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Microbial Diversity and Sensorial Properties of Malga Cheese from Trentino (Italy) after Long-Term Ageing Period

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HIGHLIGHTS

- Malga cheeses contained a very concentrated microflora after 16 months of ageing.
- No pathogen microorganisms were found in Malga cheeses aged for 6 and 16 months.
- Use of Alpine-selected lactic acid bacteria ensured quality and safety of cheeses without reducing microbial biodiversity.

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Acronyms and abbreviations
CFU=Colony Forming Unit
FBP=Fermalga Bacterial Pools
LAB=Lactic Acid Bacteria
TMC=Total Microbial Count

ABSTRACT

Background: Malga cheeses are made in artisanal and seasonal dairies located in the Alps. This study was carried out to determine microbial diversity and sensorial properties of Malga cheese from Trentino (Italy) after long-term ageing period. The effects of adding the Fermalga Bacterial Pools (FBP) on microbiota biodiversity of cheese were also evaluated.

Methods: Populations of Lactic Acid Bacteria (LAB), mesophilic/thermophilic lactobacilli, mesophilic/thermophilic lactococci, coliforms, *Enterococcus* sp., and *Streptococcus* sp. was evaluated by plate count in 38 cheese samples after 6 and 16 months of ageing. Sensorial analysis of cheeses was performed by a panel of 30 expert judges. Statistical analysis of the data was carried out using STATISTICA v. 8.0.

Results: The total microbial count of medium-aged (6 months) cheeses was significantly ($p<0.05$) higher than that of long-aged (16 months) cheeses. Coliforms as well as *Staphylococcus* sp. were not detected in all samples. Thermophilic LAB were the relevant (45%) population in cheese made by FBP; while mesophilic LAB, mainly lactobacilli were the relevant (87%) population in the non-inoculated cheeses. The sensorial scores of 16 months-aged cheeses were significantly ($p<0.05$) higher than that of 6 months samples. However, there was no significant ($p>0.05$) difference between sensorial scores of Fermalga and non-Fermalga cheeses.

Conclusion: The protection of traditional fermented foods can occur by innovative technologies, making them safe without altering their typical features. This preliminary investigation on the characteristics of Alpine Malga cheeses showed that the addition the FBP had no undesirable effect on the organoleptic characteristics of the cheeses.

Introduction

Cheese has been produced in the Alpine pastures from ancient time which is valuable in preserving a peculiar and fragile environmental and social ecosystem. In summer, the production of cheese in the Alpine pastures

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takes place in small artisanal dairies called Malghe (Bergamaschi et al., 2016). There are relevant differences among the Malga cheeses produced in the Alpine regions but, in general, cheeses are made by raw, whole or semi-fat milk. The curd is heated, usually performed with the direct heat of a wood fire, reaching a temperature not exceeding 47 °C; then, the cheeses with an average weight of 5-12 kg, are aged for a minimum of 3 months up to 2 years (Bergamaschi et al., 2016). Due to the peculiar composition of milk obtained during summer pasture and the features of native microbiota, these cheeses have distinctive organoleptic qualities and are source of nutraceutical compounds (Akalin et al., 2002; Carafa et al., 2015; Enri et al., 2017; Poznanski et al., 2004).

Today, the production of Malga cheese is threatened by two critical issues. Due to the extreme environmental conditions during cheese-making, Malga cheeses are frequently affected by some defects. Early swelling, excessive proteolysis, and appearance of unpleasant aromas or tastes are mainly due to an incorrect succession of microbes in the early stages of cheese making (Brandle et al., 2016; Doyle et al., 2015; Guzzon et al., 2017), resulting in significant economic losses. Another criticism is related to the hygienic risks for consumers. The technological limitations encountered in Malga might favor the occurrence of pathogens. In industrial dairies, the pathogens can be eliminated by thermal treatment (e.g. milk pasteurization) together the use of selected cultures of Lactic Acid Bacteria (LAB) preventing the growth of spoilage microbes (Lucchini et al., 2018; Van Hoordea et al., 2010). In Malga cheese making, milk is not pasteurized; because the thermal treatment eliminates the native microflora and finally alters the peculiar characteristics of cheeses (Nasrollahi et al., 2016). Furthermore, the biodiversity in cheese microbiota could be compromised by the use of selected cultures of LAB having high capacity of acidification of the curd. However, given the increasing numbers of food-related diseases, due to the presence of *Escherichia coli* in non-thermally treated foods of animal origin (Ombarak et al., 2016; Ranjbar et al., 2018; Sarowska et al., 2019), it is particularly important to monitor the microbiological quality of Malga cheeses and find solutions to improve their safety.

