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ORIGINAL ARTICLE

Stress response assessment of *Lactobacillus sakei* strains selected as potential autochthonous starter cultures by flow cytometry and nucleic acid double-staining analyses

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Keywords
flow cytometry, *Lactobacillus sakei*, nucleic acid double staining, stress conditions.

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Abstract

Aims: The aim of this study was to apply the flow cytometry to *Lactobacillus sakei* strains, selected as potential autochthonous starters, to investigate dynamics and physiological heterogeneity of microbial behaviour under different stress conditions.
Methods and Results: A simultaneous nucleic acid double-staining assay was applied to discriminate cell populations in different physiological states after exposure to heat (50 and 55°C) and acid (pH 2·5 and 3·0) stresses. Alive cells with intact membranes, damaged cells still alive but with injured membranes, so with even a recovery ability, and dead cells with a permanent membrane damage were differentiated with a significant increase in damaged cells after stronger stress treatments.
Conclusions: The existence and characteristics of subpopulations displaying heterogeneity in particular conditions are highly relevant, because specific subpopulations may show improved survival, changes and dynamics under stress conditions.
Significance and Impact of Study: This assay has potential for physiological research on lactic acid bacteria and for application in the food industry. The assessment of intermediate physiological states in *Lb. sakei* strains with recovery possibility could be an important criterion for application of potential starter cultures. Application of flow cytometry and characterization of sorted subpopulations may contribute to further understanding of diversity and heterogeneity in physiology of bacterial populations.

Introduction

Lactic acid bacteria (LAB) are widely used in meat fermentation, and an efficient control of these microbiological processes requires increased knowledge of bacterial behaviour under stress conditions that can affect bacterial viability and activities (Bunthof *et al.* 2000; Bonomo *et al.* 2010) and are also used as model system to test antibacterial activity of natural compounds (Padula *et al.* 2013; Russo *et al.* 2012). *Lactobacillus* (*Lb.*) *sakei* is recognized as one of the most important components of starter cultures used for Italian fermented sausage production, and its growth and survival are affected by various factors such as temperature, pH and salt concentration (Bonomo *et al.* 2008, 2010). Development of rapid and reliable methods for measuring viability is of the utmost importance for studies on bacterial physiology (Bunthof *et al.* 2000). Bacterial viability is usually defined as the ability of cells to multiply and form visible colonies on agar plates (Breeuwer and Abee 2000; Smigic *et al.* 2009), and its defined aspects are reproduction, vitality and membrane integrity. This status is conventionally assayed by colony counting. Vital cells are metabolically active. Intact cells have a cytoplasmic membrane with selective permeability. Membrane integrity, vitality degree and self-replication ability depend on environmental conditions and also on cell physiological status. Micro-organisms can adopt different states, such as a

dormant or a latent state, from which they may be resuscitated, that is, induced to return to a physiologically active state (Bunthof *et al.* 1999).

Classical techniques (turbidity measurement, dry weight determination and plate counting method) give information about microbial growth associated with cell division but do not consider physiological states. Intermediate states, for example, cell injury, are difficult to detect with the plating method but there are viability indicators based mostly on fluorescent molecules, which can be detected at the single-cell level without culturing cells with some advanced techniques, such as the flow cytometry. Each indicator is based on criteria that reflect different levels of cellular integrity or functionality. Over the last 20 years, multiparameter flow cytometry has become a powerful tool in microbiology (Joux and Lebaron 2000; Nebe-von Caron *et al.* 2000; Berney *et al.* 2006, 2007), because it is fast and it allows single-cell analysis. Flow cytometry can reveal information about dynamics and physiological heterogeneity of microbial populations and describes more accurately the state of a population than the average values obtained from traditional techniques (Malacrinò *et al.* 2001; Holm *et al.* 2004; Phe *et al.* 2005; Schellenberg *et al.* 2006).

Studies concerning the existence and characteristics of subpopulations, displaying heterogeneity in particular conditions, are highly relevant, because specific subpopulations may show improved survival, changes and dynamics under stress (Quiros *et al.* 2007). Flow cytometry application and sorted subpopulation characterization may contribute to further understanding of diversity and heterogeneity in physiology of bacterial populations present in specific foodstuffs. Previous studies (Bonomo *et al.* 2008, 2010) concluded that the LAB ecosystem is characterized by a species–site dominance, and this dominance is closely related to environmental parameters. Traditional fermented sausages show a distinctive organoleptic profile due to bacterial species, responsible for fermentation process, that possess specific physiological and technological characteristics that make these traditional products unique. For this reason, it is necessary to select appropriate cultures from indigenous micro-organisms, more competitive, well adapted to the particular product and to the specific production technology, and with high metabolic capacities that can beneficially affect product quality and safety, preserving their typicity (Bonomo *et al.* 2008). The development of autochthonous starter cultures for food fermentations is a multidisciplinary endeavour requiring not only an ecological study of the spontaneous process but also characterization of useful physiological features of the predominant strains to select those with the highest potential for industrial applications.

