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Application of flow cytometry for rapid bacterial enumeration and cells physiological state detection to predict acidification capacity of natural whey starters

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ABSTRACT

Natural whey starter cultures are undefined microbial communities mainly consisting of thermophilic lactic acid bacteria (LAB). The technological pressure that shapes the natural whey starter community before and during the back-slopping procedure can impact the amount and viability of the different thermophilic LAB. Traditional culture-dependent analytical methods are useful for evaluating natural whey cultures based on plate enumeration with various culture media and are commonly used as self-control procedures in dairy items. These methods have high variability and require days to obtain results. As the dairy industry has been searching for a solution to this problem for a long time, researchers must explore alternative methods for the technological evaluation of natural whey and assessment of the health status of the thermophilic acidifying bacteria community in the cheesemaking process. The flow cytometry approach has been considered an alternative to classical methods in this work sector. This study compared bacterial enumeration by plate counting and flow cytometry on natural whey samples. Flow cytometry results showed positive agreement with a tendency to overestimate, linearity, and correlation with plate counting. Other parameters have also been introduced for evaluating a natural whey starter, measuring the physiological state of the cells. Specifically, cell-wall damage and metabolic activity were also evaluated, allowing us to quantify the number of cells even in sub-optimal physiological conditions.

1. Introduction

Fermented cultures are commonly used in the dairy industry for cheese production, mainly to produce cheeses with Protected Designation of Origin (PDO) certification. In contrast to selected commercial starters, the control of these fermentations is often left to the experience of the cheesemaker. The natural whey starter is one of the most famous natural starters producing hard cheeses, such as Parmigiano Reggiano and Grana Padano PDOs in Italy [1]. The natural whey starter production process begins with removing the curds, leaving a sweet whey. The latter is incubated overnight in the temperature-controlled tank following a controlled cooling cycle to reach a thermophilic lactic acid bacteria content necessary for cheese production the following day [2,3].

During the first hours of hard-cheese production, the overnight grown LAB consortia acidifying lactic community determines the critical acidification phase, designing the enzymatic potential and environment that prevail for microbial growth and activity

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throughout the ripening period [4]. The typical lactic community of starter whey is called starter lactic acid bacteria, and it is well-known that this community plays a primary role in curd acidification during the first hours or days of ripening [5]. The chemical and microbiological parameters currently practiced for self-monitoring in dairy or external laboratories are the measurement of the acidity reached overnight by the natural whey (expressed in SH°/50 mL), culture methods to number the cultivable cells present, and, in some cases, the assessment of acidifying activity [6]. However, the limitations of these methods are mainly due to two key factors: the time required for analysis and the ability to provide information to make process changes. The acidity reached in the whey alone allows an understanding of the past metabolic capacity of the community. Still, it does not allow us to know the physiological state of the cells after acidification.

On the other hand, culture methods require two days to obtain a relevant result and only measure cells that can be cultured. Concerning the acidifying activity, it is a suitable but challenging parameter to use during a production run because it requires at least 4 h to obtain data [7]. Moreover, it can often have problems due to the laboratory scale and smaller volumes, even if it remains one of the most empirical parameters that can evaluate the metabolic potential of natural whey starters. Therefore, it became necessary to study such microbial populations that are viable but not cultivable. There is a share of bacteria that cannot grow in whey medium and cannot be isolated, and it becomes interesting to investigate if these non-culturable cells express significant metabolic activity during cheese curd fermentation [8]. Some works focused on this aspect using new generation techniques, such as length heterogeneity polymerase chain reaction (LH-PCR), fluorescence *in-situ* hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and fluorescence microscopy based on stained live/dead cells, which have been essential to understanding how the microbial community and physiological status change [9–15]. After the publication of ISO 19344:2015 [16], the use of flow cytometry for quantification of viable and non-viable bacteria has become more popular. This technique allows to perform a single cell-level analysis using different fluorochromes to simultaneously investigate multiple phenotypic characteristics, ranging from wall damage to specific metabolic activities [17]. The current trend in dairy sector is the implementation of faster multiparameter flow-cytometric methods to replace or integrate culture-based techniques. Flow cytometry is currently used at both research and industrial levels to ensure that milk or its derivatives meet quality requirements [17–20]. Applications for detecting pathogenic microorganisms have been tested by combining flow cytometry with fluorescent in situ hybridization (FISH) to rRNA [21]. Moreover, milk clarification techniques have been applied to improve the counting of bacteria present continuously [22]. These approaches improve flow cytometric techniques by allowing rapid, specific, and quantitative identification of the microbial diversity in milk, suitable for laboratories or dairy plants.

