POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES

http://journal.pan.olsztyn.pl
e-mail: pjfns@pan.olsztyn.pl

Pol. J. Food Nutr. Sci.
2010, Vol. 60, No. 4, pp. 363-368

FLUORESCENT MICROSCOPY AND GAS CHROMATOGRAPHY TO ASSESS THE VIABILITY AND METABOLIC ACTIVITY OF *PROPIONIBACTERIUM* SP. STRAINS

Iwona Warmińska-Radyko, Hanna Sielawa, Marta Mikš-Krajnik

Chair of Industrial and Food Microbiology, Faculty of Food Sciences, University of Warmia and Mazury, Olsztyn, Poland

Key words: *Propionibacterium*, LIVE/DEAD staining, viability, volatile fatty acids

The aim of the study was to assess usefulness of the LIVE/DEAD fluorescent staining method and gas chromatography to monitor the viability and metabolic activity of *Propionibacterium* strains in long-term cultures in milk. The effect of 4% NaCl addition and a temperature of 10°C on the growth of *Propionibacterium freudenreichii* ssp. *freudenreichii 111, 109C, 108* strains was studied for 28 days. Bacterial cells were assessed in cultures by microscopic and plate counting methods in regular intervals. The cultures were additionally determined for the content of volatile fatty acids: C_2 to C_7. The total cell counts of all strains in cultures assessed by the microscopic method were noticed to be 1 to 5 logarithmic cycles higher in comparison to those determined with the plate counting method. In following days and weeks of culture, increasing discrepancies were observed between the results obtained using microscopic and plate methods. Both methods revealed similar trends in the viability of strains under control conditions and a little impact of NaCl addition on cell growth and decrease. The cultures run at a temperature of 10°C exhibited different course of growth and decline of the number of monitored populations depending on strain and method applied. Individual strains possessed different acid formation activity. From the beginning of incubation, the highest concentrations were reported for propionic and acetic acids, whereas the other acids in number from 4 to 6 appeared subsequently. The temperature of 10°C inhibited acids formation by all strains, whereas 4% addition of NaCl stimulated the acid-forming activity and during incubation under those conditions the contents of volatile acids were recorded to be the highest.

INTRODUCTION

Among *Propionibacterium* genus, "skin" and "classical" species can be distinguished. The first group includes species of clinical significance, which can sometimes expose pathogenic activity. The second group encompasses species of industrial importance, due to properties of their metabolites formed during propionic acid fermentation [Paściak & Mordarska, 1999]. The capability of classical propionic bacteria strains to biosynthesize vitamin B_{12}, folic acid, antibacterial proteins as well as their fungistatic activity are well known [Hugenholtz *et al*., 2002; Lind *et al*., 2005]. The viability of those bacteria in the gastrointestinal tract, their adhesion to intestinal mucus as well as stimulating activity towards health-promoting microflora has also been already confirmed [Hervé *et al.*, 2007; Mantere-Alhonen, 1995; Warmińska-Radyko *et al*., 2002]. The bacteria of propionic acid fermentation play an important role in the industrial production of Swiss type cheeses. Their enzymatic activity at the product ripening stage contributes to the releasing of a substantial amount of volatile fatty acids, peptides, amino acids and CO_2, which affect proper eyes formation and characteristic flavour of cheese [Stepaniak, 2000; Panon, 1990; Gagnaire *et al*., 1999; Chamba & Perreard, 2002].

The investigation of *Propionibacterium* growth with the standard culture method causes a lot of problems due to a long time of cell generation, necessity of assuring appropriate incubation conditions as well as imperfections of plate counting [Lahtinen *et al.*, 2006]. The modern methods of microbiological analysis, including fluorescence techniques offering probes with various cell functions, enable monitoring bacteria viability and enzymatic activity directly in the medium of food material. The LIVE/DEAD® BacLight™ Bacterial Viability Kit provides information about microorganisms viability on the basis of integrality of their cell membrane. The kit contains two fluorochromes – green SYTO®9 and red PI (propidium iodide). SYTO®9 stains both live and dead cells, whereas PI penetrates only the ones with damaged membrane, as a result of which live cells exhibit green fluorescence and dead ones – red fluorescence [Joux & Lebaron, 2000; Gatti *et al*., 2006; Biggerstaff *et al*., 2006]. There is a lack of scientific data on the usage of those methods in investigations of *Propionibacterium* strains. Such studies were, however, conducted in order to assess the viability of *Bifidobacterium* sp., *Lactobacillus* sp., *Streptococcus* sp. in commercial probiotic preparations [Moreno *et al*., 2006]. This technique served to monitor the viability of encapsulated *Bifidobacterium longum* strain in starch hydrolysates, which develops both in fermented and unfermented foods [Lahtinen *et al.*, 2006].

