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Comparison of natural whey starters for Grana Padano cheese using sunray plots

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Abstract Twenty-one natural whey starters, collected from dairy factories located in six provinces of the Grana Padano production area, were characterized. Basic techniques, such as acidity evaluation and microbial plate count, together with more complex methods such as the Live/Dead® BacLight™ bacterial viability kit, have been used. Seven parameters, including pH, Soxhlet Henkel degrees, microbial plate count in Man Rogosa Sharpe medium and Whey Agar medium, together with count of total, viable and non-viable cells, have been adopted to produce sunray plots. One plot for each natural whey starter sample was obtained by characterizing the status of the microbial culture and compared with three natural whey starters prepared in the laboratory. In this way, a sunray trace is suggested to define the traits of a good natural whey starter. Another multivariate technique, principal component analysis, was applied, and it should be possible to conclude that, for this particular dataset composed of 24 objects and 7 variables, PCA allowed the highlighting of the good and bad samples, while sunray plots, even if remaining only a descriptive and explorative analysis, allowed the better visualizing of the differences among all the samples.

Keywords Natural whey starters · Grana Padano · Sunray plots · Principal component analysis

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Introduction

The Grana Padano Production disciplinary states that this Protected Designation of Origin (PDO) cheese must be produced with raw milk and natural whey starter. The starter is a natural culture of thermophilic lactic acid bacteria that grow in the whey produced at the beginning of the dairy process (http://www.granapadano.com). The whey starter is produced by culturing the non-acidified whey resulting from daily cheesemaking, called sweet whey. Whey is fermented at a naturally decreasing temperature, that in approximately 20 h goes down from about 54 to about 35°C, performing a thermophilic selection. Whey starter is added in the milk vat and sweet whey is recovered after curd cooking from each cheese production (Rossetti et al. 2008; Santarelli et al. 2008). The modality of preparation of the whey starter cultures warrants the survival of different biotypes useful to the development of the ecosystem itself, and a mixture of strains of the same species is necessary for the natural starter evolution (Gatti et al. 2004). The primary function of starter bacteria is to produce acid during the fermentation process; however, they also contribute to cheese ripening thanks to their enzymes involved in proteolysis and conversion of amino acids into flavor compounds (Fox and Wallace 1997).

Moreover, one important role of starter bacteria is to provide a suitable environment, with respect to redox potential, pH and moisture content in the cheese, allowing enzyme activity of rennet and starter, and allowing the growth of secondary flora to proceed favourably (Beresford et al. 2001). For traditional Grana Padano production, it is the experience of the cheese maker that defines the correct characteristics of a good

natural whey starter. To date, the analytical techniques traditionally used for its characterization are very basic (pH, titratable acidity, plate count agar) and do not provide exhaustive information on the performance of the starter. These data are usually regularly recorded. This study had the aim of applying a different approach to recording these data, together with the number of live/dead microbes, using a figurative method which could be useful when comparing different samples.

Sunray plots are basically a radial plot showing the importance of each variable in a sample, and are used to compare more than two endpoints, or datasets, simultaneously. Sunray plots, or star plots, traditionally used for sensory analyses, have not been reported infrequently in the literature for visualization of other data. As well as for sensory evaluation, this method has been used, for example, to show the relative importance of different descriptors chosen to predict the protein retention time in anion-exchange chromatography (Song et al. 2002; Tugcu et al. 2003) and to visualize plasma fatty acids chosen to screen and monitor the effects of infection following the use of adenoviral vectors in gene therapy (Paik et al. 2007). Sunray plots have also been useful in showing the differences between wine samples and coffee samples (Haswell and Walmsley 1998), in sensor responses of five typical malodors in fields (Romain et al. 2000), and in different types of propagation materials of banana 'Nanicão' (Scarpare Filho et al. 1998). However, the use of sunray plots for microbial parameters has rarely been reported. In particular, Hofman and collaborators characterized soil biological quality, by means of microbial biomass determination and another seven chemicals parameters, describing and comparing their status using the sunray plots (Hofman et al. 2003).

