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Patterns of Survival and Volatile Metabolites of Selected *Lactobacillus* Strains During Long-Term Incubation in Milk

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The focus of this study was to monitor the survival of populations and the volatile compound profiles of selected *Lactobacillus* strains during long-term incubation in milk. The enumeration of cells was determined by both the Direct Epifluorescent Filter Technique using carboxyfluorescein diacetate (CFDA) staining and the plate method. Volatile compounds were analysed by the gas-chromatography technique. All strains exhibited good survival in cultured milks, but *Lactobacillus crispatus* L800 was the only strain with comparable growth and viability in milk, assessed by plate and epifluorescence methods. The significant differences in cell numbers between plate and microscopic counts were obtained for *L. acidophilus* strains. The investigated strains exhibited different metabolic profiles. Depending on the strain used, 3 to 8 compounds were produced. The strains produced significantly higher concentrations of acetic acid, compared to other volatiles. *Lactobacillus* strains differed from one another in number and contents of the volatile compounds.

***Keywords*:** Direct Epifluorescent Filter Technique, CFDA staining, gas-chromatography, *L. acidophilus*, *L. crispatus*

The production and consumption of beverages fortified with bacterial strains displaying probiotic properties have been increasing dynamically in recent years (Macciola *et al.*, 2008). The great interest expressed by consumers in fermented products is linked with a demand for conventional and functional food products. Supplementation of dairy beverages or their production with microorganisms presenting health-promoting properties, e.g. *Bifidobacterium* sp., *Lactobacillus* sp. is aimed at improving these products' nutritious, therapeutic, and prophylactic properties (Banina *et al.*, 1997; Gomes and Malcata, 1999; Baron *et al.*, 2000; Vinderola and Reinheimer, 2000). The example is the use of *Lactobacillus acidophilus* in the production of acidophilus milk. Most references address the health-promoting properties of dairy products, whereas the effect of metabolites synthesized by probiotic strains determining the flavor of a product has been the subject of sparse reports (Helland *et al.*, 2004). The most common metabolites attention affecting flavor characteristics has been given to cheeses, the source of variety of volatile and non-volatile compounds (Fernandez-Garcia *et al.*, 2004). Apart from the predominating lactic acid, the most frequently determined metabolites synthesized by lactic acid bacteria (LAB) include: aldehydes, ketones, alcohols, and volatile fatty acids e.g. acetic acid, propionic acid, butyric acid, etc (Marilley and Casey, 2004). Metabolites that affect the attractive flavor of dairy beverages include: acetaldehyde, diacetyl, acetoin, ethanol and some volatile acids (Gardini *et al.*, 1999; Leroy and De Vuyst, 2004).

Still, little information is available referring to the metabolic pathways performed by probiotics or potentially probiotic bacteria, which could be further used as starter microorganisms in manufacture of probiotic milk products. A combination of positive image of probiotic and fermentation aspects should guarantee health effects as well as good sensory properties of products. Production of acceptable products based on probiotic strains has met many problems, while probiotic bacteria such as *L. acidophilus* grow slowly in milk, suggesting that milk is not very good growth medium for them. What is more, the metabolic profiles of probiotic bacteria can vary depending on a strain (Østlie *et al.*, 2003), confirming that the specific profile of produced metabolites is not only a genus-but even strain-specific characteristic dependent upon the specific activity of the bacteria and their enzymes. For this reason, a need has emerged for the monitoring of both metabolism and viability of selected strains of lactic acid bacteria in milk-based or dairy-like model products. That is why, the objective of this study was to monitor the growth and metabolic activity of potentially probiotic bacteria, such as *L. acidophilus* and *L. crispatus* (Coeuret *et al.*, 2003) during long-term incubation in skimmed milk. Two methods of enumeration: microscopic and plating were compared by monitoring cell counts of lactobacilli in milk during incubation. During the experimental period, profiles of volatile metabolites were also monitored.

Materials and Methods

Bacterial strains and growth conditions

The model systems were consisted of ultra-high temperature (UHT) milk [0.5% (w/v) fat] containing the appropriate lactobacilli culture added. Six strains were studied: *L. acidophilus* 1, *L. acidophilus* 2, *L. acidophilus* 2B, *L. acidophilus* 194, *L. acidophilus* K55/81 (Chair of Industrial and Food Microbiology, University of Warmia and Mazury, Poland), and *L. crispatus* L800 (CSK Food Enrichment, Poland). The required inoculum of individual strains to give at the level of 2 log CFU/ml in milk was calculated. Prepared milks were incubated at

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37°C for *L. acidophilus* strains and at 44°C for *L. crispatus* for 14 days. Analyses were made after 0 h, 3 h, 6 h, 9 h, 12 h, 24 h (1 day), 48 h (2 days), 72 h (3 days), 96 h (4 days), 120 h (5 days), 192 h (8 days), 264 h (11 days), 336 h (14 days).

Enumeration of LAB

Staining procedure with CFDA (carboxyfluorescein diacetate, Biochemika Fluka, Switzerland) prefluorochrome was performed using appropriate dilutions of cultured milks in sterilized phosphate-buffered saline (PBS, 0.1 M, pH 7.8). The volumes of 1 ml of cell suspensions were transferred into eppendorf tubes and stained with CFDA working solution in anhydrous dimethylsulphoxide (DMSO) to a final concentration of 10 μM in the dark for 30 min at 37°C (GFL Inkubator, Germany). The suspensions of stained cells were filtered by using a glass filter tower (Millipore, Germany) with a vacuum applied on black polycarbonate filters (13 mm diameter, 0.22 μm pore size, Millipore). Air-dried filters were mounted in nonfluorescent immersion oil (Molecular Probes, USA) on glass slides beneath cover slips. Microscopic counts were performed with image analysis software (Cell ^ F, Olympus Company) connected to a Olympus BX51 microscope (Germany). The average values of twenty fields per assay were determined. The plate method was used to estimate the number of lactobacilli. Samples were plated on MRS agar and incubated anaerobically at optimal temperature for 48 h.