The aim of this study was to assess the usefulness of the fluorescence staining technique using the LIVE/DEAD® BacLight™ Viability Kit, and gas chromatography to monitor the viability and enzymatic activity of *Propionibacterium* strains in culture conditions in milk, at 10°C and

Author's address for correspondence: Marta Mikš-Krajnik, Chair of Industrial and Food Microbiology, University of Warmia and Mazury, Plac Cieszyński 1, 10-726 Olsztyn, Poland; e-mail: marta.miks@uwm.edu.pl

© Copyright by Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences

in the presence of NaCl. The evaluation of salt and low temperature effects on the strain is essential in view of the application of propionic acid bacteria in the industrial production of Swiss type cheeses.

MATERIALS AND METHODS

The strains of propionic acid bacteria used in the study were: *Propionibacterium freudenreichii* ssp. *freudenreichii 111*, *109C*, *108*, originating from WSRO(=LCC) collection of the Chair of Industrial and Food Microbiology, University of Warmia and Mazury in Olsztyn. The culture medium for *Propionibacterium* strains was UHT milk with 0.5% fat content. Three different cultures were run on milk: (1) the control culture run at 30°C, (2) the culture run at 10°C, and (3) the culture with addition of 4% of NaCl run at 30°C. The individual strains were inoculated onto the prepared milk portions at the level of 10^6 cfu/mL and incubated for 42 days. Each culture was performed in three to five repetitions. Samples were collected after 2, 7, 14, 21, 28 and 42 days of incubation and determined for the cell count by the LIVE/DEAD microscopic method and the plate counting method (2-28 days) as well as for the content of volatile fatty acids (2-42 days).

Total cell count determined by the plate method

The total cell count by the plate method was carried out by the surface method in lactate growth medium enriched with 1% of glucose and 1.5% of yeast extract. The incubation was carried out under anaerobic conditions (anaerobic incubator Lab-Line Instruments, INC – model No. 315-1) at 30°C for 6 to 14 days. The strains investigated formed colourful colonies: *Propionibacterium freudenreichii* ssp. *freudenreichii 111* and *109C* – light beige, whereas *Propionibacterium freudenreichii* ssp. *freudenreichii 108* strain – red-brown.

Total cell count determined by the microscopic method

The cell count by the microscopic method was carried out by a diagnostic test LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes, Invitrogen) according to producer's procedures. The microscopic preparations were performed with *BacLight™ Mounting Oil* (Invitrogen, Molecular Probes), and they were stored at -20°C until analysed. In the preparations made by fluorescence staining, bacterial cells were visualized using an epifluorescence microscope OLYMPUS BX51 with an PI/FITC filter. The digital image analysis was performed using Cell^F Imaging Software for Live Science Microscopy (Olympus Company). The total bacteria cell count determined in every sample is an average of digital analysis of 10 to 20 pictures of microscopic preparations.

Determination of volatile fatty acids

The determination of volatile fatty acids C_2 to C_7 content was carried out by the headspace analysis technique using a gas chromatograph Clarus 500 (Perkin Elmer) with a flame ionization detector (FID) and a Turbomatrix 40 autosampler (Perkin Elmer). The chromatograph was calibrated for the C_2-C_7 fatty acids analysed in skimmed milk using Volatile Acid Standard Mix in deionized water (SUPELCO). The regression coefficient for calibration curves of the acids was R^2=0.99, at minimum. The chromatograph working parameters were as follows: a 60 m x 0.530 mm x 1.00 µm HP-INNOWAX column (length x i.d. x film, respectively, by Agilent Technologies); a carrier gas – helium, a flow rate – 5.0 mL/min; a temperature program: gradient from 80°C (5 min) to 220°C (5 min) at the rate of 10°C/min; FID temperature – 230°C; and injector temperature – 230°C. The headspace autosampler working conditions were as follows: the sample was thermostated at 70°C/40 min; needle temperature – 90°C; pressurization time – 1.0 min; injection time – 0.08 min. The integration of peaks recorded during chromatographic analyses was performed with Total Chrom Navigator software (Agilent Technologies).