In Trentino, an Italian region of the central Alps, at least 100 cheeses producing Malghe are still active (Franciosi et al., 2013). In recent years, the Fermalga project, initiated by the Edmund Mach Foundation, has led to the creation of 6 different bacterial cultures selected from the local Alpine pastures. The addition of the Fermalga Bacterial Pools (FBP), containing strains of *Streptococcus* sp. and *Lactobacillus* sp., to the raw milk prior to the cheese-making has been tested with the intention of reduce defects in cheeses and elimination of the food-borne pathogens (Carafa et al., 2012, 2015; Schiavon et al., 2019).

This work presents the results of a survey concerning the nature of microflora in Malga cheeses after 6 and 16 months of ageing, approaching microbiological control in accordance international standards. The effects of adding the FBP on microbiota biodiversity of cheese were also evaluated. In addition, sensory analysis was done to assess whether any relation exists between the microflora present during the ageing period and the organoleptic qualities of Malga cheese.

Materials and methods

Production of Malga cheese

Totally, 38 cheeses coming from 4 regions of Trentino Province were considered in this work (Table 1), including 18 medium-aged (6 months) and 20 long-aged (16 months) cheeses. The cheeses were made by mixing raw skimmed cow's milk obtained by evening and morning milking sessions. After the addition of commercial rennet, curdling occurs at temperatures in the range of 35-38 °C. Natural starter cultures, made by milk thermally treated at 60 °C and stored at 45 °C for 12 h, were added traditionally to the milk. The curd was cooked at about 43-47 °C, using a wood fire, for 30 min and cut into rice grain-sized particles. After cooking, the curd was separated from the whey. After two days of brine salting, the cheeses were ripened for at least two months, in a dedicated warehouse with temperature of maximum 20 °C, depending on the external weather conditions. The cheeses were seasoned on wooden shelves and turned over every two days to avoid the proliferation of molds or spoilage bacteria (Guzzon et al., 2017). Ripening period of the cheese was completed at the end of the summer.

FBP cultures

FBP were composed of a mixture of 5 bacterial strains belonging to the species *Streptococcus thermophilus* (n=3), *Lactobacillus delbruekii sup. Lactis* (n=1) and 1 non-starter strain belonging to the genera *Lactobacillus* sp. (species *L. paracasei*, *L. rhamnosus* or *L. plantarum* in function of the different pastures). Bacteria were isolated from 6 Alpine areas of the Trentino Province, Italy in the years 2006-2010. FBP pools were produced in lyophilized form by Bioagro Srl (Thiene I, Italy), with a nominal concentration of 3.3×10^{10} Colony Forming Unit (CFU)/g. After rehydration in water at a temperature of 20 °C for 30 min (dilution 10% w/w), the bacteria were inoculated (1% w/w) in the milk prior to the rennet addition.