Volatile compounds analyses

Volatile compounds were measured by gas chromatography. Milk samples were analysed using a Turbomatrix 40 Headspace Autosampler (Perkin Elmer, USA), connected to a Clarus 500 gas chromatograph (Perkin Elmer), equipped with a flame ionisation detector (FID) and a HP-INNOWAX capillary column (Agilent Technologies, USA, 60 m ×0.53 mm i.d.×1.0 μm). Helium was used as a carrier gas at a flow of 5.0 ml/min. Milk samples were placed in hermetically sealed 22-ml vials. The prepared vials were heated at 70°C for 40 min for equilibration. A chromatographic temperature programme was as follows: 5 min at 80°C, 10°C/min up to 220°C, and 5 min at 220°C. The chromatograms were integrated using TotalChrom Navigator software (Perkin-Elmer). Peak identification of volatile compounds was based on the retention times of the individual reference standards: acetaldehyde, acetone, acetoin (Fluka, Switzerland), ethanol, diacetyl (Aldrich, Germany) and the standard solution of acids ranging from C_2 to C_7 in deionized water (Supelco, USA). In order to quantify the amount of volatiles (μg/ml) the calibration curves were prepared with the final regression coefficient of R^2=0.992. The identification of compounds was verified using solid-phase microextraction-gas chromatography–mass spectrometry (SPME-GC-MS).

Statistical analyses

Pearson correlation was performed to evaluate the significance and direction of a obtainable correlation between incubation time and measurable variables. Analysis of variance (ANOVA) followed by Tukey's test (at a level of significance of 5%) was used for data comparison. The analysis was carried out using microbial strain, enumeration method and incubation time as the main parameters, in the case of data of microbiological counts and microbial strain and incubation time as the main parameters, in the case of data of volatile compounds. In addition, the Principal Component Analysis (PCA) of compounds was performed to evaluate differences in volatile compound profiles of *Lactobacillus* strains. Data were analysed using STATISTICA 8 software (StatSoft®, Poland).

Results and Discussion

Microbiological analyses

Analyses of the growth of the investigated lactic acid bacteria in the milk demonstrated differences in its dynamics as affected by the strain examined. Significant differences ($p<0.05$) were demonstrated between the strain *L. crispatus* L800 and *L. acidophilus* strains. *L. acidophilus* strains were characterized by lower growth rates in milk as compared to *L. crispatus* L800 (Fig. 1). Strains of *L. acidophilus* exhibited a similar capability to adaptation and growth in the medium of skimmed milk at the incubation temperature applied, as the cell counts of strains: 2, 2B, and K55/81 were observed to increase from a level of 2 to 7 log CFU/ml in 24 h, whereas that of *L. acidophilus* 1 and 194 - in 48 h. In the case of investigated strains, the maximum number of viable cells reached the level of 7.8-8.3 log CFU/ml. *L. acidophilus* strains sustained these

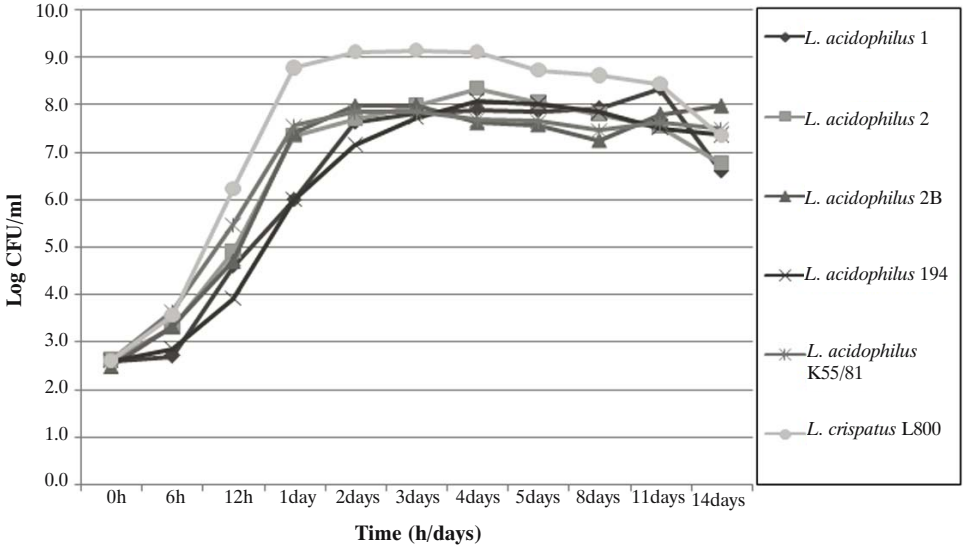

Fig. 1. Growth of *Lactobacillus* strains during long-term incubation assessed with plate method.

viable counts during the whole test period, except for the *L. acidophilus* 1 and 2, in the milk batches of which the viable counts decreased to 6 log CFU/ml in the last days of incubation (Figs. 2A, B, C, D, and E). Scientific reports indicate that *L. acidophilus* grows more slowly in milk than other lactic acid bacteria. Therefore, in practice, they are often combined into common cultivations to grow in association with other species of LAB (Hunger and Peitersen, 1993; Brasheras and Gilliland, 1995; Montes *et al.*, 1995). The *L. crispatus* L800 attained the highest cell population – the maximum cell count after 48 h of incubation reached over 9 log CFU/ml. The stationary phase of growth was maintained at this level for further 4 days, after which the number of cells started to decrease and reached the level of 7.4 log CFU/ml on the 14th day of incubation (Fig. 2F) ($R=-0.87$, $p<0.05$).