Thus, the aim of this study was to apply a flow cytometric method to *Lb. sakei* strains, selected as potential autochthonous starters, in order to investigate dynamics and physiological heterogeneity of microbial behaviour under different stress conditions.

Materials and methods

Bacterial strains and culture conditions

Five strains of *Lb. sakei* (DBPZ0062, DBPZ0098, DBPZ0338, DBPZ0329 and DBPZ0416 of the culture collection of the Dipartimento di Scienze, Università degli Studi della Basilicata, Potenza, Italy) used in this study were isolated from traditional fermented sausages of Basilicata region, identified by phenotypic and molecular analysis and technological characterization and selected as potential autochthonous starters in previous studies (Bonomo *et al.* 2008, 2010). All strains were maintained as freeze-dried stocks in reconstituted (11% w/v) skim milk, containing 0·1% (w/v) ascorbic acid and routinely cultivated in MRS broth at 30°C for 16 h, before stress treatments.

Stress treatments

Late-exponential phase cells grown overnight in MRS broth, pH 6·2, were harvested by centrifugation (12 000 ***g***, 5 min) and washed twice in sterile saline solution (0·85% w/v NaCl). Cells were resuspended in 2 ml of different media to a final OD_{600} = 1·0 to achieve the following stress conditions: (i) MRS broth, pH 2·5 and 3·0 (adjusted with HCl, acid stress); (ii) MRS broth, pH 6·2, at different temperatures (heat stress). Cell suspensions were incubated for 30 min both at 30°C for acid stress and at 50 and 55°C for heat stress. Bacterial cells incubated at 30°C for 30 min in 2 ml of MRS broth, pH 6·2, were used as control.

Fluorochromes and staining conditions

Two different fluorochromes were used to stain the bacterial cells: SYBR Green II RNA gel stain (Molecular Probes, S-7586, abbreviated as SYBR II) and propidium iodide (Sigma-Aldrich, Milan, Italy, P-4170, abbreviated as PI). Both are nucleic acid stains and are well suited for excitation by the argon laser blue line at 488 nm. SYBR II has a maximum absorption wavelength at 488 nm, and it fluoresces green with a maximum emission at 520 nm (Haugland 2002). As for SYBR II, PI maximum absorption wavelength is 493 nm but its maximum fluorescence emission is at 630 nm (red emission). To test whether a treatment caused membrane damage, cells were incubated with the impermeant nucleotide-binding dye PI. Stock

solutions of 1·0 mg of PI per ml were prepared in distilled water, stored in the refrigerator and kept in the dark. PI was added to a concentration of 44 mmol l^{-1} to a cell suspension and incubated at 30°C for 20 min. The molecular weight is not known for SYBR II, because its chemical formula is proprietary, so to find the appropriate working concentration and the best results, 1 μl of the original solution was firstly diluted 10, 20, 30, 50, 100, 150, 200 and 300 times in distilled water. Of these solutions 3 μl was added per ml of final solution (including cell buffer and PI, or water when single staining was performed). Samples were stained with either SYBR II or PI at the beginning; then, dual staining was performed measuring the emitted fluorescence in the SYBR II and PI channels, respectively, and incubated at 30°C for 20 min before being analysed with the FACScanto II cytometer (Becton Dickinson, Sunnyvale, CA, USA).

Flow cytometry analysis

Samples were run on a FACScanto II cytometer with a 15-mW, 488-nm argon ion laser and combined with BD FACSDIVA 6.1.2 software (BD Biosciences, San Jose, CA, USA). Instrumental parameters were forward light scatter (FSC, Log) and sideward light scatter (SSC, Log, 304 PMT voltage, 488/10 filter), and green fluorescent cells stained using SYBR II were collected in SYBR II channel and red fluorescent cells stained using PI were collected in PI channel. All bacterial analyses were performed at a medium flow rate setting (approximately 2·0 ml min^{-1}), and acquisition was performed over an 1-min period. Samples were run such that the event rate was below 1000 events s^{-1} to avoid coincidence. Bacteria were detected in a plot of 90° side light scatter (SSC; related to cell size) *vs* green fluorescence for SYBR II and red fluorescence for PI. Bacterial clusters were identified visually and delimited on the SSC *vs* green or red density plot by drawing a user-defined gate. Quantitative assessment of each bacterial cluster was performed by counting the number of events included inside the corresponding window. The flow cytometric noise corresponded to particles that could not be assigned to any population. For total cell percentage count, a 1-ml sample was put into a 12 × 75 mm plastic tube (Falcon plastic tube, Becton Dickinson 352054) and diluted in 2 ml of FACS flow solution. Bacterial counts by flow cytometry were obtained by normalizing the numbers of events occurring in cluster windows on a density plot to the sample volume analysed in order to account for flow rate fluctuations. Ten thousand cells per sample were analysed, and data were recorded as histograms of fluorescence. The geometric mean of fluorescence for the defined population was calculated with the Becton Dickinson software.

