and Cas_csn2 (conserved hypothetical protein) (Bourgogne et al., 2008). CRISPR-cas systems represent an immunity system against exogenous DNA such as incoming phages and plasmids (Barrangou et al., 2007; Marraffini and Sontheimer, 2008). Enterococci biotypes were positive for CRISP locus associated genes, as follows: *cas1* (21 *Ec. faecalis*, 2 *Ec. faecium* and 1 *Ec. durans*), *cas-csn1* (23 *Ec. faecalis*, 3 *Ec. faecium* and 1 *Ec. durans*) and *cas-csn2* (22 *Ec. faecalis*, 2 *Ec. faecium*, 1 *Ec. durans* and 1 *Ec. italicus*) (Table 3.2). The presence of these genes in the majority of antibiotic resistant isolates indicates that the CRISPR-cas locus does not reduce the ability of TM-cheese related enterococci to acquire exogenous DNA.

Table 3.2 Results of phenotypical and genotypical assays performed on TM-cheese resident enterococci: antibiotic resistance; histamine (His), cadaverine (Cad), putrescine (Put), tyramine (Tyr) production; bile salt resistance (BSR), bile salt hydrolysis (BSH); detection of CRISP-cas locus associated genes (*Cas1*, *Cas_Csn1*, *Cas_Csn2*).

Biotype	Species	Coliforms inhibition	BSR (%)	Antibiotic resistance	His	Cad	Put	Tyr	*Cas1*	*Cas_ Csn1*	*Cas_ Csn2*
148	*Ec. faecium*	-	0	multiple	-	-	-	+	+	-	+
149	*Ec. faecium*	+	0	multiple	-	-	-	+	-	-	-
150	*Ec. faecium*	-	124	multiple	-	+	+	-	+	+	-
151	*Ec. faecium*	-	0	none	-	+	+	-	-	-	-
152	*Ec. faecium*	-	101	multiple	-	-	-	-	-	-	-
153	*Ec. faecium*	-	103	multiple	-	-	-	-	-	+	-
154	*Ec. durans*	-	0	none	-	-	-	+	-	-	-
155	*Ec. faecium*	-	69	multiple	+	+	+	-	-	-	-
156	*Ec. durans*	-	0	single	-	-	-	+	+	+	+
157	*Ec. faecium*	-	100	multiple	-	-	-	-	-	+	-
158	*Ec. faecium*	+	100	multiple	-	+	+	-	-	-	-
159	*Ec. faecalis*	-	98	multiple	-	+	+	+	-	-	-
160	*Ec. faecalis*	+	107	multiple	-	+	+	+	-	-	-
161	*Ec. faecium*	-	0	multiple	-	+	+	-	-	-	-
162	*Ec. faecalis*	+	4	multiple	-	-	-	-	-	-	-
163	*Ec. italicus*	-	0	multiple	+	+	+	-	-	-	-
164	*Ec. italicus*	-	54	single	-	-	-	-	-	-	-
165	*Ec. italicus*	-	0	none	-	-	-	-	-	-	-
166	*Ec. faecalis*	-	107*,(**)	multiple	-	+	+	+	-	-	-
167	*Ec. faecium*	-	97	multiple	-	+	+	+	-	-	-
168	*Ec. faecalis*	-	110	multiple	-	+	+	+	-	-	-
169	*Ec. faecalis*	-	102	multiple	-	+	+	+	-	-	-
170	*Ec. faecalis*	+	140	multiple	-	-	-	-	-	-	-
171	*Ec. faecalis*	+	100	multiple	-	+	+	+	-	-	-
172	*Ec. durans*	-	0	single	-	-	-	-	-	-	-
173	*Ec. faecium*	-	99	multiple	+	+	+	-	-	-	-
174	*Ec. faecalis*	-	98	multiple	-	+	+	+	-	-	-
175	*Ec. faecalis*	-	99	multiple	-	+	+	+	-	-	-
176	*Ec. durans*	+	137*	multiple	-	-	-	-	-	-	-
177	*Ec. faecium*	-	129	multiple	-	+	+	-	-	-	-
178	*Ec. faecium*	-	95	multiple	-	-	-	-	-	-	-
179	*Ec. faecalis*	+	2	multiple	-	-	-	-	-	-	-
180	*Ec. faecium*	-	101	multiple	-	-	-	-	-	-	-
181	*Ec. faecium*	-	101	multiple	-	-	-	-	-	-	-
182	*Ec. italicus*	-	6	multiple	+	+	+	-	-	-	-
183	*Ec. faecalis*	+	105	multiple	-	+	+	-	-	-	-
184	*Ec. faecalis*	+	100	multiple	-	-	-	-	+	+	-
185	*Ec. faecalis*	+	111	multiple	-	+	+	-	+	+	+
186	*Ec. italicus*	-	1	none	-	-	-	-	-	-	-
187	*Ec. faecalis*	+	0	single	-	-	-	-	-	-	-
188	*Ec. faecalis*	+	0	multiple	-	+	+	-	-	-	+
189	*Ec. faecalis*	-	97	multiple	-	+	+	-	+	+	+
190	*Ec. faecalis*	+	104	multiple	-	+	+	-	+	+	+
191	*Ec. faecalis*	+	145	multiple	-	+	+	+	+	+	+
192	*Ec. faecalis*	+	100	multiple	-	-	-	-	+	+	+
193	*Ec. faecalis*	-	99	multiple	-	+	+	-	-	-	-
194	*Ec. italicus*	-	102	multiple	-	+	+	-	-	-	-

Biotype	Species	Coliforms inhibition	BSR (%)	Antibiotic resistance	His	Cad	Put	Tyr	*Cas1*	*Cas_ Csn1*	*Cas_ Csn2*
195	*Ec. faecalis*	-	110	multiple	-	+	+	-	-	-	-
196	*Ec. italicus*	-	110	single	+	-	-	+	-	-	-
197	*Ec. faecalis*	+	0	multiple	-	-	-	-	+	+	+
198	*Ec. faecalis*	+	0	single	-	-	-	-	+	+	+
199	*Ec. faecalis*	-	0	multiple	-	-	-	-	-	-	-
200	*Ec. faecalis*	+	0	multiple	-	-	-	+	-	-	-
201	*Ec. faecalis*	-	0	single	-	-	-	-	+	+	+
202	*Ec. faecalis*	-	103	multiple	-	-	-	-	-	-	-
203	*Ec. faecalis*	+	0	single	-	-	-	+	-	-	-
204	*Ec. faecalis*	-	0	multiple	-	+	+	-	-	-	-
205	*Ec. faecalis*	-	133	single	-	-	-	+	-	-	-
206	*Ec. faecalis*	+	6	multiple	-	-	-	-	+	+	+
207	*Ec. faecalis*	+	0	single	-	-	-	-	+	+	+
208	*Ec. faecalis*	-	0	multiple	-	-	-	-	+	+	+
209	*Ec. faecalis*	+	105	multiple	-	-	-	-	-	-	-
210	*Ec. faecalis*	+	116	none	-	-	-	-	+	+	-
211	*Ec. faecalis*	+	0	multiple	-	-	-	-	+	+	+
212	*Ec. faecalis*	-	0	multiple	-	-	-	-	+	+	+
213	*Ec. faecalis*	+	104	multiple	-	-	-	-	+	+	+
214	*Ec. faecalis*	+	103	multiple	-	+	+	-	+	+	+
215	*Ec. faecalis*	+	109	multiple	-	-	-	-	-	-	-
216	*Ec. faecalis*	+	100	single	-	-	-	-	-	+	+
217	*Ec. faecalis*	+	138	multiple	-	-	-	-	+	+	+
218	*Ec. faecalis*	+	108	single	-	-	-	+	-	-	-
219	*Ec. faecalis*	+	0	multiple	-	-	-	-	+	+	+
220	*Ec. faecalis*	-	0	multiple	-	-	-	-	-	-	-
221	*Ec. faecalis*	+	16*	single	-	-	-	-	-	-	-
222	*Ec. faecalis*	+	0	single	-	-	-	-	-	+	+
223	*Ec. faecalis*	+	0	single	-	-	-	-	-	-	-
224	*Ec. faecalis*	-	0	multiple	-	-	-	+	+	+	+
225	*Ec. faecalis*	+	101	multiple	-	-	-	+	+	+	+
226	*Ec. italicus*	-	141	single	+	+	+	-	-	-	-
227	*Ec. italicus*	-	89	multiple	+	+	+	-	-	-	-
228	*Ec. italicus*	-	97	multiple	+	+	+	-	-	-	+
229	*Ec. durans*	-	0	multiple	+	+	+	-	-	-	-
230	*Ec. italicus*	-	99	single	-	+	+	-	-	-	-
231	*Ec. faecium*	-	135	single	-	+	+	-	-	-	-

* Biotypes showing the BSH activity

(**) Biotypes showing the hemolytic activity

3.4 Conclusions

Many enterococcal strains isolated from TM-cheese showed extremely interesting technological properties such as inhibition of coliforms, bile salt resistance or bile salt hydrolysis. Unfortunately, some enterococci associated risk

factors were detected. CRISPR-cas locus genes were detected in the majority of antibiotic resistant isolates, suggesting that these genes do not reduce the ability of TM-cheese related enterococci to adcquire exogenous DNA.

Ec. durans biotype 176 and three *Ec. faecalis* strains (biotypes 170, 209, 215) showed a good BSR (the biotype 176 showed also BSH), inhibited milk-resident coliforms, never produced the tested biogenic amines, but showed multiple antibiotic resistances. The antibiotic resistance represents a risk only if its horizontal dissemination is possible, conversely the presence of virulence factors is always dangerous because is associated with the pathogenicity of enterococci. Only two *Ec. faecalis* strains (biotypes 170 and 209) free from virulence genes showed interesting properties, but further investigations about the origin (intrinsic or acquired) of the antibiotic resistance are needed.

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4. Exploitation of autochthonous *Lactococcus lactis subsp. lactis* 68 and *Streptococcus thermophilus* 93 for experimental Traditional Mountain cheese production

Abstract

The activity of *Lactococcus lactis subsp. lactis* 68 and *Streptococcus thermophilus* 93 as starter cultures was tested *in situ* through producing experimental Traditional Mountain (TM)-cheeses. Three control (CTRL) cheeses were produced according to the tradition and any starter or adjunct culture was not added; three starter (STR) and three commercial starter (CMS) cheeses were produced inoculating the vat milk with both strains and a commercial *Sc. thermophilus* strain, respectively. After 24 hours, 1 month and 7 months of ripening the microbial content of all experimental cheeses was investigated. Mesophilic cocci and lactobacilli dominated in cheese samples after 24 hours and 1 month of ripening, while cocci dominated in full-ripened cheese. The total genomic DNA was extracted, and a fragment of the V1-V3 region was amplified and pyrosequenced. Lactococci and streptococci were the most abundant species in CTRL and STR cheese, and *Lc. lactis ssp. lactis* 68 affected the proliferation of the (raw milk) indigenous *Lc. lactis ssp. cremoris* during the early fermentation. Moreover, the commercial *Sc. thermophilus* showed to be dominant towards *Lc. lactis subsp. lactis* and *cremoris* naturally present in raw milk and to be responsible in decreasing the abundance of *Lactobacillus sp.* and *Enterococcus sp.*

The survival of TM-cheese microbiota *in vitro* was investigated under simulated human gastro-intestinal (GI) conditions. The 9 full-ripened experimental TM-cheese were subjected to a model system that simulates digestive processes in the mouth, stomach and small intestine, comprising sequential incubation in human gastric and duodenal juices. Bacterial counts were performed before and after simulation: total bacterial count and thermophilic cocci significantly decreased after the simulated digestion. Thirty-six lactobacilli were isolated from cheese after

digestion: among them 1 *Lb. paracasei*, 1 *Lb. parabuchneri* and 1 *Lb. fermentum* were tested for their survival after GI transit. *Lc. lactis subsp. lactis* 68 and *Lb. parabuchneri* D34 strains were used to ferment whole milk and digested. The load of *Lb. parabuchneri* D34 decreased by about one logarithmic cycle more when grown as pure culture than fermented milk after simulated digestion, suggesting that *Lb. parabuchneri* D34 had in itself the ability to survive to digestion, but the fat content and the cheese structure might protect LAB during the GI transit.

4.1 Introduction

Raw milk cheeses harbor a heterogeneous microbial composition characterized by adventitious microorganisms proceeding from raw milk, the dairy environment and facilities (Eneroth et al., 1998). All those bacteria may be pathogens, spoilage or beneficial bacteria (Fox and McSweeney, 2004). Nowadays, lactic acid bacteria (LAB) are selected and used as starter or adjunct cultures during cheese manufacture owing to their fast acid production from the fermentation of lactose into lactic acid and their activity during cheese-ripening (McSweeney, 2004; Beresford and Williams, 2004).

Carafa et al. (2015, 2016) investigated the microbial diversity of TM-cheese by using culture-based methods, and identified twenty-nine different LAB species after 24 hours and 7 months of ripening. Analyzing the microbial evolution of cheese, the culture-based characterization techniques and the presence of non-culturable strains allow focusing the investigation on a small fraction of the complex cheese microbiota (Coppola et al., 2001; Muyzer et al., 1993). Furthermore, the stressing conditions which strains are exposed to during cheese ripening (e.g., high concentration of salt, low pH and non-optimal temperature) might induce cells to a resting or non-proliferative state (Fleet, 1999). All these factors might cause the under-estimation of cheese ecosystem. By contrast, culture-independent methods, as like PCR-denaturing gradient gel electrophoresis (DGGE) or rDNA-based methods, supply the overview of biodiversity and allow identifying the dominant species (Delbès et al., 2007; Rademaker et al., 2005). Recently, the next-generation

sequencing methods such as 454-pyrosequencing have been reported to provide more detailed information about natural fermented foods (Jung et al., 2011; Roh et al., 2010) and cheeses (Fuka et al., 2013; Masoud et al., 2012).

In this work, two LAB strains previously selected for their good phenotypical and technological properties (Carafa et al., 2016) were exploited for experimental TM-cheeses production in order to test their efficiency as starter cultures. The microbial composition of the cheese samples was investigated by 454-pyrosequencing after 24 hours, 1 months and 7 months of ripening.

Furthermore, we wanted to know if TM-cheese microbiota reaches alive the intestine; thus, the survival of the cheese-resident microbiota to simulated gastro-intestinal (GI) conditions was investigated. Any strain with functional or probiotic activity which is used as starter or adjunct cultures can provide positive effects only if is able to survive under GI conditions and to adhere to the intestinal epithelium (Huang and Adams, 2004; Kailasapathy and Chin, 2000), and are generally accepted as bioactive ingredients in food supplements (capsules and tablets) or in functional food products (Faye et al., 2012; Pfeiler and Klaenhammer, 2007; Zhu et al., 2009).