In the reported study, CFDA was used as an indicator of the cell count of bacteria displaying the activity of intracellular enzymes. CFDA is a prefluorochrome which can penetrate into the cell and is transformed into fluorescent carboxyfluorescein (CF) upon the action of non-specific esterases (Papadimitriou *et al.*, 2006; Mikš and Warmińska-Radyko, 2008). What is more, the maintenance of membrane integrity is required to sustain the fluorescent compound in the cells (Bunthof *et al.*, 2001). The technique of directly counting the cells with microscopic method restricts detection to the level of over 4 log cells/ml (Papadimitriou *et al.*, 2006), thus determinations by means of this technique were begun after 24 h of incubation. Results showed that the high number of enzymatically-active lactobacilli was maintained over the entire period of incubation. In contrast, significant differences ($p<0.05$) were demonstrated between numbers of *L. acidophilus* strains determined with the plate and the microscopic methods (Table 1). The difference between the epifluorescence and the plate methods was indicated in a study by Moreno *et al.* (2006) who evaluated the viability of lactic acid bacteria in yoghurt and fermented milk. Auty *et al.* (2001) also presented results indicating a lower number of probiotic bacteria determined by means of the plate method in respect of the direct in situ method. Inaccuracies in the quantitative analysis conducted with the plate method may result from the questionable assumption that one colony is formed from one cell. Researchers undertaking the evaluation of the physiological status of LAB in fermented products have distinguished more than two physiological states of cells in a population. Under specified conditions, the viable, metabolically-active cells may lose their capability to grow on agar-based media. This physiological status is interchangeably referred to as VNC ("viable but non-culturable"), ANC ("active but non-culturable") or "dormant cells" and is expressed by underestimation in the determination of the bacteria number with the plate method (Joux and Lebaron, 2000). The dormant state entered by a part of the population may occur in probiotic products or dairy starters, especially during long-term storage (Bunthof and Abee, 2002). Other investigators provide evidence of the dormancy state of cells exposed to the action of stress-bearing factors, e.g. starvation, osmotic stress, acid stress (Bunthof *et al.*, 1999, 2001; Lahtinen *et al.*, 2006; Rault *et al.*, 2007; Warmińska-Radyko *et al.*, 2010). According to results obtained, we can conclude that in most cases, the direct cell counts were higher than the colony counts for stationary phase cultures. The greatest differences at a level of 2-3 log units were observed on the last days of experiment for strains of *L. acidophilus* 1 and 2 (Figs. 2A and B). No significant differences between microscopic and plate counts were observed for *L. crispatus* L800 strain, which performed high enzymatic activity and propagation ability in milk (Fig. 2F).

Chromatographic analyses

Chromatographic analyses of cultured milks with the lactobacilli strains were performed to determine the following volatile compounds: acetaldehyde, acetone, ethanol, diacetyl, acetoin, acetic acid, propionic acid, and isobutyric acid. The prolongation of incubation period affected the intensification of biochemical processes, which resulted in the detection of 3 to 8 volatile compounds, depending on the strain used as an inoculum.

Among the volatile compounds obtained in the study, acetic acid was found as a dominant constituent in all milks tested, but still its concentration differed significantly ($p<0.05$) depending on the strain and ranged from 98.1 to 741.4 μg/ml (Table 1). The greatest differences were observed between the *L. crispatus* L800 which was producing the highest amount of that acid, and the *L. acidophilus* K55/81 and *L. acidophilus* 2B strains, which were generating concentrations of acetic acid accounted for 98.1 and 289.7 μg/ml, respectively. The presence of acetic acid in cultured milk is likely to result from the hydrolysis of saccharides coupled with transformations of pyruvate (Reguła, 2007). The production of acetic acid is determined by the presence of oxygen in the medium, while the acetaldehyde may be formed alternatively in the same pathway under anaerobic conditions (Fernandez-Mugara *et al.*, 1998). The aeration occurred might have affected a decrease in the content of aldehyde in favor of acetic acid.

In the pathway of metabolic transformations of lactic acid bacteria, other volatile aromatic compounds may also be formed from pyruvate, including diacetyl and acetoin (Pakdeeto *et al.*, 2003). According to scientific reports, the concentration of acetoin is 10-50 fold higher than that of diacetyl, which is related to the activity of acetolactate decarboxylase as well as of diacetyl reductase, an enzyme contributing to the increased concentration of acetoin (McSweeney and Sousa, 2000). The results obtained in the study indicate that prolonging the incubation period at a temperature optimal for the growth of bacteria contributed to the intensified accumulation of acetoin, whose content turned out to be even 40 fold higher than that of diacetyl. The milk batches tested differed in terms of concentrations of diacetyl and acetoin over the range of 0.7-4.0 and 25.2-158.6 μg/ml, respectively (Table 1), except for the strains *L. acidophilus* K55/81 and 2B which did not synthesize those metabolites. The enhanced intensification of diacetyl and acetoin synthesis was observed to occur between the 48th and 72th h of incubation, depending on the strain used as an inoculum (Table 1). Significant differences ($p<0.05$) in the concentrations of acetoin were found between *L. crispatus* L800 and *L. acidophilus* 1, 2, and 194 strains.

