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Influence of aerobic and anaerobic conditions on the growth and metabolism of selected strains of *Lactobacillus plantarum*

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Abstract: Three wild strains of *Lactobacillus plantarum* were investigated for their growth and ability to produce lactic acid, acetic acid and ethanol under aerobic and anaerobic conditions. They were tested at three different temperatures (30 °C, 37 °C and 45 °C). The growth of lactobacilli was studied by measuring optical density (OD) at λ = 600 nm and pH value at the following times. With increasing temperature difference of cell yield was observed. The final cell yield under aerobic conditions was higher. Organic acids and ethanol were analysed using an HPLC RID method. Formation of lactic acid (as the major metabolite) was the slowest during cultivation at 30 °C, but the final amount of lactic acid showed the highest values. Concentrations of metabolites produced by lactobacilli after 48th hours of cultivation were: 9.18—11.48 $g.dm^{-3}$ (lactic acid), 0.84—1.65 $g.dm^{-3}$ (acetic acid) and 2.51—4.03 $g.dm^{-3}$ (ethanol). No significant differences (p = 0.05) were found in production of lactic acid and ethanol by different bacterial strains under aerobic and anaerobic conditions. Statistically significant differences (p = 0.05) were observed in production of acetic acid by 2L2 under aerobic and anaerobic conditions and for production of ethanol under anaerobic conditions by strains 1L5 and 2L2.

Keywords: *Lactobacillus plantarum*, aerobic conditions, anaerobic conditions, growth, organic acids

Introduction

The genus *Lactobacillus* is by far the largest of the genera included in lactic acid bacteria (LAB). It is very heterogeneous, encompassing species with a large variety of phenotypic, biochemical, and physiological properties (Axelsson 2004).

Lactobacilli are generally the most acid-tolerant of the LAB and will, therefore, terminate many spontaneous lactic fermentations such as silage and vegetable fermentations. Lactobacilli are also associated with the oral cavity, gastrointestinal tract, and vagina of humans and animals (Axelsson 2004). In these environments lactobacilli are considered essential components, playing a large variety of health — promoting functions, such as immunomodulation, intestinal integrity, and pathogen resistance (Vaughan et al. 2005). For such reasons strains of some species have traditionally been used as probiotics and added as functional bacteria in various food commodities (Ljungh and Wadström 2006).

In many cases, lactobacilli are also used as starter cultures in industrial and artisanal food fermentation since they contribute to the conservation, flavour, and texture of the fermented foods. While the fermentative conversion of sugars present in the raw materials into lactic acid is their main function, production of antimicrobial peptides, exopolysaccharides and a variety of other metabolites are other important properties (De Vries et al. 2006). Production of lactic acid during fermentation can be affected by medium composition (carbohydrate source, sugar concentration and growth factors), the presence of oxygen, pH and product concentration. Production of lactic acid from lactose by *Lactobacillus plantarum* was studied and was found that cell yield to be higher under aerobic conditions whereas lactic acid production was higher under anaerobic conditions (Gupta et al. 2011).

Within the genus *Lactobacillus*, *Lactobacillus plantarum* is a member of the facultatively heterofermentative group of lactobacilli. It is a heterogeneous and versatile species that is encountered in a variety of environmental niches, including dairy, meat, fish, and many vegetable or plant fermentations. *L. plantarum* strains have also been found in many cheese varieties. Moreover, strains of *L. plantarum* have proven ability to survive gastric transit and colonize the intestinal tract of humans and other mammals (Zago et al. 2011).

Fundamental and applied research is still needed to improve starter cultures in the existing production technology and to obtain quantitative data that may yield precious information about the relationship between the cheese environment and bacterial functionality, thus contributing to optimal

strain selection. Quality, safety, and acceptability of both traditional and industrial fermented dairy products may be significantly improved through the use of starter cultures implemented on a multifunctional basis (e.g., efficient acid and aroma production, overproduction of bacteriocins, effective proteolytic systems) taking into consideration also the probiotic aspects and health – promoting properties.
The aim of this study was to characterize the ability of new-isolated strains of *Lactobacillus plantarum* to produce lactic acid as a major property (at the different temperatures and presence of oxygen). Currently, there is an effort to find potential starter cultures from a group of wild strains obtained from raw milk or cheese from traditional. Because sheep lumb cheese is made from raw milk, it is used as useful source of new microorganisms. First, they must be tested for their technological, safety and probiotic properties and then they may be used as a primary starter cultures or adjunct cultures.
The professional literature substantiates the need for continuous search of new strains for achievement diversity of products. Starter cultures of lactic acid bacteria are used primary because of their ability to convert sugars into lactic acid. In addition, they have also other important functions such as inhibition of undesirable organisms, improve sensory and textural properties, as well as contributing to health benefits.