Recent studies have been carried out to evaluate the microbial diversity of natural whey starters for Grana Padano cheese. Applying advanced microbiological techniques, such as random amplified polimorphic DNA PCR, temporal gradient gel electrophoresis (Andrighetto et al. 2004) and length heterogeneity PCR (Rossetti et al. 2008), the thermophilic dominant microflora characteristic of Grana Padano whey culture have been described. This study suggests a new approach for easily comparing and evaluating the quality of natural whey starters for Grana Padano cheese. To reach this goals, an alternative and easy way to visualize each sample in order to recognize the best or the worst characteristics has been considered. To implement this experimental study, 21 natural whey starters were characterized by three traditional parameters, such as count on Man Rogosa Sharpe (MRS), titratable acidity and pH, as well as four new parameters including Whey Agar (WAM) plate count agar and direct assessment of the total microbial population discriminating viable and non-viable cells.

Materials and methods

Determination of the parameters

Twenty-one natural whey starters collected from 21 dairy factories involved in Grana Padano cheese production and located in the six main provinces of the Grana Padano production area (Brescia, Mantova, Piacenza, Padova, Vicenza and Verona) were obtained. Just before being added in the milk vat, samples were collected, cooled to 4°C, quickly transported to the laboratory and analyzed. Measurements of pH were performed in duplicate by using the pH212 pH meter (Hanna Instruments, Padova, Italy). Titratable acidity was obtained by titrating 100 ml of sample with NaOH 0.25 M, using phenolphtalein as indicator, and the results are expressed as Soxhlet Henkel degrees (°SH). The analysis was carried out in duplicate.

Agar plate counts were performed using MRS agar pH 5.4 (Biolife, Milano, Italy) and whey agar medium (WAM; Gatti et al. 2004). Plates of MRS and WAM were incubated under anaerobic conditions (Anaerogen, Oxoid, Basingstoke, UK) at 42°C for 48 h. The counts were carried out in duplicate.

Fluorescence microscopy counts to assess the total (T), viable (V), and non-viable (NV) bacterial population were carried out using a Leica DMSL (Leica Microsystems, Wetzlar, Germany) and LIVE/DEAD® BacLight™ bacterial viability kit, based upon SYTO®9 and Propidium iodide as previously described (Gatti et al. 2006). Each sample was prepared in duplicate and average values were calculated.

A traditionally measured variable in dairy factories is °SH which is used to calculate the amount of natural whey starter to add to the milk in the vat. Depending on milk acidity, the higher the acidity of the natural culture, the lower the amount to add. Moreover, positive traits of natural whey starters are a high number of T linked to a high number of V.

With the aim of obtaining samples to be considered positive and negative, three whey starters were prepared in the laboratory (samples G, B1 and B2). The production started from the incubation at 45°C of one sweet whey obtained from the nearest dairy factory at less than 30 km. Values of pH and titratable acidity were monitored and microbial growth evaluated by optical density at 650 nm (OD_{650}). Sample G was collected, and refrigerated (4°C), at the end of the

exponential phase, after 20 h of incubation (high acidity and high OD_{650}), sample B1 was collected after 15 h (low acidity and low OD_{650}), and sample B2 after 25 h (high acidity and high OD_{650} in stationary phase) (data not shown).

Sunray plots

All the seven parameters obtained, count on MRS (MRS), on WAM (WAM), number of total cells (T), number of viable cells (V), number of non-viable cells (NV), tritrable acidity (°SH), and pH, were centered by average subtraction and normalized by dividing by standard deviation in the framework of the natural whey starters set evaluated. The standardized values were plotted into sunray plots with seven axes using Statistica 6.1 (StatSoft Italia, Padova, Italy). Sunray plots are a subclass of circular icon plots in which the rays tend to form a circle. Each variable is represented by one ray or direction and all rays start in the center. The value of each variable is reflected by the distance from the center. These plots are basically a radial plot showing the importance of each variable in the sample. In this way, a unique plot for each whey starter sample was obtained, characterizing the status of the microbial culture. Samples G, B1 and B2 were used to represent one good and two bad natural whey starters, respectively.

Principal component analysis

The PCA analysis was performed using Statistica 6.1 (StatSoft Italia).

