

Full Length Research Paper

The physiological state of *Lactococcus lactis* and *Propionibacterium freudenreichii* strains in co-cultures induced by low temperature and osmotic stress

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The bacterial physiological state, type of interactions and changes in metabolism of *Lactococcus lactis* and *Propionibacterium freudenreichii* strains in co-culture were studied in skimmed milk in response to osmotic [3% (w/v) NaCl] and low temperature (10°C) stress during long-term incubation. Changes in the integrity of cell membrane were examined by LIVE/DEAD[®] BacLight[™] staining, and culture viability and bacterial interactions studies were performed with the use of the plate count technique. The profiles of volatile organic compounds were assessed by static headspace-gas chromatography. During the stationary growth phase, the number of LIVE cells with intact membranes was reduced in the presence of NaCl in comparison with control conditions, and a longer adaptation phase was observed at 10°C. The viability of starter cultures was high at $\sim 10^8$ to 10^9 CFU/ml at the end of the experiment in all tested conditions. Our results point to the possibility of commensalisms interactions between lactic acid and propionic acid bacteria. Prolonged culture incubation contributed to the accumulation of acetoin, and it enhanced acetic acid and propionic acid synthesis during the stationary growth phase of propionic acid bacteria.

Key words: *Lactococcus lactis*, *Propionibacterium freudenreichii*, viability, volatiles compounds, osmotic stress, low temperature stress.

INTRODUCTION

Lactic acid bacteria (LAB) and propionic acid bacteria (PAB) are widely used as starter cultures in fermented dairy products, including Swiss-type or Swiss-Dutch type ripened cheeses (Borawska et al., 2010; Kerjean et al., 2000). LAB, mainly *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus*, *Streptococcus thermophilus*, are used to acidify the cheese curd under press (pH \sim 5.4) by producing D

and L lactic acid from lactose during first stage of ripening (in the “cold” room \sim 12°C), which is afterwards catabolized by PAB into propionic acid (C₃). PAB grow during the second stage of ripening in the “warm” room (\sim 22-24°C), and ferment lactic acid to acetic acid (C₂), propionic acid (C₃) and CO₂. Propionic and acetic acids contribute to the nutty and sweet flavour of the cheese; whereas, CO₂ is responsible for eye formation (Piveteau et al.,

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Abbreviations: LAB, Lactic acid bacteria; PAB, propionic acid bacteria; VBNC, viable but not culturable; PI, propidium iodide; UHT, ultra high temperature; DEFT, direct epifluorescent filter technique; HS, headspace; GC, gas chromatography; FID, flame ionization detector; FISH, fluorescent *in situ* hybridization.

1995). During ripening, the activity of LAB creates the environment for further PAB growth by providing essential amino acids and carbohydrates, modulation of the oxidoreduction potential of cheese matrix and the production or/and destruction of certain inhibitors (for example, bacteriocins, free fatty acids) or activators (proteins, amino acids) before PAB growth (Kerjean et al., 2000). However, the interactions that occur between LAB and PAB present in cheese are poorly understood and the compounds involved in these interactions have not been identified.

In the literature, many divergent theories on LAB/PAB interactions were developed, starting from bacterial growth stimulation (Condon and Cogan, 2000; Pérez Chaia et al., 1995; Piveteau et al., 1995; Warminska-Radyko et al., 2002) through commensalisms (Liu and Moon, 1982; Rymaszewski et al., 1995) ending with inhibition and antagonism (Jimeno et al., 1995; Pérez Chaia et al., 1995). Whereas, the interaction between LAB and PAB, in particular the genera: *Lactobacillus*, *Streptococcus* and *Propionibacterium* has been investigated extensively (Kerjean et al., 2000; Thierry et al., 2004; Pérez Chaia et al., 1995); lactic acid cocci such as *Lactococcus* spp. combined with *Propionibacterium* spp. are rarely used and remain weakly researched (Treimo et al., 2006; Rymaszewski et al., 1995). The latter starter culture was successfully applied in Norwegian Jarlsberg cheese and in Swiss-Dutch type cheese produced in Poland (Mikš-Krajnik, 2012; Treimo et al., 2006). Pérez Chaia et al. (1995) suggested that behaviour of LAB and PAB in mixed culture depends on the strain involved. Stimulation of growth of *L. helveticus* ATCC 15009 and CRL 581 strains in co-culture with *Propionibacterium acidipropionici* CRL 756 was noticed, while growth of *Lactobacillus casei* LC3 was not affected by mixed culturing. On the other hand, *Propionibacterium freudenreichii* AP8 was inhibited in co-cultures with *L. helveticus* CRL 581 and *L. casei* LC3 but stimulated by *L. helveticus* ATCC 15009. Kerjean et al. (2000) reported that in more than 100 pairs of LAB/PAB cultures tested in whey model system, the stimulation of PAB by LAB was noticed. The strongest activation was observed after 12 h of incubation when complex nitrogenous sources were supplemented to the whey. While addition of vitamins, minerals and free amino acids did not show a stimulatory effect, Treimo et al. (2006) showed that *Lactococcus lactis* subsp. *lactis* INF L2 autolyzed first, followed by *P. acidipropionici* ATCC 4965, *P. freudenreichii* ISU P59 and then *Propionibacterium jensenii* INF P303. They found that the effect was highly strain specific, with all the strains responding differently. They also analyzed autolysis of *L. lactis* subsp. *lactis* INF L2 and *P. freudenreichii* ISU P59 in a liquid cheese model and detected it later in this cheese model system than in broth media.