Materials and methods

Tested microorganisms
We tested three strains of *Lactobacillus plantarum* that were isolated from the lump sheep's cheeses. These cheeses were produced from raw sheep's milk on the different farms of Slovak Republic (Table 1.). These strains were identified by MALDI-TOF MS.

Tab. 1. Tested strain of *Lactobacillus plantarum.*

Sample	Classification	Origin (village)
115	*Lactobacillus plantarum*	Beňuš
1L5	*Lactobacillus plantarum*	Hanigovce
2L2	*Lactobacillus plantarum*	Podolínec

Conditions of cultivation
The samples of lactobacilli were cultivated in MRS broth at the temperature 30 °C, 37 °C and 45 °C during 48 hours under aerobic and anaerobic conditions. Initial concentration of lactobacilli was 10^7 CFU.ml^{-1}. The samples were analyzed at 0, 2, 4, 6, 8, 10, 12, 24 and 48 h under aerobic and anaerobic cultivation.

Monitoring of growth
The growth of lactobacilli was studied twice by measuring optical density (OD) at λ = 600 nm and pH value. Measured values of optical density were plotted on growth curves. The specific growth rates of individual strains were calculated as:

$$\mu = \frac{\ln X - \ln X_0}{\Delta t} \qquad (1)$$

Where: X – optical density in the end of the exponential growth phase, X_0 – optical density in the beginning of the exponential growth phase, Δt – the time interval between observations (Kask et al. 2003).
Measured pH values were plotted on graphs. From graphs the rates of decrease of pH values were calculated (1).

Monitoring of production of organic acids and ethanol
At the following times (0, 6, 8, 12, 24 a 48 h), lactobacilli were centrifugated to obtain cell-free supernatant.
Cell-free supernatant was analysed using an HPLC-apparatus consisting of a DeltaChrom™ SDS 030 pump (Watrex, Bratislava, Slovakia), a manual injector Rheodyne 7725i, a Polymer IEX H^+ (250 × 8 mm) column (Watrex, Bratislava, Slovakia), a column heater DeltaChrom™ Temperature Control Unit (50 ± 0.1 °C). One mmol/L sulphuric acid was used as the mobile phase at a flow rate of 1 mL/min. For detection of organic acids a refractometric detector RI K-2301 (Knauer, Berlin, Germany) was used. Recordings were made on Clarity (DataApex, Praha, Czech Republic). Concentrations of produced metabolites were evaluated using calibration curves.

Statistical analysis
One way analysis of variance (ANOVA) and multiple range test – Fisher's least significant differences (LSD) at level of p = 0.05 were applied to describe the significance of differences between production lactic acid, acetic acid and ethanol by various bacterial strains under aerobic and anaerobic conditions. Statgraphic Plus, Version 3.1 (Statsoft; Tulsa, Oklahoma, USA) was used as statistical analysis software.

Results and discussion

Monitoring of growth
Three selected strains of *Lactobacillus plantarum* were studied for their growth in MRS broth. From measured growth curves (for example: sample 2L2, Fig. 1 and Fig. 2) specific growth rate were