Results and discussion

Determination of the parameters

As expected, total cell counts (T) of the 21 natural whey starters for Grana Padano cheese were high and very similar to each other (CV 18%) (Table 1). As for the total count, the viable populations (V) were high and similar to each other, representing from 98 to 73% of the T population. The G sample, as expected, was characterized by a rather high percentage of viable cells (98%), showing that 20 h of incubation at 45°C were an optimal condition for producing the whey starter beginning from the selected sweet whey. Sample B1 showed the highest percentage of viable cells (99%) but a lower number of total cells because the culture was in stationary phase. The B2 sample exhibited the lowest percentage of viable cells (58%) as well as a low level of total cells, suggesting that the longer incubation, i.e. 10 h more than the B1 sample, had affected the microbial cells. The non-viable population (NV) gave a more variable result (CV 56%), representing, on PC2, almost one-third of the T population (Table 1).

Plate counts in MRS at pH 5.4 were more variable (CV of 68%) than those in WAM, ranging from 4% in PC2 to 46% in MN4. With respect to T, cultivable population in WAM ranged from 21% in PC2 to 100% in MN6, VI1 and VR1, and in MRS at pH 5.4 from 1% in PC2 to 44% in MN4. Cultivability of samples G and B1 in MRS at pH 5.4 and in WAM were similar, showing that this parameter depends upon the microbial biodiversity of the sample. In sample B2, cultivability was lower than for samples B1 and G, suggesting that acid stress can modify the capability of cells to duplicate in the agar medium.

The measures of pH and titratable acidity are two different methods for evaluating the whey starter acidity. As expected, the two parameters were not strongly correlated (correlation coefficient, −0.60). pH varied from 3.15 for BS4 to 3.49 for VI2. The highest value of pH in sample B1 was linked with low number of T and V values and high value of °SH. Differently from this laboratory sample, for the real samples, the same correlation was not found. It was observed that. in samples characterized by a low percentage of viable bacteria (BS3, VI1, MN5 and MN2), the °SH were higher than 31.0, but in other samples where the viable population was less then 86% (PC1, VI2 and PD1). the °SH was 29.0. In contrast, MN4 had 96% of viable cells and 32°SH. In the laboratory samples, similar percentages of viable cells were observed when acidity was 26.0 and 31.5°SH (in B1 and G samples, respectively 99 and 98%), whereas the percentage of viable cells decreased in sample B2, when the acidity was 34.0°SH. The relationship between acidity and cell viability could depend on the acid resistance of the dominant microbial population.

Sunray plots

Each sample plot was constructed by averaging the sample replicates first and normalizing the data to scale the plots correctly later. In this way, it is possible to ensure that no distortion in the plots results due to relative magnitude. The sunray plots produced demonstrate the differences between samples fairly well.

To evaluate the quality of a natural whey starter, it is necessary to consider that its role during cheese manufacture is to produce the lactic acid that influences important quality characteristics such as texture, moisture content, absence of pathogenic microorganisms, and taste (Fox and Wallace 1997). The rate of acid production, generally measured as °SH and/or pH, is critical for the production of cheese. Starters may also be required to produce acid at a

Table 1 Microbiological and chemical determinations and standard deviations (*SD*) of 21 natural whey starters for Grana Padano cheese collected in six different provinces and three laboratory samples (positive control G, negative controls B1 and B2)