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Microbiological analysis

All microbiological analysis was performed in the laboratory of Edmund Mach Foundation (San Michele all' Adige, TN) by plate count assay (ISO, 2001; 2007; 2013). Cheeses were aseptically sampled through cutting 3 different portions for each cheese (1 kg in total for each cheese) and submitted to microbiological analysis within 24 h. Samples were placed in a sterile Stomacher bag (Seward, Worthing, UK), diluted 1:10 w/w by a 20 g/L K_2HPO_4 solution (Sigma-Aldrich, St. Louis, USA), and homogenized for 3 min. After preparation of the first dilution, the samples were appropriately diluted by decimal dilutions using peptone water solution (1 g/L of Mycological Peptone). Total Microbial Count (TMC) was performed on Plate Count Agar (PCA) supplemented by 1 g/L of skimmed milk extract and incubated at 30 °C for 72 h. *Lactobacillus* sp. was quantified on De Man, Rogosa and Sharpe (MRS) agar, at 45 °C for thermophilic species and 30 °C for mesophilic species under anaerobiosis using Anareogen Kit (Oxoid, Basingstoke, UK). The same conditions were applied for *Lactococcus* sp., enumerated on M17 agar. The hetero fermenting character was essayed placing grew colonies into vials containing 10 ml of MRS broth, and observing the development of gas after 48 h at 37 °C. Coliforms were quantified on Violet Red Bile Agar after 24 h of incubation at 37 °C. *Enterococcus* sp. was counted on Kanamycin Aesculin Azide agar after 24 h at 37 °C. *Staphylococcus* sp. was enumerated on Baird-Parker agar base in addition 50 ml/L egg yolk tellurite emulsion. Incubation was performed at 37 °C for 24 h. All media and chemicals were purchased from Oxoid, Basingstoke, UK.

Sensorial analysis

Sensory evaluation of Malga cheeses was carried out by a panel of 30 trained participants using a 1-10 rating scale considering descriptors, including external aspect, color, cheese holes, consistency, flavor, taste, and texture. Three cheese samples were replicated twice in order to check the sensitivity and reliability of each judge (Stone et al., 2008).

Statistical analysis

Statistical analysis of the data was carried out applying Anova and Tukey's HSD tests using STATISTICA v. 8.0 (Stat Soft Inc., Tulsa).

Results

The results of the TMC of the 38 cheese samples were shown in Table 1. As illustrated in Figure 1, the TMC of medium-aged (6 months) cheeses was significantly ($p<0.05$) higher than that of long-aged (16 months) cheeses.

Table 2 shows the distribution of LAB groups quantified in the cheeses, grouped by pasture and ageing time. Coliforms and *Staphylococcus* sp. were not detected in all samples. Distribution percentages of LAB in various cheeses are shown in Figure 2.

Figure 3 compares the distribution of cheese populations produced with or without FBP in experimental cheese-making performed in the Vezzena pasture (mean data, n=4). Thermophilic LAB were the relevant (45%) population in cheese made by FBP; while mesophilic LAB, mainly lactobacilli were the relevant (87%) population in the non-inoculated cheeses.

The sensorial scores of 16 months-aged cheeses were significantly ($p<0.05$) higher than that of 6 months ones. However, there was no significant ($p>0.05$) difference between sensorial scores of Fermalga and non-Fermalga cheeses. The addition of FBP had no effect on the typical feature of Malga cheese, but also positively influenced the physical characteristics of cheeses, including consistency, holes, color, and external aspect (Figure 4).

Discussion

Considering the peculiarity of the Malghe, there are little available information about the microbiology of cheeses obtained in Alpine pastures reported by Bergamaschi et al. (2016), Franciosi et al. (2013), and Lucchini et al. (2018). We found too much variability in LAB population of cheese samples. The main objective of addition of FBP Fermalga bacteria was the reduction of technological defects and prevention of the pathogen microbes in cheeses which both these objectives appear to have been accomplished. The absence of pathogen microbes in all 38 samples of Malga cheeses allowed them to be considered safe if consumed after an adequate ripening period of at least 6 months (Table 2). In agreement with our findings, Lucchini et al. (2018) found no pathogenic bacteria, including *E. coli*, *Listeria monocytogenes*, and *Salmonella* spp. in alpine Malga cheese samples in Italy. However, they found 5 Log CFU/g of coagulase-positive staphylococci in the curd in the two Malga without starter cultures.