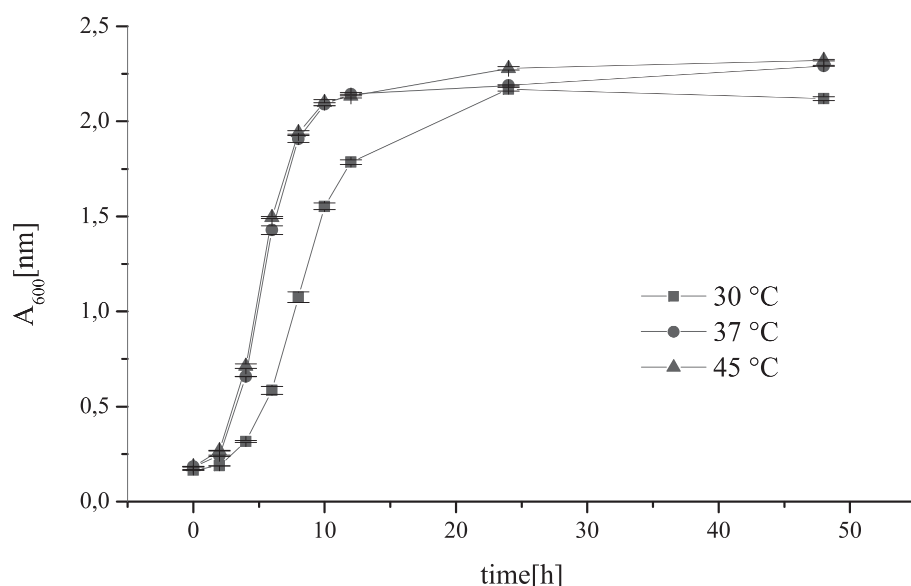

Fig. 1. Growth curves of *Lactobacillus plantarum* (2L2) cultivated under aerobic conditions at the different temperatures (30 °C, 37 °C and 45 °C).

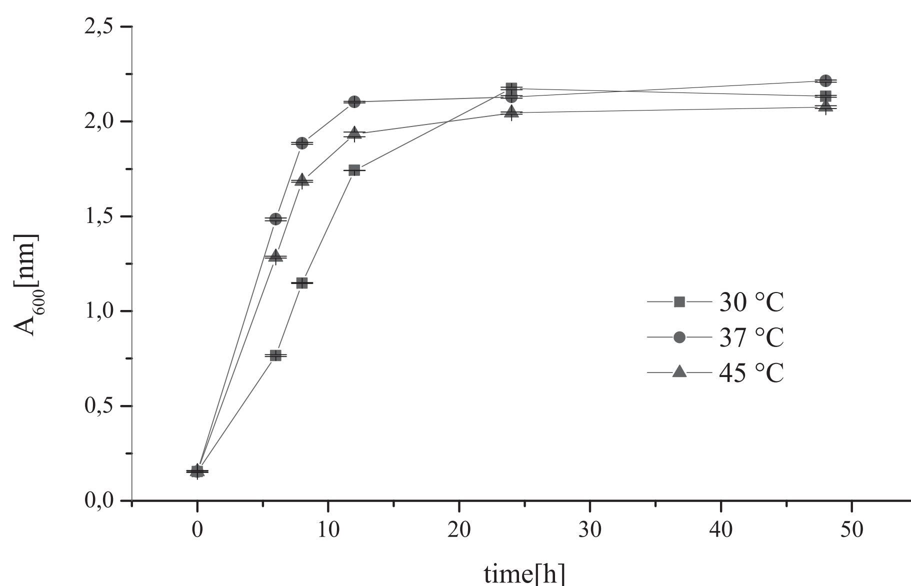

Fig. 2. Growth curves of *Lactobacillus plantarum* (2L2) cultivated under anaerobic conditions at the different temperatures (30 °C, 37 °C and 45 °C).

calculated. For the temperature 37 °C and 45 °C, the exponential growth phase was observed between 2nd and 8th hours. The exponential growth phases of lactobacilli cultivated at 30 °C were extended up to 10th hour of fermentation. The growth of lactobacilli was affected by the temperature and by the presence of oxygen.

The calculated values for specific growth rates were in the range 0.18—0.67 OD/h (Fig. 3). In general, these strains of lactobacilli grew faster under aerobic conditions. The samples 115 and 2L2 grew under anaerobic conditions at 37 °C faster as under aerobic conditions.

Fu and Mathews (1999) compared the cell growth of *Lactobacillus plantarum* under anaerobic and aerobic conditions using synthetic lactose medium at 37 °C. The cell growth rate under anaerobic conditions was faster during the exponential growth period from the 15th to 25th hour of fermentation. The final cell yield was lower for the anaerobic system, reaching a cell concentration of 10 $g.dm^{-3}$ after 40 h. However, the final cell yield under aerobic conditions was higher, reaching a value of 12 $g.dm^{-3}$ at about 48 h of fermentation. The same trend (better growth in the presence of oxygen) was observed by us.