Sample[a]	Plate count on		Direct count			Acidity	
	WAM	MRS pH5.4	Total	Viable	Non viable	Titratable	
	(CFU mL^{-1}) *SD*	(CFU mL^{-1}) *SD*	(cells mL^{-1}) *SD*	(cells mL^{-1}) *SD*	(cells mL^{-1}) *SD*	°SH *SD*	pH *SD*
BS 1	9.50×10^{8}	2.60×10^{8}	1.58×10^{9}	1.45×10^{9}	1.25×10^{8}	28.0	3.31
	9.90×10^{7}	*2.26×10^{7}*	*3.54×10^{7}*	*1.41×10^{7}*	*2.83×10^{6}*	*0.14*	*0.01*
BS 2	1.40×10^{9}	2.21×10^{8}	1.55×10^{9}	1.47×10^{9}	7.50×10^{7}	31.0	3.48
	1.06×10^{8}	*8.49×10^{6}*	*3.89×10^{7}*	*2.83×10^{7}*	*1.06×10^{6}*	*0.35*	*0.02*
BS 3	7.80×10^{8}	1.18×10^{8}	1.59×10^{9}	1.37×10^{9}	2.20×10^{8}	31.5	3.35
	5.66×10^{7}	*5.66×10^{6}*	*6.36×10^{7}*	*2.12×10^{7}*	*7.07×10^{6}*	*0.35*	*0.02*
BS 4	9.40×10^{8}	2.14×10^{8}	1.35×10^{9}	1.25×10^{9}	1.00×10^{8}	33.0	3.51
	3.54×10^{7}	*1.20×10^{7}*	*3.54×10^{7}*	*4.24×10^{7}*	*1.41×10^{6}*	*0.14*	*0.02*
MN 1	6.40×10^{8}	7.20×10^{7}	1.50×10^{9}	1.35×10^{9}	1.50×10^{8}	30.0	3.42
	6.36×10^{7}	*5.66×10^{6}*	*1.41×10^{7}*	*4.24×10^{7}*	*7.07×10^{5}*	*0.14*	*0.02*
MN 2	9.80×10^{8}	8.30×10^{7}	1.90×10^{9}	1.70×10^{9}	2.00×10^{8}	31.5	3.38
	4.95×10^{7}	*5.66×10^{6}*	*4.24×10^{7}*	*2.83×10^{7}*	*5.66×10^{6}*	*0.07*	*0.01*
MN 3	4.82×10^{8}	1.60×10^{8}	1.40×10^{9}	1.35×10^{9}	5.00×10^{7}	32.0	3.48
	2.90×10^{7}	*1.27×10^{7}*	*2.12×10^{7}*	*3.54×10^{7}*	*1.34×10^{6}*	*0.35*	*0.01*
MN 4	1.32×10^{9}	6.04×10^{8}	1.37×10^{9}	1.27×10^{9}	1.00×10^{8}	27.0	3.49
	8.49×10^{7}	*4.67×10^{7}*	*1.77×10^{7}*	*2.12×10^{7}*	*1.41×10^{6}*	*0.28*	*0.01*
MN 5	4.05×10^{8}	9.86×10^{7}	1.00×10^{9}	8.75×10^{8}	1.25×10^{8}	33.0	3.38
	1.77×10^{7}	*7.35×10^{6}*	*3.54×10^{7}*	*1.27×10^{7}*	*3.54×10^{6}*	*0.35*	*0.01*
MN 6	1.61×10^{9}	2.11×10^{8}	1.61×10^{9}	1.53×10^{9}	7.50×10^{7}	33.0	3.39
	1.84×10^{8}	*1.56×10^{7}*	*3.18×10^{7}*	*4.24×10^{7}*	*1.06×10^{6}*	*0.35*	*0.01*
PC 1	9.28×10^{8}	1.27×10^{8}	1.00×10^{9}	8.25×10^{8}	1.75×10^{8}	29.0	3.49