The use of techniques based on flow cytometry is turning towards those typical productions that use natural starter cultures. For instance, monitoring and knowing cell viability could increase the success of dairy fermentations. Arioli et al. (2017) exploited flow cytometry to study specific metabolic interactions within a simulated yogurt consortium [23]. Michelsen et al. (2007) used a flow cytometric approach to discriminate bacteriophage infecting *Lactococcus lactis* by combining scattering and fluorescence signals. This method was a preliminary attempt to replace phage enumeration culture methods with a faster technique, decreasing the probability of fermentation failures [24]. A recent study has used the flow cytometry approach to enumerate live and dead bacterial cells in natural whey starters subjected to mycotoxin-detoxifying agents [25].

This study aims to implement a flow cytometric approach with two different staining protocols directed to natural whey starters to quantify the total number of cells and investigate their physiological status at the industrial level. This rapid technique was compared to the classical cultural-dependent method to understand if a replacement of the time-consuming plate-count approach could be possible for routine analyses in the dairy sector. Finally, we aimed to exploit the information about cells physiological state measured by flow cytometry to predict the ability of the natural whey starter to acidify the milk.

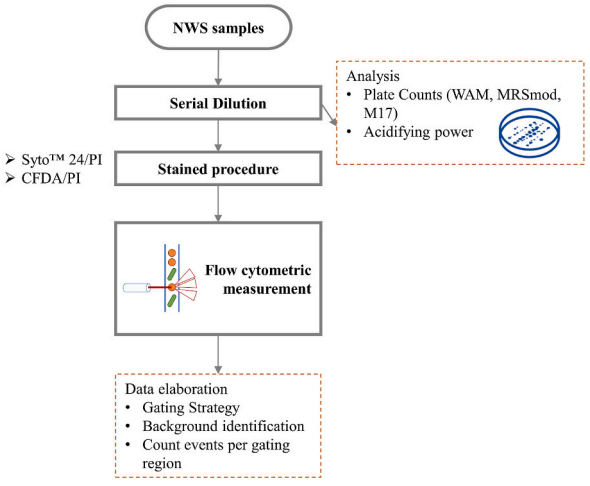

Fig. 1. Flow chart of procedures applied to natural whey starter (NWS) samples to compare bacterial counts using culture-dependent and flow cytometric methods.

2. Materials and methods

2.1. Samples collection

A total of 28 batches (100 mL each) of natural whey starters (NWS) were collected from dairy companies within the production area of Grana Padano and Parmigiano Reggiano PDOs and kept at 4 °C until analysis. The analysis took place within 5 h of sampling, and triplicates were considered for each batch, for a total of 84 samples (Supplementary Table 1). The collected NWS samples were immediately analyzed by culture-dependent methods. The scheme of the experimental procedure is more accurately illustrated in the flow chart below (Fig. 1).

2.2. Plate count enumeration method

NWS samples were serially diluted using 9 g/L of NaCl solution. The colony-forming units of the lactic acid bacteria community present in the sample were counted using agar plate count method. deMan Rogosa Sharpe pH 5.4 (MRS; BD™, Difco™, Franklin Lakes, NJ, USA) was supplemented with 40 g/L yeast extract (MRSmod) [26]; while whey agar medium (WAM) was prepared according to Gatti et al. (2003), [27,28]. For enumeration of the *Streptococcus* group, M17 selective medium (M17 Agar; BD™, Difco™, Franklin Lakes, NJ, USA) was used. The plates WAM, MRSmod and M17 were incubated under anaerobic conditions (Anaerogen, Oxoid, Basingstoke, UK) at 45 °C for 48 h.