The TMC cultured in cheeses were considerably high both for medium-aged cheeses and for the long-aged samples, respect to other Alpine cheese productions. In analogues condition, the raw milk cheeses produced in the eastern regions of Trentino, Poznanski et al. (2004) observed microbial loads in the interval between the 5 and 7 log CFU/g only up to 60 days of ageing. It is also generally known that the bacterial load of cheese decays

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Table 1: The characteristics of Malga cheeses, including time of ageing, geographical origin, and total microbial count considered in the current work

Malga type	Period of ageing (Month)	Pasture origin	Total microbial count (log colony forming unit/g)
Arpaco	6	Lagorai	7.4
Cagnon	6	Lagorai	8.3
Cambroncoi	6	Lagorai	8.1
Casabolenga	6	Lagorai	8.0
Casapinello	6	Lagorai	8.3
Cavallara	6	Lagorai	7.6
Cenon	6	Lagorai	6.7
Colo	6	Lagorai	7.9
M. Granda	6	Lagorai	7.6
Setteselle	6	Lagorai	7,7
Stramaiolo	6	Lagorai	8.5
Trenca	6	Lagorai	7.8
Valfontane	6	Lagorai	8.0
Valpiana	6	Lagorai	7.4
Valsolero	6	Lagorai	7.7
Campo capra	6	Vezzena	7.8
Palu'	6	Vezzena	7.0
Scura	6	Vezzena	8.2
Valcoperta	6	Vezzena	7.3
Zochi	6	Vezzena	7.5
Casapinello	16	Lagorai	6.4
Cenon	16	Lagorai	7.0
Colo	16	Lagorai	5.9
M. Granda	16	Lagorai	6.8
Setteselle	16	Lagorai	6.4
Stramaiolo	16	Lagorai	6.5
Valfontane	16	Lagorai	5.9
Valsolero	16	Lagorai	6.0
Bordolona	16	Val di Non	6.8
Fondo	16	Val di Non	5.9
Tovre	16	Val di Non	4.7
Bolentina	16	Val di Sole	6.9
Dimaro	16	Val di Sole	6.5
Mondent	16	Val di Sole	6.1
Strino	16	Val di Sole	3.3
Villar	16	Val di Sole	6.9
Basson	16	Vezzena	4.0
Palu'	16	Vezzena	5.2

Table 2: Occurrence of microbial groups in Malga cheeses after 6 and 16 months of ageing. Mean±SD (log colony forming unit/g) subdivided by Alpine pasture.

Alpine pasture	Mesophilic lactobacilli	Mesophilic lactobacilli hetero fermentation	Thermophilic lactobacilli	Thermophilic lactobacilli hetero fermentation	Mesophilic lactococci	Thermophilic lactococci	*Enterococcus* sp.
Lagorai (LA)	6.2±0.6	6.1±0.6	4.2±0.4	3.8±0.4	6.2±0.6	5.4±0.5	5.5±0.5
Val di Non (LA)	ND	ND	2.7±0.2	3.3±0.4	4.7±0.3	4.4±1.4	4.7±1.7
Val di Sole (LA)	4.4±0.3	ND	ND	ND	5.3±0.5	3.8±0.3	4.5±2.8
Vezzena (LA)	4.5±0.4	4.4±0.3	ND	ND	5.3±0.5	3.9±0.4	4.4±0.4
Lagorai (MA)	7.8±0.8	7.8±0.8	5.8±0.6	5.8±0.6	7.5±0.7	6.3±0.6	7.1±0.7
Vezzena (MA)	7.2±1.4	7.4±1.2	7.5±0.3	7.7±0.6	5.5±0.9	7.8±0.4	6.9±1.1