Rymaszewski et al. (1995) found neither synergic nor antagonistic activity in mixed cultures of *L. lactis* and *Leuconostoc mesenteroides* (022 and CH-N-01) with

Propionibacterium spp. (108 and Bioprop). Lactic acid cocci and propionic acid rods were characterized by similar growth dynamics suggesting commensal relationship. Mikš-Krajnik (2012) described that biotechnological processes occurring in the ripened cheese under the industrial conditions mainly depend on the physiological state of the microorganisms applied in Swiss-Dutch type cheese. The metabolism of bacterial cells governs the biochemical and enzymatic changes occurring in the cheese environment, thus, it creates the desirable characteristics of the product. Furthermore, stress conditions can lead to changes in the bacterial physiological state of starter culture, such as sub-lethal injury or lysis of cells, the loss of the ability to grow (cultivability) on agar media or enter a reversible anabiosis or dormant state, also called VBNC state – viable but not cultivable (Moreno et al., 2006; Oliver 2005; Olszewska and Łaniewska-Trokenheim, 2011). Various stressors can also affect cells' membrane transport system, intracellular enzyme activity, bacterial interactions and metabolism (Ganesan et al., 2007; Warminska-Radyko et al., 2010). The physiological state of starter cultures influences the overall flavour of many fermented dairy products (Marilley and Casey, 2004). In order to detect different physiological states of bacteria, culture-dependent and culture-independent techniques should be adopted; whereas, bacterial culturing techniques can be used to detect the bacteria cultivability, resuscitation from dormant state, as well as the bacterial interactions between the strains.

Fluorescent staining can be applied for physiological studies at single-cell level, while the changes in metabolic pathways can be tracked by the analysis of volatile organic compounds of microbial origin using gas chromatography techniques. However, there is no available information on the influence of stress conditions on physiological state, metabolism and bacterial interactions of *L. lactis* and *P. freudenreichii* strains in co-cultures.

The aim of this study was to analyze the interactions of *L. lactis* and *P. freudenreichii* bacterial strains in co-culture in response to selected stress factors in skimmed milk, including osmotic stress and low temperature, relative to their viability, physiological state and metabolic changes in long-term culture in skimmed milk. The conditions applied in this experiment are characteristic of the production and ripening (cold and warm room) of Swiss-Dutch type cheese.

MATERIALS AND METHODS

LAB / PAB co-culture selection

During the preliminary study, 7 *L. lactis* and 13 *Propionibacterium* strains belonging to species: *P. freudenreichii* n = 6, *P. thoenii* n = 3, *P. acidipropionici* n = 3, *P. jensenii* n = 1 were carefully chosen in view of their ability to lower pH, alter the volatile compound profile in skimmed (0.5% fat) UHT milk, ferment substrates, produce gas. The bacterial interactions between investigated strains were carried with well diffusion agar assay, as previously described (Warminska-Radyko et al., 2002). The influence of PAB metabolites present in

Table 1. Number of 24 h and 7 days-old LAB cultures on skimmed milk incubated at 22°C influencing the growth of *Propionibacterium* strains examined with well diffusion agar assay on a lactate agar growth medium.

<i>Propionibacterium</i> (n=13)	<i>Lactococcus lactis</i> cultures incubation time			
	24 h		7 days	
	No influence/stimulation	Inhibition	No influence/stimulation	Inhibition
<i>P. freudenreichii</i> (n=6)	36 (86%)	6 (14%)	38 (90%)	4 (10%)
<i>P. acidipropionici</i> (n=3)	16 (76%)	5 (24%)	19 (90%)	2 (10%)
<i>P. thoenii</i> (n=3)	19 (90%)	2 (10%)	21 (100%)	0 (0%)
<i>P. jensenii</i> (n=1)	2 (29%)	5 (71%)	5 (71%)	2 (29%)
Total	73 (80%)	18 (20%)	83 (91%)	8 (9%)