2.3. Acidifying capacity

The acidifying capacity of the NWS sample was evaluated according to the method of Coloretti et al. (2016), [7]. A 1.5 mL NWS sample was inoculated into 50 mL skim milk (Oxoid) reconstituted at 10% (w/v). After 4 h of incubation at 52 °C, the increase in titratable acidity was estimated: the rate of acidification at a given temperature was expressed as the difference between the final and initial acidity (Δ°SH/50 mL).

2.4. Flow cytometry-stained procedure

Flow cytometric analysis was conducted using a BD Accurri™ C6 sampler Plus (BD; Franklin Lakes, NJ, USA) on NWS samples. Samples were diluted in phosphate-buffered saline (PBS) to obtain a final concentration between 10^5 and 10^6 cells/mL. Samples were stained separately with two pairs of fluorescent markers of propidium Iodide (PI; Life Technologies, Carlsbad, CA, United States)/ Syto™ 24 (Life Technologies, Carlsbad, CA, United States) and 6-Carboxyfluorescein diacetate (CFDA; Merck, USA)/PI. Treated samples were incubated at 37 °C while shaking and protected from light for 15 min. All Samples were analyzed by performing Protocol A and Protocol B according to ISO 19344:2015 [16] specifications. A 50 μL of NWS diluted samples was taken for instrument reading. The thresholds for side scattering (SSC) and forward scattering (FSC) were 1000 and 3,000, respectively. Moreover, an excitation laser at 488 nm and an emission filter at 533/30 (FL1) and 675/25 (FL3) were used to visualize the stained events. Data was collected and analyzed using BD Accuri™ C6 software version 1.0 (BD Biosciences, USA).

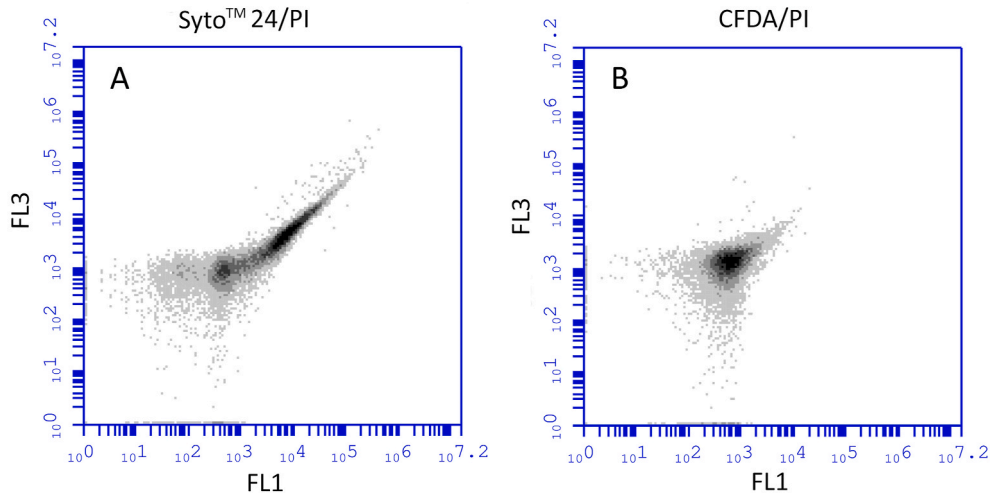

Fig. 2. Two-dimensional graph representing the cell-free simulated natural whey starter background noise: (A) FL1 vs. FL3 channels of simulated natural whey starter stained with Syto™24/PI; (B) FL1 vs. FL3 channels cell-free simulated natural whey starter stained with CFDA/PI.

2.5. Flow cytometry gating strategy

The Syto™24/PI marker pair can discriminate cell groups based on wall integrity (ISO 19344:2015, [16]). Syto™24 is permeable to the bacterial wall and intercalates intracellular DNA from all cells present in a sample, while PI is impermeable to the cell wall and only intercalates intracellular DNA when the bacterial wall is damaged. Specifically, the Syto™24/PI marker pair in the FL1/FL3 visualization, three regions were identified: cells with a low FL3 fluorescence value and a high FL1 fluorescence value were classified as presumed live cells (LIVE), while the region with a high FL3 fluorescence value was classified as presumed dead cells (DEAD), and, between the two regions, there was an intermediate zone which was classified as presumed damaged cells (DAMAGE). The gating process was carried out in accordance with ISO 19344:2015 [16], (Fig. 2A). The CFDA/PI marker pair is able of discriminating cell groups based on intracellular esterase activity [16,29]. CFDA is permeable to cells; when intracellular non-specific esterase hydrolyze the diacetate (DA) groups, fluorescent carboxyfluorescein (CF) is released, while the addition of PI as mentioned before, discriminates cells with the damaged wall.