LA: Long Ageing (16 months)
MA: Medium Ageing (6 months)
ND: Not Detected

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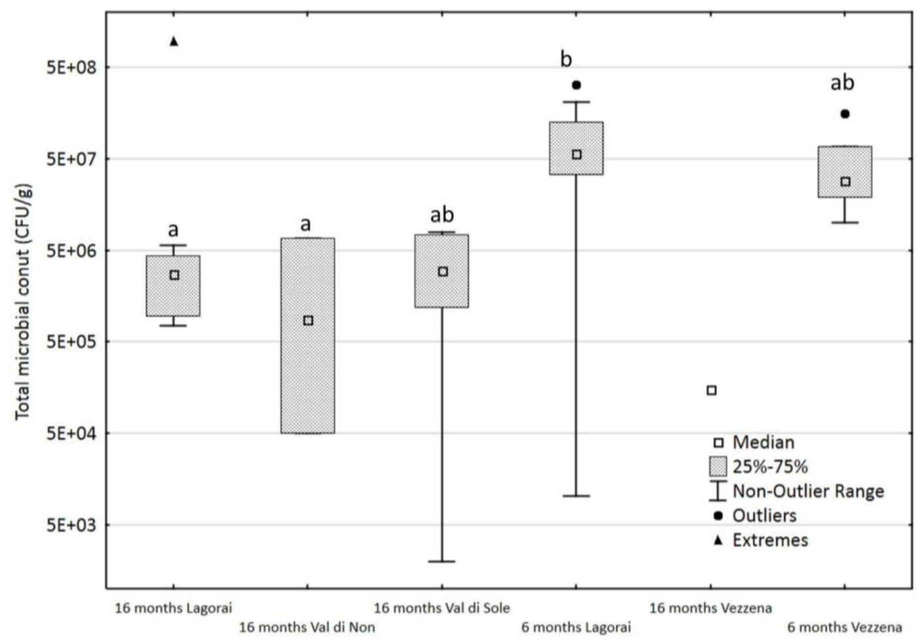

Figure 1: Box plot of the Total Microbial Count (TMC) as log Colony Forming Unit (CFU)/g in Malga cheeses after 6 and 16 months of ageing. Having no similar letter above the separated sections indicates significant ($p<0.05$) difference.

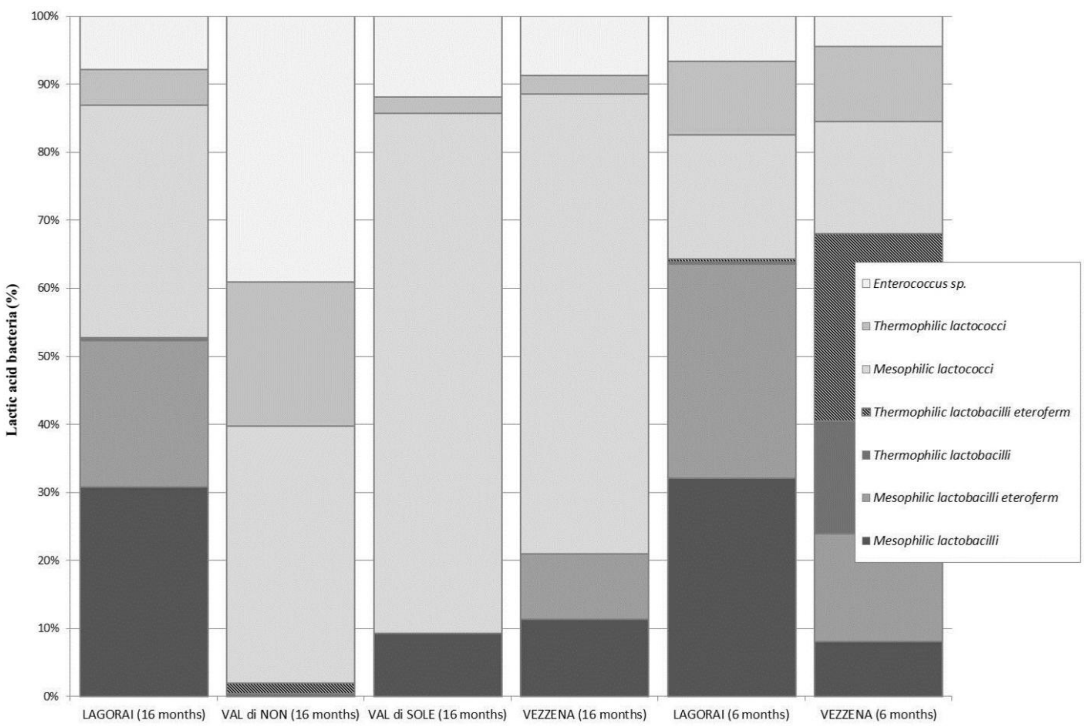

Figure 2: Distribution (%) of different microbial groups of the lactic acid bacteria population of Malga cheeses produced in 4 Alpine pastures of the Trentino region after 6 or 16 months of ageing (mean data).