Among volatile compounds determined, ethanol, and acetone were identified in all investigated milks from the very beginning of the experiment (Table 1). In 3 batches, an increase was observed in the content of ethanol after 24 h of incubation in the case of *L. acidophilus* 194 (an increase by almost 30 μg/ml) after 72 h in the case of *L. acidophilus* 1 and 2 by ca. 9

Table 1. Production of volatile compounds (μg/ml) by different strains during long-term incubation

Time h (days)	*L. acidophilus* 1								*L. acidophilus* 2								*L. acidophilus* 2B							
	Acal	Ac	ET[a]	DI[a]	ACET[a]	C2[a]	C3	iC4	Acal	Ac	ET[a]	DI[a]	ACET[a]	C2[a]	C3	iC4	Acal	Ac[b]	ET	DI	ACET	C2	C3	iC4
0	1.2	4.2	38.4	-	-	-	-	-	2.5	4.9	110.0	-	-	-	-	-	1.2	4.8	30.4	-	-	-	-	-
3	1.3	4.7	38.5	-	-	-	-	-	2.1	4.8	110.9	-	-	-	-	-	1.1	4.8	30.0	-	-	-	-	-
6	1.2	4.7	37.2	-	-	-	-	-	2.1	4.9	110.1	-	-	-	traces	-	0.1	4.8	29.0	-	-	-	-	-
9	1.2	4.5	37.0	-	-	-	-	-	1.6	3.7	88.4	-	-	-	-	-	1.3	4.6	28.4	-	-	-	-	-
12	1.2	4.6	37.6	-	-	-	-	-	2.9	4.8	109.1	-	-	-	traces	-	1.1	4.6	28.6	-	-	-	-	-
24(1)	1.3	4.6	37.8	-	-	-	-	-	2.0	4.6	109.2	-	-	-	-	-	1.2	4.6	28.3	-	-	-	-	-
48(2)	6.4	4.6	37.5	1.0	8.6	-	-	-	3.3	4.7	109.1	-	-	-	-	-	0.6	4.7	28.7	-	-	-	-	-
72(3)	14.1	4.5	38.2	1.4	20.9	-	-	-	1.6	4.7	110.0	-	8.0	-	-	-	0.0	4.6	28.4	-	-	-	-	-
96(4)	65.5	4.7	41.1	1.9	28.2	-	-	-	33.9	4.9	134.4	0.9	13.5	-	-	-	0.0	4.5	28.9	-	-	-	-	-
120(5)	3.9	4.5	41.3	2.5	38.5	-	-	-	2.1	4.9	125.1	1.1	18.8	-	-	-	0.0	4.5	28.7	-	-	-	-	-
192(8)	0.2	4.4	43.5	2.5	44.9	212.7	-	-	1.0	4.8	135.6	1.5	22.7	287.6	-	-	1.2	4.6	28.0	-	-	-	-	-
264(11)	0.0	4.5	47.1	1.4	50.2	349.5	-	-	0.0	4.8	141.0	0.6	24.0	311.6	-	-	0.0	4.5	29.0	-	-	-	-	-
336(14)	1.5	4.5	47.6	1.5	50.9	323.4	-	-	0.2	4.7	138.5	0.7	25.2	357.4	-	-	0.0	4.5	28.8	-	-	98.1	traces	traces

Time h (days)	*L. acidophilus* 194								*L. acidophilus* K55/81								*L. crispatus* L800							
	Acal	Ac[b]	ET[a]	DI	ACET	C2[a]	C3	iC4[a]	Acal	Ac	ET	DI	ACET	C2	C3	iC4	Acal	Ac	ET	DI[a]	ACET[a]	C2[a]	C3[a]	iC4[a]
0	1.2	4.7	44.6	-	-	-	-	-	1.2	4.9	52.6	-	-	-	-	-	1.2	4.6	27.4	-	-	-	-	-
3	1.3	4.8	46.2	-	-	-	-	-	0.2	4.8	54.5	-	-	-	-	-	1.1	4.8	27.6	-	-	-	-	-
6	1.3	4.8	44.9	-	-	-	-	-	1.4	4.8	53.2	-	-	-	-	-	1.2	4.7	25.6	-	-	-	-	-
9	1.3	4.8	44.5	-	-	-	-	-	1.3	4.6	53.0	-	-	-	-	-	0.9	3.9	22.2	-	-	-	-	-
12	1.2	4.6	44.4	-	-	-	-	-	0.2	4.5	52.2	-	-	-	-	-	1.1	4.6	25.9	-	-	-	-	-
24(1)	27.6	4.6	43.5	-	-	-	-	-	1.7	4.6	53.5	-	-	-	-	-	18.0	4.6	29.9	-	27.8	-	-	-
48(2)	4.0	5.6	57.1	0.5	-	-	-	-	0.7	4.6	52.8	-	-	-	-	-	63.0	4.7	35.7	0.3	49.8	-	-	-
72(3)	0.0	4.2	66.6	0.3	6.3	88.7	-	-	0.0	4.5	52.5	-	-	-	-	-	122.2	4.6	35.8	0.3	78.6	-	-	-
96(4)	0.0	2.7	67.5	0.4	8.2	308.1	-	-	0.0	4.5	53.1	-	-	-	-	-	43.5	4.5	35.9	0.0	76.8	303.5	83.2	-
120(5)	0.5	1.9	68.2	0.0	8.9	339.2	-	-	0.0	4.6	53.0	-	-	-	-	-	5.8	4.6	36.2	0.3	90.3	395.1	93.4	-
192(8)	0.7	0.4	69.8	0.0	5.1	123.4	-	-	2.2	4.7	52.6	-	-	-	-	-	0.0	4.5	34.8	1.8	92.7	682.0	131.2	-
264(11)	0.0	0.1	74.7	0.0	0.0	407.2	-	66.9	0.0	4.5	53.1	-	-	-	-	-	0.4	4.8	37.1	6.2	129.6	590.8	140.5	68.4
336(14)	0.0	0.1	72.9	0.0	0.0	578.5	traces	77.4	0.0	4.5	52.9	-	-	289.7	-	-	3.5	4.0	30.6	4.0	158.6	741.4	106.4	69.5