On the other hand, four regions were identified for the CFDA/PI marker pair. The rightmost region with a high fluorescence value in FL1 was classified as a presumed active cell (ACTIVE). On the left side of the graph, three regions were identified. Starting from the bottom of the graph, the region of presumed inactive cells (INACTIVE) was observed, while the above two regions representing the extent of cell wall damage that could be identified through the intensity of FL3 given by the fluorescent marker PI (INACTIVE-WALL DAMAGE and DEAD-2) were evidenced(Supplementary Fig. 1).

2.6. Background identification

One of the problems encountered during the flow cytometric analysis of NWS samples is the presence of interferents, such as fat globules and protein aggregates [22], that reach similar or larger sizes than the bacterial cells and affect the scattering of light. Similarly, they can bind fluorescent dyes, masking bacterial cell readings. Furthermore, it was impossible to obtain cell-free NWS samples while preserving the background of non-cellular particles, such as fat globules and protein aggregates. To solve this problem, we simulated a lab-scale cheesemaking process according to the procedure described by Franceschi et al. (2019) [30], with some modifications. Specifically, 500 mL of raw milk sample was taken from a farm within the Po Valley's Grana Padano and Parmigiano Reggiano PDO areas. Raw milk was heated to 33 °C and immediately added both 2.5 g per 100 kg of milk of calf rennet (1:125,000 units) and NWS (3% v/v). The mixture was incubated for 10–12 min at 33 °C. Specifically, the added NWS was cell-free due to filtration on filters at 0.22 μm.

After an incubation period, the curd was broken into granules and heated at 53 °C for 45 min with continuous stirring. Finally, after depositing the caseous mass on the bottom, we filtered the liquid with linen filters (typical in the GRANA cheeses process) and recovered the resulting whey. At this point, we instantly acidified the whey by adding lactic acid up to SH° 33 and pH 3.3, simulating the acidification of whey by thermophilic bacteria. The cell-free NWS samples obtained were subjected to flow cytometric analysis (see 2.5 and 2.6).

2.7. Statistical analysis

The coefficients of variation (CV) were determined after ten measurements of the same sample by a single operator. Bland-Altman analysis was carried out to study the accordance between the two methods of measurement [31]. The relative difference between a pair of measurements based on the two data methods was displayed in relation to the mean of the paired measurements. The agreement parameters between the two methods were assessed by evaluating the average difference and the limits of agreement (LOA). In addition, the relationship between flow cytometric parameters and the plate counts method was assessed using univariate linear regression by calculating R^2. Moreover, a Pearson correlation (r) was generated for all cultural-dependent, cultural-independent, and acidification power data. A multiple linear regression model using the number of cells in the different physiological states was implemented to predict acidification power.

3. Results and discussion

3.1. Background identification

The background noise of non-cellular particles was highlighted by constructing a simulated cell-free natural whey starter (section 4.3.5). Background noise plot displayed that the use of Syto™24/PI double labeling generated a particle scatter that was centrally located in the graph (Fig. 2A). We recognized the same dot scatter in the outputs generated from the data obtained by analyzing the NWS samples (Supplementary Fig. 1).Similarly, CFDA/PI background noise plot evidenced a different shape dispersion. In this case, a dispersion of points was observed from the simulated free-cells NWS output in the lower right part of the graph that generated possible overlap between the background noise and the region labeled "INACTIVE" (Fig. 2B). The results obtained on the simulated free-cell NWS samples suggest a partial interaction of the fluorescent molecules used to mark the cells with the non-cellular particles. Analyzing a simulated cell-free NWS did not allow us to eliminate the background a priori. However, it made us aware of its theoretical position by improving the gating strategy used in the enumeration phase of cells at a different physiological state.