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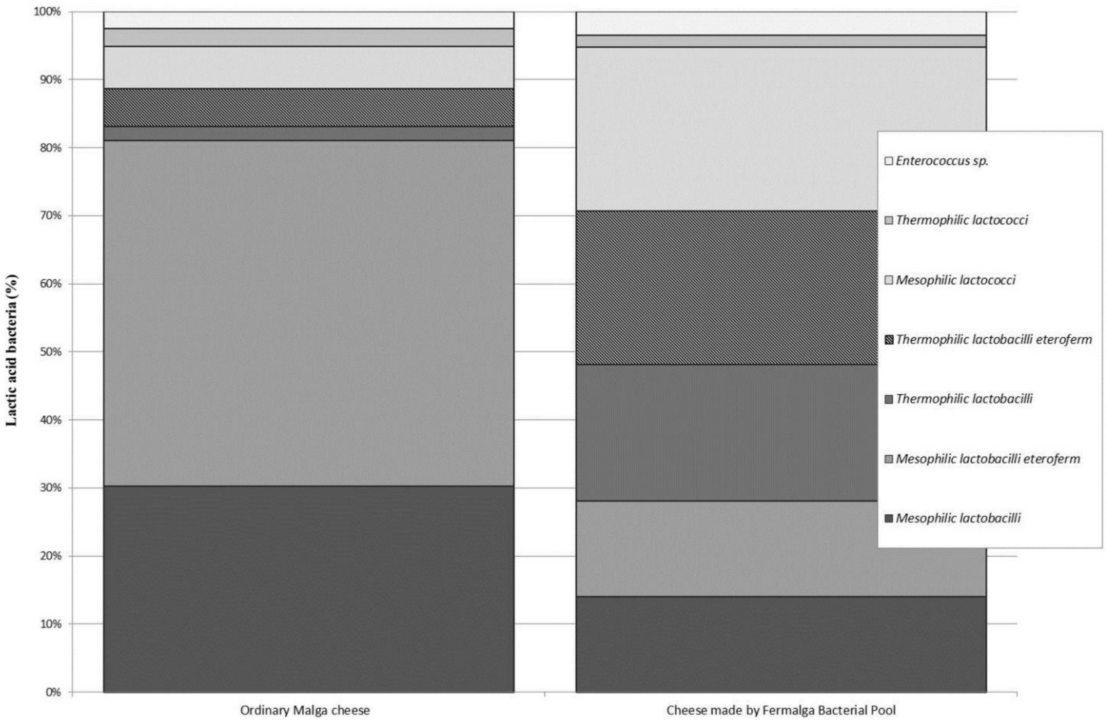

Figure 3: Distribution (%) of different microbial groups of the lactic acid bacteria population of Malga cheeses produced with or without the addition of the Fermalga bacterial pool. Malga cheeses were produced in the Vezzena pasture and analyzed after 6 months of ageing (mean data).