4.2 Materials and methods

4.2.1 Microorganisms and culture conditions

Lc. lactis subsp. lactis 68 and *Sc. thermophilus* 93, previously isolated from TM-cheese samples were selected as starter culture for cheese productions because fast acidifiers, able to grow at non optimal temperatures and producing pleasant flavours (Carafa et al. 2016). The strains, belonging to the culture collection of the Food Quality and Nutrition Department at Fondazione Edmund Mach (FEM, San Michele all'Adige, Trento, Italy), were grown overnight as follows: *Lc. lactis* in M17 broth incubated at 30 °C, *Sc thermophilus* in M17 broth incubated at 45 °C. The media were purchased from Oxoid (Milan, Italy).

4.2.2 Production of experimental cheese and sample collection

Nine experimental cheese-making processes were carried out and followed in August 2012 during three consecutive weeks as follows: i) control cheeses (CTRL, n = 3), produced without addition of any starter and following the traditional cheese-making process in the Malga-farm; ii) starter cheeses (STR, n = 3), produced inoculating the vat milk with the freeze-dried *Lc. lactis* 68 and *Sc. thermophilus* 93 at the final concentration of 5.0 x 10^6 CFU/mL); iii) commercial starter cheeses (CMS, n = 3) belonging to *Sc. thermophilus.*

The experimental cheese making trials were carried out in a Malga-farm (Malga Juribello, Tonadico, Trento, Italy), in collaboration with the Technology Transfer Centre, Fondazione Edmund Mach (San Michele all'Adige, Italy). Briefly, 150 L of raw cow's milk were heated at 37 °C, inoculated with the corresponding bacterial mixture, and 5.3 g of calf rennet paste 1:115,000 (Clerici Sacco International, Cadorago, Italy) were added. After coagulation, curd was cut into nut-size grains, cooked at 45 °C for 25 min and held at this temperature for 20 min further. After extraction and moulding, curds were stored for 24 h at room temperature. The pH was monitored using the portable pH meter PT1000 (Knick, Berlin, Germany) equipped with a Hamilton electrode (Hamilton Bonaduz, Bonaduz, Switzerland) every hour for 24 h, and after 1 and 7 months of ripening. During the all cheese-making process, the temperatures of the cheese and room were monitored by a 175-T2 data logger (Testo, Settimo Milanese, Italy). Twenty-four hours following production, the cheese wheels were salted for 24 h in a saturated brine solution, air dried for 24 h, stored for about 2 months in the Malga and moved for 5 more months to the FEM's experimental dairy store at 18 °C and 85% humidity.

Vat milk, and cheese after 24 hours, 1 month and 7 months of ripening were sampled for each of the 9 cheese-making days for a total of 36 samples. Twenty mL of milk were collected from the vat: 10 mL were immediately processed for microbial analysis, and 10 mL were stored at −80 °C for DNA extraction. Milk and cheese samples were homogenized and plated onto selective agar media as described by Carafa et al. (2016).

4.2.3 Total DNA extraction from milk and cheese samples

Samples of vat milk (n = 9) and cheese (24 hours, 1 month and 7 months of ripening, n = 27) were processed for DNA isolation as follows: 4 mL of milk or 4 g of cheese were added to 36 g of sterile Na-citrate 2% (w/w) solution and homogenized by a ULTRA-TURRAX® for 5 min at speed 3. The obtained mixture was centrifuged at 4,000 g for 10 min. The genomic DNA was extracted from the pellet using the Power Food™ Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. All DNA samples were purified by PowerClean DNA Clean-up Kit (Mo Bio Laboratories Inc.) and quantified by Nanodrop3300 Fluorospectrometer (Thermo Scientific, USA) using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Life Technology, USA).

4.2.4 DNA amplification and 454-pyrosequencing analysis

To create an amplicon library for the Genome Sequencer FLX+ system (Roche, Mannheim, Germany), Multiplex Identifiers (MIDs; Roche) were used to label each PCR product from the 36 samples, according to the manufacturer's instructions.

For each sample of the 9 analysed cheese-making days, a 525-nucleotide sequence of the V1-V3 region (Liu et al., 2008) of the 16S rRNA gene (*Escherichia coli* positions 8 to 533) was amplified starting from the extracted genomic DNA using special fusion primers for the Genome Sequencer FLX+ System. Unique multiplex identifier adaptors were attached between the 454 adaptor sequences and the forward primers to facilitate the pooling and subsequent differentiation of samples. For each sample, a PCR mix of 25 µL was prepared containing 1X PCR buffer, 1.25 U of FastStart High Fidelity polymerase and dNTPs from the FastStart High Fidelity PCR system (Roche), 400 nM primers and 5 ng genomic DNA. Thermal cycling consisted of initial denaturation at 94 °C for 3 min, followed by 30

cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 45 s and extension at 72 °C for 1 min, with a final extension of 8 min at 72 °C.

To prevent the preferential sequencing of the smallest amplicons, amplicons were cleaned using the Agencourt AMPure kit (Beckman coulter) according to the manufacturer's instructions; subsequently, the amplicons DNA concentrations were determined using the Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen) following the manufacturer's instructions. In order to ensure the absence of primer dimers and to assay the purity, the generated amplicon libraries quality was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using the High Sensitivity DNA Kit (Agilent). Following the quantification, the cleaned amplicons were mixed and combined in equimolar ratios into one pools. Pyrosequencing was carried out by a 454 Life Sciences Genome Sequencer FLX instrument (Roche) following titanium chemistry at DNA Sequencing and Genomics Lab, FEM.

4.2.5 Pyrosequencing data analysis and sequence identification by QIIME

The sequences obtained from pyrosequencing were processed using the Quantitative Insights Into Microbial Ecology (QIIME) software package version 1.7 (Caporaso et al., 2010). Briefly, quality trimming of dataset removed sequences with a mean quality score less than 25, length of < 150 or > 700 bps, without primer sequence, containing ambiguous characters, homopolymer run exceeding 6 nt and with mismatches in the primer region. The sequences were de-multiplexed based on their respective barcode. The primer sequences were excluded before alignment. Chimeric sequences were removed using ChimeraSlayer script implemented in QIIME pack. The remaining sequences were assigned into operational taxonomic units (OTUs) at a threshold of 97% pair-wise sequence identity with uclust. OTU representative sequences were then classified taxonomically using Ribosomal Database Project (RDP) classifier 2.0.1 (Maidak et al., 2001). The OTUs were aligned using PyNAST with a minimum alignment length of 150 bp and a minimum identity at 80% (Caporaso et al., 2010). After alignment, PH LANE mask

(http://greengenes.lbl.gov) was used to screen away the hypervariable regions. Rarefaction plots were constructed and diversity indices were estimated as implemented in QIIME. Finally, manual sequence comparison of some dominant selected OTUs using the NCBI sequence database located at http://www.ncbi.nlm.nih.gov, was also performed to have a more detailed species identification (Altschul et al., 1990).

The species richness within each sample was estimated by QIIME pipeline using Chao1 metric that is non-parametric abundance-based estimator of species richness (Chao and Bunge, 2002).

4.2.6 In vitro simulation of human gastro-intestinal digestion and microbiological counts

The nine experimental cheese samples were subjected to simulated human gastro-intestinal digestion, in triplicate. The preparation of phospholipid vesicles, the *in vitro* gastric and duodenal digestion were performed following the method described by Mandalari et al. (2008). All enzymes used in this study were purchased from Sigma Chemical Co. (USA).

After digestion, the microbial load was calculated by plate counting onto selective media, as described by Carafa et al., 2016.

4.2.7 Isolation and identification of strains resistant to the in vitro digestion

Thirty-six colonies were randomly picked up from countable MRS and M17 agar plates for bacterial isolation and purified by subsequent culturing. Pure cultures were stored at -80 °C in glycerol (20%, v/v) stocks. LAB were selected, purified and genotypically identified by using RAPD–PCR and partial 16S rRNA gene sequencing as previously described (Carafa et al., 2016): the biotype clustering was done setting 85% similarity cutoff after testing the RAPD-PCR repeatability as previously determined by Foschino et al. (2008).

Lc. lactis subspp. lactis and *cremoris*, *Lb. casei*, *Lb. paracasei* and *Lb. rhamnosus* species were also confirmed by means of species-specific PCR species according with Corroler et al. (1998) and Ward and Timmins (1999), respectively. All amplifications were performed with a T100^{TM} ThermalCycler (Bio-Rad Laboratories).

4.2.8 Milk fermentation by LAB isolated from experimental TM-cheese

Lc. lactis subsp. lactis 68 and three lactobacilli (*Lb. paracasei* D20, *Lb. fermentum* D25 and *Lb. parabuchneri* D34) isolated from the experimental TM-cheeses after digestion were cultured at 30 °C in triplicate in M17 and MRS, respectively. Then, *Lc. lactis subsp. lactis* 68 and *Lb. parabuchneri* D34 were grown and inoculated (1%, v/v) in 50 mL of UHT whole milk (3.5% fat) incubated overnight at 30 °C in triplicate, according to the method described by Vinderola et al. (2002). All grown cultures (n_{tot} = 12) and fermented milk samples (n_{tot} = 3) were digested as described above. The survival of the tested strains after the simulated GI transit was investigated by plate counting the viable cells onto MRS and M17 agar media before and after digestion.

4.2.9 Statistical analysis

All data were subjected to one-way analysis of variance (one-way ANOVA) using STATISTICA data analysis software system, version 9.1 (StatSoft, Inc. 2010 ww.statsoft.com). Differences were considered significant when $p < 0.05$.

4.3 Results and discussion

4.3.1 Effect of bacteria inocula on the cheese acidification

The aim of this study was to evaluate *Lc. lactis* 68 and *Sc. thermophilus* 93 as autochthonous starter cultures during TM-cheese manufacture. The traditional

cheese-making process and conditions were accurately followed in a Malga-farm. The room temperature recorded in the Malga-farm during the first 24 h of acidification ranged between 18.4 and 28.3 °C (mean value of 25 ± 1.3 °C). Eight hours following curd extraction the pH values of CTRL curd samples were higher than 5.8, and lower than 5.5 and 5.0 in STR and CMS curd samples, respectively (data not shown). The pH of the three CTRL samples decreased to less than 5.8 during the first 24 hours of ripening; conversely, the pH values of the STR and CMS cheese samples were equal to or lower than 5.0 (Table 4.1). At the end of ripening the maximum pH value registered was 5.84, suggesting that the wild bacteria of cheese (probably mesophilic cocci in STR and lactobacilli in CMS cheese, which were the bacterial group with the highest load at 7 months of ripening) might have modified the cheese pH owing to their hydrolytic activity on the high molecular weight peptides (Sousa et al., 2001). The pH evolution of the experimental CTRL cheeses was similar to spontaneously fermented TM-cheese (Carafa et al., 2016), and the pH values registered in the experimental STR and CMS 24h-cheeses was lower than TM-cheese, likely owing to the presence of wild and commercial starter strains.

4.3.2 Microbial characterization of milk, CTRL and inoculated cheeses

The microbial loads of total bacterial counts (TBC), mesophilic and thermophilic lactococci, mesophilic lactobacilli, enterococci and coliforms enumerated in milk, curd and cheese after 24 hours, 1 month and 7 months of ripening are shown in Table 4.1. During cheese-making production an increase of all microbial groups was recorded, likely owing to the retention of microorganisms in the curd structure and the microbial growth during coagulation.

TBC of vat milk samples was 4.7 ± 0.75 Log CFU/mL without significant differences amongst the trials. A similar result has been detected in milk samples used for spontaneously fermented TM-cheese, as reported by Carafa et al. (2016). The highest total bacterial load (9 logarhitmic units) was recorded in cheese 24 hours following production and thereafter gradually decreased at the end of ripening

without significant differences between CTRL and inoculated samples. The bacteria enumerated on M17 at 30 °C presented a ripening evolution similar to TBC. In particular, the load of mesophilic cocci in STR curd samples (7.9 ± 0.46 Log CFU/g) was significantly higher than CTRL (6.1 ± 1.26 Log CFU/g) and CMS (7.0 ± 0.59 Log CFU/g) samples, and this could be related to the addition of *Lc. lactis subsp. lactis* 68 as starter culture. The highest counts were registered 24 hours following production and significantly ($p< 0.05$) decreased at the end of ripening, without any difference between CTRL, STR and CMS cheeses.

Themophilic cocci had a similar evolution in CTRL and CMS cheeses, which were significantly different from STR cheeses: they reached the maximum load in CTRL (9.0 ± 0.29 Log CFU/g) and CMS (8.8 ± 0.31 Log CFU/g) cheeses 24 h following production and after 1 month of ripening in STR cheeses (8.4 ± 0.39 Log CFU/g). Themophilic cocci gradually decreased at the end of ripening, to 7.0 ± 0.49, 6.6 ± 1.33 and 8.1 ± 0.95 Log CFU/g, in CTRL, STR and CMS cheeses, respectively. These results suggested that the wild selected strain *Sc. thermophilus* 93 had a slower development than the commercial *Sc. thermophilus* strain, but was more adapted to dairy environment: in fact, it was able to survive and dominate the cheese microbiota together with mesophilic cocci through the first month of ripening at the ripening room temperature (18 °C). The drop of both *Sc. thermophilus* and *Lc. lactis* during cheese ripening might be due to their autolytic activity (Sandholm and Sarimo, 1981; Thomas and Pritchard, 1987). Mesophilic rod-shaped LAB in milk were 3.4 ± 0.45 Log CFU/mL and increased during production and ripening without significant differences between CTRL and inoculated cheeses. Enterococci in milk were 2.7 ± 0.54 Log CFU/mL, reached the maximum concentration 24 h following production in all cheeses and gradually decreased after 1 and 7 months of ripening. Enterococci were always significantly lower in CMS than in CTRL and STR cheeses. The presence of enterococci in cheese has been reported desirable by some authors (Giraffa, 2003; Franz et al., 1999; Suzzi et al., 2000) because they play an important role during cheese ripening.