All experiments were performed with at least three batches of cells. It was measured the fluorescence of alive, dead and injured cells to increase and measure the usefulness of the double staining. A positive control (100% of dead cells obtained heating cell solutions up to 90°C for 1 h) was used, and cells were only PI positive.

Device settings of flow cytometer

With flow cytometer analysis, the detected fluorescence signals are converted to voltages and can be amplified and further analysed. Each fluorescence signal was treated individually. The amplification of these signals had a significant influence on the final result because different combinations of these signal voltages produced variation in the fluorescence ratios, thereby affecting the resolution of the cell fluorescence distribution. It was necessary to find a combination of these signal voltages, where fluorescence intensities of both samples and the calibration substances were within the measuring range. The combination of 348 V for SYBR II and 227 V for PI was determined to be optimal for flow cytometric analyses.

Plate counting

After exposure of *Lb. sakei* cells to different stresses, number of viable bacterial cells was determined by the standard counting method, measuring colony-forming units (CFU) on MRS agar plates. Cell counts were determined by serial decimal dilutions with sterile saline/peptone water (8·5 g l^{-1} NaCl and 0·1 g l^{-1} bacteriological peptone), plating on MRS agar, and after incubation at 30°C in anaerobic jar, colonies from plates containing 30–300 colonies were counted. All measurements about cell growth were taken in three independent assays, and the mean log values as well as standard deviation were calculated.

Statistical analysis

Statistical analysis was performed using Systat 10.0 for Windows (SPSS, Chicago, IL, USA). All counts by standard plate count method and flow cytometry were performed with at least three batches of cells. The relationship between the bacterial counts obtained with both methods was evaluated, the average of triplicate results tested by each method was compared, and the correlation coefficient (r) was calculated. Significant differences and correlations between counts of two methods were evaluated by analysis of variance (ANOVA) followed by a Student's t-test. The correlation between the methods was tested at a significance level of 0·05. The effect of each stress treatment and the differences among

treatments were tested for significance with the Student's *t*-test. Furthermore, comparison of two methods was made by the Student's *t*-test (*P* values). These *P* values were taken into consideration to evaluate the general correspondence between the two methods.

Reagents and media

Unless otherwise specified, all reagents were obtained from Sigma-Aldrich (Milan, Italy), while bacteriological media and ingredients were obtained from Oxoid (Basingstoke, Hampshire, UK).

Results

In this study, we proposed a nucleic acid double-staining assay based on flow cytometry technique, which allows to distinguish viable from damaged and membrane-compromised bacteria and to sort out noise and detritus. Firstly, a single staining was performed using bacterial cells after exposure to different stress conditions to find the right staining conditions, as no data were previously reported about flow cytometric analyses applied to this bacterial species. Different SYBR II dilutions were used to find the most useful in detecting cell fluorescence and at the same time that was not so strong to interfere with the instrument detector. PI was used as recommended from the BD manual, after that dual staining was performed. The same SYBR II dilutions were used together with PI staining. The higher ones (10, 20, 30, 50, 100, 150) overlapped or influenced PI fluorescence emission, and it was not possible to evidences three different populations (data not shown). The dilution of 300 times gave consequently a lower emission, optimized for our bacteria and conditions. Applying the ratio described above, all experiments were performed.

All events observed for each *Lb. sakei* strain were represented as a population in density plots (cytograms). Moreover, they were converted in related cell percentages; in each cytogram, generated for each bacterial stress condition, the green, green plus orange-red and orange-red *Lb. sakei* cells were identified as alive, damaged and dead cells, respectively.

Figure 1 gives an example of the optimized flow cytometric analyses for *Lb. sakei* DBPZ0146 strain, and it represents the density plot of SYBR II green fluorescence (SYBR II channel) *vs* PI red fluorescence (PI channel). Cytograms clearly shows two bacterial clusters that can be discriminated from one another after single staining with SYBR II. The first cluster was a highly fluorescent population after SYBR II staining, which corresponds to alive cells, while the second cluster is less fluorescent and corresponds to dead cells, which are difficult to stain using SYBR II. These results were confirmed by PI staining. Moreover, the double staining with SYBR II and PI gave the same fractions of green- and red-labelled cells that the SYBR II labelling and PI labelling gave separately, but with an important difference that dead cells were separated in cells killed because of a permanent membrane damage, and in damaged cells with even a recovery ability. The bacterial cell per cent results determined by nucleic acid double-staining flow cytometry analysis for each *Lb. sakei* strain after heat and acid stress treatments are shown in Table 1.