RESULTS AND DISCUSSION

The observations of *Propionibacterium* strains cultured in various conditions revealed different effects of those conditions on the growth and death of the population depending both on strain and cell counting method applied. The growth of *P. freudenreichii* ssp. *freudenreichii 111* in cultures incubated at 30°C was similar, when controlled by both microscopic and plate counting method. In the culture run at 10°C the result obtained by the plating method showed a strong reduction of the population by 3 logarithmic cycles from day 7 to day 28 of incubation. During that period, the results obtained by the LIVE/DEAD method revealed the stability of cell count in the range of 1 logarithmic cycle (Figures 1A and 1B). The development of *P. freudenreichii* ssp. *freudenreichii 109C* strain was inhibited by NaCl and low temperature over the entire examined period, which was confirmed by results obtained by the LIVE/DEAD method (Figure 1C). However, the plate counting method showed the stimulating effect of salt between day 7 and 14 of incubation of this strain (Figure 1D). The development of *P. freudenreichii* ssp. *freudenreichii 108* was slightly inhibited by salt addition and the temperature of 10°C, as recorded by both counting methods, but still the plate counting method revealed 10 to 100 times lower number of cells (Figures 1E and 1F). The number of cells determined by the plate counting method in the culture incubated at 10°C was the same during the whole period of experiment, which indicates that the strain is uncapable to develop under those conditions. The growth of propionic acid bacteria in skimmed milk medium proceeded differently than in the complex microbiological media. Depending on *Propionibacterium* strain and inoculum applied, either inhibition or lack of growth was observed in milk and whey [Boyaval *et al.*, 1999; Piveteau *et al.*, 2000]. It is linked with the presence of inhibiting substances in milk, with the most commonly mentioned being: immunoglobulins, transferrin, lactoferrin and peptides originating from decomposition of lactoferrin and casein [Piveteau *et al.*, 2000]. Generally, it can be concluded that in response to 4% NaCl addition an increase was observed in the count of *Propionibacterium* strains population in the range of 1-2 logarithmic cycles and in the rate of its death at the final stages of incubation. The addition of NaCl changes osmotic pressure of the medium and simultanously decreases its a_w, which is a stressing factor to developing cells. Bacteria of *Propionibacterium* genus are characterised by mod-