Table 4.1 Mean Value (MV) and related standard deviations (SD) of pH and microbial counts of milk. CTRL, STR and CMS cheeses at different stages of ripening (curd. 24 hours, 1 month, 7 months) collected during the different trials. for a total of 42 samples. MV is expressed in Log CFU/mL for milk samples and Log CFU/g for cheese samples.

	pH		**TBC**		**Mesophilic cocci**		**Thermophilic cocci**		**Lactobacilli**		**Enterococci**		**Coliforms**	
	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD
MILK (n=9)	6.57	0.06	4.7	0.75	4.34	0.53	3.1	0.70	3.4	0.45	2.7	0.54	2.2	0.41
CURD														
CTRL (n=3)	6.43	0.05	5.7a	0.30	6.1a	1.26	3.2a	2.17	3.8	0.58	3.6	0.53	2.7	0.79
STR (n=3)	6.36	0.03	7.8b	0.41	7.9b	0.46	7.7b	0.83	3.8	0.74	4.2	0.95	2.5	0.96
CMS (n=3)	6.46	0.03	7.0b	0.38	7.0a	0.59	8.0b	0.33	4.2	0.67	3.8	0.91	2.1	0.55
24h Cheese														
CTRL (n=3)	5.64a	0.55	9.1	0.45	8.7	0.75	9.0a	0.29	8.8	0.61	7.6a	0.27	2.7	0.47
STR (n=3)	5.01b	0.26	9.4	0.40	9.4	0.32	8.2b	0.62	9.3	0.38	8.0b	0.30	2.5	0.62
CMS (n=3)	4.96b	0.33	9.3	0.52	9.0	0.41	8.8a	0.31	8.9	0.54	5.6c	0.46	2.1	0.97
1M Cheese														
CTRL (n=3)	5.61	0.32	8.2	0.77	8.4	0.56	7.5a	0.51	8.3	0.75	7.5a	0.33	2.9a	1.78
STR (n=3)	5.56	0.18	8.9	0.36	8.9	0.39	8.4b	0.39	8.7	0.38	8.0b	0.61	2.0a	1.38
CMS (n=3)	5.58	0.31	8.5	0.21	8.4	0.43	6.8a	0.35	8.7	0.36	5.5c	1.93	0.0b	0.00
7M Cheese														
CTRL (n=3)	5.59	0.28	7.2	0.23	7.2	0.25	7.0a	0.49	6.5	0.74	6.7a	0.47	0.0	0.00
STR (n=3)	5.84	0.17	8.0	0.66	8.1	0.77	8.1b	0.95	7.0	0.46	7.5b	1.12	0.0	0.00
CMS (n=3)	5.78	0.19	7.2	0.43	7.2	0.11	6.6a	1.33	7.1	0.12	4.4c	0.36	0.0	0.00
7M Cheese (n=9)														
Before digestion	n.c.	n.c.	7.3a	0.40	7.5	0.48	7.2a	0.18	6.8	0.54	6.0	1.17	0.00	0.00
After digestion	n.c.	n.c.	6.7b	0.52	7.4	0.42	6.9b	0.14	6.9	0.52	6.4	0.41	0.00	0.00

a, b, c: Different letters in the same column indicate significant statistical differences (Tukey's Test, $p < 0.05$). n.c.: not calculated.

Coliforms are associated with the hygienic quality of a food product, and high loads in milk and cheese indicate poor hygiene practices during milk collection and cheese production (Farkye, 2000). Low counts of coliforms were detected in milk (2.2 ± 0.41 Log CFU/mL), and their concentation did not significantly change in the first month of cheese ripening, with the exception of CMS cheeses (where no coliforms were detected after 1 month of ripening), suggesting that the commercial *Sc. thermophilus* strain inhibited the growth of coliforms during the first month of ripening owing to its fast acidifying activity. However, at the end of ripening coliforms were not detected in any sample.

4.3.3 Taxonomic characterization of the microbial communities of milk, CTRL, STR and CMS cheese samples by 454-pyrosequencing analysis

The total composition of the TM-cheese ecosystem was analyzed during the cheese-making process, ripening and in presence or absence of starter cultures. The 16S rRNA gene covering the variable regions 1, 2 and 3 was amplified after DNA extraction from the 9 milk and 36 cheese samples and submitted to 454-pyrosequencing. About 593,000 sequences were obtained: after barcode splitting and quality trimming on raw data; 93,286 and 203,648 reads per milk and cheese samples, respectively, were classified by means of the RDP Classifier implemented in the QIIME pipeline.

The bacterial genera in milk and cheese samples belonged mainly to the phyla *Firmicutes* and *Proteobacteria*. In particular milk samples were dominated by the genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Macrococcus*, *Leuconostoc*, *Enterococcus* and *Staphylococcus* among *Firmicutes* and *Alphaproteobacteria*, *Betaproteobacteria*, *Acinetobacter*, and *Pseudomonas* among *Proteobacteria* (Fig. 4.1). These genera, with the exception of *Pseudomonas* and *Macrococcus*, were also detected by Delbès et al. (2007) in milk for Saint-Nectaire cheese production. *Firmicutes* are commonly found in milk: the incidence of lactococci, streptococci and enterococci was also confirmed by colony enumeration onto selective media (Table 4.1). The presence of *Macrococcus* and *Acinetobacter spp.* has been

previously detected in alpine raw milk (Franciosi et al., 2011; Panelli et al., 2013), while the presence of *Staphylococcus* might be related to the cow's teat apex and the canal contamination (Braem et al., 2012).

In CTRL and STR cheeses samples a high bacterial richness was observed similarly to that recorded in milk samples: up to 12 different genera were detected, but the percentages showing the relative abundance of milk, CTRL and STR cheeses were different (Fig. 4.1). In particular, we observed that *Lactococcus* (28-79% in CTRL, 73-80% in STR cheese) and *Streptococcus* (2-33% in CTRL, 2-16% in STR cheese) were the most abundant bacterial genera in CTRL and STR cheese, and the incidence of the genera *Lactobacillus* species, which was detectable (with a relative abundance higher than 0.1%) only after 1 month of ripening. By contrast, CMS curd and cheese samples were composed by only five genera and *Streptococcus* was the dominant one (80 and 90% of relative abundance). Surprisingly, we detected the genera *Sphingobacteria* only in CMS samples that reached the 13% in curd samples: this genus have been reported by other authors as component of raw milk microbiota and dairy environment (Quigley et al., 2013; Schmidt et al., 2012), but its role in cheese ripening is still unknown. The OTUs number suggested that the fermentation of CTRL cheeses was carried on by lactococci and streptococci during the first 24 hours following production; during fermentation, the number of OTUs assigned to the genus *Lactococcus* increased, while those assigned to the genus *Streptococcus* decreased noticeably after 1 month of ripening. The early fermentation of STR cheeses was lead by lactococci, which was the most abundant genus in the first 24 hours after curd extraction. This result suggested that amongst the two inoculated starter strains, *Lc. lactis subsp. lactis* 68 was more suitable for the dairy environment, able to dominate on *Sc. thermophilus* 93, and to carry on the early fermentation process, lowering the milk pH. Surprisingly, the relative abundance of streptococci increased only after 1 month of ripening: in fact, we expected to detect more sequences assigned to streptococci than we did, because *Sc. thermophilus* 93 was inoculated in milk as starter culture together with *Lc. lactis subsp. lactis* 68. The relative abundance of the genus *Lactobacillus* increased in all cheese samples after 1 month of ripening.

All data obtained from the 454-pyrosequencing were in agreement with the microbiological counts, except for mesophilic cocci of CMS cheeses, which were detected as dominant in 24h-cheese by plate counting but not by 454-pyrosequencing. This result might be due to the limited selectivity of the medium M17 were lactobacilli can grow, as reported by other authors (Abriouel et al., 2008; Franciosi et al., 2015), and the ability of many streptococci strains to grow at 30 °C (Carafa et al., 2016). Furthermore, the bacterial mix inoculated in STR cheeses influenced the content of non-LAB bacteria, decreasing the abundance of *Escherichia* and *Enterobacteriaceae sp.* 24 hours following production (Fig. 4.1).

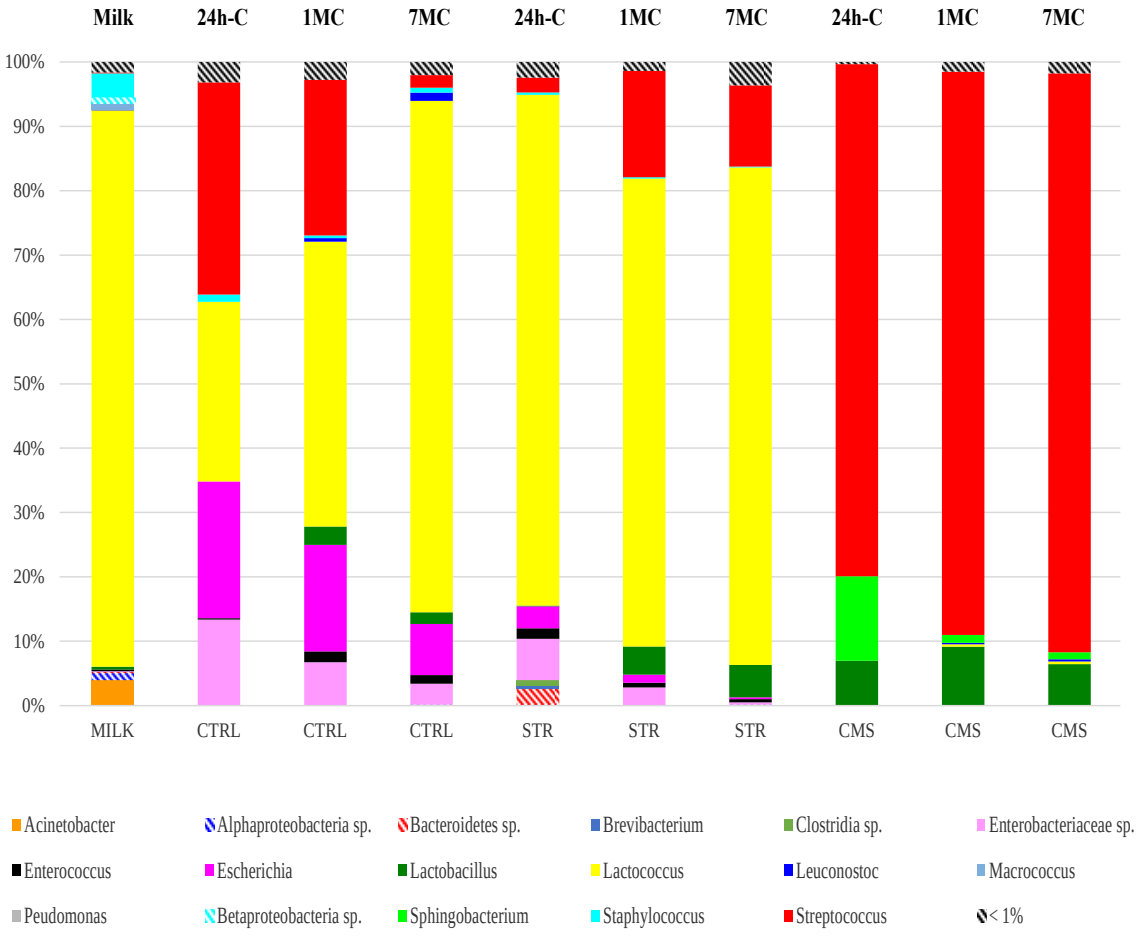

Fig. 4.1 Bacterial genera identified after operational taxonomic units (OTUs) assignment with 97% identity with pair-wise sequences. The number of OTUs for each genus was normalised for the total number of reads in each sample analysed by 454 pyrosequencing and expressed in percent.

In order to identify *Lactobacillales* at species level, a BLASTn analyses of milk and cheese samples OTUs was performed against the NCBI bacterial database, with a sequence similarity cutoff of 98%. The LAB species with at least 1% of relative abundance are shown in Table 4.2. In milk samples the OTUs belonging to *Lc. lactis ssp. lactis* and *cremoris* accounted for most of *Lactobacillales* reads of the milk samples, suggesting that these species were dominant, as reported by Franciosi et al. (2009) who analyzed raw milk samples from alpine regions. *Ec. raffinosus* and *Lb. coryniformis* were detected in milk but disappeared during cheese production and ripening, suggesting that they might not be adapted to the cheese-making conditions. By contrast, *Lb. animalis*, *Lc. garviae*, *Leuconostoc lactis*, *Sc. dysgalactiae* and *Sc. gallolyticus* grew in CTRL cheeses but not in the inoculated samples, suggesting that the use of both commercial and autocthonous starter strains, might dominate over the local microbial population. *Lb. casei/paracasei* and *Ln. lactis* were found in traces in milk, according with Franciosi et al. (2009), and increased in cheese after 1 month of ripening, suggesting that these strains proceeded from the Malga and ripening room environment.

The species identified in cheese belonged to the genera *Streptococcus* (*Sc. dysgalactiae, gallolyticus, uberis* and *thermophilus*), *Lactococcus* (*Lc. garviae* and *lactis*), *Enterococcus* (*Ec. faecalis* and *faecium*), *Leuconostoc* (*Ln. mesenteroides* and *lactis*) and *Lactobacillus* (*Lb. casei/paracasei, parabuchneri* and *plantarum*). The BLAST analysis indicated the prevalence of *Lc. lactis ssp. lactis* and *cremoris* in CTRL, *Lc. lactis ssp. lactis* in STR and *Sc. thermophilus* in CMS cheeses. The fermentation process in CTRL cheeses has been probably leaded by *Sc. thermophilus* and *Lc. lactis ssp. cremoris* which accounted for the highest number of reads 24 hours following production (Table 4.2); after 1 month of ripening *Lc. lactis ssp. lactis* was dominant together with *Lc. lactis subsp. cremoris* and *Sc. thermophilus*, and was the main species at the end of ripening. By contrast, the fermentation of ST and CMS cheeses was carried on by *Lc. lactis ssp. lactis* and *Sc. thermophilus*, respectively, which dominated during the whole ripening process. We expected these results because the starter strains added during cheese production belonged to the species *Lc. lactis subsp. lactis* and *Sc. thermophilus*.

Table 4.2 Identification at the species level of representative *Lactobacillales* in the microbiota of milk, CTRL and STR. CMS cheeses after 24 hours (24hC), 1 (1MC) and 7 months (7MC) of ripening obtained by 454-pyrosequencing. In each column is signed the presence in reads number for each species.

Closest species	Milk	CTRL			STR			CMS		
		24hC	1MC	7MC	24hC	1MC	7MC	24hC	1MC	7MC
E. faecalis	94	48	363	310	338	129	109	n.r.	n.r.	n.r.
E. faecium	85	8	n.r.	n.r.	22	33	15	n.r.	n.r.	n.r.
E. raffinosus	58	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Enterococcus sp.	n.r.	n.r.	n.r.	n.r.	8	13	n.r.	n.r.	n.r.	n.r.
Lb. animalis	106	18	13	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Lb casei/paracasei	35	n.r.	495	262	13	922	1,050	n.r.	1,464	982
Lb. coryniformis	232	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Lb. parabuchneri	n.r.	n.r.	n.r.	42	n.r.	n.r.	35	n.r.	n.r.	n.r.
Lb. plantarum	n.r.	n.r.	20	115	n.r.	18	32	n.r.	39	20
Lactobacillus sp.	n.r.	n.r.	101	28	n.r.	84	117	1,251	636	419
Lc. garviae	749	26	52	30	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Lc lactis ssp. cremoris	73,611	6,196	5,644	1,857	8	8	n.r.	n.r.	8	n.r.
Lc. lactis ssp. lactis	5,195	1,033	4,087	17,444	17,815	16,984	18,913	11	82	82
Lactococcus sp.	53	9	17	61	54	63	87	n.r.	n.r.	n.r.
Ln. lactis	40	n.r.	19	47	n.r.	n.r.	n.r.	n.r.	13	8
Ln. mesenteroides	110	n.r.	103	264	n.r.	7	n.r.	n.r.	42	68
Sc. dysgalactiae	39	21	67	75	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Sc. gallolyticus	n.r.	625	180	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.
Streptococcus sp.	n.r.	30	26	n.r.	n.r.	49	44	39	127	97
Sc. thermophilus	96	7,858	4.830	344	496	3,790	3,041	14,258	20,379	19,786
Sc. uberis	12	50	243	46	15	32	14	n.r.	n.r.	n.r.