When a nontreated cell suspension (negative control sample) was incubated with SYBR II and PI, nearly all (94·8–98·6%) the cells showed bright green fluorescence and very few of them (0·3–1·4%) showed red fluorescence (Table 1 and Fig. 1a). The small fraction, which was not labelled with SYBR II but was stained with PI, represented the dead cells naturally present in cell cultures and also due to harvesting procedure. On the other hand, cells deliberately killed (positive control) were incubated with both dyes and were 100% stained with PI (data not shown). Cytograms 1B–1E were obtained with bacterial cells exposed to heat and acid stress treatments. These cytograms pointed out different physiological states and the microbial ability to modify their behaviour for self-adjustment to different growth conditions. Four different quadrants were differentiated using dual staining technique and represented bacterial cells in different physiological states. Quadrant Q1 was populated with alive cells with intact membranes that were stained with SYBR II, quadrant Q2 represented an intermediate physiological state that corresponded to damaged cells that were still alive but exhibited injured membranes, quadrant Q3 corresponded to a low amount of debris obtained after treatment, and quadrant Q4 included dead cells stained with PI.

After stress treatments, all conditions but highly fluorescent SYBR II-stained bacterial cells were converted to cells with brightly red fluorescence, indicating that those cells were killed for membrane damage. Also after heat stress exposure (50°C), the *Lb. sakei* DBPZ0062, DBPZ0146 and DBPZ0098 strains showed cell percentages similar to those present in the control sample with a slight decrease in alive cells and a doubling of damaged cells number in the quadrant Q2, while in DBPZ0329 and DBPZ0338 strains, this stress caused a reduction over 30% of cell events number compared with the control (Table 1 and Fig. 1b). After exposure to 55°C, all strains (except DBPZ0098) presented any or very low alive cell percentages, and for this reason, two important populations were distinguished: cells (ranging from 1·7 to 4·6%) with a membrane damage with a recovery possibility (quadrant Q2) and cells (from 98·2 to 93·4%) with a