24 h and 7 days-old cultures in milk (at 22°C) on the growth of 7 *L. lactis* strains was examined using a lactate agar growth medium; whereas, the influence of LAB metabolites present in 24 h and 7 days-old cultures in milk (at 22°C) on the growth of 13 *Propionibacterium* strains was studied on M17 agar (Merck, Germany). Based on the lack of antagonistic interactions, 7 *L. lactis* and 11 *Propionibacterium* strains belonging to species: *P. freudenreichii* n = 5, *P. thoenii* n = 3, *P. acidipropionici* n = 2, *P. jensenii* n = 1, were selected and combined in 20 mixed cultures (1:1:10, v: v: v, 2 *L. lactis* strains of low and high acidifying activity - 10⁷ CFU/ml and 1 *Propionibacterium* strain - 10⁶ CFU/ml, respectively). The most promising culture composed of *L. lactis* subsp. *lactis* biovar. *diacetylactis* 2M5/2M (low acidifying activity), *P. freudenreichii* 111 [WSRO (= LCC) collection of the Chair of Industrial and Food Microbiology, University of Warmia and Mazury in Olsztyn, Poland] and *L. lactis* subsp. *lactis* biovar. *diacetylactis* C75 (high acidifying activity) (CSK Food Enrichment, Poland) was selected for further evaluation.

Co-culture assay and stress conditions

Model systems were prepared by inoculating skimmed (0.5% fat) UHT milk with 10⁷ CFU/ml of LAB (1:1, v:v) and 10⁶ CFU/ml of PAB (1:10, v:v, LAB/PAB). The following stress conditions were applied: osmotic - 3% (w/v) NaCl + 22°C, low-temperature - 10°C, and incubation at 22°C as control conditions. Sampling was performed at selected time intervals of 0, 2, 7, 14, 21 and 28 days.

Bacteria staining

The direct epifluorescent filter technique (DEFT) was applied with the LIVE/DEAD[®] BacLight™ Bacterial Viability kit (Molecular Probes Inc., USA) for the enumeration of bacteria with intact (LIVE) or damaged (DEAD) cell membrane. LIVE/DEAD[®] BacLight™ staining was performed according to the manufacturer's protocol and as previously described (Warminska-Radyko et al., 2010). Microscopic slides were analyzed under the OLYMPUS BX51 epifluorescent microscope equipped with CCD ColorView II bundle CELL-F camera and set of fluorochrome filters: SYTO[®]9 (U-MNB2, 470-490 nm), IP (U-MNG2, 530-550 nm) and Cell[®] Soft Imaging System (Olympus Optical Ltd.). Prepared slides were stored at -20°C until microscopic analysis. Stained cells from 10 to 15 microscopic fields were counted per assay.

Plating technique

The plate method was used to estimate the interactions and cultivability of lactococci and propionibacteria. Samples were plated on M17 agar (Merck, Germany) and incubated at 30°C/48 h for *L. lactis* subsp. *lactis* biovar. *diacetylactis* strains enumeration (Terzaghi and Sandine, 1975) and on a lactate agar growth medium enriched

with 4 µg/ml of cloxacilin and incubated anaerobically at 30°C/72 h for *Propionibacterium* strains enumeration (Drinan and Cogan, 1992).

Gas chromatography analysis

Volatile compounds were measured by headspace analysis using a gas chromatograph Clarus 500 (Perkin Elmer, USA) with a flame ionization detector (FID) and a Turbomatrix 40 autosampler (Perkin Elmer, USA) according to the method described by Warminska-Radyko et al. (2010). The chromatograph was calibrated for acetaldehyde, acetoin (Fluka, Switzerland), diacetyl (Aldrich, Germany) and the standard solution of acetic acid and propionic acid (Supelco, USA). Calibration curves were developed with the final regression coefficient of R² = 0.985 to quantify the amount of volatiles. Data were analyzed using STATISTICA 8 software (StatSoft[®], Poland).

RESULTS

LAB / PAB co-culture selection

The LAB strains can stimulate or inhibit *Propionibacterium* strains, as it was described in the introduction. In presented research, among 7 examined 24 h-old and 7 days-old *L. lactis* cultures, no influence or stimulation of *Propionibacterium* strains growth was observed, in respectively 80 and 91% of the cultures tested (Table 1). The stimulating activity of *L. lactis* or their metabolites of 7 days-old cultures increased in the majority of PAB cultures, regardless of the species studied (Table 1). On the other hand, most of 24 h-old *Propionibacterium* cultures or their metabolites inhibited the growth of *L. lactis* strains (Table 2). However, after 7 days, the inhibiting activity of PAB culture decreased and in 69% cultures the inhibition was not observed (Table 2). This may suggest differences in sensitivity of *L. lactis* strains towards anti-bacterial metabolites produced by the PAB cultures during different culture time. Of the examined 13 PAB strains, 11 of them did not inhibit at least one of the *L. lactis* strains in long-term cultures, therefore, they were chosen for further assessment.