A high number of *Lb. casei* and *paracasei* reads was detected in all cheese samples after 1 and 7 months of ripening, suggesting their intervention during cheese maturation, as previously reported by Martìn-Platero et al. (2008). *Lb. plantarum* and *parabuchneri* were not detected in raw milk samples but were present in all cheeses after 1 or 7 months of ripening, suggesting that they proceeded from the Malga ripening environment.

As expected, *Ec. faecalis*, which is the most common microbial group in artisanal cheeses together with *Ec. faecium* (Gelsomino et al., 2002), was detected in both CTRL and STR cheese samples (at the end of ripening were found 310 and 109

reads, respectively), but not in CMS cheeses. In CTRL cheeses after 24 hours and 1 months of ripening the species *Sc. gallolyticus* was detected: this species was found by Fuka et al. (2013) in Croatian cheeses and is reported to be pathogen (Dumke et al., 2015). Thus, the use of both autocthotonous (*Lc. lactis subsp. lactis* 68 and *Sc. thermophilus* 93) and commercial (*Sc. thermophilus) s*tarter strain reduced the presence of undesirable species, such as *Sc. gallolyticus, Sc. dysgalactiae* and *Lc. garviae* that were found in milk and CTRL cheeses, but not in STR and CMS cheeses, suggesting that these species were resident in raw milk and did not proceed from the Malga-ripening environment.

Finally, looking at the relative abundance of each bacterial group, we might deduce that the inoculated starter cultures which led the early fermentation process, decreased the total availability of nutrients and consequently reduced the development of other species. This occurred especially when the commercial starter strain was added: in fact, in CTRL and STR cheeses after 7 months of ripening we detected 11 LAB species, while in CMS cheeses the number of species decreased to 8. This result is in accordance with Coppola et al. (2001) who reported that the biodiversity of spontaneously fermented cheeses was higher than cheeses inoculated with industrial starters.

4.3.4 Enumeration and isolation of TM-cheese resident LAB after simulated GI digestion

In Table 4.1 are shown the microbial counts of the 9 experimental TM-cheese samples before and after simulated gastro-intestinal digestion. After digestion, 6.7 ± 0.52 CFU/g of total bacteria were detected in the cheese samples, suggesting that TM-cheese resident LAB survived to digestion. The intrinsic characteristics of cheese (high fat content and semi-hard structure) might be responsible of LAB survival after gastro-intestinal transit, as much as the LAB strains' ability to resist to the extreme gastric and intestinal conditions.

In particular, TBC and thermophilic cocci significantly decreased from 7.3 ± 0.40 and 7.2 ± 0.18 CFU/g to 6.7 ± 0.52 and 6.9 ± 0.14 CFU/g, respectively. The

load of mesophilic cocci, lactobacilli and enterococci did not change significantly, suggesting that since these populations survived better *in vitro* during GI transfer, they might persist alive after *in vivo* digestion as well. This result is partially in accordance with Sumeri et al. (2012), who analyzed the microbial population of ripened semi-hard cheeses before and after transit through a GI tract simulator and observed that *Lb. casei*/*paracasei* species showed the highest survival and only 3% of mesophilic cocci resisted to digestion *in vitro*. Coliforms were not detected neither before nor after digestion.

The thirty-six isolates were considered putative LAB and were genotypically characterized after total DNA extraction. Twelve biotypes with 85% of similarity index were identified by analysing the RAPD patterns (data not shown): 3 *Lc. lactis subsp. lactis*, 2 *Lc. lactis subsp. cremoris* and 3 *Lb. paracasei*, were identified by species specific PCR. Conversely, 1 *Leuconostoc mesenteroides*, 1 *Ec. faecalis*, 1 *Lb. parabuchneri* and 1 *Lb. fermentum* were identified by partial 16S rRNA gene sequencing. The species *Lc. lactis subsp. lactis* and *Lb. paracasei* were also found in Finnish and Estonian cheese (Sumeri et al., 2012) after simulated GI digestion. Also the study conducted by Faye et al. (2012) proved that lactobacilli had better tolerance overall to the conditions mimicking the environment in the digestive tract and three strains belonging to *Lactococcus lactis* ssp. *cremoris* and *Enterococcus* species showed a good survival rate in fermented milk.

4.3.5 Survival of selected LAB strains during digestion

The microbial loads of pure cultures and fermented milk samples are shown in Table 4.3. All pure cultures contained between 8.9 and 9.9 Log CFU/mL bacteria; in fermented milk 10.2 ± 0.32 and 9.1 ± 0.27 CFU/mL mesophilic cocci and lactobacilli were registered, respectively. After the *in vitro* digestion, the load of the pure cultures decreased by 2.9-4.5 Log CFU/mL: *Lb. parabuchneri* D34 was the best resistant strain and, for this reason, was selected as inoculum for milk fermentation. The fermented whole milk samples showed a reduction of 4.3 ± 0.49 and 1.7 ± 0.45

Log CFU/mL onto M17 and MRS agar media, respectively (Table 4.3). These data suggested that *Lc. lactis subsp. lactis* 68 had a lower tolerance to GI tract than *Lb. parabuchneri* D34, which survived better in fermented milk than in pure culture. This result is in accordance with other authors who reported that bacterial strains used for the production of fermented milk, survived better than the same strains grown in culture media, and that some food matrices can protect bacteria from stress injury and increase the stress tolerance to the digestive tract (Faye et al., 2012; Huang and Adams, 2004; Leverrier et al., 2005). In particular, the good survival of some LAB strains within a model cheese system might be due to their ability to attach to the cheese matrix during digestion, indicating the production of extracellular polysaccharides (Pitino et al., 2012).

Table 4.3 Bacterial loads registered in the pure cultures (n = 12) and fermented milk samples (n = 3) are reported as mean value (MV, expressed in Log CFU/mL) and related standard deviations (SD). The milk samples were fermented for 24 h by *Lc. lactis subsp. lactis* 68 and *Lb. parabuchneri* D34, isolated from experimental TM-cheese after digestion. The microbial reduction detected after digestion is showed as value of delta (Δ= load before digestion−load after digestion).

	***Lb. paracasei* D20**		***Lb. fermentum* D25**		***Lb. parabuchneri* D34**		**Fermented milk**			
							M17		MRS	
	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD
Before digestion	9.9a	0.39	9.4a	0.52	8.9a	0.45	10.2a	0.35	9.1a	0.27
After digestion	5.4b	0.42	5.9b	0.26	6.0b	0.39	5.9b	0.41	7.4b	0.38
Δ	4.5	0.61	3.5	0.57	2.9	0.46	4.3	0.49	1.7	0.45

a. b: Different letters in the same column indicate significant statistical differences (Tukey's Test $p < 0.05$).

Furthermore, comparing the microbial counts of experimental cheese and fermented whole milk samples after digestion, is evident that *Lc. lactis subsp. lactis* 68 benefited significantly from the presence of the cheese as food matrix and vehicle during digestion.

4.4 Conclusions

The study showed that the addition of *Lc. lactis ssp. lactis* 68 and *Sc. thermophilus* 93 as starter culture kept the traditional high biodiversity of TM-cheese (see Carafa et al., 2015, 2016), did not standardized the final product at the microbiological level and suppressed the incidence of the pathogen *Sc. gallolyticus.* In particular, the starter strain *Lc. lactis ssp. lactis* 68 lead the early fermentation process of STR cheeses, whereas the role of *Sc. thermophilus* 93 remains unclear. The inoculated *Lc. lactis ssp. lactis* 68 might have a better fitness for Malga cheese environment, which affected the proliferation of both autochtonous and inoculated *Sc. thermophilus* strains. The results obtained after simulating *in vitro* the digestive processes of experimental cheeses, pure cultures and fermented milk samples suggested that TM-cheese might acts as protecting matrix during the GI transit because of the presence of fat (about 21%) and its semi-hard structure, but further investigations are needed.

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5. *Lactobacillus brevis* BT66 for the development of GABA-enriched cheese

Abstract

Probiotic microorganisms have recently been shown to impact on brain development and function through the gut:brain axis. *Lactobacillus brevis* BT66 isolated from Traditional Mountain cheese has been reported produce high concentrations of gamma-aminobutyric acid (GABA) and to possess bile salt hydrolysis activity *in vitro*. GABA is synthesized from glutamate, which is the most common amino acid in cheese. The aim of this study was to test the ability of the strain to convert glutamate to GABA during cheese production. Twenty experimental micro-cheeses were produced using a commercial starter strain (10^7 CFU/mL) and *Lb. brevis* BT66 as adjunct culture. Four different concentrations (10^2, 10^3, 10^4, 10^5 CFU/mL) of *Lb. brevis* BT66 were tested in quadruplicate. In order to follow the microbial evolution, samples of milk, curd and cheese after 20 days of ripening were enumerated in selective media. The control and experimental samples showed a similar trend, suggesting that both milk-resident and starter strains grew during ripening. However, the load of mesophilic lactobacilli in all experimental curd samples was higher than the control. The concentration of GABA and glutamic acid in cheese samples after 20 days of ripening was quantified by UHPLC-HQOMS. The amino acidic profiles showed that while the concentration of *Lb. brevis* BT66 in milk increased, the amount of both glutamic acid (from 284±97 to 202±44) and GABA (from 154±48 to 83±28) significantly decreased during cheese production. These results suggested that the experimental strain converted the glutamic acid to GABA, but that GABA may have subsequently been converted to succinate by GABA transaminases.

5.1 Introduction

Lactic acid bacteria (LAB) able to produce health-promoting metabolites are increasingly used in dairy applications for developing *multifunctional* products, which may have a regulatory activity in the human organism (Diplock et al., 1999; Leroy and de Vuyst. 2004; Settanni and Moschetti, 2011).

Notably, GABA impacts on brain function through the gut:brain axis system, regulates immune system, the inflammation process and the energy metabolism (Dhakal et al., 2012; Jin et al., 2013). The bioactivity of GABA has been demonstrated *in vivo* in both animals and humans: it plays an important role in preventing or alleviating anxiety, depression, hypertension, sleeplessness, memory loss, diabetes, increasing immunity, protecting against the effects of alcohol toxicity (Adeghate and Ponery, 2002; Hayakawa et al., 2004; Kajimoto et al., 2004; Miura et al., 2006; Okada et al., 2000). GABA is widely present in microorganisms, plants and animals (Ueno, 2000), but at low levels. Since the consumption of chemical GABA or its addition to food is considered unsafe (Li and Cao, 2010), it is necessary to develop new natural ways to increase the concentration of GABA in foods, and the exploitation of GABA-producing LAB could be a valid option. Actually, the increased GABA production has been already proven in tea leaves treated under anaerobic conditions (Tsushida et al., 1987), water-soaked rice germs (Saikusa et al., 1994), red mold rice (Kono and Himeno, 2000), fermented soy beans (Aoki et al., 2003), yoghurt (Park and Oh, 2007) and kimchi (Kim and Kim, 2012), and these products are already marketed.

As reported recently, many LAB strains- mainly belonging to *Lactobacillus* genus- are able to produce GABA (Carafa et al., 2015; Cho et al., 2007; Franciosi et al., 2015; Li and Cao, 2010; Siragusa et al., 2007). In particular, the species *Lb. brevis* has been found to produce high levels of GABA in dairy products (Hou et al., 2013) and has been successfully used for producing GABA-enriched black raspberry juice (Kim et al., 2008), milk (Wu et al., 2015) and yoghurt (Park and Oh, 2007). During milk fermentation, a high level of L-glutamate, which is present at high concentration in native caseins (Zoon & Allersma, 1996) may be theoretically

liberated as consequence of their proteolysis. For this reason, cheese might be a perfect vehicle for GABA, even though the manufacture process, the use of starter and/or adjunct cultures, the wild microbiota and the ripening conditions play a fundamental role on the production and the final content of GABA (Siragusa et al., 2007), as well as the pH and the amount of the precursor L-glutamic acid (Dhakal et al., 2012). Previously, *Lb. brevis* BT66 isolated from Traditional Mountain cheese showed to be able to produce high amount of GABA *in vitro* (Carafa et al., 2015). Consequently, in this study we decided to use this strain as adjunct culture for micro-cheeses production and test its ability to convert glutamic acid to GABA *in situ*, during cheese ripening.

5.2 Materials and Methods

5.2.1 Culture preparation and storage

Lb. brevis BT66 and a commercial *Sc. thermophilus* strain were grown anerobically in MRS at 30 °C and M17 at 45 °C, respectively. Cells were washed twice with phosphate buffer solution (PBS) 50 mM, pH 7 and suspended in NaCl 0.85%. The bacterial suspensions were diluted until reaching the optical density at 600 nm ranging between 2 and 2.5 (corresponding to about 9 Log CFU/mL). *Lb. brevis* BT66 was then inoculated (10%, v/v) in UHT whole milk (Latte Trento Sca, Trento) at 30 °C, and incubated overnight at 30 °C. Afterwards, 1 g/L of glutamic acid was added to the fermented milk and incubated at 30 °C for 24 h. Aliquots (8 Log CFU/mL) of fermented milk were frozen in liquid nitrogen and stored at -80 °C. Conversely, the commercial starter strain was diluted to 3 x 10^9 Log CFU/mL in a cryoprotectant solution composed of skim milk 10% (w/v), lactose 3% (w/v), yeast extract 0.1% (w/v), frozen in liquid nitrogen and stored at -80 °C, after the OD_{600nm} measurements.

5.2.2 Experimental "micro-cheeses" production and microbiological enumeration

Twenty experimental micro-cheeses were produced using the *Sc. thermophilus* starter and *Lb. brevis* BT66 as adjunct culture during two consecutive days at Department of Agronomy, Food, Natural Resources, Animals, and Environment (DAFNAE), University of Padova (Legnaro, Italy).