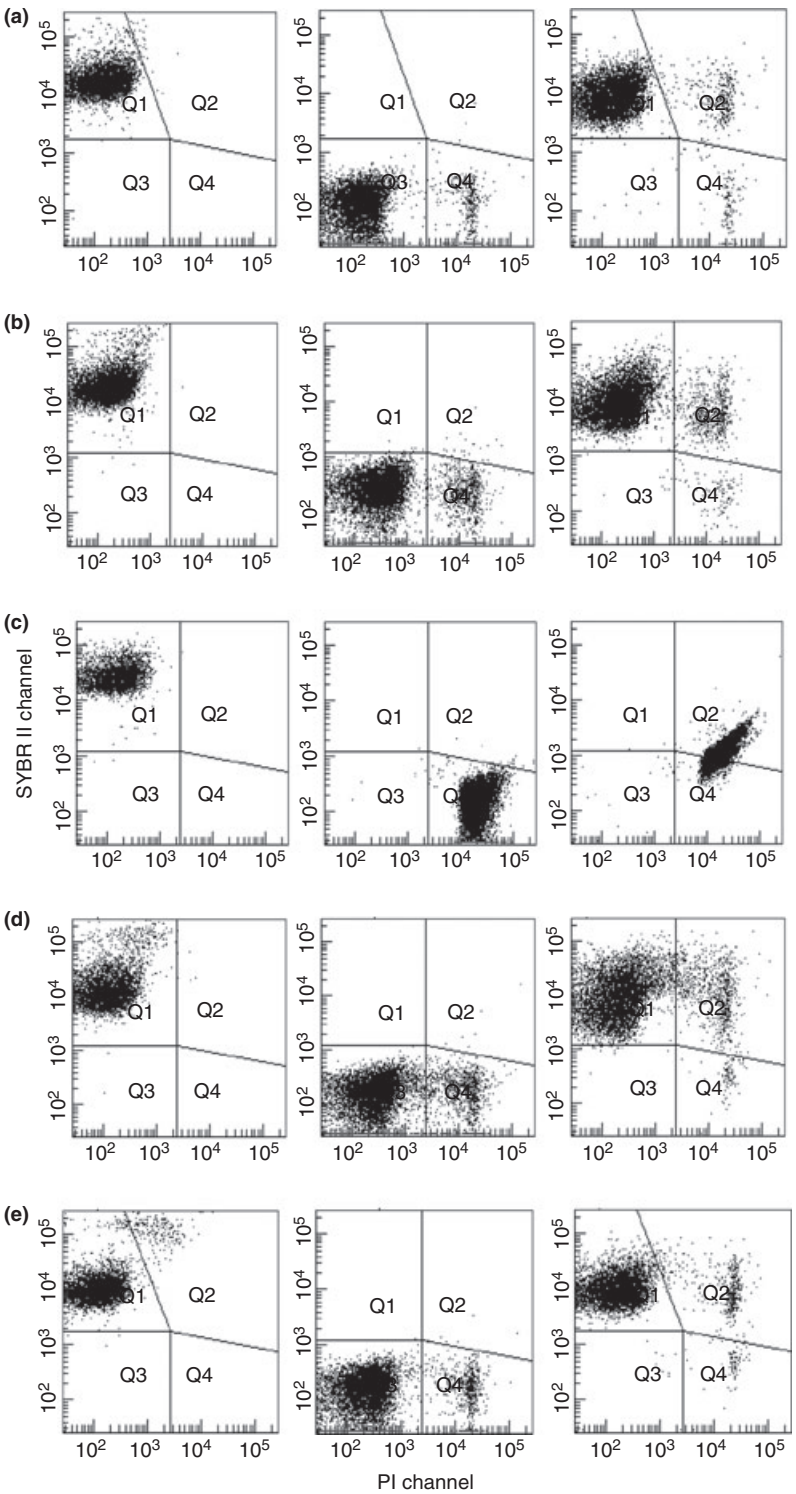

Figure 1 Example of the optimized flow cytometric analyses for *Lactobacillus sakei* DBPZ0146 strain. Flow cytometry density plots of green fluorescence (SYBR II channel) *vs* red fluorescence (PI channel) of cells stained with SYBR II and PI. In all cytograms group, from left to right: single SYBR II staining cells, single PI staining cells, double SYBR II and PI staining cells. Cytograms (a) control sample; (b) stressed sample at 50°C; (c) stressed sample at 55°C; (d) stressed sample at pH 2·5; (e) stressed sample at pH 3. Events in different quadrants correspond to alive cells with intact membranes and stained with SYBR II (Q1), damaged cells in an intermediate physiological state, still alive but with injured membranes (Q2), a low amount of debris obtained after treatment (Q3) and dead cells stained with PI (Q4).

Table 1 Bacterial cell numbers determined by nucleic acid double-staining flow cytometry and plate counts methods after heat and acid stress treatments