[a] statistically significant upward trend (Pearson correlation)
[b] statistically significant downward trend (Pearson correlation)
Acal, acetaldehyde; Ac, acetone; ET, ethanol; DI, diacetyl; ACET, acetoin; C2, acetic acid; C3, propionic acid; iC4, isobutyric acid

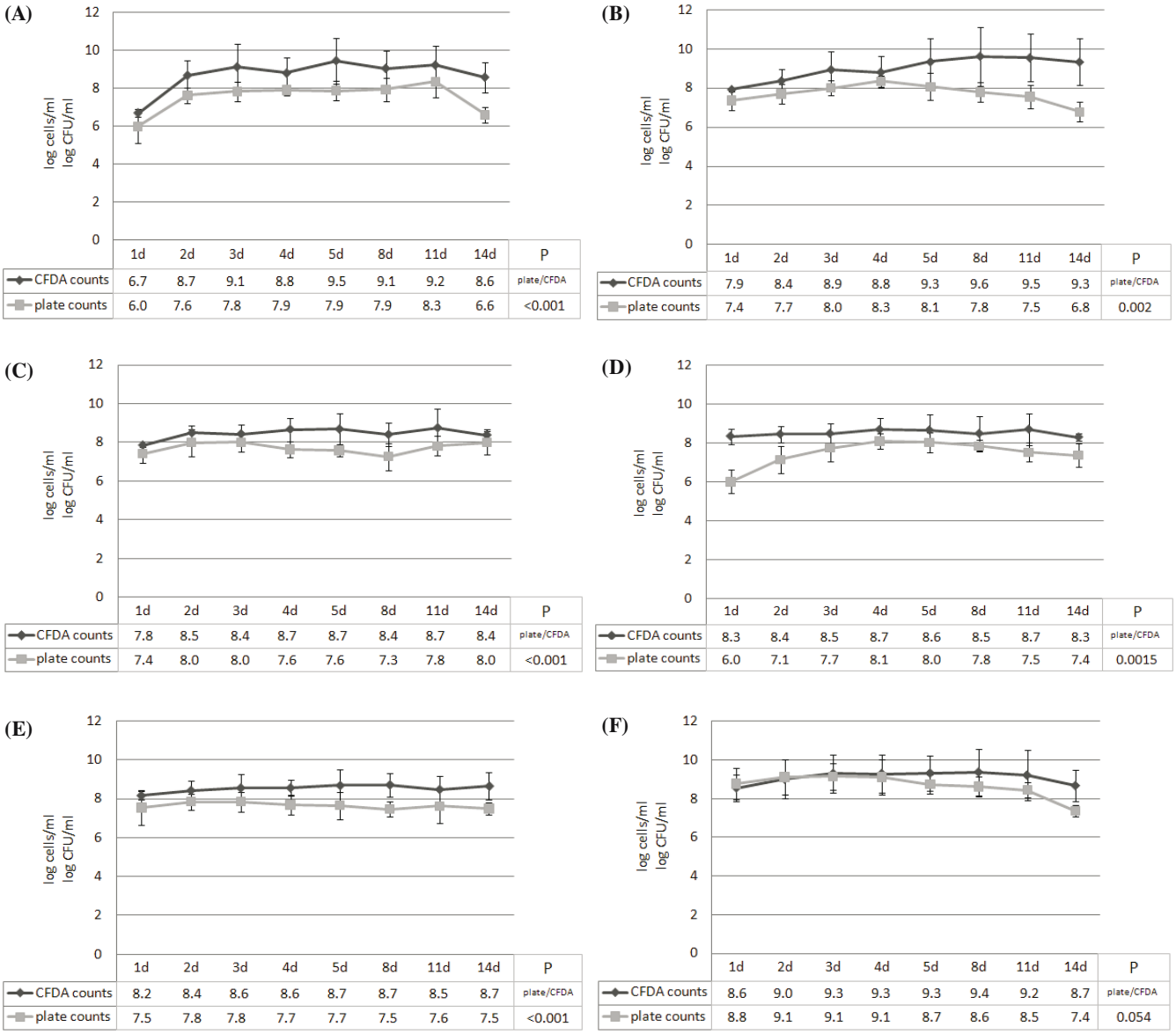

Fig. 2. The enumeration of *Lactobacillus* in cultured milks inoculated with the use of (A) *L. acidophilus* 1, (B) *L. acidophilus* 2, (C) *L. acidophilus* 2B, (D) *L. acidophilus* 194, (E) *L. acidophilus* K55/81, (F) *L. crispatus* L800 assessed by: CFDA and plate method during 14-days incubation period, including the significances of the differences between two enumeration methods for examined strains.

and 28 μg/ml, respectively ($p<0.05$). The synthesis of ethanol in the metabolic pathway of pyruvate is ascribed to alcohol dehydrogenase which is produced by some strains of *L. acidophilus* (Marshall and Cole, 1983). In turn, in most of the samples the content of acetone was not subject to any statistically significant changes during incubation, except for the culture of the strain *L. acidophilus* 194, which the content of acetone was demonstrated to decrease along with incubation time (Table 1). An analogous tendency of a diminishing concentration of acetone by *L. acidophilus* was observed by Gardini *et al.* (1999).