	4.45×10^{7}	*5.66×10^{6}*	*7.07×10^{6}*	*6.36×10^{7}*	*1.70×10^{7}*	*0.28*	*0.02*
PC 2	2.45×10^{8}	1.00×10^{7}	1.16×10^{9}	1.13×10^{9}	2.50×10^{7}	30.0	3.53
	2.40×10^{7}	*7.78×10^{5}*	*3.18×10^{7}*	*3.54×10^{7}*	*8.49×10^{5}*	*0.35*	*0.01*
PC 3	1.64×10^{9}	4.32×10^{8}	1.65×10^{9}	1.52×10^{9}	1.25×10^{8}	30.0	3.47
	9.19×10^{7}	*2.33×10^{7}*	*4.60×10^{7}*	*2.83×10^{7}*	*5.66×10^{6}*	*0.14*	*0.01*
PC 4	9.19×10^{8}	1.69×10^{8}	1.16×10^{9}	1.08×10^{9}	7.50×10^{7}	32.0	3.39
	6.43×10^{7}	*1.34×10^{7}*	*2.62×10^{7}*	*3.25×10^{7}*	*7.78×10^{5}*	*0.07*	*0.02*
PC 5	9.55×10^{8}	2.65×10^{8}	1.18×10^{9}	9.50×10^{8}	2.25×10^{8}	30.5	3.50
	6.72×10^{7}	*2.05×10^{7}*	*2.47×10^{7}*	*2.12×10^{7}*	*4.24×10^{6}*	*0.07*	*0.04*
PD 1	9.46×10^{8}	2.16×10^{8}	1.40×10^{9}	1.20×10^{9}	2.00×10^{8}	29.0	3.45
	6.65×10^{7}	*1.27×10^{7}*	*1.20×10^{8}*	*1.41×10^{7}*	*2.26×10^{7}*	*0.28*	*0.03*
PD 2	1.30×10^{9}	1.59×10^{8}	1.48×10^{9}	1.08×10^{9}	4.00×10^{8}	30.0	3.57
	1.27×10^{8}	*5.66×10^{6}*	*4.24×10^{7}*	*2.12×10^{7}*	*1.13×10^{7}*	*0.35*	*0.02*
VI 1	1.93×10^{9}	9.80×10^{7}	1.93×10^{9}	1.68×10^{9}	2.50×10^{8}	31.0	3.49
	1.20×10^{8}	*3.89×10^{6}*	*3.54×10^{7}*	*1.06×10^{8}*	*7.78×10^{6}*	*0.14*	*0.03*
VI 2	1.11×10^{9}	1.20×10^{8}	1.73×10^{9}	1.48×10^{9}	2.50×10^{8}	29.0	3.58
	8.49×10^{7}	*9.90×10^{6}*	*4.95×10^{7}*	*3.54×10^{7}*	*8.49×10^{6}*	*0.11*	*0.01*
VR 1	1.62×10^{9}	1.89×10^{8}	1.62×10^{9}	1.47×10^{9}	1.50×10^{8}	30.0	3.50
	1.27×10^{8}	*1.91×10^{7}*	*1.13×10^{8}*	*3.54×10^{7}*	*1.41×10^{6}*	*0.18*	*0.01*
VR 2	1.43×10^{9}	1.74×10^{8}	1.45×10^{9}	1.30×10^{9}	1.50×10^{8}	30.0	3.36
	1.20×10^{8}	*9.90×10^{6}*	*1.70×10^{8}*	*1.27×10^{8}*	*2.83×10^{6}*	*0.11*	*0.02*
Sample G	1.45×10^{9}	9.80×10^{8}	1.90×10^{9}	1.87×10^{9}	5.00×10^{7}	31.5	3.30
	7.78×10^{7}	*8.77×10^{7}*	*5.66×10^{7}*	*2.83×10^{7}*	*1.70×10^{6}*	*0.04*	*0.01*
Sample B1	6.20×10^{8}	3.78×10^{8}	8.00×10^{8}	7.95×10^{8}	5.00×10^{6}	26.0	3.82