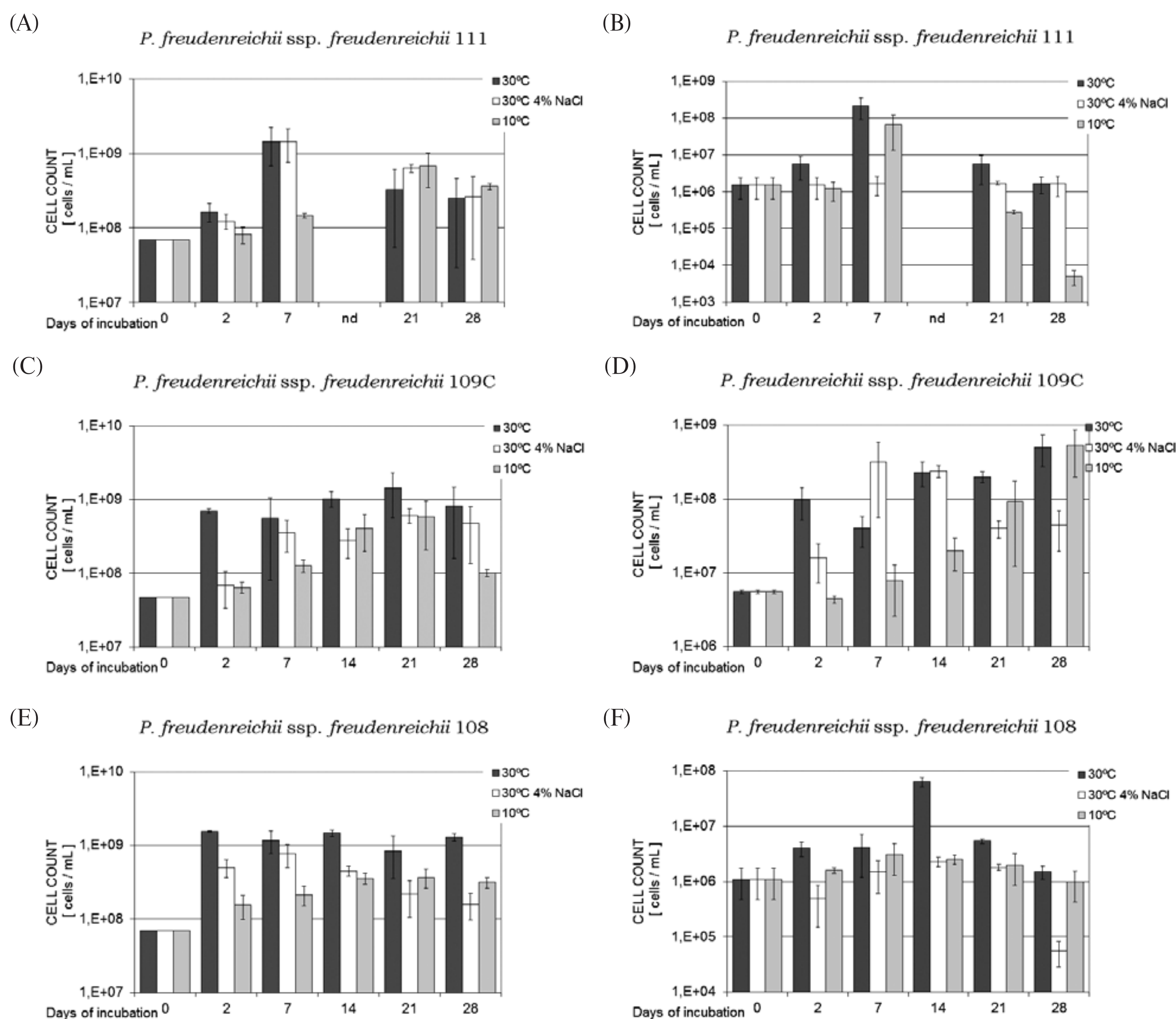

FIGURE 1. The number of viable *Propionibacterium* cells determined by LIVE/DEAD method (A, C, E) and plate counting method (B, D, F) in the cultures incubated in milk under various conditions.

erate halotolerance in the range from 0.5 to 3.0% of NaCl, and it has been established that this is a strain-, pH- and medium-dependent feature [Boyaval *et al.*, 1999]. Cells in a stationary growth phase exhibit higher tolerance to stressing conditions in comparison to cells in a logarithmic growth phase [Leverrier *et al.*, 2005]. The strains applied in the experiment were growing in the presence of 4% of salt, and their population counts were only slightly reduced after 28 days of incubation. Higher susceptibility to salt was noticed for *Propionibacterium* 109 strain whose development was inhibited at the initial stage of incubation, which was established by both microscopic and plate counting method. The microscopic method revealed that *Propionibacterium* 111 and 108 strains tolerated better the described conditions (Figures 1B, 1F).

Presumably, high tolerance of salt addition by the strains studied resulted from the protective activity of milk constituents, such as choline and its derivatives, or carnitine [Boyaval *et al.*, 1999; Leverrier *et al.*, 2005]. In turn, the application of a temperature of 10°C resulted in diminished intensity of metabolic processes of cells and extended time of their regeneration, which eventually caused the maximum number of population on day 21 or 28 of long-term incubation.

In cultures of all strains the total cell counts assessed by the microscopic method were noticed to be 1 to 5 logarithmic cycles higher in comparison to results of the plate counting method. Such a great difference in bacteria number may result from the lost ability to form colonies in solid medium of some cells of the population, since the plate counting method is based only on that ability. Cells called VNC (viable but non-culturable) or ANC (active but non-culturable) are problematic because their viability was not confirmed by the growth as a colony [Biggerstaff *et al.*, 2006; Gatti *et al.*, 2006; Lahtinen *et al.*, 2006]. Their presence in the populations of *Propionibacterium* growing in milk could have given rise to diminished cell counts determined by the plate method. The results of the plate counting method are burdened with an error, which results from a lower number of repetitions, the possibility of formation of cells conglomerates, as well as from the low ability of the strain to grow