The content of acetaldehyde in milks investigated was found to be diversified. In such products as hard cheeses, buttermilk and butter, acetaldehyde is responsible for untypical taste, whereas its high concentration in yoghurt is desirable (Beshkova *et al.*, 1998). Although no significant ascending tendencies were observed, in the case of strains *L. acidophilus* 1 and *L. crispatus* L800 an increase in acetaldehyde content occurred between the 24 and 96 h of incubation and for the strains *L. acidophilus* 2 and 194 some substantial peaks of acetaldehyde levels were observed, following which these concentrations were reduced (Table 1). The differences in concentrations of this metabolite are likely to result from the previously-mentioned competition for an acetyl-CoA substrate, also being a precursor of acetic acid. The aldehyde may also be synthesized from threonine occurring in milk under the influence of threonine aldolase as that enzyme is responsible for the capability to form acetaldehyde by lactic acid bacteria (Beshkova *et al.*, 1998). The evaluation of production yield of this metabolite by the investigated bacterial strains is difficult owing to the fact that, apart from aldolase, those strains also

probably contain alcohol dehydrogenase which utilizes the aldehyde for the conversion into ethanol. The shifts in acetic acid/acetaldehyde/ethanol production in general can be affected by environmental changes. It is known that lactose is the primary substrate for these compounds production in milk. Theirs production could be limited by the drop in pH, which inhibits the microorganisms, but on the other hand could be facilitated by a increase in number of injured cells, as a result of higher cell membrane permeability and hence a better entrance of the substrate into the cells. Availability of substrate seems to be the stimulating factor in expressing enzyme activity, e.g. β-galactosidase, which is implicated in degradation of lactose, what was shown by Fernandez-Mugra *et al.* (1998). Still, the mechanism of acetaldehyde conversion is complicated to interpret, arising from limited information concerning the specific activities of enzymes, which under particular conditions may be higher than another.

Volatile short chain fatty acid profiles reflect the specific role in flavor properties of fermented dairy products (Alewijn *et al.*, 2003). Apart from acetic acid, propionic and isobutyric acids were also found in the experimental model systems. The highest concentrations of these acids were detected in milks with the addition of *L. crispatus* L800 and *L. acidophilus* 194 strains. These acids appeared in the later stages of incubation during the stationary phase of growth of microorganisms, especially isobutyric acid, which is a proteolytic metabolite of valine (Tavaria *et al*., 2006). The strains examined are characterized by homofermentative metabolism of pyruvate with the major metabolite being lactic acid. Trace amounts of the other metabolites may result from the activity of native enzymes of milk secreted extracellularly with the advancing lysis of cells.

In conclusion, to determine an overview of compound profiles, a PCA analysis was carried out. Figure 3 shows the loading plots of variables – volatile compounds (Fig. 3A) and mean factor scores – strains of *Lactobacillus* (Fig. 3B), defined by principal components 1-PC1 and 2-PC2, which explained 96,4% of the total variance. Acetaldehyde, diacetyl, acetoin, acetic acid, and propionic acid showed high negative loading coefficients (0.97, 0.98, 0.99, 0.93, 0.94, respectively) with PC1. Ethanol presented a high positive loading coefficient (0.94) with PC2. *L. acidophilus* 2 strain showed a positive loading coefficient with the second vertical axis, which means that this strain produced greater amounts of ethanol. On the other hand, *L. crispatus* L800 strain-negative loading coefficient with the first horizontal axis with loading coefficients of acetaldehyde, diacetyl, acetoin, acetic, and propionic acids taken under consideration indicated the highest metabolic activity, with the greatest amounts of these metabolites produced. *L. acidophilus* 194, 1, K55/81, 2B strains were characterized by lower content of volatile compounds.

In this report, the growth and metabolism of selected *Lactobacillus* strains were investigated in milk by monitoring the levels of populations development and profiles of metabolites produced. The first conclusion, arising from this study is linked with good survival of examined strains, which were producing specific metabolites during long-term incubation in milk. In detail, the viable cell counts of lactobacilli varied depending on the strain and technique used for quantitative analyses. Therefore, determination of viability of bacteria may be challenging due to difficulties in selection of suitable enumeration methods. Still, accurate model research and application of alternative methods based on detection of different physiological functions and performing a high sensitivity at the single-cell level are connected with new findings leading to progress in microbiology. Our approach focused on direct *in situ* detection of two physiological parameters of lactobacilli with application of microscopy and fluorescence staining procedure, suggesting the possibility of potentially probiotic bacteria being dormant. What is more, in this investigation, examined strains had performed relatively different metabolic profiles, ranging from 3 to 8 compounds produced, varying in theirs amounts, important for flavor of dairy products. As milk is a specific environment for probiotic bacteria, it is necessary to select strains that are capable of