Table 1 (continued)

Sample[a]	Plate count on		Direct count			Acidity	
	WAM	MRS pH5.4	Total	Viable	Non viable	Titratable	
	(CFU mL^{-1}) *SD*	(CFU mL^{-1}) *SD*	(cells mL^{-1}) *SD*	(cells mL^{-1}) *SD*	(cells mL^{-1}) *SD*	°SH *SD*	pH *SD*
	3.54×10^{7}	*2.97×10^{7}*	*1.56×10^{7}*	*5.66×10^{6}*	*9.19×10^{4}*	*0.14*	*0.01*
Sample B2	5.14×10^{8}	3.13×10^{8}	9.12×10^{8}	5.24×10^{8}	3.88×10^{8}	34.0	3.15
	1.72×10^{7}	*1.92×10^{7}*	*2.05×10^{7}*	*9.90×10^{6}*	*5.66×10^{6}*	*0.35*	*0.01*

[a] *BS* Brescia province, *MN* Mantova province, *PC* Piacenza province, *PD* Padova province, *VI* Vicenza province, *VR* Verona province

consistently fast rate every day throughout the manufacturing period (Hugenholtz 2008). To reach this goal, the cells should be numerous, viable, and able to replicate in the milk in the vat. These are the reasons why high values of T and V are considered positive traits. Correspondingly, a high value of NV is a negative trait. High °SH and low pH values can be considered as positive features only if they are linked with a high percentage of V. In a sunray plot, this positive aspect is easily visualized in the two triangles: T–centre–V and V–centre–SH: the bigger they are, the better is the starter quality, as exemplified by sample G (Fig. 1). At the same time, the triangle pH–centre–VN must be smaller. According to the present approach, MN2, MN6, PC3 and VI1 can be considered good-quality