Cell membrane integrity

Figure 1 shows the changes of LIVE cell count (cells with

Table 2. Number of 24 h and 7 days-old PAB cultures on skimmed milk incubated at 22°C influencing the growth of *Lactococcus lactis* strains studied with well diffusion agar assay on M17 agar.

<i>Lactococcus lactis</i> (n=7)	<i>Propionibacterium</i> cultures incubation time			
	24 h		7 days	
	No influence/stimulation	Inhibition	No influence/stimulation	Inhibition
Total	20 (22%)	71 (78%)	63 (69%)	28 (31%)

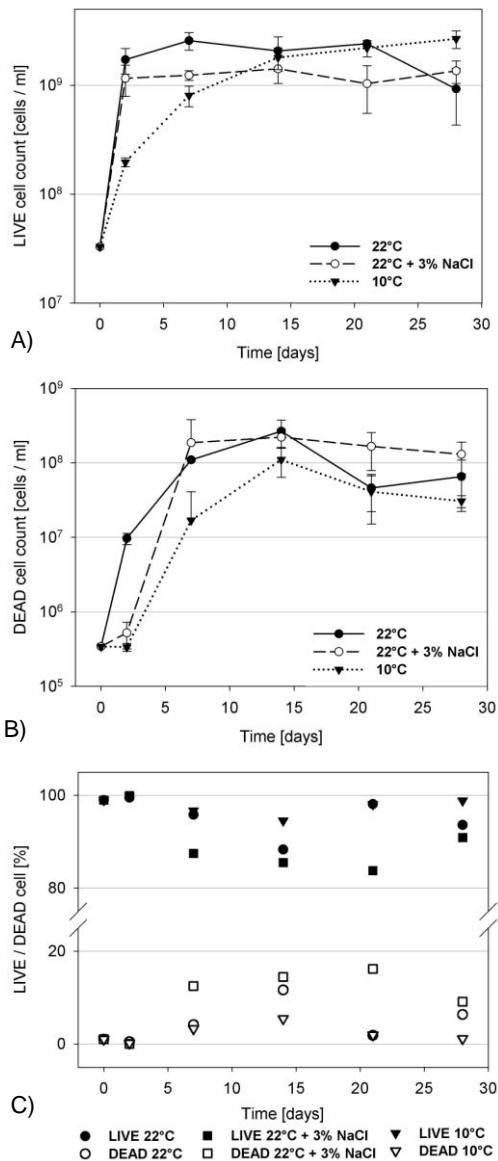


Figure 1. Cell counts of *L. lactis* subsp. *lactis* biovar. *diacetylactis* 2M5/2M, *L. lactis* subsp. *lactis* biovar. *diacetylactis* C75 and *P. freudenreichii* 111 in co-culture in skimmed milk incubated at the following stress conditions: osmotic – 3% (w/v) NaCl + 22°C, low-temperature – 10°C, and at 22°C as control conditions. Cells were stained with LIVE/DEAD[®] BacLight[™] kit to determine the number of intact – LIVE (A) and injured – DEAD cells (B) and the percentage of LIVE and DEAD cells in a population (C).

intact cell membrane) and DEAD cell count (cells with damaged cell membrane) and the percentage of LIVE and DEAD cell count in the population during long-term incubation of LAB/PAB co-culture in skimmed milk under various conditions. The LAB/PAB co-culture inoculum presented very high viability reaching 3.3×10^7 of LIVE and 3.4×10^5 cells/ml of DEAD cells (Figure 1A and B). At 22°C, the LIVE cells count reached ~ 1.0 to 2.0×10^9 cells/ml during first 48 h of incubation, and it was not affected by the addition of salt in the logarithmic phase. In the presence of NaCl, a steep increase in the LIVE cell count was observed on the second day of incubation, followed by minor inhibition of growth until day 28 of the experiment (stationary growth phase) in comparison with control conditions (Figure 1A). The percentage of LIVE cells exposed to NaCl also differed, and it was $\sim 10\%$ lower than in the control sample (Figure 1C). At 10°C, the number of LIVE cells reached 1.8×10^9 cells/ml on incubation day 14. Under the aforementioned conditions, the adaptation phase was prolonged in response to lower temperature (Figure 1A). Throughout the experiment, the number of DEAD (injured) cells was estimated between 3.5×10^5 to 2.7×10^8 cells/ml (Figure 1B). During the first 48 h of incubation, the DEAD cell count was determined at $\sim 10^5$ cells/ml and it was not affected by salt and low temperature stressors. In control conditions at 22°C, the number of injured cells was higher at $\sim 10^7$ cells/ml (Figure 1B). In long-term LAB/PAB co-cultures, the DEAD cell count was lower at 10°C than at 22°C on day 7 (both with and without NaCl).