on solid medium. Propionic acid bacteria demonstrate a very slow growth of colonies on solid media, and for that reason determination of their number by the plating method is problematic. The investigated *Propionibacterium* strains formed colonies after a long time of incubation, which could have affected the inaccuracy of determination. The weakest growth on the lactate medium was noticed for *P. freudenreichii* ssp. *freudenreichii 108,* which formed colonies after 2 weeks, whereas the other strains cultured on the same medium formed colonies in 5 to 10 days. The application of the LIVE/DEAD staining method in the study on the development of *Propionibacterium* population enabled fast and, due to the possibility of performing many replications, more reliable assessment of live bacterial cells count in long-term cultures in milk. That technique allowed also more precise determination of the effect of the conditions applied, regardless the physiological state of cells and their ability to multiply. It results from the fact that the data used for determination of *Propionibacterium* cell count by the microscopic method were acquired from 5 parallel cultures in milk under different conditions. Each result was an average from the digital analyses of 20 pictures of microscopic preparations obtained from individual cultures. In order to avoid false signal detection in determinations made in milk cultures, resulting from background emission, the curd was standardized in drained cultures by dilution with a solution of sodium citrate heated up to 46°C. The possibility of comparing numerous results and alike values of standard deviations computed for results obtained by the LIVE/DEAD method indicate a stable growth of the strains and the usefulness of the method in monitoring real changes in the *Propionibacterium* population count in milk (Figures 1A, 1C, 1E).

On the basis of parallel chromatographic analyses it was noticed that all *Propionibacterium* strains cultured in milk in the established conditions were able to produce short-chain volatile acids, *i.e.* propionic and acetic. The formation of other acids was a strain-dependent feature. Individual acids appeared in various stages of incubation depending on culture conditions. There is no literature data on volatile fatty acids production in *Propionibacterium* monocultures cultivated in milk. However, numerous authors describe the profiles of volatile compounds produced by propionic acid and lactic acid bacteria during cheese ripening [Chamba *et al.*, 2002; Gagnaire *et al.*, 1999; Panon *et al.*, 1990]. In the cultures run in control conditions and with salt addition incubated at 30°C, detectable concentration of propionic acid (C_3) was noticed after 48 hours, except for *P. freudenreichii* ssp. *freudenreichii 108* strain which at the optimal temperature formed a measurable concentration of that acid C_3 after 2 weeks. In the cultures run at 10°C, propionic acid at measurable concentration appeared late – on day 21 of incubation, except for *P. freudenreichii* ssp. *freudenreichii 109C* strain which formed the acid C_3 on day 7 and in a higher concentration than the other strains. Acetic acid (C_2) at measurable concentration was determined in the investigated cultures from day 14 to 21 of incubation, or on day 7 but only in the cultures with salt addition. Butyric (C_4), isobutyric (C_4iso), and isovaleric (C_5iso) acids were formed by the strains examined at various concentrations, but usually after 20 days of incubation and only in the control culture and that with salt addition. In contrast, the ability of acid formation was not observed at a temperature of 10°C corresponding to cold room cheese ripening conditions. Thierry *et al.* [2005] reported that a low temperature of cheese ripening intensified the synthesis of isovaleric acid by *Propionibacterium* strains in the catabolism of leucine and isoleucine. In addition, they noted that a 1% addition of salt in cheese inhibited the production of $C5_{iso}$ acid [Thierry *et al.*, 2004]. Our study revealed that, in long-term cultures in skimmed milk, the *Propionibacterium* monoculture formed the same volatile fatty acids as those determined in Swiss-type ripening cheeses [Thierry *et al.*, 2005]. However, different proportions were observed in the concentrations of these acids formed under the action of the stress factors applied, in comparison with cheeses. Hexanoic acid (C_6) appeared in the cultures of *P. freudenreichii* ssp. *freudenreichii 111* and *109C* cultured in the optimum conditions and with the addition of salt after 7 or 21 days of incubation. Valeric (C_5) and isocapronic (C_6iso) acids appeared occasionally only at minimal concentrations on day 42 of incubation of *P. freudenreichii* ssp. *freudenreichii 108* strain. In turn, enanthic acid (C_7) was identified only on day 42 of incubation of *P. freudenreichii* ssp. *freudenreichii 109C* strain at the optimal temperature and with salt. Under conditions applied in the study, none of the strains produced at the same time all the fatty acids examined, *i.e.* from C_2 to C_7.