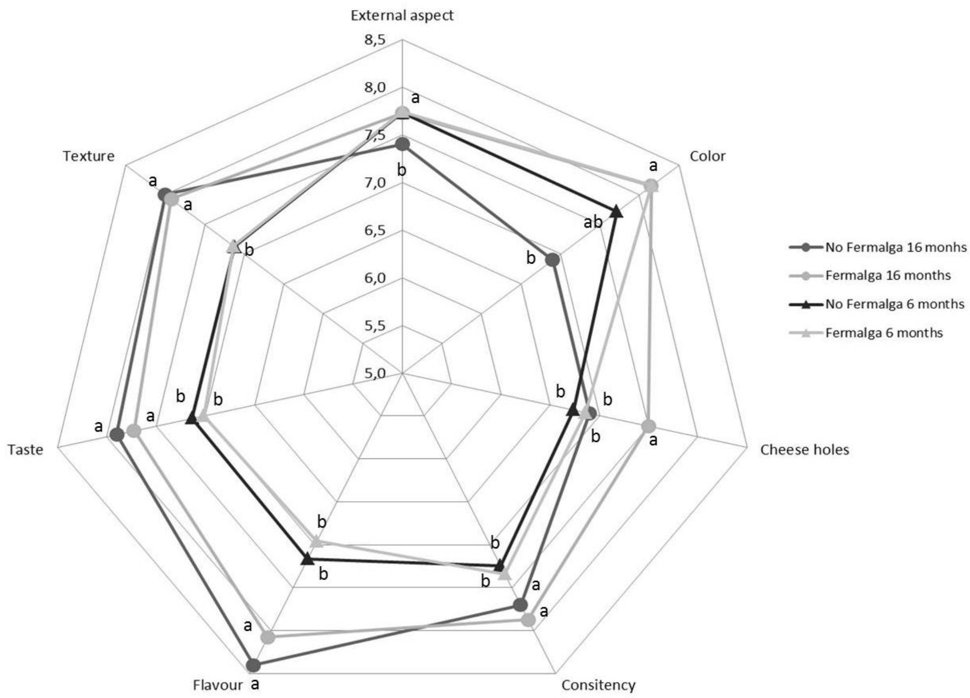

Figure 4: Results of the sensory evaluation of Malga cheeses produced with or without the addition of the Fermalga bacterial pool

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after 3 months of ageing (McSweeney, 2007). The analysis of the data provided in this study dispelled any doubts concerning reduction of microbial biodiversity. It is very interesting to observe how the differences in the distribution of the groups of LAB can be correlated with the technological variables adopted in the cheese-making in the different pastures. As example, comparing the distribution of LAB population of medium ageing samples of Vezzena and Lagorai, two pastures with a few kilometers away, it is evident the variation in the ratio between mesophilic and thermophilic bacteria. This difference may be attributed to the cheese-making procedures, given that in Vezzena, the heating of the curd typically happens at higher temperatures, up to 47 °C, respect to the 43 °C of Lagorai (Schiavon et al., 2019).

In general, it seems that the addition of the FBP ensures a long-permanence (at least 6 months) of thermophilic LAB which is recognized to be able to drive the acidification of curd and preventing the development of pathogens (Delgado et al., 2013; Lucchini et al., 2018). In the long ageing cheeses, the biodiversity of the microbiotas of samples made from the 4 pastures was greater, and mainly concerned mesophilic lactococci, and *Enterococcus* sp. It has been stated that *Enterococcus* sp. is capable of influencing the sensory profile of cheeses (Sarantinopoulos et al., 2002), characterized the samples coming from Val di Non in the present survey. The prolonged permanence of non-starter LAB plays an important role in the development of peculiar taste of raw-milk made cheeses, due to the production of amino acids and fatty acids, together to a careful management of ageing conditions such as the temperature (Poznanski et al., 2004; Rahman et al., 2000). Similarly, Franciosi et al. (2013) obtained a total of 431 LAB isolates from spontaneously fermented Malga cheese in Trentino. These researchers found that *L. lactis*, *S. thermophilus*, and *E. faecium* were the major non-putative starter strains.

Sensory analysis is an important test to assess how the evolution of the microbiota can impact on the acceptability of cheeses by consumers. Our trained panel found no difference between sensorial characteristics of cheese made with and without FBP. Long time ageing up to 16 months was appeared to be the main variable that influenced the sensorial properties of Malga cheese samples. This result which has already been revealed in Cheddar cheese (Rahman et al., 2000) and goat milk cheese (Moreira et al., 2018), is part of the expected evolution of the cheese-making.

Conclusion

The protection of traditional fermented foods can occur by innovative technologies, making them safe without altering their typical features. This preliminary investigation on the characteristics of Alpine Malga cheeses showed that the addition the FBP had no undesirable effect on the organoleptic characteristics of the cheeses. On the other hand, using the FBP in the cheeses-making process could be effective in reduction of technological defects and prevention of pathogenic microorganisms.

Author contributions

R.G., S.S., and T.R. designed the study; S.S. conducted the experimental work; R.G. analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest. All experiments were performed in total autonomy and without any pressure or interference from external companies or financiers.

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