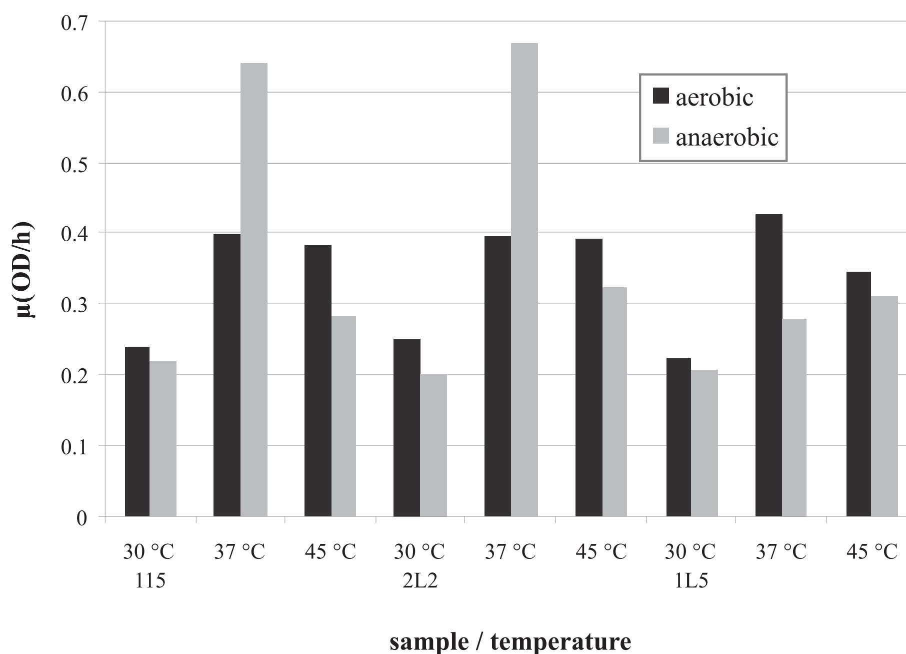

Fig. 3. Specific growth rates of lactobacilli during 48 hour of cultivation.

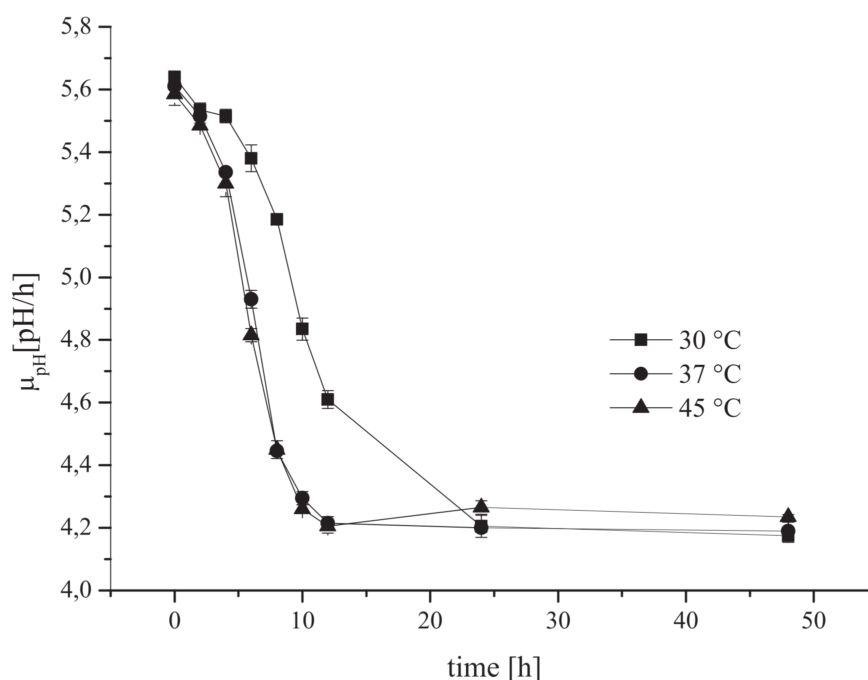

Fig. 4. Decrease of pH values of *Lactobacillus plantarum* (2L2) cultivated under aerobic conditions at the different temperatures (30 °C, 37 °C and 45 °C).