After 14 days of incubation, the average percentage of DEAD cells in the population was 13% under exposure to NaCl, 6% at 22°C (control) and only 2% at 10°C (Figure 1C).

LAB / PAB interactions and cultivability

The plating technique was applied because non-specific staining methods do not support observations of LAB and PAB interactions between strains and their viability (Figure 2). Intensity growth of LAB was observed during 48 h of incubation at 22°C. On day 2, the LAB plate count reached $\sim 10^9$ CFU/ml, and it entered the stationary phase of growth. After 21 days of incubation, the number of LAB plate count decreased slightly in all conditions tested. Simultaneously, moderate growth of PAB was observed in co-cultures incubated at 22°C with or without

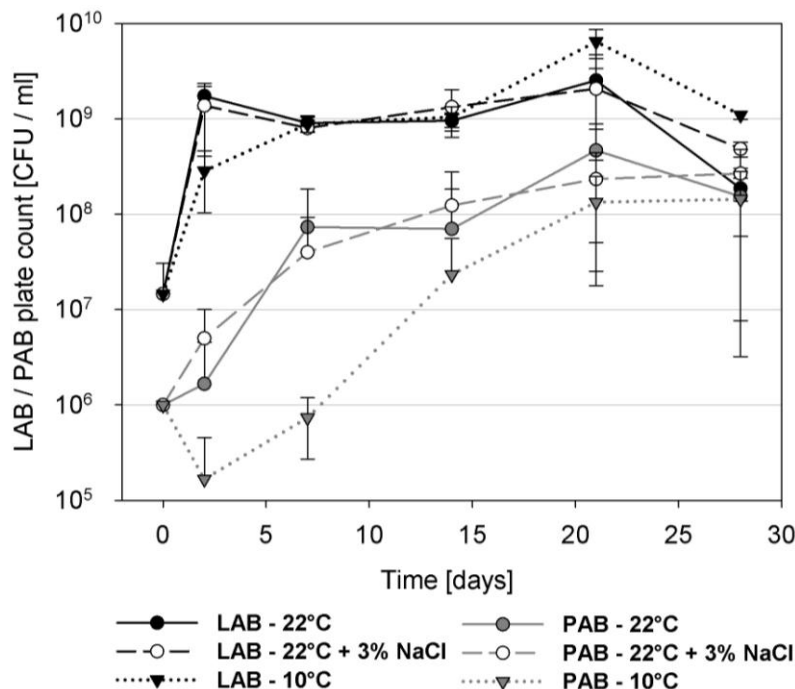


Figure 2. Plate counts of *L. lactis* subsp. *lactis* biovar. *diacetylactis* 2M5/2M, *L. lactis* subsp. *lactis* biovar. *diacetylactis* C75 (LAB) and *P. freudenreichii* 111 (PAB) in co-culture in skimmed milk incubated at the following stress conditions: osmotic – 3% (w/v) NaCl + 22°C, low-temperature - 10°C, and at 22°C as control conditions.

the addition of salt. On incubation day 21, LAB and PAB plate counts reached the maximum level of $\sim 10^9$ and $\sim 10^8$ CFU/ml, respectively (Figure 2). A minor inhibition of LAB growth was observed and PAB plate counts were considerably reduced after 48 h of incubation at the lower temperature. PAB continued to grow at 10°C between incubation days 7 and 21 (Figure 2).

LAB / PAB metabolites

GC analyses of LAB/PAB co-cultures were performed to determine the following volatile compounds: acetaldehyde, diacetyl, acetoin, acetic acid (C_2) and propionic acid (C_3) (Table 3). In the group of volatiles identified in this study, fatty acids were found to be the predominant constituent of all fermented milks. A considerable increase in fatty acids synthesis was observed during the stationary phase of PAB growth in all conditions tested. The addition of NaCl decreased the synthesis of both C_2 and C_3 . The highest content of C_2 and C_3 was observed in control conditions, and the lowest – at 10°C (Table 3). At 10°C, C_2 content was lower than C_3 concentrations, and the (C_2/C_3) molar ratio was 1:0.76 on experimental day 7, and 1:0.64 on day 28. Initially, the concentrations of C_2 and C_3 were similar at 22°C in both control conditions and under exposure to NaCl. The content of C_2 increased over time to reach 1527.8 $\mu\text{g/ml}$ on day 28 at 22°C (C_3/C_2 molar ratio of 1:1.62). Beginning on day