3.2. Comparison between the flow cytometric approach and plate count method

The repeatability of the method performed by a single operator on WAM and LIVE counts showed a higher coefficient of variation for data obtained with the plate method (CV = 10.59%) than the flow cytometer (CV = 3.74%). The agreement of the cell count measurements between the two methods was compared using the Bland-Altman analysis (Fig. 3). For the NWS samples, the relative difference between a pair of measurements based on the two indicated methods was displayed compared to the mean of the pair of measurements. The LOA indicates the overall correspondence between the two methods. However, if the differences are slight (close to zero) and are congruent over the range of measurement values with a narrow LOA, they are considered similar. The mean relative differences comparing LIVEvsWAM and LIVEvsMRSmod were $4.6 \times 10^8 \pm 1.72 \times 10^8$ and $9.38 \times 10^8 \pm 2.70 \times 10^8$, with LOAs of 95% from 1.23×10^8 to 7.97×10^8 and from 4.09×10^8 to 1.47×10^9, respectively. (Fig. 3A and B).

Based on the results obtained, an average overestimation of the flow cytometric method using SytoTM24/PI was observed by the near absence of negative differences. LIVEvsWAM had the narrowest LOAs, while LIVEvsMRSmod generated wide LOAs. Thus, using MRSmod as the medium for NWS bacterial count was not as effective as WAM. This finding is in accordance with previous works [32,