The pH decrease during the fermentation of dairy products affects a number of aspects of the manufacturing process, including the quality, texture, and composition of the products. From measured pH values, graphs were created and specific rates of pH decrease were calculated (for example: sample 2L2, Fig. 4 and Fig. 5). Comparison of specific rates of pH decrease under aerobic and anaerobic conditions is illustrated on Fig. 6. Their numeric values were in the range 0.11–0.31 pH/h. Only for the samples 115 (37 °C) and 1L5 (30 °C), decrease of pH value was faster under anaerobic conditions as under aerobic conditions.

Monitoring of production of organic acids and ethanol

The acidic condition and the reduced pH of the fermented dairy products, as well as the antimicrobial activity of undissociated lactic acid molecules,

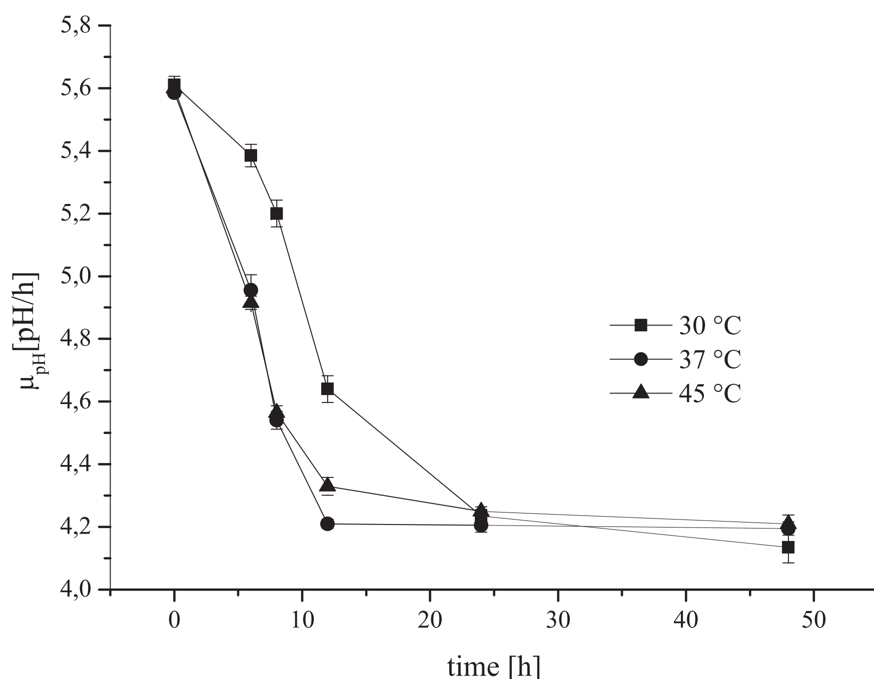

Fig. 5. Decrease of pH values of *Lactobacillus plantarum* (2L2) cultivated under anaerobic conditions at the different temperatures (30 °C, 37 °C and 45 °C).

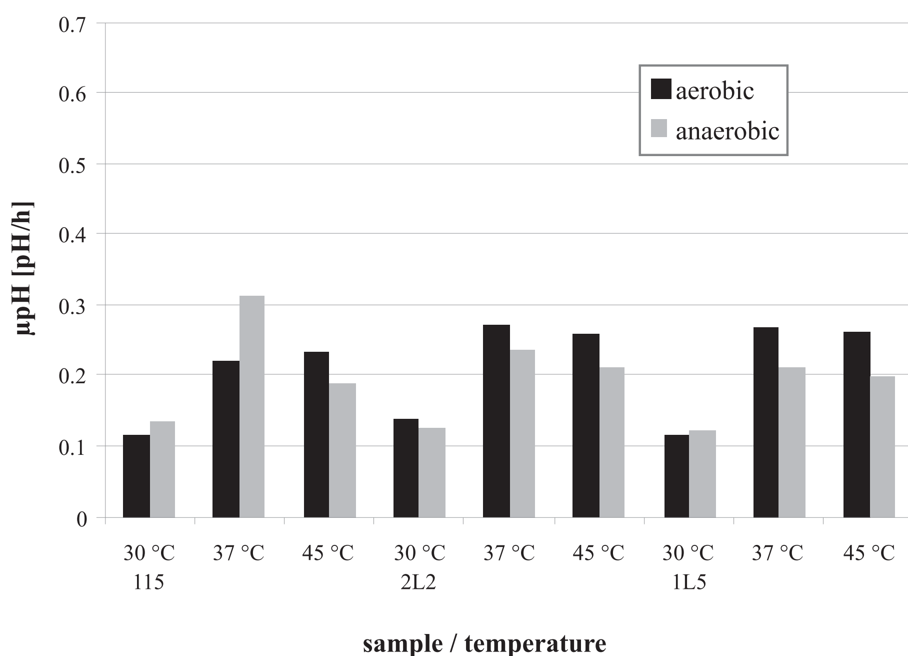

Fig. 6. Specific rates of pH decrease during 48 hour of cultivation.