The final concentration of the commercial *Sc. thermophilus* was always 3.0 x 10^7 CFU/mL; whereas, the adjunct culture *Lb. brevis* BT66 was tested at different concentrations (10^2; 10^3; 10^4; 10^5 CFU/mL. Five experimental cheeses were produced in quadruplicate (2 cheese replicate/day), as follows: i) control cheeses (CTRL, n = 4), produced without addition of any starter; ii) BT66a cheeses (n = 4), produced inoculating the vat milk with the commercial *Sc. thermophilus* starter culture and *Lb. brevis* BT66 at the final concentration of 3.0 x 10^2 CFU/mL; iii) BT66b cheeses (n = 4), produced inoculating the vat milk with the commercial *Sc. thermophilus* starter culture and *Lb. brevis* BT66 at the final concentration of 3.0 x 10^3 CFU/mL; iv) BT66c cheeses (n = 4), produced inoculating the vat milk with the commercial *Sc. thermophilus* starter culture and *Lb. brevis* BT66 at the final concentration of 3.0 x 10^4 CFU/mL; v) BT66d cheeses (n = 4), produced inoculating the vat milk with the commercial *Sc. thermophilus* starter culture and *Lb. brevis* BT66 at the final concentration of 3.0 x 10^5 CFU/mL. All five experimental cheeses were produced simultaneously in 5 *micro-vats*, following the method described by Cipolat-Gotet et al. (2013), with some modifications. Briefly, 1,500 mL of raw cow's milk were heated at 37 °C, inoculated with the corresponding bacterial mixture, and 0.053 g calf rennet paste 1:115,000 (Clerici Sacco International, Cadorago, Italy) was added. After coagulation, curd was cooked at 55 °C for 10 min, cut into nut-size grains and held at this temperature for 10 more minutes. After extraction and moulding, the curds were pressed for 30 min at room temperature and salted for 30 min in a saturated brine solution (20%, w/v NaCl), and stored for 24 h at 28 °C and 85% of relative humidity and 1 week at 18 °C and 85% of relative humidity. Afterwards, the wheels were vacuum-packed and kept for 2 more weeks at 18 °C. The pH of milk, curd and cheese after 3 weeks of ripening was monitored using the

portable pH meter PT1000 (Knick, Berlin, Germany) equipped with a Hamilton electrode (Hamilton Bonaduz, Bonaduz, Switzerland).

Samples of vat milk (1 aliquot/replicate, n = 4), curd (n = 20) and cheese after 3 weeks of ripening (n = 20) were sampled for a total of 44 samples. Ten mL of milk were collected from the vat: 10 mL were immediately processed for microbial analysis: the microbial populations of milk, curd and cheese after 21 days of ripening was evaluated by plate counting onto selective agar media as described by Carafa et al. (2016).

5.2.3 GABA and glutamic acid detection by UHPLC-HQOMS

The amino acid composition of the micro-cheese samples was quantified by UHPLC-HQOMS (Ultra High Performance Liquid Cromatography - Orbitrap Q-Exactive Mass Spectrometry) after 3 weeks of ripening, at the Technology Transfer Centre, Fondazione Edmund Mach (FEM, San Michele all'Adige, Italy).

The samples were prepared as follows: 2 g of cheese were added to 0.4 g of sulfosalicylic acid, suspended in 29.7 mL of perchloric acid and 0.3 mL of β-glutamic acid and homogenized with a ULTRA-TURRAX® (IKA® e Werke GmbH & Co.KG, Staufen, Germany) for 10 min at speed 3. The suspensions were submitted to sonication for 30 min and centrifuged at 4,000 rpm for 20 min. The supernatant was filtered through a 0.22 µm pore size filter (Minisart, Sartorius Stedim Biotech, Goettingen, Germany). Six mL of this supernatant were added with an equal volume of pure methanol, mixed and centrifuged at 4,000 rpm for 10 min. Samples were filtered through a 0.22 µm pore size filter (Minisart, Goettingen) and diluted 1:50. The separation was carried out with formic acid 0.1% (v/v; eluent A; Sigma) and methanol with formic acid 0.1% (v/v; eluent B; Sigma) using a column Acclaim Trinity P1 3µm (2.1 x 100mm; Merk, Germany) at 35 °C. The flow rate was set at 0.4 mL/min. The analytical gradient for eluent B was: 1 min at 2%, 4 min at 30%, up to 50% in 0.5 min, to 100% in 0.5 min, held at 100% for 3 min for cleaning, and to

2% for reconditioning in 0.5 min. GABA and glutamic acid were detected by a UHPLC Ultimate 3000RS (ThermoScientific; Rodano, Italy).
Mass spectra were acquired in positive mode through a full MS analysis at mass resolving power of 70,000. For ionization, HESI II parameters were set as follow: heated capillary temperature to 330°C; sheath gas flow rate at 40 arbitrary units; auxiliary gas flow rate at 20 arbitrary units; spray voltage at 3.0 kV; auxiliary gas heater temperature at 300 °C.

5.2.4 Statistical analysis

All data were subjected to one-way analysis of variance (one-way ANOVA) using STATISTICA data analysis software system, version 9.1 (StatSoft, Inc. 2010 ww.statsoft.com). Differences were considered significant when $p < 0.05$.

5.3 Results and discussion

In this study, we evaluated the possibility to use the GABA-producing *Lb. brevis* BT66 as autochthonous adjunct culture for cheese production. The experimental cheese manufacture was laboratory-scale and performed in *micro-vats* in order to produce two cheese replicates per day (in two consecutive days) by using the same milk, reducing as much as possible the variability among samples. Thus, all the differences observed amongst experimental cheeses might be attributed to the increasing inoculum of *Lb. brevis* BT66. The ripening period was set at 3 weeks because the small size (diameter: 10 cm; weight: 100 g) of the micro-cheese wheels likely promotes a faster microbial development than in a medium (diameter: 20 cm; weight: 1 kg) or big (diameter: 40 cm; weight: 8 kg). After 7 days of ripening, all wheels were vacuum-packed in order to avoid an excessive water loss and the formation of a thick rind.

5.3.1 pH evolution during cheese production

pH values of milk, cheese 24 hours and 3 weeks following production are shown in Table 5.1. CTRL, BT66a, BT66b, BT66c, BT66d cheeses showed a similar pH evolution during cheese production and ripening, in fact no significant differences ($p>0.05$) were observed when *Sc. thermophilus* was added as starter and *Lb. brevis* BT66 as adjunct culture. The pH of all curd samples significantly decreased to less than 5.10 during the first 24 hours of ripening and to less than 4.82 at the end of ripening suggesting that the commercial *Sc. thermophilus* starter strain led the early fermentation process.

CTRL	**pH MV**	**SD**
Milk	6.68a	0.048
Curd	6.52a	0.058
24h-cheese	5.06b	0.068
3weeks-cheese	4.82b	0.061
BT66a		
Milk	6.68a	0.048
Curd	6.49a	0.031
24h-cheese	5.10b	0.13
3weeks-cheese	4.80b	0.15
BT66b		
Milk	6.68a	0.048
Curd	6.46a	0.029
24h-cheese	4.95b	0.079
3weeks-cheese	4.68b	0.081
BT66c		
Milk	6.68a	0.048
Curd	6.43a	0.025
24h-cheese	4.83b	0.051
3weeks-cheese	4.66b	0.060
BT66d		
Milk	6.68a	0.048
Curd	6.44a	0.034
24h-cheese	4.54b	0.17
3weeks-cheese	4.64b	0.067

Different letters (a, b) on the same column indicate significant differences ($p < 0.05$).

Table 5.1 pH dynamic of 5 experimental micro-cheeses (ST, BT66a, BT66b, BT66c, BT66d) during processing and ripening. All values are mean values (MV) and standard deviations (SD) of 4 samples of milk, curd, cheese 24 hours and 3 weeks following production.

The microbial biosynthesis of GABA is mainly regulated by pH, which ideally might range between 4.5 e 5.0, with an optimal value of 4.7 (Nomura et al., 1998). Thus, the registered pH evolution was definitively appropriate for GABA production. Therefore, the optimal pH value for the maximum GABA production is species-dependent (Dhakal et al., 2012): in fact, Soo et al. (2006) reported that *Lb. brevis* GABA 057 converted monosodium glutamate to GABA at pH 4.2.

5.3.2 Microbial content of milk, CTRL and inoculated cheeses

The microbial evolution of total bacteria, mesophilic and thermophilic cocci, mesophilic rod-shaped, enterococci and coliforms in milk, curd and cheese after 21 days of ripening are shown in Table 5.2. We did not analyze the microbial content of cheese 24 hours following production in order to avoid any contamination of the micro-wheels and influence the ripening process.

The load of all groups registered in curd was higher than milk, likely because of the physical retention of microorganisms in curds in addition to the microbial development during cheese production. The control and the test samples showed a similar trend in all fermentations, suggesting that milk-resident and starter strains grew during production and ripening. The mean TBC registered in vat milk samples was 6.4 ± 0.95 and increased between 7.8 ± 0.24 and 8.9 ± 0.32 Log CFU/mL in curd and between 8.0 ± 0.08 and 8.8 ± 0.52 Log CFU/mL in cheese after 21 days of ripening. The bacteria enumerated on M17 presented a similar trend and evolution throughout the ripening of the TBC. The load of mesophilic lactobacilli in both experimental curd (between 7.7 ± 0.11 and 7.9 ± 0.29 Log CFU/g) and cheese (between 8.3 ± 0.6 and 8.6 ± 0.7 Log CFU/g) samples was significantly higher than CTRL samples (6.7 ± 0.3 and 7.8 ± 0.30 Log CFU/g, respectively), confirming the growth of *Lb. brevis* BT66. Any significant difference in mesophilic lactobacilli counts was not observed increasing the inoculum of *Lb. brevis* BT66.

Lactobacilli, together with mesophilic and thermophilic cocci, showed the highest counts in inoculated cheese after 3 weeks of ripening, whereas CTRL cheese was dominated by only mesophilic and thermophilic cocci. These data suggested that *Lb. brevis* BT66 co-dominated in BT66a, BT66b, BT66c and BT66d cheeses and was not sub-dominated by the commercial starter strain.

Enterococci were detected in milk (4.4 ± 1.4 Log CFU/mL) and increased during production and ripening of about 3.3 - 3.7 logarithmic units. Some authors have highlighted the important role of enterococci during cheese ripening in developing the final sensorial characteristics (Giraffa, 2003; Franz et al., 1999; Suzzi et al., 2000).

Table 5.2 Mean Value (MV) and related standard deviation (SD) of microbial counts of milk (n_{tot} = 4), curd (n_{tot} = 20) and cheese after 3 weeks of ripening (n_{tot} = 20). MV is expressed in Log CFU/mL for milk and Log CFU/g for cheese samples.

	Total bacterial count		Mesophilic cocci		Thermophilic cocci		Mesophilic lactobacilli		Enterococci		Coliforms	
	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD	MV	SD
Milk	6.4	1.0	6.6	1.2	4.8	1.2	6.3	1.3	4.4	1.4	3.5	1.4
Curd												
CTRL	7.8	0.2	8.2	0.5	8.0	0.2	6.7a	0.3	7.9	0.2	2.5a	0.5
BT66a	8.0	0.2	7.9	0.3	7.8	0.2	7.7b	0.01	7.8	0.2	3.0a	1.1
BT66b	7.9	0.1	7.9	0.3	7.8	0.2	7.9b	0.3	7.9	0.1	0.9b	1.0
BT66c	7.9	0.2	7.9	0.4	7.8	0.3	7.7b	0.1	7.8	0.1	1.0b	0.1
BT66d	7.9	0.3	7.8	0.1	8.0	0.1	7.7b	0.1	7.9	0.1	3.1a	0.8
3 weeks-cheese												
CTRL	8.4	0.4	8.3	0.4	8.2	0.6	7.8a	0.3	8.1	0.5	0.0	0.0
BT66a	8.0	0.1	8.6	0.7	8.1	0.001	8.3b	0.6	7.7	0.3	0.0	0.0
BT66b	8.6	0.5	8.5	0.3	8.5	0.6	8.4b	0.5	7.8	0.5	0.0	0.0
BT66c	8.7	0.7	8.8	0.2	8.3	0.7	8.6b	0.7	7.8	0.7	0.0	0.0
BT66d	8.8	0.5	8.6	0.1	8.6	0.1	8.6b	0.3	8.1	0.2	0.0	0.0

Different letters (a. b) on the same column indicate significant differences ($p < 0.05$).

High concentration of coliforms in dairy products are related to inadequate hygiene practices during milk collection and cheese production (Farkye, 2000): 3.5 ± 1.4 Log CFU/mL of coliform were detected in milk samples but after 3 weeks of ripening, and fell below the limit of detection both in CTRL and inoculated cheeses, suggesting that the resident microbiota and the strains used as starter and adjunct cultures competed for the available energy sources and inhibited the coliforms growth.

5.3.3 GABA and glutamic acid concentration in micro-cheese samples

The amino acid profile of micro-cheese samples after 21 days of ripening showed that the CTRL cheese contained 324 ± 37 mg/kg of glutamic acid and 154 ± 31 mg/kg of GABA (Figure 5.1). In the experimental samples the amount of both glutamic acid and GABA decreased while increasing the concentration of *Lb. brevis* BT66: more specific, glutamic acid decreased from 287 ± 50 mg/kg to 202 ± 62 mg/kg, while GABA decreased from 136 ± 16 mg/kg to 91 ± 20 mg/kg in BT66a cheese and BT66d cheese, respectively. These reductions were significant ($p<0.05$) in BT66c and BT66d, where higher concentration (10^4 and 10^5 CFU/mL, respectively) of *Lb. brevis* BT66 was added during cheese manufacture (Figure 5.1).

The concentration of GABA in CTRL cheese is similar to that detected in Gouda (177.0 mg/kg), but much higher than Cheddar (48.0 mg/kg), blue (7.1 mg/kg) and Edam (4.2 mg/kg), all commercial cheeses made by using a starter culture, as reported by Nomura et al. (1998). Thus, GABA might have been produced in CTRL cheese by the autochtonous lactobacilli found in milk, which were not dominated during cheese production and ripening by the *Sc. thermophilus* used as starter strain, as proven by the enumeration of the microbial populations in cheese. The amount of glutamic acid detected in CTRL cheese in this study is similar to that detected in Cheddar cheese (400.0 mg/kg) after 30 days of ripening (Pouliot-Mathieu et al., 2013). As expected, increasing concentrations of *Lb. brevis* BT66 corresponded to

decreasing amount of glutamic acid and incresing glutamic acid consumption rate was observed in experimental cheeses.

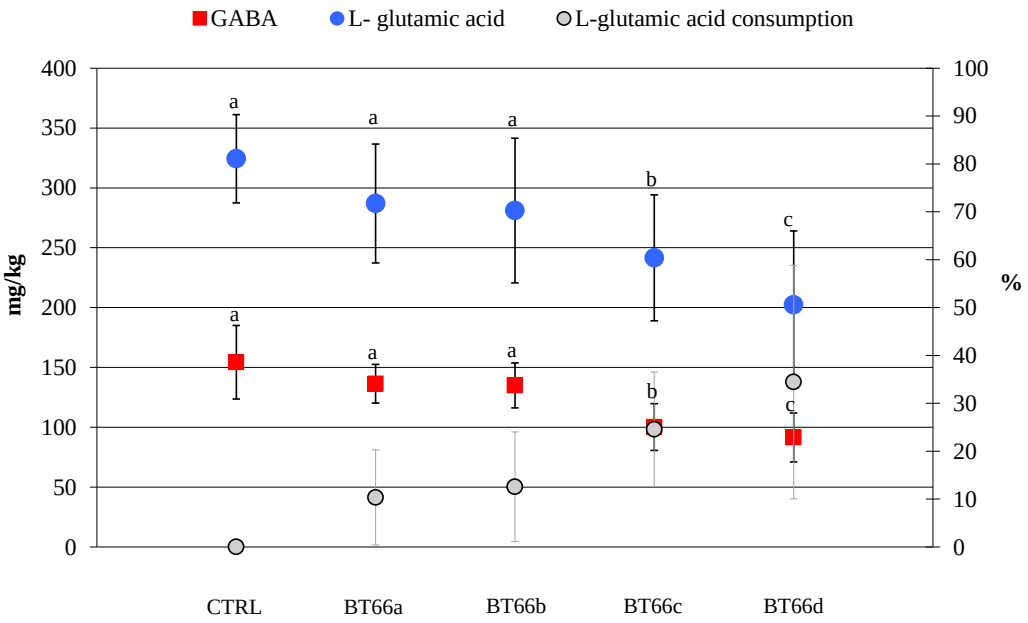

Fig. 5.1 GABA and L-glutamic acid concentration detected in micro-cheese samples after 3 weeks of ripening and L-glutamic acid consumption. The amount of both amino acids is expressed in mg/kg and the L-glutamic acid consumption is indicated as %. Different letters (a, b, c) on the same column indicate significant differences ($p<0.05$).