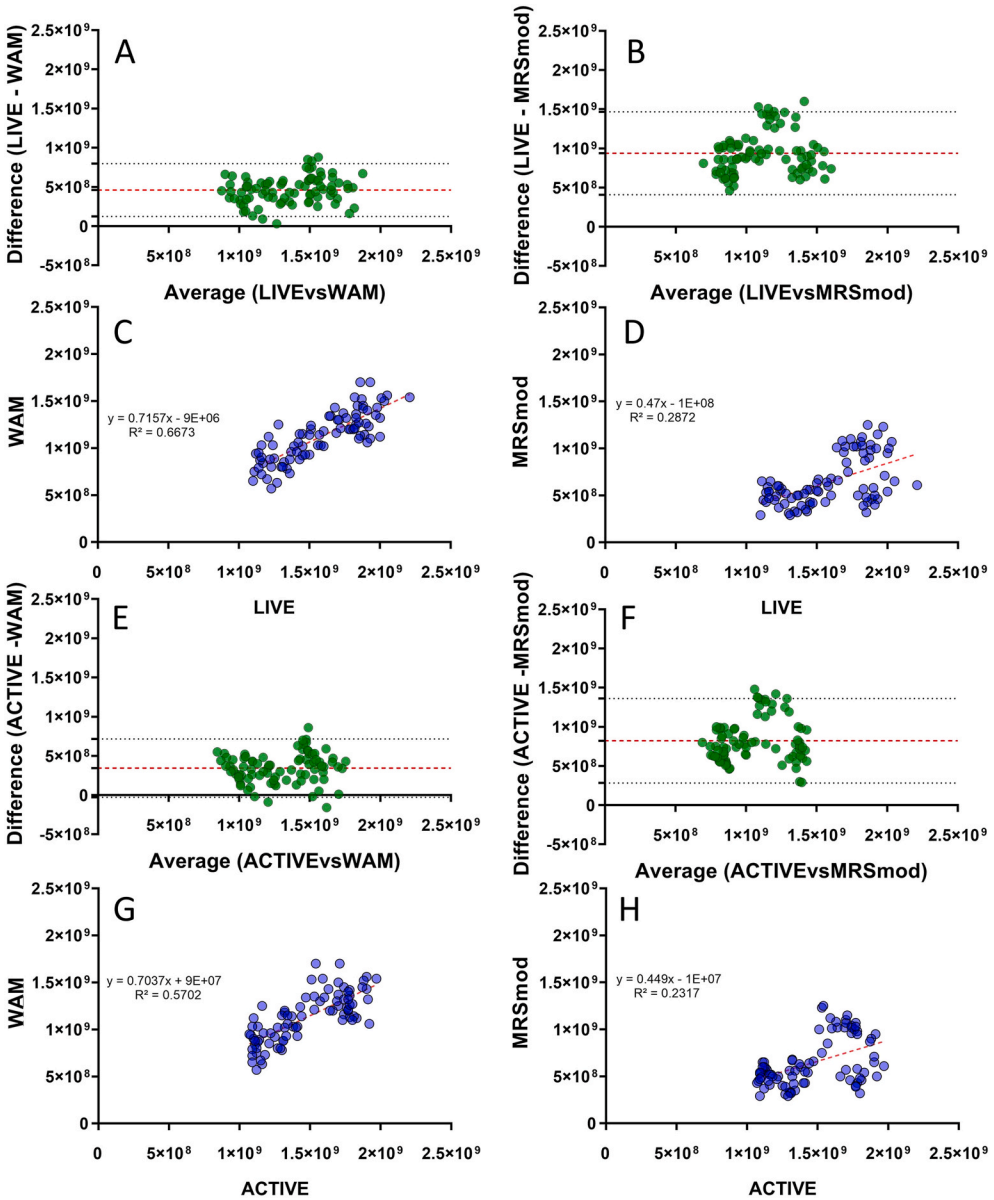

Fig. 3. Bland Altman (A, B, E and F; dotted line means limits of agreement (LOA); segmented line indicates the average difference between the two methods) and univariate linear regression (C, D, G and H; dotted line means trendline) plots between culture-dependent and quantitative flow cytometric methods for enumeration bacteria in natural whey starters samples.

33], indicating that WAM can maximize the number of cells that can be grown.

Aware of the overestimation of the cytofluorimetric method evidenced by Syto™24/PI dyes, we investigated whether using a CFDA/PI might better agree with the culture data. Comparisons were made by assessing the concordance between active cells (ACTIVE) measured by flow cytometry and the number of cells measured by culture methods. The mean relative differences comparing ACTIVEvsWAM and ACTIVEvsMRSmod were $3.44 \times 10^8 \pm 1.92 \times 10^8$ and $8.22 \times 10^8 \pm 2.75 \times 10^8$, with LOAs of 95% from 2.81×10^7 to 7.16×10^8 and from 2.82×10^8 to 1.36×10^9, respectively. (Fig. 3E and F).

Based on the results obtained with CFDA/PI staining, similar agreement parameters were observed compared to those obtained with Syto™24/PI staining. A lower overestimation of the flow cytometric method was maintained in ACTIVEvsWAM compared to ACTIVEvsMRSmod, also confirmed by the lower negative LOA value. Syto™24/PI discriminates cell viability based on wall permeability (see 2.5). The principle of stained procedure and the results suggested that the overestimation of the flow cytometry method is due to the number of viable but non-culturable cells [34] within the region identified as LIVE; there may be cells with an intact wall, but which are unable to generate a colony on a plate. CFDA/PI, on the other hand, can discriminate intracellular esterase activity and assume cell viability. However, the overestimation observed again suggests that within the region identified as ACTIVE, there may be cells with esterase activity that are not able to generate a colony on a plate. These results suggest the presence of putative viable cells measured by flow cytometry but not culturable. Whether viable but non-culturable cells can be considered significant in the technological evaluation of natural whey starters is still unclear. Confirming the almost overlap between the two stained procedure protocols used with flow cytometry, we observe through the Bland Altman plot and linear regression (Supplementary Fig. 2) a strong concordance between the two staining methods with a mean difference close to zero and a coefficient of determination $R^2 = 0.90$.

Finally, the linearity between the two flow cytometric methods and the cultural-dependent approach was assessed by applying a linear regression between the values obtained with the flow cytometric methods and those obtained with the culture methods. R^2 was used to estimate the linearity between the two methods (Fig. 3C, D, 3G, and 3H). Confirming the Bland-Atman method, the highest R^2 was found in the LIVEvsWAM ($R^2 = 0.66$) and ACTIVEvsWAM ($R^2 = 0.57$) comparisons. The only work comparing culture-dependent and flow cytometer methods on NWS samples was carried out by Ref. [26]. The study compared three counting methods: flow cytometric measure of sterile milk samples inoculated with a known amount of NWS, microscope counts, and plate counts using MRSmod. R^2 obtained between the flow cytometer and plate culture method was in accordance with the results obtained in this work.