The highest concentrations of total volatile fatty acids were found in *P. freudenreichii* ssp. *freudenreichii 111* strain, with the addition of salt (Table 1). The largest contribution to the production of fatty acids was observed for propionate and acetate acids, at the ratio of 1.4:1 which was the most favorable ratio compared with the other strains. In the culture with NaCl, that strain was characterised by the production of the same set of acids: C_2, C_3, C_4, C_4iso, C_5iso and C_6 in the period from day 7 till day 42 of incubation, and in the control culture – from day 28. At the low temperature from day 21 of incubation the strain produced simultanously C_2, C_3, C_4, C_4iso, and on day 42 traces of C_5iso were recorded as well. The strain of *P. freudenreichii* subsp. *freudenreichii 109C* produced the highest quantity of the acids in the culture run under the optimal conditions, with the largest contribution of propionic acid and acetic acid at the ratio of 1.3:1 (Table 1). In the early stages of incubation, salt stimulated the acid-forming activity of the strain, while the low temperature delayed and strongly inhibited the formation of acids. This strain compared with the others, was characterised by the ability to produce the largest number (*i.e.* 8) of different volatile fatty acids. In turn, the strain *P. freudenreichii* ssp. *freudenreichii 108* formed the highest quantity of the acids in the culture with salt addition, but under these conditions it produced more acetic than propionic acid. The molar ratio of C_3:C_2 was 0.28:1 on day 28 of incubation and 0.9:1 – on day 42. In contrast, in 6-week Swiss-type cheeses those acids occurred in higher proportions from 0.5:1 to 2.8:1 [Thierry *et al*., 2005]. Our study demonstrated that the acid-forming activity of *P. freudenreichii* ssp. *freudenreichii 111* and *108* strains was stimulated in the cultures with salt, however a temperature of 10°C inhibited the formation of acids by all strains (Table 1).

In addition, it was observed that the content and number of acids formed were increasing gradually (Table 1). After 21 days of incubation in all cultures of *P. freudenreichii* ssp.

TABLE 1. The content of volatile fatty acids $C_2 – C_7$ in cultures of *P. freudenreichii* ssp. *freudenreichii 111, 109C* and *108* incubated in milk under various conditions.

			Content of VFA C_2-C_7 (mg/mL)									
Strain	Incubation conditions	Day	C_2	C_3	C_{4iso}	C_4	C_{5iso}	C_5	C_{6iso}	C_6	C_7	Total
111	30°C	2	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.000
		7	nd	0.109	nd	nd	nd	nd	nd	nd	nd	0.109
		14	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		21	0.438	0.482	0.069	nd	nd	nd	nd	0.110	nd	1.098
		28	0.541	0.630	0.081	0.117	0.086	nd	nd	0.111	nd	1.567
		42	0.544	0.706	0.109	0.116	0.084	nd	nd	0.138	nd	1.742
	30°C, 4% NaCl	2	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.000
		7	0.398	0.503	0.082	0.167	0.127	nd	nd	0.118	nd	1.394
		14	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		21	0.531	0.704	0.090	0.255	0.121	nd	nd	0.133	nd	1.833
		28	0.514	0.721	0.089	0.266	0.117	nd	nd	0.132	nd	1.838
		42	0.532	0.767	0.113	0.284	0.126	nd	nd	0.160	nd	1.981
	10°C	2	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.000
		7	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.000
		14	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
		21	0.288	0.128	0.095	0.121	nd	nd	nd	nd	nd	0.632
		28	0.319	0.281	0.100	0.137	nd	nd	nd	nd	nd	0.838
		42	0.382	0.380	0.116	0.170	0.063	nd	nd	nd	nd	1.112
109C	30°C	2	nd	0.117	nd	nd	nd	nd	nd	nd	nd	0.117
		7	nd	0.215	nd	nd	nd	nd	nd	nd	nd	0.215
		14	0.365	0.294	nd	nd	nd	nd	nd	nd	nd	0.659
		21	0.422	0.455	0.093	nd	0.099	nd	nd	0.112	nd	1.182
		28	0.356	0.476	0.095	0.141	0.114	nd	nd	0.116	nd	1.299
		42	0.457	0.576	0.094	0.152	0.105	nd	0.098	0.124	0.140	1.746
	30°C, 4% NaCl	2	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.000
		7	0.286	0.166	nd	nd	nd	nd	nd	nd	nd	0.452
		14	0.287	0.237	nd	nd	nd	nd	nd	nd	nd	0.525
		21	0.310	0.275	nd	nd	nd	nd	nd	nd	nd	0.586
		28	0.372	0.475	0.075	nd	0.062	nd	nd	0.104	nd	1.088
		42	0.353	0.518	0.084	nd	0.060	nd	nd	0.098	0.103	1.217
	10°C	2	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.000
		7	nd	0.094	nd	nd	nd	nd	nd	nd	nd	0.094
		14	0.210	0.096	nd	nd	nd	nd	nd	nd	nd	0.305
		21	0.239	0.121	nd	nd	nd	nd	nd	nd	nd	0.360
		28	0.269	0.290	nd	nd	nd	nd	nd	0.088	nd	0.647
		42	0.332	0.311	nd	nd	nd	nd	nd	0.093	nd	0.736
108	30°C	2	nd	nd	nd	0.105	nd	nd	nd	nd	nd	0.105
		7	nd	nd	nd	0.104	nd	nd	nd	nd	nd	0.104
		14	0.189	0.397	0.069	0.105	nd	nd	nd	nd	nd	0.760
		21	0.292	0.105	0.069	0.109	nd	nd	nd	nd	nd	0.575
		28	0.277	0.095	0.068	0.106	nd	nd	nd	nd	nd	0.575
		42	0.560	0.657	0.094	0.133	0.131	0.083	nd	nd	nd	1.658
	30°C, 4% NaCl	2	nd	0.687	nd	0.116	nd	nd	nd	nd	nd	0.803
		7	0.513	0.386	nd	0.107	nd	nd	nd	nd	nd	1.006
		14	0.471	0.084	nd	0.094	nd	nd	nd	nd	nd	0.649
		21	0.281	0.109	0.114	0.105	nd	nd	nd	nd	nd	0.609
		28	0.300	0.085	0.085	0.109	0.060	nd	nd	nd	nd	0.639
		42	0.410	0.386	0.094	0.168	0.130	nd	0.109	nd	nd	1.297
	10°C	2	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.000
		7	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.000
		14	0.072	nd	0.066	nd	nd	nd	nd	nd	nd	0.138
		21	0.102	0.088	0.070	nd	nd	nd	nd	nd	nd	0.260
		28	0.212	0.212	0.067	0.100	nd	nd	nd	nd	nd	0.591
		42	0.111	0.113	0.117	0.103	nd	nd	nd	nd	nd	0.444