Strains	Population* All events	# Events (% total)† 10·000	Plate counts‡ LOG (CFU/ml) (%)
Lactobacillus sakei DBPZ0062			
Control	Q1	9·481 ± 0·11 (94·8)	8·22 ± 0·05 (100)
	Q2	375 ± 0·08 (3·7)	
	Q3	10 ± 0·07 (0·1)	
	Q4	134 ± 0·05 (1·3)	
Heat stress at 50°C	Q1	9·158 ± 0·21 (91·6)	8·05 ± 0·15 (97·91)
	Q2	665 ± 0·08 (6·6)	
	Q3	12 ± 0·06 (0·1)	
	Q4	165 ± 0·07 (1·7)	
Heat stress at 55°C	Q1	108 ± 0·43 (1·1)	2·78 ± 0·83 (31·63)
	Q2	304 ± 0·08 (3·0)	
	Q3	11 ± 0·09 (0·1)	
	Q4	9577 ± 0·95 (95·8)	
Acid stress at pH 2·5	Q1	7012 ± 0·59 (70·1)	4·14 ± 0·02 (50·38)
	Q2	2652 ± 0·15 (26·5)	
	Q3	84 ± 0·11 (0·8)	
	Q4	252 ± 0·23 (2·5)	
Acid stress at pH 3	Q1	9193 ± 0·22 (92·0)	8·09 ± 0·04 (98·33)
	Q2	683 ± 0·35 (6·8)	
	Q3	3 ± 0·14 (0·0)	
	Q4	121 ± 0·08 (1·2)	
Lactobacillus sakei DBPZ0146			
Control	Q1	9·531 ± 0·67 (95·3)	8·23 ± 0·05 (100)
	Q2	317 ± 0·10 (3·2)	
	Q3	11 ± 0·59 (0·1)	
	Q4	141 ± 0·33 (1·4)	
Heat stress at 50°C	Q1	9·171 ± 0·89 (91·7)	7·96 ± 0·82 (96·74)
	Q2	671 ± 0·44 (6·7)	
	Q3	12 ± 0·65 (0·1)	
	Q4	146 ± 0·25 (1·5)	
Heat stress at 55°C	Q1	0 ± 0·01 (0·0)	1·38 ± 0·25 (16·26)
	Q2	170 ± 0·09 (1·7)	
	Q3	13 ± 0·11 (0·1)	
	Q4	9·817 ± 0·98 (98·2)	
Acid stress at pH 2·5	Q1	6·816 ± 0·88 (68·2)	4·10 ± 0·38 (48·80)
	Q2	2925 ± 0·43 (29·2)	
	Q3	120 ± 0·33 (1·2)	
	Q4	139 ± 0·25 (1·4)	
Acid stress at pH 3	Q1	9·461 ± 0·75 (94·6)	8·08 ± 0·14 (98·19)
	Q2	425 ± 0·31 (4·2)	
	Q3	5 ± 0·02 (0·0)	
	Q4	109 ± 0·12 (1·1)	
Lactobacillus sakei DBPZ0329			
Control	Q1	9735 ± 0·98 (97·4)	8·38 ± 0·05 (100)
	Q2	157 ± 0·54 (1·6)	
	Q3	5 ± 0·03 (0·0)	
	Q4	103 ± 0·22 (1·0)	
Heat stress at 50°C	Q1	6831 ± 0·89 (68·3)	6·79 ± 0·85 (81·09)
	Q2	164 ± 0·09 (1·6)	
	Q3	10 ± 0·02 (0·1)	
	Q4	2995 ± 0·78 (30·0)	
Heat stress at 55°C	Q1	174 ± 0·44 (1·8)	2·71 ± 0·39 (32·36)
	Q2	463 ± 0·57 (4·6)	
	Q3	21 ± 0·05 (0·2)	
	Q4	9342 ± 0·88 (93·4)	
Acid stress at pH 2·5	Q1	9470 ± 0·90 (94·7)	8·25 ± 0·27 (96·23)
	Q2	380 ± 0·26 (3·8)	
	Q3	13 ± 0·08 (0·1)	
	Q4	137 ± 0·05 (1·4)	
Acid stress at pH 3	Q1	9547 ± 0·78 (95·4)	8·23 ± 0·19 (98·20)
	Q2	305 ± 0·09 (3·1)	
	Q3	12 ± 0·24 (0·1)	
	Q4	136 ± 0·31 (1·4)	
Lactobacillus sakei DBPZ0338			
Control	Q1	9857 ± 0·87 (98·6)	8·71 ± 0·05 (100)
	Q2	100 ± 0·65 (1·0)	
	Q3	11 ± 0·07 (0·1)	
	Q4	32 ± 0·06 (0·3)	
Heat stress at 50°C	Q1	6640 ± 0·55 (66·4)	6·52 ± 1·41 (74·87)
	Q2	221 ± 0·32 (2·2)	
	Q3	12 ± 0·07 (0·1)	
	Q4	3127 ± 0·78 (31·3)	
Heat stress at 55°C	Q1	95 ± 0·05 (1·0)	2·43 ± 0·08 (27·92)
	Q2	382 ± 0·44 (3·8)	
	Q3	15 ± 0·06 (0·1)	
	Q4	9508 ± 0·96 (95·1)	
Acid stress at pH 2·5§	Q1	6573 ± 0·87 (65·7)	4·16 ± 0·43 (47·79)
	Q2	3083 ± 0·77 (30·8)	
	Q3	25 ± 0·04 (0·3)	
	Q4	319 ± 0·21 (3·2)	
Acid stress at pH 3	Q1	9689 ± 0·98 (96·9)	8·53 ± 0·36 (97·98)
	Q2	168 ± 0·08 (1·7)	
	Q3	11 ± 0·02 (0·1)	
	Q4	132 ± 0·21 (1·3)	
Lactobacillus sakei DBPZ0098			
Control	Q1	9839 ± 0·96 (98·4)	8·60 ± 0·05 (100)
	Q2	108 ± 0·05 (1·1)	
	Q3	7 ± 0·02 (0·0)	
	Q4	46 ± 0·06 (0·5)	
Heat stress at 50°C	Q1	9517 ± 0·89 (95·2)	8·32 ± 0·15 (96·68)
	Q2	395 ± 0·27 (3·9)	
	Q3	10 ± 0·10 (0·1)	
	Q4	78 ± 0·26 (0·8)	
Heat stress at 55°C§	Q1	6749 ± 0·85 (67·5)	6·27 ± 0·84 (72·92)
	Q2	3122 ± 0·66 (31·2)	
	Q3	13 ± 0·04 (0·1)	
	Q4	116 ± 0·11 (1·2)	
Acid stress at pH 2·5	Q1	7543 ± 0·98 (75·5)	5·35 ± 0·92 (62·17)
	Q2	2203 ± 0·77 (22·0)	
	Q3	21 ± 0·04 (0·2)	
	Q4	233 ± 0·27 (2·3)	
Acid stress at pH 3	Q1	9652 ± 0·99 (96·5)	8·26 ± 0·04 (95·98)
	Q2	248 ± 0·22 (2·5)	
	Q3	13 ± 0·05 (0·1)	
	Q4	87 ± 0·14 (0·9)	

*Population represents all events observed. Q1 quadrant represents the alive cells with intact membranes and stained with SYBR II; Q2 quadrant represents the damaged cells in an intermediate physiological state, still alive but with injured membranes; Q3 quadrant represents a low amount of debris obtained after treatment; and Q4 quadrant represents dead cells stained with PI.

†Events number observed in the density plots (cytograms) and the related percentages of cells obtained by flow cytometry analysis.