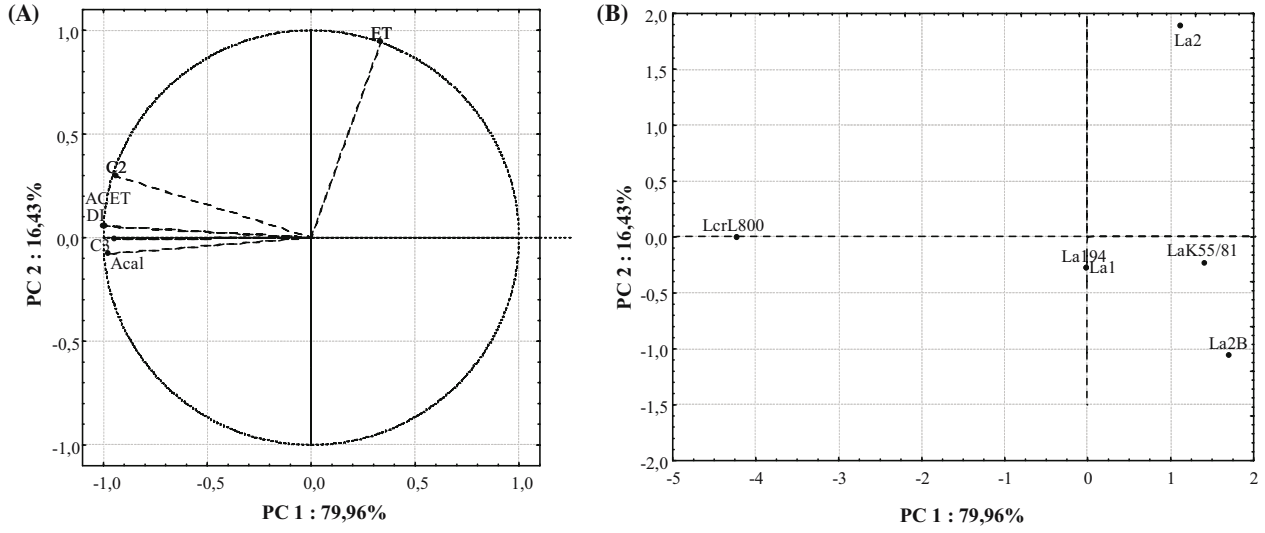

Fig. 3. The diagrams of PCA analysis: loading plots of volatile compounds (A) and mean factor scores of *Lactobacillus* strains (B). Acal, acetaldehyde; Ac, acetone; ET, ethanol; DI, diacetyl; ACET, acetoin; C2, acetic acid; C3, propionic acid; iC4, isobutyric acid; La1, *L. acidophilus* 1; La2, *L. acidophilus* 2; La2B, *L. acidophilus* 2B; LaK55/81, *L. acidophilus* K55/81; La194, *L. acidophilus* 194; LcrL800, *L. crispatus* L800.

affecting desirable flavor characterictics by specific metabolites formed. It is known that for lactic acid bacteria the growth and metabolism can be uncoupled, what was shown for different *L. acidophilus* strains by Østlie *et al.* (2003) and by our findings, where increased production of various metabolites occurred after reaching the stationary phase of growth. Taken together, our studies indicated that the proper selection of *Lactobacillus* strains belonging to the same species is still of great importance mostly from the industrial point of view.

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References

Alewijn, M., E.L. Sliwinski, and J.T.M. Wouters. 2003. A fast and simple method for quantitative determination of fat-derived medium and low-volatile compounds in cheese. *Int. Dairy J.* 13, 733-741.

Auty, M.A.E., G.E. Gardiner, S.J. McBrearty, E.O. O'Sullivan, D.M. Mulvihill, J.K. Collins, G.F. Fitzgerald, C. Stanton, and R.P.M. Ross. 2001. Direct *in situ* viability assessment of bacteria in probiotic dairy products using viability staining in conjunction with confocal scanning laser microscopy. *Appl. Environ. Microbiol.* 67, 420-425.

Banina, A., M. Vukasinovic, S. Brankovic, D. Fira, M. Kojic, and L. Topisirovic. 1997. Characterization of natural isolate *Lactobacillus acidophilus* BGRA43 useful for acidophilus milk production. *J. Appl. Microbiol.* 94, 593-599.

Baron, M., D. Roy, and J.C. Vuillemard. 2000. Biochemical characteristics of fermented milk produced by mixed-cultures of lactic starters and bifidobacteria. *Le Lait* 80, 465-478.

Beshkova, D., E. Simova, G. Frengova, and Z. Simov. 1998. Production of flavour compounds by yogurt starter cultures. *J. Ind. Microbiol. Biotechnol.* 20, 180-186.

Brasheras, M.M. and S.E. Gilliland. 1995. Survival during frozen and subsequent refrigerated storage of *Lactobacillus acidophilus* cells as influenced by the growth phase. *J. Dairy Sci.* 78, 2326-2335.

Bunthof, C.J. and T. Abee. 2002. Development of a flow cytometric method to analyze subpopulations of bacteria in probiotic products and dairy starters. *Appl. Environ. Microbiol.* 68, 2934-2942.

Bunthof, C., K. Bloemen, P. Breeuwer, F. Rombouts, and T. Abee. 2001. Flow cytometric assessment of viability of lactic acid bacteria. *Appl. Environ. Microbiol.* 67, 2326-2335.