These results suggested that *Lb. brevis* BT66 was able to metabolize the glutamate naturally present in raw milk and to convert it to GABA in response to increasing inocula. Unexpectedly, GABA did not cumulate during ripening: it might have been converted to succinate by GABA-transaminases and used as energetic source by the cheese-resident microbiota.

5.4 Conclusions

The first experimental production of GABA-enriched cheese supported the hypothesis that *Lb. brevis* BT66 could be exploited as adjunct cultures, facilitating the *in situ* bio-synthesis of GABA during cheese ripening and providing an option to replace chemical GABA with natural GABA. Therefore, more tests need to be performed in order to confirm and optimize the GABA production in cheese. Many

parameters may affect the concentration of GABA in cheese (Siragusa et al., 2007): thus, micro-cheeses could be manufactured with thermized or pasteurized milk in order to avoid that GABA production is influenced by the wild microbiota during ripening, and microbiological and aminoacid content analysis of cheeses have to be performed at different ripening times.

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6. GABA-producing *Lactobacillus brevis* BT66 and DPC6108: animal trial set up

Abstract

Lactobacillus brevis BT66 and DPC6108 isolated from Traditional Mountain Italian cheese and the human gastrointestinal tract, respectively, were previously reported to produce high concentrations of γ-aminobutyric acid (GABA) and to possesses Bile Salt Hydrolysis (BSH) activity *in vitro*. Those strains were selected for testing in mice suffering obesity-associated type-2-diabetes. Both strains were sub-cultured at increasing concentrations of rifampicin in order to generate the rifampicin resistant mutants (rif). The genotypic profile of each mutant strain was obtained by RAPD-PCR and PFGE and was identical to the native strain. The conversion rates of monosodium glutamate to GABA were investigated by next-generation amino acid analysis: *Lb. brevis* BT66 produced 840.5 ± 266 µg/mL of GABA with about 73% of bioconversion and *Lb. brevis* DPC6108 produced 1,218.0 ± 393.2 µg/mL with about 87% of bioconversion. The BSH activity was positive to both qualitative and quantitative assays and the results were similar in both native and mutant strains. The rifampicin resistant strains were freeze-dried and tested for their stability at room temperature, +4 and -20 °C. Both spectrophotometer and plate count methods revealed that freeze-dried strains survived at room temperature during 24 hours after suspending in sterile water. The stability of freeze-dried strains at +4 and -20 °C was investigated enumerating the viable cells in selective medium during 10 weeks and no significant load reduction was detected in the first 4 weeks following freeze-drying. Both pharmabiotic-producing *Lb. brevis* BT66rif and DPC6108rif were resistant to freeze-drying, survived transit through the mouse gastrointestinal tract, and their therapeutic efficiency is being assessed *in vivo* to treat metabolic obesity and type-2-diabetes.

6.1 Introduction

Metabolic syndrome is a collection of cardio-metabolic risk factors that includes obesity, insulin resistance, hypertension, and dyslipidemia which is unequivocally linked to an increased risk of developing type 2 diabetes (T2DM) and cardiovascular disease (Roberts et al., 2013). Around 3.4 million adults die each year as a result of being overweight or obese. In addition, 44% of the diabetes burden, 23% of the ischemic heart disease burden and between 7% and 41% of certain cancer burdens are attributable to overweight and obesity (World Health Organization, 2014). Many medications are available for the management of hyperlipidemia and hyperglycemia but they have adverse effects. Therefore, the discovery and development of new substances that can safely inhibit obesity development and improve glucose metabolism will be of great benefit for slowing the development of T2DM and limiting its long-term complications (Tian et al., 2011).

The non-protein amino acid GABA has been reported to harbor an anti-obesity and anti-diabetogenic effect, as well as many other physiological functions in mammals including induction of hypotension, diuretic and tranquilizer effects, stimulation of immune cells (Hagiwara et al., 2004; Jakobs et al., 1993; Oh et al., 2003; Wong et al., 2003). Oral supplementation with GABA has previously demonstrated therapeutic effects against diet-induced type-2-diabetes in murine studies (Tian et al., 2011).

The BSH activity is also considered a health promoting effect because this activity may positively influence the host physiology (Jones et al., 2008), confers a selective advantage on the probiotic strains establishing in the human intestinal tract (Begley et al., 2006) and reduces the cholesterol level in the host blood (De Smet et al., 1994).

Two probiotic *Lb. brevis* strains, have been recently reported to convert monosodium glutamate (MSG) to high concentrations of GABA and to possess BSH activity *in vitro* (Barret et al., 2012; Carafa et al., 2015). Thus, they might increase glucose tolerance and insulin sensitivity and reduce the cholesterol levels *in vivo*. For this reason they were selected to be tested in an animal trial. The aims of this study

were: i) to generate *Lb. brevis* BT66 and DPC6108 rifampicin resistant strains (rif); ii) to compare the genotypic profile of the native and mutant strains, and their health-promoting properties; iii) to investigate the resistance and stability after freeze-drying.

6.2 Materials and methods

This study was done at the Alimentary Pharmabiotic Centre of the Teagasc Food Research Centre (Moorepark, Fermoy, Ireland).

6.2.1 Growth conditions and production of the rifampicin resistant mutants

Cells were grown over night in MRS (deMan-Rogosa-Sharp) broth medium pH 5.5 at 30 °C, anaerobically. The rifampicin resistant mutants were obtained by inoculating the grown cells (1%; v/v) in MRS with increasing concentrations of the rifampicin (10, 100, 250 and 500 μg/mL; Sigma Chemical Co., USA) and incubating them at optimal conditions. Pure cultures were kept at -80 °C in glycerol (20%; v/v) stocks.

6.2.2 Genotypic comparison of native and mutant strains

Both native and rifampicin resistant strains were subjected to Pulsed Field Gel Electrophoresis (PFGE) and Randomly Amplified Polymorphic DNA–PCR (RAPD–PCR).

6.2.2.1 PFGE

The cell suspension was mixed with an equal volume of 2% (w/v) low-melting-point agarose (Bio-Rad Laboratories, Richmond, Calif.) in 0.125 M EDTA (pH 7.6), dispensed into molds 10 mm long by 5 mm wide by 1 mm deep, and allowed to solidify at 4 °C. The agarose-cell mixture set within each mold was

referred to as a plug. Up to three plugs per strain were added to 1 mL of 1 M NaCl–6 mM Tris-HCl–100 mM EDTA–1% (w/v) Sarkosyl (Sigma), pH 7.6, containing 10 mg/mL of lysozyme (Sigma) and incubated overnight at 37 °C. The lysozyme solution was replaced with 1 mL of 0.5 M EDTA–1% (w/v) Sarkosyl, pH 8.0, containing 0.5 mg of proteinase K/mL (Sigma) and incubated at 37 °C overnight. This step was repeated with a fresh proteinase K solution. After two 1-h washes with 1 mM phenylmethylsulfonyl fluoride in 10 mM Tris-Cl–1 mMEDTA (pH 8.0) at 37 °C, the plugs were stored in 10 mM Tris-HCl–100 mM EDTA (pH 8.0) at 4 °C. Prior to incubation with the selected restriction enzyme, a 1-mm slice of the plug was washed three times for 15 min in 1 mL of 10 mM Tris-HCl–0.1 mM EDTA (pH 8.0) at room temperature. Each slice was incubated with 100 µl of the restriction buffer for 30 min at 4 °C and then replaced with 100 µl of fresh buffer containing 20 U of ApaI. Restriction digestion was performed for 30 min at 37 °C. Plug slices were loaded directly into the wells of a 1% (w/v) pulsed-field grade agarose (Bio-Rad Laboratories) gel and sealed with 1% agarose. DNA fragments were resolved with a contour-clamped homogeneous electric field DRIII pulsed-field system (Bio-Rad Laboratories) at 6 V/cm for 15 h with 0.5xTris base-borate-EDTA running buffer maintained at 14 °C. The pulse times were linearly ramped from 1 to 15 s. Gels were stained in distilled water containing 0.5 µg/ml of ethidium bromide for 30 min and destained for 60 min in distilled water.

6.2.2.2 RAPD-PCR

DNAs were extracted from MRS broth cultures using the GenElute™ Bacterial Genome DNA Kit (Sigma) following the manufacturer's instruction. RAPD–PCRs were carried out using the primer M13 (GAGGGTGGCGGTTCT; Huey and Hall, 1989), in an Applied Biosystems® 2720 Thermal Cycler (Thermo Fisher Scientific Inc.). 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 and stained with ethidium bromide (0.5 µg/L).

6.2.3 GABA production

The ability to produce GABA of both native and rifampicin resistant strains was investigated. Grown cells were inoculated (1%; v/v) in MRS broth pH 5.5 containing 3 mg/mL of MSG (from a stock solution of 300 mg/mL; Oxoid) and incubated anaerobically for 24 h at 30 °C. The native strains were tested using 3 and 30 mg/mL of MSG. Cells were harvested by centrifugation at 12,000 rpm for 5 min and discarded. Amino acids present in the supernatants were quantified using a Jeol JLC-500/V amino acid analyser (Jeol UK Ltd., Welwin Garden City, Herts, UK) fitted with a Jeol Na+ high performance cation exchange column. GABA production was expressed as μg/mL and percentage of bioconversion, the latter calculated by:

cGABA/cMSG x 100 = % bioconversion

cGABA = GABA concentration following fermentation

cMSG = consumed MSG

6.2.4 BSH activity

The BSH activity of both native and mutant strains was investigated qualitatively and quantitatively. The qualitative activity was tested on MRS-Thio agar plates supplemented with 0.5% (w/v) of taurodeoxycholic acid sodium or glycocholic acid sodium salt (Oxoid), as reported by Kumar et al. (2011). The quantitative BSH activity was tested following the method reported by Kumar et al. (2011), with some modifications: the cultures were inoculated at 1% (v/v) into fresh MRS-Thio broth and incubated at 30 °C for 16–18 h. Exponentially grown cells were harvested (12,000 g for 30 min at 4 °C), washed twice with peptone saline and suspended in 0.1 M-sodium acetate buffer (pH 5.0) to give an optical density of 2.5 at 600 nm. The bacterial suspensions (100 μL) were incubated at 37 °C with 100 μl of 20mM-conjugated bile salt (sodium salt of glycocholic or taurodeoxycholic acid). Reaction mixtures were incubated at 37 °C for 30 min. The enzymatic reaction was terminated by the addition of an equal volume (200 μl) of 15% TCA (w/v) followed

by centrifugation at 12,000 g for 15 min. The release of amino acids from hydrolysis of conjugated bile salts was measured using a Jeol JLC-500/V amino acid analyser (Jeol UK Ltd.) fitted with a Jeol Na+ high performance cation exchange column. The BSH activity was tested also for the *Streptococcus thermophilus* 84C, which has been recently reported as GABA producing strain (Franciosi et al., 2015). *Lb. mucosae* DPC6426 was used as positive control.

6.2.5 Freeze-drying process

The rifampicin resistant strains were grown and subcultured three times in MRS pH 5.5 with 500 μg/mL of rifampicin at optimal conditions, allowing the strains to grow to 10^9 CFU/mL. Cells were harvested by centrifugation at 7,000 g for 15 min at 4 °C (Sorvall RC5B Plus, SL-3000 rotor), washed twice in PBS and concentrated x20 by resuspending in 15% (w/v) trehalose. One-millilitre aliquots were dispensed into 2 mL vials & half-cap and freeze-dried by VirTis Wizard 2.0 Freeze Dryer (SP Industries Corporate Headquarters, Warminster, PA USA). The freeze-drying cycle had the following setting parameters: freeze temperature 240 μC, condenser set point 260, vacuum set point 600 mTorr. At the end of freeze-drying cycle, half of aliquot was stored at +4 °C, the other half at -20 °C.

6.2.6 Stability of strains to the freeze-drying process

The survival of both mutant strains following freeze-drying was tested in triplicate: at the end of the freeze-drying cycle, each aliquot was suspended in 1 mL of sterile water and three decimal dilutions were plated onto MRS agar pH 5.5 and incubated at optimal conditions.

Their viability at room temperature was evaluated during 24 h; the optical density (OD_{600nm}) of the bacterial solution was registered continuously with a GENion Plus plate reader (GENion Plus, Tecan, USA). In addition, three decimal dilutions were plated onto MRS agar pH 5.5 with rifampicin and incubated at

optimal conditions 0, 4, 8, and 24 h following freeze-drying. Their stability at +4 and -20 °C was evaluated enumerating the viable cells every week during 10 weeks by using the spread plate enumeration method onto MRS agar pH 5.5.

6.2.7 Animal trials

Two animal studies were designed in order to test the survival of *Lb. brevis* BT66rif during gastro-intestinal transit and the incidence of both *Lb. brevis* BT66rif and DPC6108rif on obese and T2 diabetic mice (Fig. 6.1 and 6.2).
The University College Cork Ethics Committee, under a license issued from the Department of Health and Children, approved all experimental procedures. The numbers of animals has been calculated using G power analyses (aiming for a power of 0.8-0.9 and significance value of $p<0.05$), and the numbers were increased by 15% in order to compensate any loss of animals due to natural causes or culling.

6.2.7.1 Survival of Lb. brevis BT66rif in mice: the pilot study

A pilot study was performed in order to test *in vivo* the survival of *Lb. brevis* BT66rif after gastro-intestinal digestion, as shown in Fig. 6.1. Six healthy C57BL/6J (males, 3 weeks old) mice were housed under standard conditions (room temperature of 21°C, 12-h light–dark cycle, lights on at 07:00) and allowed to acclimatise for one day before starting the pilot study. All animals had free access to food and water: fresh water was daily replaced and contained *Lb. brevis* BT66rif (each mouse was expected to drink at least 5 mL of water/day and to ingest 1 x 10^9 CFU/day).