‡Total counts (log CFU/ml) of late-exponential phase cells after exposure to different stresses for 30 min. All experiments were performed three times. Results are the average values ± standard deviations, and the percentages of viable cells relative to that for cell no treated (set at 100) are shown in parentheses.

§These treatments were significantly different from the others.

permanent damage that are not able to remedy injuries suffered (quadrant Q4) (Table 1 and Fig. 1c). On the contrary, the response of *Lb. sakei* DBPZ0098 strain to 55°C stress was completely different if compared with other strains. In fact, alive cell percentage was 67·5% (quadrant Q1), and among cell population, a large portion of damaged cells (31·2%) was detected. In the case of acid stress, after exposure to pH 3, all strains had a similar behaviour with almost the same cell percentages than their corresponding control sample (Table 1 and Fig. 1d). In contrast, the acid stress at pH 2·5 caused a reduction over 20% of alive cells with a significant increase in damaged cells (from 22·0 to 30·8%) compared with the control in all tested strains but DBPZ0329 that showed 94·7% of alive cells able to withstand the low pH stress (Table 1 and Fig. 1e). These results were compared with those obtained using the standard counting method to observe the bacterial growing and developing ability in particular stress conditions.

Comparing stress growth of *Lb. sakei* cells *vs* non-stressed cells, total counts, determined on MRS agar plates, after heat stress at 50°C were similar to the three strain controls, while DBPZ0329 and DBPZ0338 strains showed a lower count of 2-log cycles than other strains (Table 1). At the highest tested temperature, 55°C, it was observed a decline of 5·4–6·8-log cycles for all tested strains, but one, DBPZ0098, whose count was dropped only for 2·3-log cycles (Table 1). Moreover, after exposure to pH 3, counts of all strains had no differences with the control, while *Lb. sakei* cells grown under pH 2·5 showed reductions in counts ranged between 3·2- and 4·1-log cycles in four strains, while for the other one, the count was higher of 4·5-log cycles than other strains counts and similar to that of the control (Table 1).

Discussion

In this study, we investigated the use of the flow cytometry to characterize and to assess the physiological states of *Lactobacillus sakei* strains exposed to different stress conditions. The aim was to apply a rapid assay that provides a valid method for studying and assessment of bacterial viability and membrane integrity. Flow cytometry showed to possess the highest potential for characterizing viable (active or inactive) cells, distinguishing them from damaged and dead cells, after exposition to stress treatments (Gregori *et al.* 2001).

To distinguish between viable and dead cells in various bacterial species, membrane integrity analysis has been proposed in many studies (Joux and Lebaron 2000; Hoefel *et al.* 2003; Leuko *et al.* 2004; Stocks 2004; Berney *et al.* 2007). Most of them used single staining to evaluate cell viability by high or low green fluorescence intensity, and moreover, in this research, the PI staining was singularly performed to confirm the results obtained. Few assays are based on double staining, combining Syto 9 and PI (Alonso *et al.* 2002; Allegra *et al.* 2008), as far as we know only one study used a nucleic acid double staining with SYBR II and PI to analyse bacteria of freshwater and marine waters (Gregori *et al.* 2001).

Our purpose was achieved using an approach based on nucleic acid double-staining protocol to count bacterial cells and to rapidly determine clusters according to their cell membrane permeability together with advances in the field of fluorescent probes. Its principle is to use simultaneously a permeant (SYBR II) and an impermeant (PI) dyes and to take advantage of the energy transfer that occurs between them when both probes are staining nucleic acids. SYBR II is a widely used fluorochrome that has a better quantum fluorescence yield for both DNA and RNA than other fluorochromes such as SYTO-13 or SYTO-9 (Lebaron *et al.* 1998; Phe *et al.* 2005). A previous study (Phe *et al.* 2005) showed that SYBR II, applied as single staining, is suitable for assessing damage caused by chlorination on cell membranes evidencing both membrane and nucleic acid damages.

Because of the complexity of the physiological status and heterogeneity of bacterial cells in a culture, especially after stress, multiparameter analysis is preferable (Nebe-von Caron and Badley 1995; Davey and Kell 1996; Bunthof *et al.* 1999). Examples indicate that after exposure to stress, cell cultures may contain dormant and injured subpopulations. Dormant cells may regain growth by resuscitation, while damaged cells may recover from injury and regain growth (Lloyd and Hayes 1995; Kell *et al.* 1998). Study of growth, recovery, dormancy and adaptation is important for understanding bacterial physiology.

In the food industry, the standard plate count method is widely used as the reference method for rapid microbiological analyses (Holm *et al.* 2004). The number of colony-forming units counted with the standard plate count method depends on parameters such as the bacterial species, culturing conditions, aggregates, the physiological status of the bacteria and sublethal injuries (Holm *et al.* 2004). Also for the flow cytometric technique, various factors will influence the results obtained. The parameters that will influence the results are expected to include the bacterial species, the staining procedure, possible aggregates, the metabolic status and sublethal injuries. This means that both methods are only estimative of the actual number of bacteria, and when the flow cytometric method is compared with the standard plate count, full conformity is not necessarily a requirement (Holm *et al.* 2004).