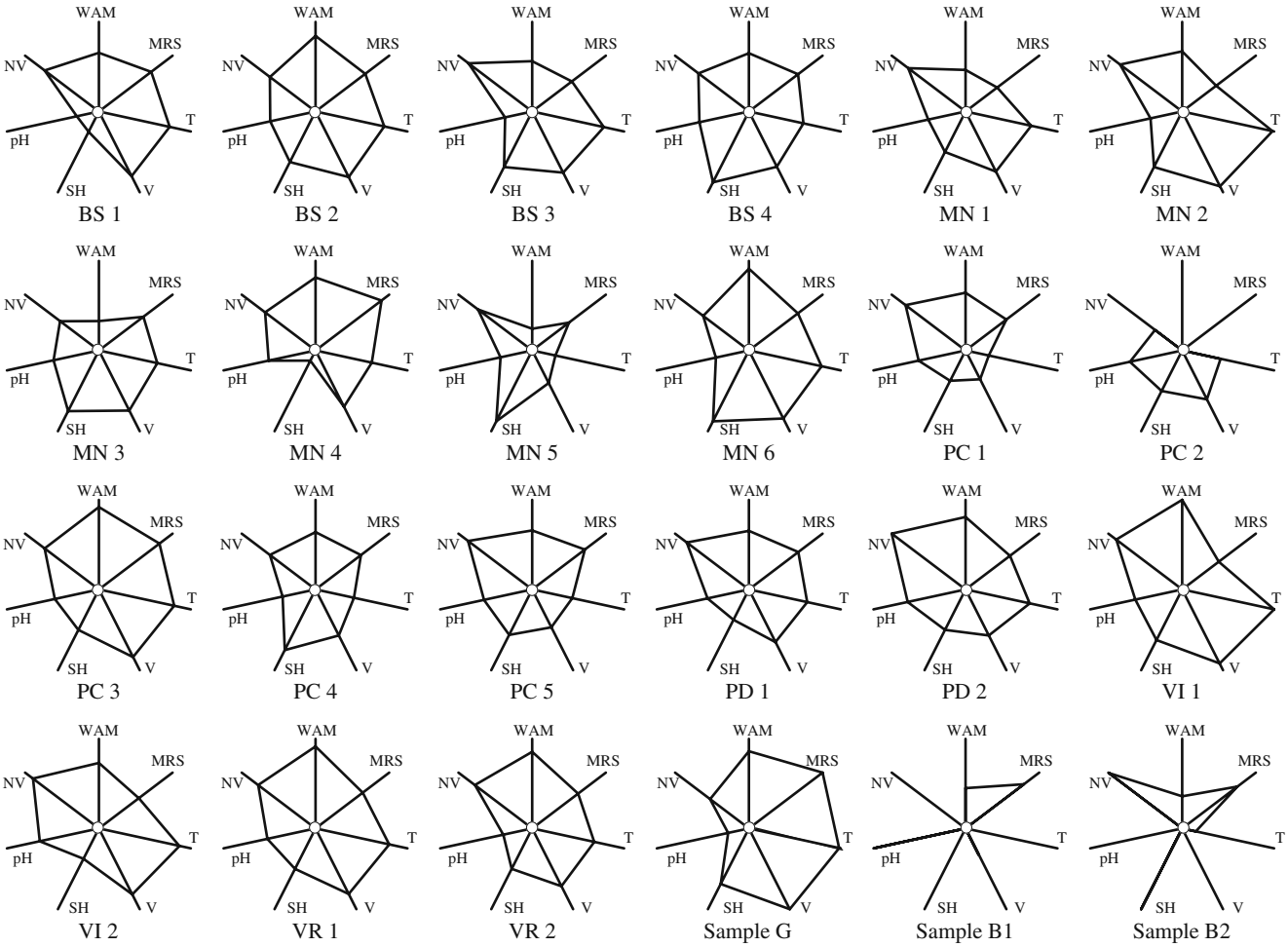

Fig. 1 Sunray plots of the standardized chemical and microbial parameters for 21 natural whey starters for Grana Padano cheese and three laboratory samples (G, B1 and B2). Each axis represent one parameter: CFU mL^{-1} in *WAM*, CFU mL^{-1} in *MRS* at pH 5.4, total cells number mL^{-1} (*T*), viable cells number mL^{-1} (*V*), titrable acidity expressed as *SH*, *pH* and not viable (*NV*) cells number mL^{-1}

samples, MN5, PC1 and PC2 can be considered low-quality samples, with the latter one appearing to be particularly poor.

The capacity of the cells to grow in the synthetic commercial medium MRS, even when it is acidified, is generally lower than their capacity to grow in WAM. As previously discussed (Lazzi et al. 2004), the reason for this difference is related to the cells' nutritional requirements and to the greater complexity of the WAM which attempts to reproduce the natural system of the whey. The capability of the cells to grow in MRS and WAM could be interpreted as a different possibility of adaptation to the curd and cheese environment. Even high count values in WAM and in MRS at pH 5.4 can be considered as positive traits, while low levels of these parameters may be considered as biodiversity, rather than as a negative factor.

PCA analysis

To confirm the efficiency of this unusual approach, the same pool of data was analyzed by means of a more