14, C_2 concentrations in cultures supplemented with NaCl were twice as high in comparison with control, and the C_3/C_2 molar ratio was determined at 1:0.91 (Table 3). In our study, on experimental day 14, average acetoin concentrations reached ~ 1000 $\mu\text{g/ml}$ (in control conditions) and ~ 550 $\mu\text{g/ml}$ (with NaCl addition) and afterwards no acetoin increase were noticed (Table 3). However, an inhibitory effect of NaCl addition on acetoin synthesis was observed in comparison to control culture. At 10°C, a steep increase in acetoin content was observed between days 14 and 28 (Table 3), and the aforementioned can be associated with increased growth and metabolism of PAB (Figure 2).

Slight variations in diacetyl content were observed during incubation in all conditions tested (Table 3). Acetaldehyde concentrations increased after 48 h of incubation at 22°C, followed by a sharp decrease (Table 3). The addition of NaCl decreased acetaldehyde synthesis, whereas, the highest acetaldehyde concentrations were observed at 10°C between incubation days 7 and 14, after which they decreased steadily.

DISCUSSION

Osmotic stress and bacterial physiological state

The studies on bacterial physiology changes, metabolism, cultivability and strains interactions are difficult to verify since there is no standard methodology to measure

Table 3. The content^a of volatile compounds determined by HS-GC-FID in *L. lactis* subsp. *lactis* biovar. *diacetylactis* 2M5/2M, *L. lactis* subsp. *lactis* biovar. *diacetylactis* C75 and *P. freudenreichii* 111 co-culture in skimmed milk incubated at the following stress conditions: osmotic – 3% (w/v) NaCl + 22°C, low-temperature - 10°C and at 22°C as control conditions.

Culture	Day	Acetaldehyde (µg/ml)		Diacetyl (µg/ml)		Acetoin (µg/ml)		Acetic acid (C ₂) (µg/ml)		Propionic acid (C ₃) (µg/ml)		C ₂ :C ₃ molar ratio
22°C	0	1.0	± 0.0	0.0	± 0.0	0.0	± 0.0	0.0	± 0.0	0.0	± 0.0	0
	2	51.4	± 1.4	0.3	± 0.0	59.5	± 8.6	260.2	± 60.1	367.9	± 21.1	0.87
	7	10.0	± 0.7	1.5	± 0.2	524.1	± 14.7	746.2	± 28.8	740.5	± 16.1	1.24
	14	4.8	± 0.1	0.4	± 0.1	948.1	± 61.9	1210.1	± 132.7	895.3	± 26.2	1.67
	21	1.8	± 0.1	0.6	± 0.9	991.7	± 32.7	1190.9	± 37.7	914.1	± 6.9	1.61
	28	0.9	± 0.7	0.4	± 0.1	996.1	± 27.3	1527.8	± 85.1	945.3	± 10.6	1.99
22°C + 3% NaCl	0	1.0	± 0.0	0.0	± 0.0	0.0	± 0.0	0.0	± 0.0	0.0	± 0.0	0
	2	32.3	± 1.7	0.3	± 0.0	47.8	± 2.6	320.0	± 16.8	297.5	± 36.9	1.33
	7	1.0	± 0.7	0.7	± 0.1	316.9	± 13.1	586.6	± 287.0	636.2	± 125.4	1.14
	14	0.0	± 0.0	0.3	± 0.0	549.9	± 28.1	622.6	± 95.3	713.2	± 27.5	1.08
	21	0.0	± 0.0	0.3	± 0.0	559.2	± 32.6	622.8	± 37.5	723.0	± 13.4	1.06
	28	0.0	± 0.0	0.2	± 0.0	542.4	± 44.0	656.9	± 40.4	724.7	± 5.4	1.12
10°C	0	1.0	± 0.0	0.0	± 0.0	0.0	± 0.0	0.0	± 0.0	0.0	± 0.0	0
	2	4.8	± 0.6	0.1	± 0.0	11.4	± 0.6	0.0	± 0.0	55.7	± 96.5	0
	7	64.3	± 2.6	0.6	± 0.0	136.1	± 5.2	146.0	± 132.4	236.0	± 51.9	0.76
	14	53.8	± 2.0	0.4	± 0.1	164.2	± 4.6	344.4	± 28.2	438.4	± 33.0	0.97
	21	18.8	± 9.7	0.8	± 0.2	536.3	± 56.6	339.4	± 21.1	552.1	± 8.6	0.76
	28	7.7	± 1.0	0.3	± 0.1	685.2	± 40.4	389.0	± 33.8	607.6	± 22.2	0.79