6.2.7.2 Enumeration of Lb. brevis BT66 in *faecal samples*

Mice faecal samples were collected 0, 3 and 7 days following the beginning of the treatment. *Lb. brevis* BT66 strain was enumerated after plating serial dilutions on MRS agar supplemented with 100 μg/mL of rifampicin and incubating anaerobically for 48 h at 30 °C. Colonies isolated from MRSrif agar were genotypically compared

by RAPD-PCR (as described above) with both wild and mutant *Lb. brevis* BT66 and tested for GABA production in order to ensure that gastric transit did not affect the ability of *Lb. brevis* BT66 to produce GABA.

6.2.7.3 GABA detection in serum and mice tissues

After 1 week of treatment, mice were euthanised by decapitation and blood samples were collected, allowed to clot at 4 °C, and centrifuged for 20 min at 2000 x g for serum collection. Duodenum, jejunum, ileum, caecum, proximal colon, distal colon, liver, adipose tissue were removed and flash-frozen on dry ice. All samples were stored at -80 °C prior to analyses. The tissues were diluted (10%, w/v) in a Tissue Lysis Solution (0.01 N HCl, 1 mM EDTA, 4mM sodium metabisulphite) and homogenized by using a Tissue Lyser II (QIAGEN, Germany). GABA was detected in serum and tissue homegenates by using the GABA ELISA kit (Immusmol, France), following the manufacturer instructions.

6.2.7.4 Influence of GABA-producing Lb. brevis BT66rif and DPC6108rif on mice with high fat diet induced type 2 diabetes

Lb. brevis BT66rif and DPC6108rif were selected for the treatment of obesity and T2-diabetes and are currently under investigation. The animal trial (still in progress) includes six groups of C57BL/6J mice (14 mice/group): mice are housed under standard conditions (room temperature of 21°C, 12-h light–dark cycle, lights on at 07:00) and allowed to acclimatise for one week before starting the study. As shown in Fig. 6.2, five groups are fed daily for up to 28 weeks with a high fat diet in order to induce obesity and T2 diabetes, and one group is fed with a control diet. After 12-16 weeks of high fat feeding, each group is treated for 8-12 weeks with *Lb. brevis* BT66rif, *Lb. brevis* DPC6108rif, the drugs metformin and vildagliptin (generally used for T2 diabetes therapy) and placebo, respectively. The beginning of the treatment depends on how quickly animals develop glucose intolerance and obesity. All animals had free access to food and water: fresh water was daily replaced and

contained *Lb. brevis* BT66rif or DPC6108rif (each mouse was expected to drink at least 5 mL of water/day and ingest 1 x 10^9 CFU/day), or metformin (300 mg/kg body weight) or vildagliptin (15 mg/kg body weight).

The body weight is assessed weekly. Faecal samples are collected weekly and analysed for metabolomics and microbiological content by plate count and next generation sequencing (Illumina system). Samples of serum, duodenum, jejunum, ileum, caecum, proximal colon, distal colon, liver, adipose tissue will be collected at the end of the study, after culling, and will be processed for metabolomics, GABA quantification, next generation sequencing, insulin, cholesterol, triglyceride, cytokines and chemokines detection, fat composition, gene expression.

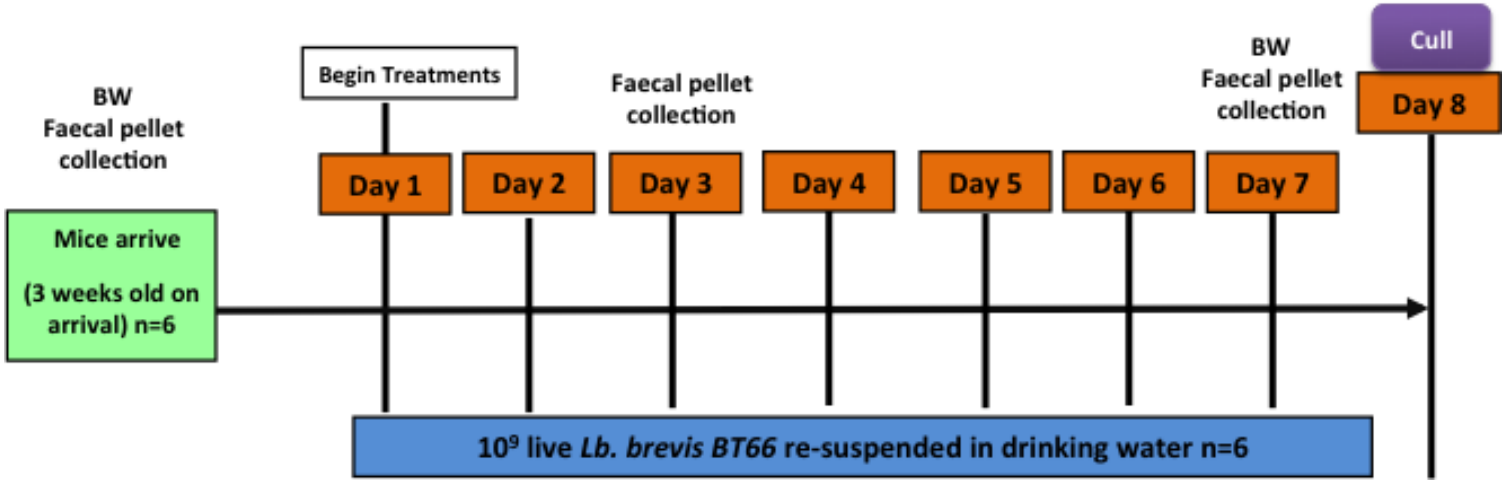

Figure 6.1 Schematical view of the pilot study. Six healthy C57BL/6J (males, 3 weeks old) mice were administered with *Lb. brevis* BT66rif (1 x 10^9 CFU/day) during 1 week after one day acclimatisation. Faecal samples were collected 0, 3 and 7 days following the beginning of the treatment. At day 8 animals were culled.

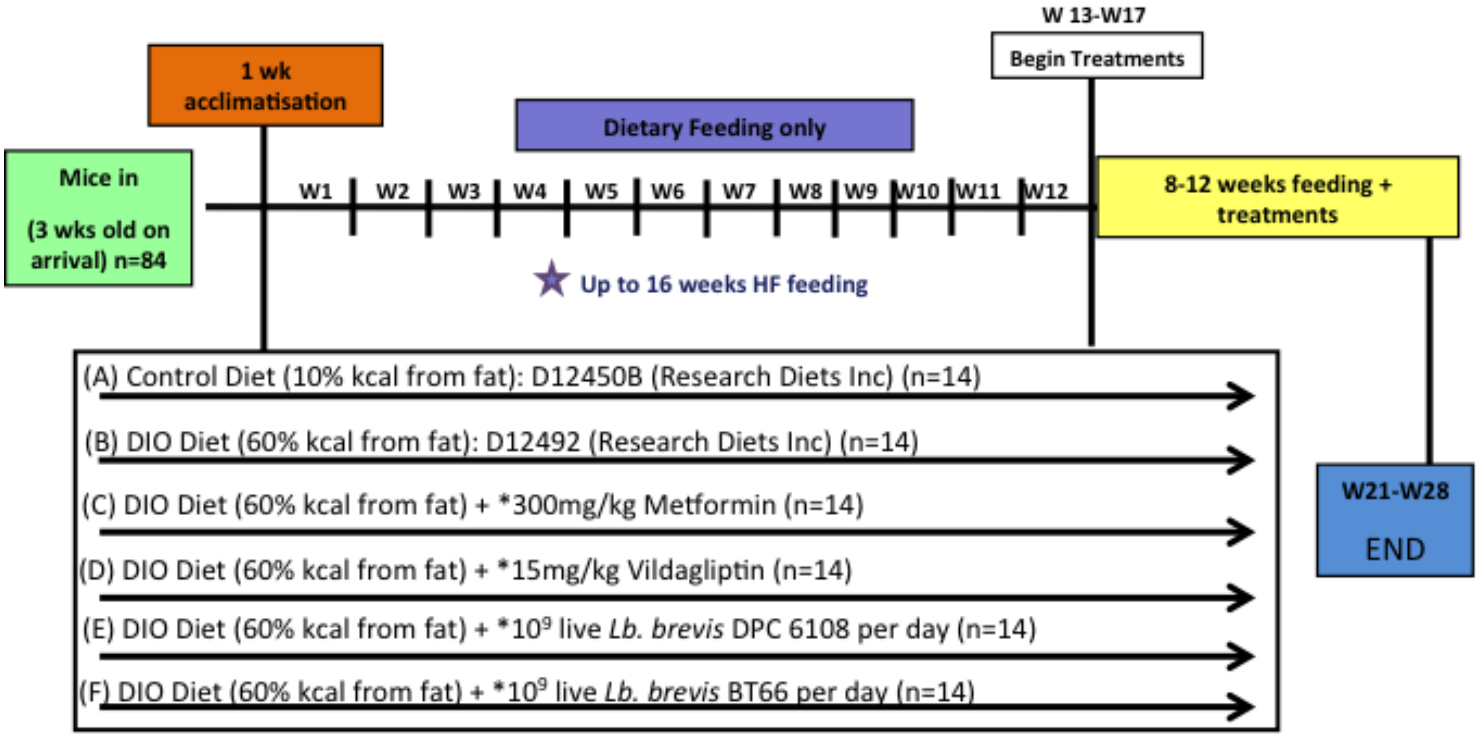

Figure 6.2 Schematical view of the animal trial that is currently in progress. Six groups of healthy C57BL/6J (males, 3 weeks old) mice (n_{tot}=84; 14 mice/group) are allowed to acclimatise during 1 week. Then, one group are fed with a control diet, and five groups were fed with high fat diet (60% kcal from fat). After 12 weeks (up to 16), the five groups are administered during 8 up to 12 weeks with *Lb. brevis* BT66rif, *Lb. brevis* DPC6108rif (1 x 10^9 CFU/day), Metformin, Vildagliptin and placebo, respectively.

6.3 Results and discussion

6.3.1 Genotypic comparison of native and mutant strains

The genotypic profile of both *Lb. brevis* BT66 and DPC6108 rifampicin resistant mutants was identical to the respective native strain. This result suggests that the acquisition of the antibiotic resistance, via antibiotic resistance gene acquisition by the plasmid or chromosome or via vertical transmission by resistant cells (Levy, 1998) did not affect the strains' genotype.

6.3.2 GABA production

The tested strains produced high concentrations of GABA (Fig. 6.3). The ability to convert MSG to GABA was investigated 24 hours following incubation because both strains reached the logarithmic growth phase between 11 and 24 hours (data not shown). In detail, better bioconversion rate of MSG by the native strains was observed when the concentration of precursor was 3 mg/mL than 30 mg/mL (Fig. 6.3a). The native strain BT66 produced 840 ± 266 μg/mL of GABA with a bioconversion percentage of 73 ± 28; the respective rifampicin resistant strain generated 964 ± 180 μg/mL of GABA with a bioconversion percentage of 70 ± 13 (Fig. 6.3b). The native strain *Lb brevis* DPC6108 converted 87 ± 19% of MSG and produced 1,218 ± 393 μg/mL; DPC6108 mutant strain produced 1,034 ± 265 μg/mL with a bioconversion of 75 ± 20% (Fig. 6.3b). The concentration of GABA produced by the mutant strains was not significantly different compared with the native strain, suggesting that the rifampicin resistant strains did not lose the ability to convert MSG to GABA (Fig. 6.3b).

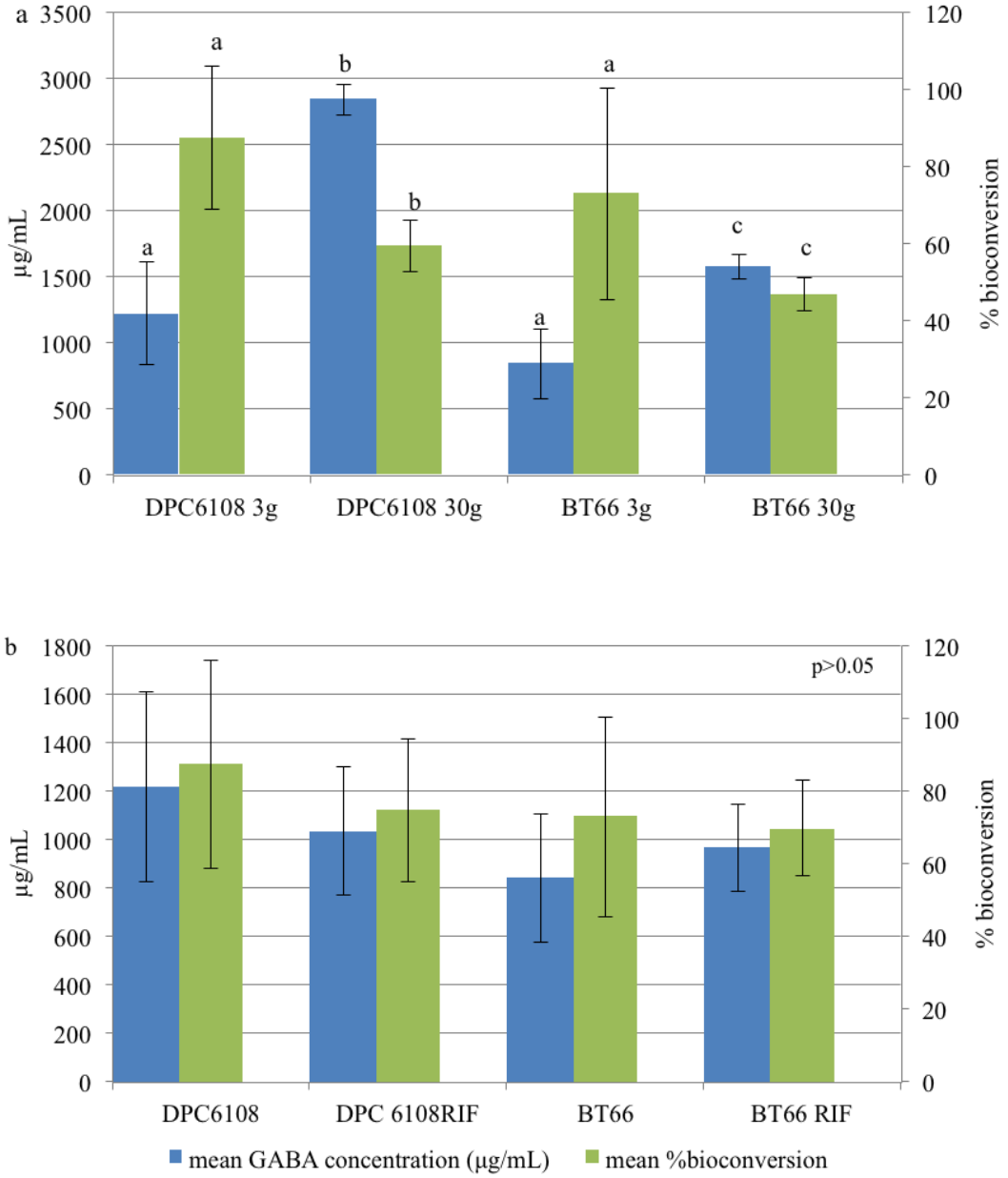

Fig. 6.3 (a) Concentration of GABA produced by *Lb. brevis* BT66 and DPC6108 after 24 hours of incubation in MRS pH 5.5 containing 3 and 30 mg/mL of MSG at 30 °C. Different letters (a, b, c) on the same column indicate significant differences ($p < 0.05$). **(b)** The ability of the rifampicin resistant strains to produce GABA in MRS pH 5.5 containing 3 mg/mL of MSG was compared with the native strains. The bioconversion rate was calculated by: cGABA/cMSG x 100 = % bioconversion (cGABA = GABA concentration following fermentation; cMSG = consumed MSG). The amount of GABA detected in each mutant strain was not significantly different ($p>0.05$) from the respective native strain.