Bunthof, C., S. Braak, P. Breeuwer, F. Rombouts, and T. Abee. 1999. Rapid fluorescence assessment of the viability of stressed *Lactococcus lactis*. *Appl. Environ. Microbiol.* 65, 3681-3689.

Coeuret, H., S. Dubernet, M. Bernardeau, M. Gueguen, and J.P. Vernoux. 2003. Isolation, characterization and identification of lactobacilli focusing mainly on cheeses and other dairy products. *Lait* 83, 269-306.

Fernandez-Garcia, E., M. Carbonell, P. Gaya, and M. Nunez. 2004. Evolution of the volatile components of ewes raw milk Zamorano cheese. *Int. Dairy J.* 14, 701-711.

Fernandez-Murga, M.L., A.P. De Ruiz Holgado, and G.F. De Valdez. 1998. Survival rate and enzyme activities of *Lactobacillus acidophilus* following frozen storage. *Cryobiology* 36, 315-319.

Gardini, F., R. Lanciotti, M.E. Guerzoni, and S. Torriani. 1999. Evaluation of aroma production and survival of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus* in fermented milks. *Int. Dairy J.* 9, 125-134.

Gomes, A.M.P. and F.X. Malcata. 1999. *Bifidobacterium* spp. and *Lactobacillus acidophilus*: biological, biochemical, technological and therapeutical properties relevant for use as probiotics. *Trends Food Sci. Technol.* 10, 139-157.

Helland, M.M., T. Wicklund, and J.A. Narvhus. 2004. Growth and metabolism of selected strains of probiotic bacteria in milk- and water-based cereal puddings. *Int. Dairy J.* 14, 957-965.

Hunger, W. and N. Peitersen. 1993. New technical aspects of the preparation of starter cultures. *Int. Dairy Fed. Bull.* 277, 1-27.

Joux, F. and F. Lebaron. 2000. Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. *Microbes Infect.* 2, 1523-1535.

Lahtinen, S., M. Gueimonde, A. Ouwehand, J. Reinikainen, and S. Salminen. 2006. Comparison of methods to enumerate probiotic bifidobacteria in a fermented food product. *Food Microbiol.* 23, 571-577.

Leroy, F. and L. De Vuyst. 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends Food Sci. Technol.* 15, 67-78.

Macciola, V., G. Candela, and A. De Leonardis. 2008. Rapid gas-chromatographic method for the determination of diacetyl in milk, fermented milk and butter. *Food Control* 19, 873-878.

Marilley, L. and M. Casey. 2004. Flavours of cheese products: metabolic pathways, analytical tools and identification of producing strains. *Int. J. Food Microb.* 90, 139-159.

Marshall, V.M. and W.M. Cole. 1983. Threonine aldolase and alcohol dehydrogenase activities in *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* and their contribution to flavour production in fermented milks. *J. Dairy Res.* 50, 375-379.

McSweeney, P. and M. Sousa. 2000. Biochemical pathways for the production of flavour compounds in cheeses during ripening. *Le Lait* 80, 293-324.

Mikš, M.H. and I. Warmińska-Radyko. 2008. Selected fluorescent techniques in the research of the physiological state and viability of bacteria cells in food. *Medycyna Wet.* 64, 623-627.

Montes, R.G., T.M. Bayless, J.M. Saavedra, and J.A. Perman. 1995. Effects of milks inoculated with *Lactobacillus acidophilus* or a yoghurt starter culture in lactose-maldigesting children. *J. Dairy Sci.* 78, 1657-1664.

Moreno, Y., M.C. Collado, M.A. Ferrus, J.M. Cobo, E. Hernandez, and M. Hernandez. 2006. 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.* 41, 275-280.

Østlie, H.M., M.H. Helland, and J.A. Nervhus. 2003. Growth and metabolism of selected strains of probiotic bacteria in milk. *Int. J. Food Microb.* 87, 17-27.

Pakdeeto, A., N. Naranong, and S. Tanasupawat. 2003. Diacetyl of lactic acid bacteria from milk and fermented foods in Thailand. *J. Gen. Appl. Microbiol.* 49, 301-307.

Papadimitriou, K., H. Pratsinis, G. Nebe-Von-Caron, D. Kletsas, and E. Tsakalidou. 2006. Rapid assessment of the physiological status of *Streptococcus macedonicus* by flow cytometry and fluorescence probes. *Int. J. Food Microbiol.* 111, 197-205.

Rault, A., C. Beal, S. Ghorbal, J.C. Ogier, and M. Bouix. 2007. Multiparametric flow cytometry allows rapid assessment and comparison of lactic acid bacteria viability after freezing and during frozen storage. *Cryobiology* 55, 35-43.

Reguła, A. 2007. Free fatty acid profiles of fermented beverages made from ewe's milk. *Le Lait* 87, 71-77.

Tavaria, F.K., T.G. Tavares, A.C. Silva-Ferreira, and F.X. Malcata. 2006. Contribution of coagulant and native microflora to the volatile-free fatty acid profile of an artisanal cheese. *Int. Dairy J.* 16, 886-894.

Vinderola, C.G. and J.A. Reinheimer. 2000. Enumeration of *Lactobacillus casei* in the presence of *L. acidophilus*, bifidobacteria and lactic starter bacteria in fermented dairy products. *Int. Dairy J.* 10, 271-275.

Warmińska-Radyko, I., M. Olszewska, and M. Mikš-Krajnik. 2010. Effect of temperature and sodium chloride on the growth and metabolism of *Lactococcus* strains in long-term incubation of milk. *Milchwissenschaft* 65, 32-35.