111 – *Propionibacterium freudenreichii* ssp. *freudenreichii 111*, 109C – *Propionibacterium freudenreichii* ssp. *freudenreichii 109C,* 108 – *Propionibacterium freudenreichii* ssp. *freudenreichii 108,* nd – not detected.

freudenreichii 109C and *108* strains from 2 to 5 different acids were determined. On day 28, *i.e.* the last day of quantitative assessment of cells by LIVE/DEAD and plate counting methods, the presence of 3 to 6 acids was recorded. The additional analysis of the composition of the headspace phase performed on day 42 of incubation revealed that the contents of C_2 and C_3 acids in *P. freudenreichii* ssp. *freudenreichii 111* and *109C* strains cultures increased slightly, whereas from 2 to 5 times higher contents of those acids were recorded in *P. freudenreichii* ssp. *freudenreichii 108* strain cultures. No dependency was confirmed between the number of viable cells in particular stages of incubation and the content of volatile fatty acids. Probably, the accumulation of dead cells, the phenomenon of cell autolysis and release of intracellular enzymes contributed to the increased concentration and diversity of the identified volatile compounds [Gatti *et al.*, 2006].

Based on the results obtained, it may additionally be stated that the *Propionibacterium* strains applied in the study tolerated the 4% addition of salt and the temperature of 10°C. The highest contents and diversity of volatile fatty acids were recorded in *P. freudenreichii* ssp. *freudenreichii* 111 and *P. freudenreichii* ssp. *freudenreichii* 108 cultures with salt addition. It proves the ability of those strains to develop flavour under conditions similar to those occurring during cheese ripening. The features like: activity at a low temperature and salt tolerance or stimulation resulting from salt addition are especially useful in industrial production.

CONCLUSIONS

1. The LIVE/DEAD method enables observation of subtle changes in viable and dead cells number of *Propionibacterium* in long-term cultures on milk at population levels of 10^6 to 10^9 cells/mL.

2. The numbers of viable cells determined by the plate counting were always lower in comparison to those assessed by the microscopic method. The difference, if any, may indicate the presence of the so-called dormant cells in the population, which are not able to form colonies on solid growth media.

3. The study demonstrated the stimulating effect of NaCl on the formation of propionic and other volatile fatty acids as well as the inhibiting effect of low temperature on the metabolic activity of *Propionibacterium* strains.