In previous studies, the flow cytometry was compared with traditional techniques to validate this method as an

alternative quantitative assay for cell viability assessment (Bunthof *et al.* 1999; Malacrinò *et al.* 2001; Holm *et al.* 2004; Phe *et al.* 2005; Saito *et al.* 2005; Flint *et al.* 2006; Pils *et al.* 2006; Quiros *et al.* 2007; Smigic *et al.* 2009). All research works validated the flow cytometry against plate counts mainly to enumerate, assess and evaluate both bacteria and yeasts vitality, their optimal growth conditions and their response to different treatments. The comparison of flow cytometry with the standard plate count method highlighted a good agreement (r value of 0·76) between the total bacteria counts obtained with both techniques, and it also proved a good quantitative and qualitative repeatability, which are parameters indicating a reliable performance of the technique.

Compared with single staining cell viability approaches, the novelty of the present work is the ability to use a different approach based on nucleic acid double staining that was introduced to characterize and to assess the physiological states of micro-organisms in conjunction with the classical plate counting method. Therefore, our approach was directed to evaluate the validity of the fluorescence assays as indicators for particular practical aspects of viability. In fact, we have applied and optimized a protocol able to detect the intermediate physiological states that are, somehow, activated to face difficult conditions changing the behaviour and cell dynamics.

The presence of damaged cells with injured membranes, but still with a recovery ability, and the possibility to monitor the changes in these bacterial cells represent the validation of the flow cytometry technique and the optimization of nucleic acid dual staining with SYBR II and PI as an more reliable, accurate and alternative quantitative assay, compared with traditional methods, for viability assessment.

The compared analysis of results obtained with both methods allowed to confirm flow cytometry as a technique able to study important physiological parameters with high sensitivity, high time resolution and with the potential of single-cell analysis. It also requires short incubation times and gives more accurate information. Moreover, the application of flow cytometry to evaluate the bacterial response to stress conditions highlighted the existence of different cell physiological states and so different cell behaviours towards particular situations and how these react by modifying their structure and physiology (Quiros *et al.* 2007; Smigic *et al.* 2009).

After milder treatments, the methods comparison revealed a survival percentage of bacterial cells by plate count that included both viable and damaged cell percentages determined by flow cytometry analysis. Instead, severe stresses had a stronger effect on bacterial viability involving a different behaviour. It has been observed that, removing the stress and incubating under more favourable conditions, the cell suspension was able to recover from injury and regain growth. In the flow cytometry study, cells able to grow on agar plates corresponded to damaged cells and to a part of cells that resulted not viable but that, in optimal growth conditions, resulted in an immediate increase in cell population by plate count, as already observed in another study on the effect of lactic acid on the pH_i in *Campylobacter jejuni* (Smigic *et al.* 2009).

After severe stresses, such as pH 2·5, comparison of the two methods highlights that a small part of alive cells are not able to grow on plates but detectable by flow cytometry as evidence of quality and reliability of the technique. According to these results, it has been observed that the flow cytometry is a more immediate and sensitive method than plate count because there are not middle steps but the cell sample is analysed soon. Usually, the ability of microbial cells to reproduce is considered as the benchmark method for determination of cell viability, and this is most commonly determined by the plate count method. The time needed to form visible colonies, however, is relatively long. On the other hand, microorganisms that do not form colonies, because they are dead, sublethally damaged, viable but noncultivable, dormant and inactive, are not counted. Moreover, the viable plate count method can be frustrated by clumping, inhibition by neighbouring cells and composition of the used growth media. Therefore, there is an increasing interest in rapid methods that exploit criteria other than reproduction (Breeuwer and Abee 2000).

For this reason, in this study, we compared the two methods to strengthen the use of flow cytometry for viability assessment of bacteria that is relevant for a wide variety of applications in industry, including quality assessment of starter cultures for different food production.

In conclusion, this rapid assay has the potential for physiological research on LAB and for the application in the food industry. The assessment of intermediate physiological states in *Lb. sakei* strains with recovery possibility could be an important criterion for application of potential starter cultures. Application of flow cytometry and characterization of sorted subpopulations may contribute to further understanding of diversity and heterogeneity in physiology of bacterial populations.

Studies concerning the existence and characteristics of subpopulations displaying heterogeneity in particular conditions are highly relevant, because specific subpopulations may show improved survival, changes and dynamics under stress. Finally, the nucleic acid dual staining with SYBR II and PI may be used in the selection of LAB to test the effect of different stress conditions and may enable fast measurement of microbial behaviour present

in cultures and of subpopulations and individual cells of LAB in food industry research and applications.

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