^a The data were expressed as mean ± standard deviation (n=3).

the phenomenon (Ganesan et al., 2007; Oliver, 2005). In our study, the LIVE/DEAD[®] BacLight™ kit was applied to determine the number of intact (LIVE) and injured (DEAD) cells in the population. Our experiments revealed high viability and metabolic activity of the LAB/PAB co-culture during long-term incubation in skimmed milk under exposure to osmotic stress conditions (3% NaCl). Salt, as an osmotic active agent, is commonly added to cheese for flavour, bacterial growth or acidification control purposes. In ripened cheeses, NaCl concentrations can reach up to 2% (w/v) (Marilley and Casey, 2004). External osmotic pressure can affect bacterial shape and envelope

composition, membrane permeability, transport system and sensitivity to cell lysis (Sanders et al., 1999). In hypertonic solutions, many survival mechanisms can be induced (Ganesan et al., 2007). Moreover, the protective effect of milk and cheese ingredients, such as choline or carnitine, on the physiological state of cells exposed to osmotic stress was also indicated (Boyaval et al., 1999; Leverrier et al., 2004). In order to maintain optimum pressure in the cytoplasm, bacterial cells in hypertonic solution accumulate compatible solutes such as betaines, proline or sugars like lactose which are available in the environment. At high concentrations, these particles enhance the

stability of enzymes and preserve the integrity of biological membranes (Sanders et al., 1999).

In our study, LIVE cell count remained high until the end of experiment, the bacterial cell membrane integrity was not affected by applied stress conditions and cell lysis of LAB/PAB starter co-culture was not observed. Interestingly, Ganesan et al. (2007) also showed that the cellular integrity of *L. lactis* subsp. *lactis* strains was maintained via intact membranes and the lack of induction of the lytic system was noticed. Bacterial resistance to osmotic stress depends also on the growth phase. When incubated in monocultures, stationary-phase cells are generally more resistant to NaCl than

exponential-phase cells (Warminska-Radyko et al., 2010). However, our results point to a reverse trend. The aforementioned can be attributed to higher resistance of the LAB/PAB co-culture to osmotic stress or its mutually stimulating growth effects, as observed during preliminary examinations.

Low temperature stress and bacterial physiological state

Bacteria exposed to low temperature stress have a capacity to adapt. Cells enter a stationary phase during which they cease to grow and retain their cultivability (Sanders et al., 1999). Both LAB and PAB are mesophilic bacteria which grow at a temperature of $\sim 10^{\circ}\text{C}$, and the aforementioned conditions are encountered during cheese ripening in a cold room (Mikš-Krajnik, 2012). In our study, the adaptation to low temperature stress was observed regardless of the technique applied and it was longer for PAB than LAB (as indicated by the results of cultivability studies). Our results showed that LAB and PAB cells in co-culture remained membrane integrity at 10°C , and it probably induced pathways to maintain its long-term survival. Only during first 7 days of incubation, noticeable inhibition of growth (adaptation) and reduction in the number of intact cells were observed, pointing to possible physiological changes such as longer generation time (Sanders et al., 1999), beginning of dormancy (Olszewska and Łaniewska-Trokenheim, 2011) and preservation of membrane integrity (Warminska-Radyko et al., 2010).

LAB / PAB interactions and cultivability

According to earlier studies, during cheese ripening, LAB/PAB interactions are bound by commensalisms relationship (Kerjean et al., 2000; Liu and Moon, 1982; Rymaszewski et al., 1995). During cold room ripening (10 to 14°C), the oxidation-reduction potential of cheese is decreased due to LAB metabolism, and lactate is synthesized to enhance PAB growth at the warm room stage (18 to 24°C) (Piveteau, 1999). PAB have high nutritional requirements and a low ability to utilize lactose as a carbon source (Kujawski et al., 1995), therefore, usually, their growth is delayed in comparison with LAB (Treimo et al., 2006). Additionally, Kerjean et al. (2000) suggested that the LAB/PAB interaction in Swiss cheese can change from commensalisms to mutualistic relationship when lactic acid is accumulated and LAB begins to benefit from its removal by PAB. However, as summarized in the introduction, many types of bacterial interactions between LAB and PAB were noticed by different authors (Condon and Cogan, 2000; Jimeno et al., 1995; Liu and Moon, 1982; Pérez Chaia et al., 1995; Piveteau et al., 1995; Rymaszewski et al., 1995; Warminska-Radyko et al., 2002). Our results support the theory on commensal relationship between tested LAB and PAB strains. Moreover, no influence of NaCl on LAB/PAB

growth in co-culture was demonstrated; whereas, low temperature induced only adaptation mechanisms, the cultivability of PAB at 10°C was significantly reduced in comparison to 22°C (Figure 2).