ACKNOWLEDGEMENTS

The work was financially supported by the grant from the Polish Ministry of Science and Higher Education– N312 081 32/4016.

One of the authors was supported by the European Union within the European Social Fund.

REFERENCES

1. Biggerstaff J.P., Le Puil M., Weidow B.L., Prater J., Glass K., Radosevich M., White D.C., New methodology for viability testing in environmental samples. Mol. Cell Probes, 2006, 20, 141–146.
2. Boyaval P., Deborde C., Corre Ch., Blanco C., Bégué Ã., Stress and osmoprotection in propionibacteria. Lait, 1999, 79, 59–69.
3. Chamba J., Perreard J.E., Contribution of propionic acid bacteria to lipolysis of Emmental cheese. Lait, 2002, 82, 33–44.
4. Gagnaire V., Mollé D., Sørhaug T., Léonil J., Peptidases of dairy propionibacteria. Lait, 1999, 79, 43–57.
5. Gatti M., Bernini V., Lazzi C., Neviani E., Fluorescence microscopy for studying the viability of micro-organisms in natural whey starters. Lett. Appl. Microbiol., 2006, 42, 338–343.
6. Hervé Ch., Fondrevez M., Chéron A., Barloy-Hubler F., Jan G., Transcarboxylase mRNA: A marker which evidences *P. freudenreichii* survival and metabolic activity during its transit in the human gut. Int. J. Food Microbiol., 2007, 113, 303–314.
7. Hugenholtz J., Hunik J., Santos H., Smid E., Nutraceutical production by propionibacteria. Lait, 2002, 82, 103–112.
8. Joux F. Lebaron P. Use of fluorescent probes to asses functions of bacteria at single-cell level. Microbes Infect., 2000, 2, 1523––1535.
9. Lahtinen S. J., Gueimonde M., Ouwehand A.C., Reinkainen J.P., Salminen S.J., Comparison of four methods to enumerate probiotic bifidibacteria in a fermented food product. Food Microbiol., 2006, 23, 571–577.
10. Leverrier P., Fremont Y., Rouault A., Boyaval P., Gwénaël J., *In vitro* tolerance to digestive stresses of propionibacteria: influence of food matrices. Food Microbiol., 2005, 22, 11–18.
11. Lind H., Jonsson H., Schnorer J., Antifungal effect of dairy propionibacteria- contribution of propionic acids. Int. J. Food Microbiol., 2005, 98, 157–165.
12. Mantere–Alhonen S. Propionibacteria used as probiotics – A review. Lait, 1995, 75, 447–452.
13. Moreno Y., Collado M.C., Ferrus M.A., Cobo J.M., Hernandez E., Hernandez M., Viability assessment of lactic acid bacteria in commercial dairy products stored at 4°C using LIVE/DEAD® BacLight™ staining and conventional plate counts. Int. J. Food Sci. Technol., 2006, 41, 275–280.
14. Panon G., Purification and characterization of proline iminopeptidase from *Propionibacterium shermanii* 13673. Lait, 1990, 70, 439–452.
15. Paściak M., Mordarska H., *Propionibacterium* genus – taxonomic and biological heterogeneity. Post. Mikrobiol., 1999, 38, 245–256 (in Polish).
16. Piveteau P., Condon S., Cogan T., Inability of dairy propionibacteria to grow in milk from low inocula. J. Dairy Res., 2000, 67, 65–71.
17. Stepaniak L., Isolation and characterization of proline iminopeptidase from *Propionibacterium freudenreichii ATCC 9614.* Nharung, 2000, 44, 102 -106
18. Thierry A., Maillard M-B., Richoux R., Kerjean J-R., Lortal S., *Propiobnibacterium freudenreichii* strains quantitatevely affect production of volatile compounds in Swiss cheese. Lait, 2005, 85, 57–74.
19. Thierry A., Richoux R., Kerjean J-R., Isovaleric acid is mainly produced by *Propionibacterium freudenreichii* in Swiss cheese. Int. Dairy J., 2004, 14, 801–807.
20. Warmińska-Radyko I., Łaniewska-Moroz Ł., Babuchowski A., Possibilities for stimulation of *Bifidobacterium* growth by propionibacteria. Lait, 2002, 82, 113–121.

Received April 2009. Revision received September 2009 and accepted July 2010.