prevent the growth or survival of many spoilage and pathogenic bacteria. The capability of a few strains to produce secondary metabolites having an inhibitory activity (i.e., bacteriocins such as nisin and other inhibitory peptides, hydrogen peroxide, diacetyl) can enhance the preservative effects (Carminati et al. 2010).

Lactic acid imparts a distinctive and fresh acidic flavour of fermented milks. The fermentation process has to be controlled (e.g., by proper cooling) to avoid excessive acid concentration that can mask more delicate flavour like diacetyl. The lactic acid in cheese making is responsible for the milk coagulation and the texturizing (Carminati et al. 2010).

The organic acid (lactic acid, acetic acid) and ethanol were analyzed by HPLC RID at the following times. Rate of formation of lactic acid as the major metabolite reached the lowest level during cultivation at 30 °C, but the concentration values obtained the highest level. Amount of detected

lactic acid produced by lactobacilli was in the range 9.83—11.48 g.dm^{-3} under aerobic conditions and 9.18—10.75 g.dm^{-3} under anaerobic conditions after 48 hours.

Fu and Mathews (1999) observed fermentation of lactose to lactic acid. The fermentation was conducted using the strain *Lactobacillus plantarum* under aerobic and anaerobic conditions. Synthetic lactose medium was used as the culture medium. Anaerobic fermentation gave a higher lactic acid yield of about 2.3 times that for aerobic fermentation at optimum pH (between 5 and 6). We found no significant differences in production of lactic acid by tested strains of *L. plantarum* under aerobic and anaerobic conditions.

Isotachophoresis was used for analysis of organic acids by Hudáček et al. (2006). The strains of *Lactobacillus plantarum* produced 76.5—56.8 g.dm^{-3} of lactic acid. Metabolites were analysed after 18 hours of cultivation at 37 °C under aerobic conditions. These results (higher concentration of lactic acid) confirm the specificity of individual strains.

Tejero-Sariñena et al. (2012) studied the antimicrobial activity of some strains of lactic acid bacteria. They analyzed lactic and acetic acid as the main antimicrobial agents. *L. plantarum* produced lactic acid to about 16.12 g.dm^{-3} and acetic acid to about 4.26 g.dm^{-3} after 24 hours.

Freitas et al. (1999) observed the production of acetic acid in goat and sheep's milk inoculated with cultures of *Lactobacillus plantarum* and *Lactobacillus paracasei* at 30 ° C. After 24 h cultivation were amounts of acetic acid 0.92 to 6.61 g.dm^{-3}.

Concentration of acetic acid produced by three new-isolated strains of *L. plantarum* was in the range 1.03—1.65 g.dm^{-3} under aerobic conditions and 0.84—1.16 g.dm^{-3} under anaerobic conditions after 48 hours. This concentration was little smaller but comparable with the above-mentioned literature.

Lactobacilli produced ethanol in the range 2.71—3.65 g.dm^{-3} under aerobic conditions and 2.51—4.03 g.dm^{-3} under anaerobic conditions.

Final concentrations of metabolites (after 48 hours of cultivation) produced by tested strains of *Lactobacillus plantarum* are illustrated on the Fig. 7.

Conclusions

Three new isolated strains of *Lactobacillus plantarum* showed a good capability to grow in MRS broth (cultivation at 30 °C, 37 °C, 45 °C; aerobic and anaerobic conditions). For the temperatures 37 °C and 45 °C, the exponential growth phase was observed between 2nd and 8th hours, for 30 °C the exponential growth phase was extended up to 10th hour of fermentation. With increasing temperature difference of cell yield was observed. The final cell yield under aerobic conditions was higher. In general, these strains of lactobacilli grew faster under aerobic conditions, except for samples 115 and 2L2 at 37 °C. The pH value of broth was decreased on the optimal level (approximately pH 4.2 after 24 hours cultivation) for using in milk industry. Amount of produced metabolites (lactic acid, acetic acid and ethanol) showed potentially good antimicrobial properties of tested strains. No significant differences were found in production of lactic acid and ethanol by tested strains of *L. plantarum* under aerobic and anaerobic conditions. Within individual strains were found statistically significant differences in production of acetic acid by 2L2 under aerobic and anaerobic conditions. In