3.3. Correlation power between plate technique and flow cytometric techniques

Pearson correlation coefficients (r), which measure the tendency of two numerical variables to vary together (co-vary), were calculated by comparing all variables measured with a culture approach (WAM, MRSmod, and M17) and variables measured with a flow cytometric approach (LIVE, DEAD, DAMAGE, ACTIVE, INACTIVE, INACTIVE WALL DAMAGE, DEAD-2). Pearson correlation coefficients were calculated and represented in the heat map (Fig. 4) and were considered significant with a P-value <0.05 (Supplementary Table 1). In agreement with previous comparative analyses between culture and flow cytometric methods, where a threshold value of ± 0.7 indicates a good correlation [35], we obtained a Pearson correlation index >0.7 for LIVEvsWAM, ACTIVEvsWAM, and LIVEvsACTIVE comparisons.

Particular attention should be paid to the variables measured with the flow cytometric representing suboptimal cell physiological states and cell death, such as DEAD and INACTIVE-WALL DAMAGE. Both showed a negative correlation greater than 0.7 with CFU/mL in the Streptococcus group (M17). This correlation suggests a tendency to co-vary inversely between the *Streptococcus* group and cells having a suboptimal physiological state within the sample. We did not know the curd-cooking temperatures and temperature gradient

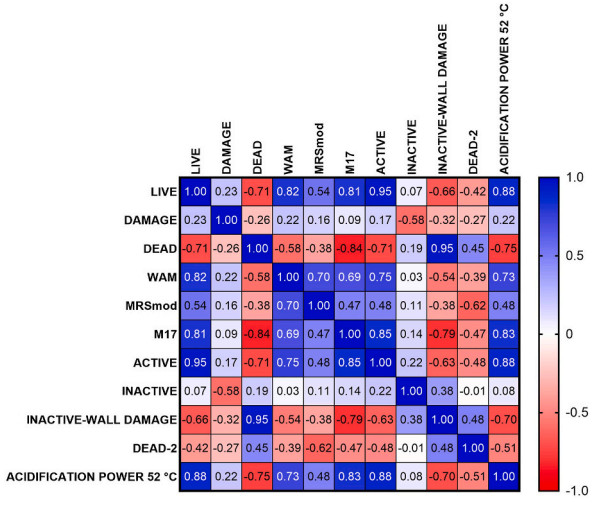

Fig. 4. Correlation matrix obtained all parameters derived by flow cytometric and cultural-dependent techniques. Pearson's correlation was used. Negative correlations are shown in red, and positive correlations are in blue.

used in the whey temperature-controlled tanks, unshared company information. We can advance the hypothesis that stress conditions occurring during the temperature gradient applied to the temperature-controlled tanks overnight impact the population of *Streptococcus* group [8]. Therefore, the negative correlation between dead cells measured by flow cytometry and the number of cells belonging to the *Streptococcus* group could be used as a thermal stress index. However, a more in-depth investigation of the relationship between natural whey fermentation and different cooling gradients was needed to confirm this preliminary hypothesis. Interestingly, acidifying power was positively correlated with cell counts based on culture-dependent techniques and variables based on flow cytometric techniques. Specifically, a positive correlation greater than 0.7 was found with plate count WAM, LIVE, and ACTIVE counts, and a negative correlation was evidenced between DEAD and INACTIVE-WALL DAMAGE counts since acidifying capacity was a parameter of the technological goodness of natural whey starters to carry out its function of acidifying the curd [6].

3.4. Physiological state of natural whey starter cells as a predictive estimation of the acidifying capacity

In the previous section, we showed a good correlation between the flow cytometry data and the cultivable bacterial counts with the acidification power of a natural whey starter at 52 °C.

To test whether the rapid flow cytometry method was able to predict the acidification capacity, we carried out two multiple linear regression models by combining the measurement of LIVE and DEAD cells obtained with Syto™24/PI for the first model (Fig. 5A) and ACTIVE and INACTIVE-WALL DAMAGE cells with CFDA/PI for the second model (Fig. 5B). Models had a R^2 greater than 0.8, and a root mean square error and deviation (RMSE and RMSD) lesser than 0.3 (Table 1). These findings suggested that combining the number of cells in different physiological states could predict the acidifying power of the natural whey starter. However, further investigations are needed to validate the correlation between RMSD and RSME values and the NWS acidification performance at the industry level. Indeed, the error explained by RSMD and RMSE indexes should be evaluated *in-situ* by following the acidification of the curd to understand whether the model error is negligible. The predictive approach of NWS acidification power based on detecting the cells physiological state by flow cytometry could be a valuable tool for the dairy industry to rapidly evaluate the stability and performances of different natural whey starter cultures over time.