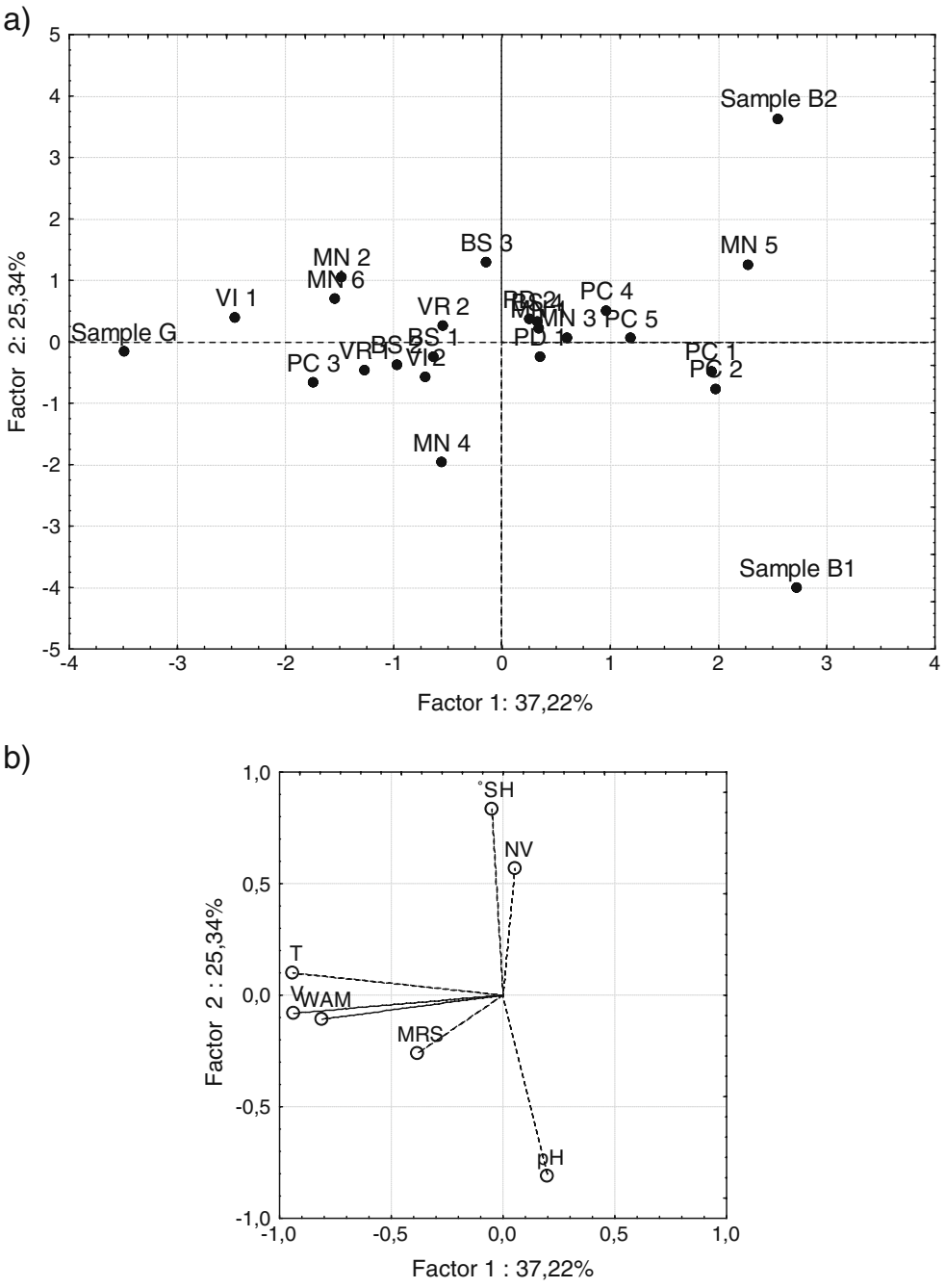

Fig. 2 Scores (**a**) and loadings (**b**) plots for the first and second factors of principal component analysis carried out on 21 natural whey starters for Grana Padano cheese and three laboratory samples (G, B1 and B2) and 7 variables: CFU mL^{-1} in *WAM*, CFU mL^{-1} in *MRS* at pH 5.4, total cells number mL^{-1} (*T*), viable cells number mL^{-1} (*V*), titrable acidity expressed as *SH*, *pH* and not viable cells (*NV*) number mL^{-1}

common statistical method, principal components analysis. Observing the biblot in Fig. 2, it is possible to evaluate the difference of the sample G from samples B1 and B2 along factor 1, mainly composed by variables T, V and WAM. On the other hand, samples B1 and B2 lay differently on the biblot along Factor 2 composed by variables °SH, NV (towards sample B2) and pH (towards sample B1). Sample G and samples B1 abd B2 are at the extremities of the biblot and, as expected, variables T, V and WAM discriminate between the good and bad samples. Bad samples differ from each other for their different acidity. According to the sunray plot, samples VI1, MN2, MN6 and PC3 are closest to G whereas samples MN5, PC1 and PC2 lay in the bad samples zone. Samples PD2, MN1 and BS4 overlap in the middle of the biblot but their sunray plots are different. Similarly, in the "bad zone", PC1 and PC2 are very close and, in the "good zone", MN2 is very close to MN6, but their sunray plots are different. The not very high value of variance of the PCA analysis (62,56%) could be the reason of this discrepancy (Massart et al 1988). During PCA transformation from 7 original variables to 2 new ones, some information can be lost, information fully considered by sunray plots. Comparing the two explorative techniques, it could be concluded that, for this particular dataset composed by 24 objects and 7 variables, PCA allowed the highlighting of the good and bad samples, while sunrays plots, even if remaining only a descriptive and explorative analysis, allowed the better visualizing of the differences among all the samples.

Conclusion

The interpretation of the chemical and microbiological data in natural whey starters for Grana Padano cheese has been simplified. Considering their significance for natural whey starter quality, a new way for evaluating these parameters is proposed. This approach could be useful to summarize and outline the microbiological and chemicals data and to readily compare different samples, as well as compiling and monitoring a data archive, for example to control their time stability.

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