Applied stress conditions did not disturb the typical interactions between LAB and PAB strains. It can be concluded that our starter LAB/PAB co-culture composed of *L. lactis* subsp. *lactis* biovar. *diacetyllactis* 2M5/2M, *L. lactis* subsp. *lactis* var. *diacetyllactis* C75 and *P. freudenreichii* 111 can be applied to Swiss-Dutch type cheese production.

LAB / PAB metabolites

Fatty acids ratios such as the ratio of the C_3/C_2 are controlled for thermodynamic reasons, ATP production and entropy generation (Babuchowski 1995; Marilley and Casey, 2004). Changes in the C_3/C_2 ratio are induced by growth conditions. Theoretically, the C_3/C_2 molar ratio is 2; nevertheless, the C_3/C_2 ratio has been observed to vary extensively from 2:1 to 10:1 for glucose as a carbon source (Boyaval et al., 1999). Our results indicate that the highest C_3/C_2 ratio was observed in control conditions (an average $\sim 1:1.6$) and the lowest at 10°C (an average $\sim 1:0.8$) (Table 3). The reported values are comparable to the fatty acid ratio in Swiss-type cheese which ranges from 1:0.5 to 1:2.8 (Thierry et al., 2004). Moreover, no branched chain fatty acids synthesis (isobutyric acid or isovaleric acid) was determined (data not shown), which normally suggest the entrance of bacterial cells to dormant state or VBNC state induced by carbohydrate starvation and its swift to amino acid catabolism as previously described (Ganesan et al., 2006; Thierry et al., 2004). Diacetyl (2,3-butanedione) and acetoin (3-hydroxy-2-butanone) are the main flavour compounds responsible for the buttery aroma of many dairy products, including certain types of cheese (Marilley and Casey, 2004). Both compounds can be produced by LAB during citrate metabolism, and they can be derived from α -acetolactate:acetoin by the activity of α -acetolactate dehydrogenase and diacetyl – by oxidative decarboxylation (Mikš-Krajnik, 2012).

In our study, slight variations in diacetyl content during experiment can be attributed to the activity of diacetyl reductase, temperature, pH and aeration level. Diacetyl can be irreversibly reduced to acetoin by diacetyl reductase, while acetoin can be reduced to 2,3-butanediol by acetoin reductase (Marilley and Casey, 2004). In general, the results of our study indicate that acetoin synthesis was inhibited by NaCl addition and prolonged incubation intensified the accumulation of acetoin in culture at 10°C . Acetaldehyde is an important determinant of yoghurt flavour and aroma, and it is found in many fermented dairy products, mainly yogurt (5 to 40 mg/L). Acetaldehyde content of $1\ \mu\text{g/g}$ gives dairy products a characteristic “natural style” or “green apple-like” flavour (Thierry et al., 2004). In our studies, the initial increase in acetalde-

hyde content leading to its accumulation in milk was observed. It can be probably attributed to the limited or lack of alcohol dehydrogenase activity in studied *L. lactis* subsp. *lactis* biovar. *diacetylactis* strains (Boylston, 2012). The subsequent sudden drop in acetaldehyde concentrations after 2 day (at 22°C) and 14 day (at 10°C) of incubation coincides with the increase in PAB population in each condition tested. It can be caused by the reduction of acetaldehyde to ethanol by NAD-linked alcohol dehydrogenase possibly as a result of greater metabolic activity of PAB (Keenan and Bills, 1968). Another explanation may be the shift from homolactic to mixed fermentation pathway, which has been observed under different environmental conditions such as the lack of carbon source, change in pH or NADH/NAD⁺ ratio, oxygen content or cofactors presence (Oliveira et al., 2005).

In conclusion, our results indicate that co-culture composed of *L. lactis* subsp. *lactis* biovar. *diacetylactis* 2M5/2M, *L. lactis* subsp. *lactis* biovar. *diacetylactis* C75 and *P. freudenreichii* 111 retained their activity under all culturing conditions in skimmed milk. In presence of NaCl, the bacterial cultivability and physiological state were not affected, while the metabolites synthesis (acetaldehyde, acetoin, acetic and propionic acids) were slightly reduced, except for a single drop of acetaldehyde content. Accurate monitoring of changes in the physiological state, growth and death dynamics of microbial populations in dairy products requires the application of culture-independent techniques. However, it must be underlined that fluorescent LIVE/DEAD[®] BacLight[™] staining has limited application to mixed starter cultures studies as it determines only the overall cell membrane permeability of bacterial population, not individual species. For this purposes and further studies, the genetic techniques, such as fluorescent *in situ* hybridization (FISH) could be applied.

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