4. Conclusion

A flow cytometric assay was tested with natural whey starter samples and compared with classical culture-dependent techniques. The work was designed as a first step to implementing a flow cytometry method to support dairy farms. Agreement results of the method shown an overall overestimation of the flow cytometric method, but linearity scores reveal good correlations with data obtained with classical techniques. Furthermore, a direct correlation was observed between the health status of bacterial cells and the ability to acidify milk by natural whey starter under thermophilic conditions. Finally, the results obtained on the predictive ability of the flow cytometric method regarding the acidifying power of a natural whey starter were promising and deserve future validation on an industrial level. Moreover, it is essential to highlight that the flow cytometric approach can provide different levels of cell health within a time compatible with industrial timing.

In future work, we could confirm the potential of this approach by increasing the sample size and finding other technological parameters to correlate with the physiological state of bacterial cells (e.g., acidification curve in the first 24 h after cheese molding). Moreover, it would be interesting to understand the metabolic and technological role of viable but non-culturable cells.

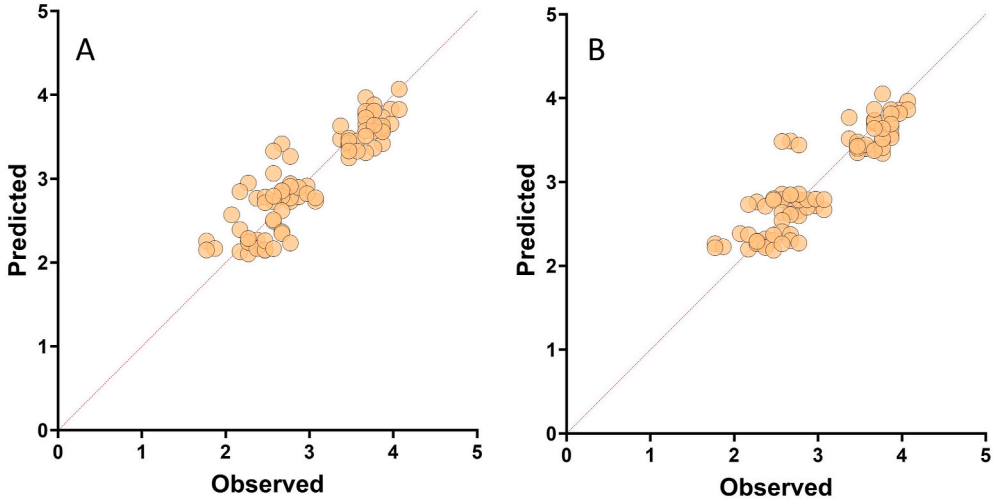

Fig. 5. Observed vs. predicted acidification capacity at 52 °C resulted from multiple linear regression analysis models using the amount of cells at different physiological states (A- LIVE and DEAD, B- ACTIVE and INACTIVE-WALL DAMAGE) detected by flow cytometric approach with two staining procedures.

Table 1
Goodness-of-fit parameters of regression models for predicting the acidifying power of natural whey starters.

Goodness of Fit	Syto™24/PI (LIVE and DEAD)	CFDA/PI (ACTIVE and INACTIVE-CELL WALL DAMAGE)
Adjusted R^2	0.80	0.81
RMSE	0.28	0.27
RSMD (Observed vs. Predicted)	0.28	0.27
Variance Inflation Factor (VIF)	2.00	1.66

We believe that natural whey cultures used in cheese production are often controlled only by the experience of cheesemakers, so it is appropriate for researchers to support this experience.

Author contributions

Paolo Bellassi: Conceptualization; Formal analysis; Data curation; Investigation; Writing – original draft. Alessandra Fontana: Data curation; Investigation; Writing – review & editing; Writing- Original draft. Lorenzo Morelli: Supervision; Writing – review & editing.

Declarations

The acquiring of raw milk was conducted according to established animal welfare guidelines.

Data availability statement

Data included supplementary material.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e19146.

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