6.3.3 BSH activity

The BSH activity of *Lb. brevis* BT66, *Lb. brevis* DPC6108 and *Sc. thermophilus* 84C is shown in Figure 6.4.

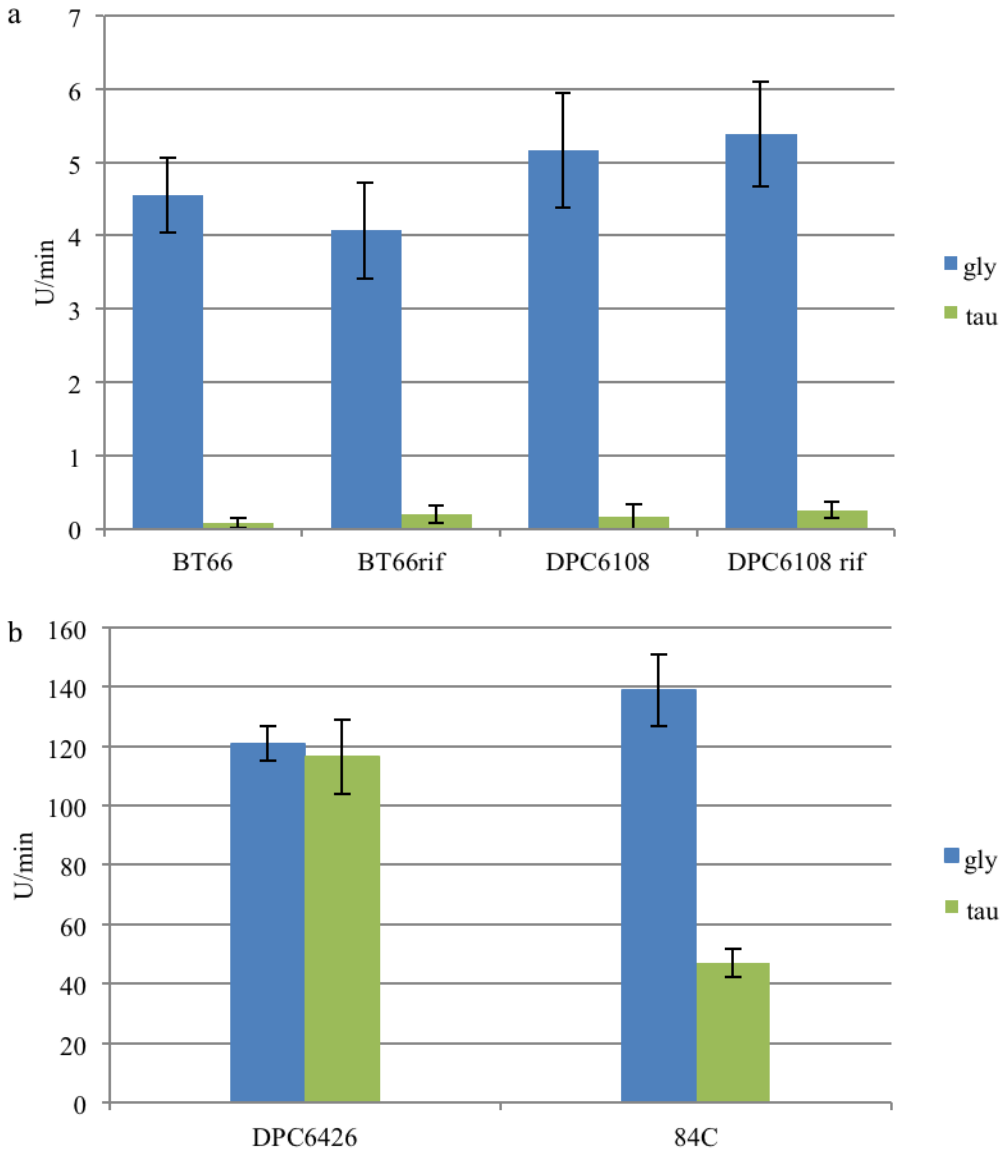

Figure 6.4 BSH activity: the release of amino acids from hydrolysis of conjugated bile salts was detected by HPLC and expressed in U/min. **(a)** BSH activity of *Lb. brevis* BT66 and DPC6108: any significant difference was not detected between native and mutant strains. **(b)** BSH activity of *Sc. thermophilus* 84C.

Strain BT66 had 4.5 ± 0.5 U/min of activity releasing glycine and 0.080 ± 0.006 releasing taurine; strains DPC6108 had 5.1 ± 0.8 U/min of activity releasing glycine

and 0.160 ± 0.002 releasing taurine. The mutant strains did not show significant differences for BSH activity against glycocholic and taurodeoxycholic acid salts ($p > 0.05$), confirming that no loss of activity occurred after acquiring the antibiotic resistance. *Sc. thermophilus* 84C showed a good BSH activity with 139 ± 12 U/min against glycine and 47 ± 5 U/min against taurine. This value was similar to the positive control DPC6426.

6.3.4 Stability of strains to freeze-drying process

Both mutant strains showed to be resistant to freeze-drying process. Furthermore, they were stable at room temperature during 24 hours and at +4 and -20 °C, as shown in Table 6.1 and 6.2. The load of both *Lb. brevis* BT66rif and DPC6108rif strains was not significantly different before and after freeze-drying (Table 6.1). The stability of strains in water at room temperature during 24 hour was confirmed by measuring the OD of the bacterial suspension and by plate counting. No significant differences ($p > 0.005$) were observed after 4, 8 and 24 hours.

Table 6.1 Stability of *Lb. brevis* BT66rif and DPC6108rif post-freeze-drying at room temperature during 24 hour. Freeze-dried strains were suspended in water and enumerated after 0, 4, 8 and 24 hours. The load of both strains is shown as mean value (MV) and standard deviation (SD), and did not significantly changed before and after freeze-drying ($p > 0.05$).

Strain	Time (h)	Log CFU/mL	
		MV	SD
BT66 rif	b.f.	10.1	0.2
	0	10.1	0.3
	4	10.1	0.3
	8	10.1	0.1
	24	10.1	0.2
DPC6108 rif	b.f.	10.0	0.2
	0	10.1	0.1
	4	10.0	0.2
	8	9.8	0.4
	24	9.8	0.2

b.f. = before freeze-drying process

Lb. brevis BT66rif was stable at both +4 and -20 °C during 10 weeks: in fact, the microbial load was not significantly different to that registered immediately after freeze-drying (time 0) (Table 6.2). Conversely, the load of the strain *Lb. brevis* DPC6108rif did not significantly change at +4 °C during 5 weeks and at -20 °C

during 6 weeks. This stability allows the production of freeze-dried strain aliquots and store them at +4 or -20 °C without any significant reduction of viability.

Table 6.2 Survival of BT66rif and DPC6108rif at +4 and -20 °C during 10 weeks post-freeze-drying. The freeze-dried strains were suspended in water and enumerated after 0, 1, 2, 3, 4, 5, 6, 8 and 10 weeks .

Strain	**Time (weeks)**	**Log CFU/mL**		**Log CFU/mL**	
		MV	SD	MV	SD
BT66rif	t0	9.48	0.98		
		+4 °C		**-20 °C**	
	t1	8.9	0.9	8.8	0.9
	t2	8.2	0.9	9.4	1.0
	t3	8.4	0.9	9.4	1.0
	t4	9.2	1.0	9.2	1.0
	t5	9.1	1.0	9.5	1.0
	t6	8.3	0.9	9.4	1.0
	t8	8.6	0.9	9.0	1.0
	t10	9.4	1.0	9.4	1.0
DPC6108rif	t0	9.15	0.96		
		+4 °C		**-20 °C**	
	t1	8.1a	0.9	8.5a	0.9
	t2	9.5a	1.0	8.9a	0.9
	t3	9.5a	1.0	8.2a	0.9
	t4	9.2a	1.0	9.1a	1.0
	t5	9.0b	0.4	8.8a	1.0
	t6	8.3b	0.4	9.2b	0.4
	t8	8.7b	0.4	8.5b	0.5
	t10	8.7b	0.6	8.6b	0.4

Different letters (a, b) on the same column indicate significant differences ($p < 0.05$).

6.3.5 Lb. brevis BT66rif survived mouse gastro-intestinal transit

The presence of the administered rifampicin-resistant *Lb. brevis* BT66 strain in the faeces of mice confirmed its survival during gastro-intestinal transit. As expected, any rifampicin resistant strain was not detected at day 0 (before starting the treatment), while 7.01 ± 0.38 Log CFU/g of *Lb. brevis* BT66rif were enumerated in faeces after 3 days of treatment and remained at similar load until the end of the pilot study (the load at day 7 was 7.44 ± 0.26 Log CFU/g) (Fig. 6.5). All colonies isolated from MRSrif agar had the same RAPD-PCR profile and produced similar quantities of GABA *in vitro* when compared to the wild type strain. These results demonstrated

that the administred *Lb. brevis* BT66rif strain survived gastro-intestinal transit in mice and did not lose the ability to produce GABA after digestion.

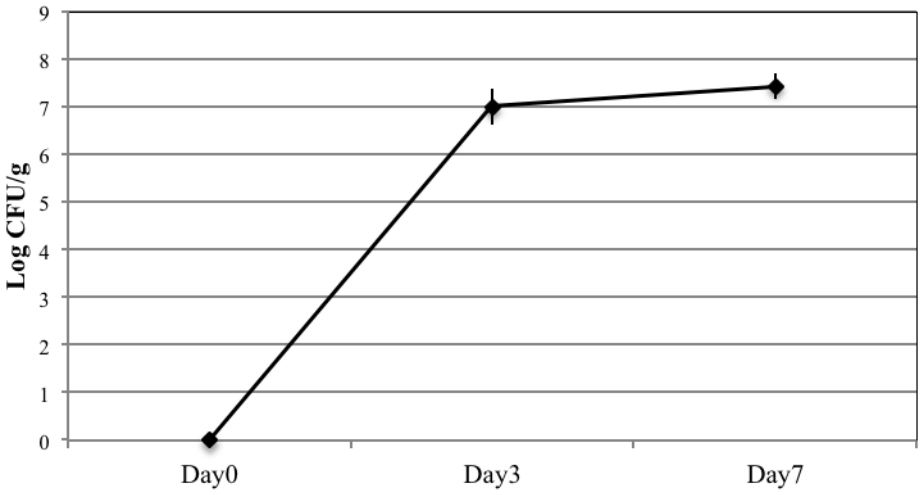

Figure 6.5 Load of *Lb. brevis* BT66rif in mice faecal samples enumerated onto MRSrif 0, 3 and 7 days following the commencement of the pilot study.

6.3.6 GABA in mice tissues

Several authors investigated the presence of GABA in the brain tissue and pancreatic islets of mice and rats, or the expression of GABA receptors in other murine organs (Borden et al., 1992; Enna and Snyder, 1976; Okada et al., 1976; Watanabe et al., 2002). In this study, we explored the concentration of GABA *in vivo* in different mice tissues and the results are shown in Table 6.3.

In serum 149 ± 85 μg/mL GABA were detected; amongst the analysed tissues, the highest concentration of GABA was recorded in the intestine, in particular in the duodenum (31,126 ± 205 μg/g), ileum (32,288 ± 134 μg/g) and caecum (26,429 ± 209 μg/g).

Table 6.3 Concentration of GABA in serum, intestine (duodenum, jejunum, ileum, cecum, proximal and distal colon), liver and adipose tissue detected by ELISA kit. The mean values (MV) and the standard deviations (SD) are expressed in μg/mL (serum) or mg/g (tissues).

Sample	**MV (μg/mL)**	**SD**
Serum	149	85
	MV (μg/g)	**SD**
Duodenum	31,126	205
Jejunum	17,899	133
Ileum	32,288	134
Caecum	26,429	209
Proximal	8,153	419
Distal	5,090	299
Liver	14,912	707
Adipose tissue	9,151	409

The limitation of this analysis was the absence of a negative control group, since the main objective of the pilot study was testing the survival of *Lb. brevis* BT66 after transit through the mouse gastro-intestinal tract. However, these data might be useful in future and compared with those obtained at the end of the animal trial.

6.4 Conclusions

Lb. brevis BT66 and DPC6108, previously reported for their ability to produce GABA, were examined for their use in an animal trial. Both strains exhibited optimal attributes to be used as probiotic in a preclinical study, survive transit through mouse gastro-intestinal tract and their therapeutic efficiency is being assessed *in vivo* for treating metabolic obesity and T2 diabetes.

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Final remarks

This study was aimed to characterize the autochthonous LAB established in TM-cheese, and to select strains to be used as starter or adjunct cultures for cheese production. The results confirmed that the Malga environment harbors a high biodiversity of wild LAB with good attitude to dairy production and bioactive properties. Among all tested isolates, *Lc. lactis subsp. lactis* 68 and *Sc. thermophilus* 93 showed the best technological performance and were tested as starter cultures during experimental TM-cheese production. The use of these strains did not reduce the traditional high biodiversity of TM-cheese, did not standardize the sensory attributes of the final product and suppressed the incidence of the pathogen *Sc. gallolyticus*. Furthermore, *Lb. paracasei* BT18 and *Lb. rhamnosus* BT68 harbored the most interesting technological and health-promoting properties and are currently in use as adjunct cultures for the production of experimental TM-cheeses, in combination with *Lc. lactis ssp. lactis* 68 and *Sc. thermophilus* 93.

The study became more complex and interesting when the one and only *Lb. brevis* (strain BT66) isolated from TM-cheese produced high concentrations of GABA *in vitro*. Thus, *Lb. brevis* BT66 was used as adjunct culture during micro-cheese-making processes: the first results supported the hypothesis that it might be exploited for producing GABA-enriched cheese, and more tests need to be performed. Furthermore, *Lb. brevis* BT66 showed to survive transit through mouse gastro-intestinal tract during a pilot study, and was selected to be used *in vivo* (together with the GABA producing *Lb. brevis* DPC6108). Currently, its therapeutic efficiency is being assessed for treating metabolic obesity and T2 diabetes in mice.

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Memento audere semper

(Gabriele D'Annunzio)