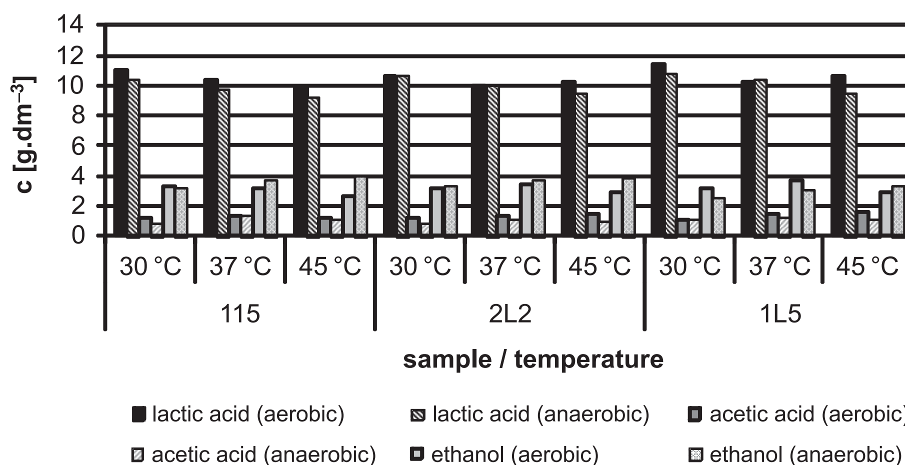

Fig. 7. Concentrations of lactic acid, acetic acid and ethanol produced by lactobacilli at the different temperature (30 °C, 37 °C and 45 °C) after 48 hours of cultivations. Comparison of aerobic and anaerobic condition.

comparison of ethanol production under anaerobic conditions by strains 1L5 and 2L2 was found also statistically significant differences.

The difference in growth rates and concentration of metabolites can be attributed to differences in metabolic pathways under aerobic and anaerobic conditions. The possible application of the tested wild strains of *L. plantarum* as starter cultures is conditional by other technology, antimicrobial and probiotic properties. The ability to produce biogenic amines and antibiotic resistance are undesirable properties for the food industry.

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References

Axelsson L (2004) In: Salminen S, von Wright A, Ouwehand A (Ed) Lactic Acid Bacteria, Marcel Dekker, Inc., New York – Basel.

Carminati D, Giraffa G, Quiberoni A, Binetti A, Suárez V, Reinheimer J (2010) In: Mozzi F, Raya RR, Vignolo GM (Ed) Biotechnology of Lactic Acid Bacteria: Novel Applications, Wiley-Blackwell, Ames-Singapore. ISBN 978-0-8138-1583-1.

De Vries MC, Vaughan EE, Kleerebezem M, De Vos W (2006) International Dairy Journal 16: 1018–1028.

Freitas C, Pintado AE, Pintado ME, Malcata FX (1999) European Food Research and Technology 209: 434–438.

Fu W, Mathews AP (1999) Biochemical Engineering Journal 3: 163–170.

Gupta S, Abu-Ghannam N, Scannell AGM (2011) Food and Bioproducts Processing 89: 346–355.

Hudáček J, Zalán Z, Štětina J, Chumchalová J, Halász A (2006) In: Proceedings "Mléko a sýry 2006", 234–239. ISBN 80-7080-620-6, Praha.

Kask S, Adamberg K, Orłowski A, Vogensen FK, Møller PL, Ardö Y, Paalme T (2003) Food Research International 36: 1037–1046.

Ljungh A, Wadström T (2006) Current Issues in Intestinal Microbiology 7: 73–89.

Tejero-Sariñena S, Barlow J, Costabile A, Gibson GR, Rowland I (2012) Anaerobe 18: 530–538.

Vaughan EE, Heilig HG, Ben-Amor K, de Vos WM (2005) FEMS Microbiology Reviews 29: 477–490.

Zago M, Fornasari ME, Carminati D, Bums P, Suàrez V, Vinderola G, Reinheimer J, Giraffa G (2011) Food Microbiology 28: 1033–1040.