In vitro growth competition of *Lactobacillus plantarum* HM1 with pathogenic and food spoilage microorganisms

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Summary

Studying the growth competition of novel strains of *Lactobacillus plantarum* in synthetic media followed with real one allows various possibilities to control the development of undesirable microorganisms in food. Thus, the competitive effect of different inoculum concentrations of *Lb. plantarum* HM1 on selected pathogenic and food spoilage microorganisms was studied in an individual experiments in Brain Heart Infusion broth. While the effect of co-cultivation with *Lb. plantarum* at 5% (v/v) (8.49 ± 0.14 log CFU·ml^{-1}) on *Escherichia coli* and *Pseudomonas aeruginosa* was not proven after 24 h (change of final density compared to the control was only 0.53 log CFU·ml^{-1} and 0.96 log CFU·ml^{-1}, respectively), the decrease in gram-positive spore-former *Bacillus cereus* and in *Staphylococcus aureus* was 3.73 log CFU·ml^{-1} and 2.41 log CFU·ml^{-1}, respectively. Further, *Lb. plantarum* exhibited varying degrees of inhibitory activity on *Candida parapsilosis* and *Aspergillus niger* counts (99.9 % and 98.0 % after 24 h and 72 h, respectively). The growth of *A. niger* within 1%, 2% and 5% (v/v) concentration of *Lb. plantarum* (7.93 log CFU·ml^{-1}, 8.09 log CFU·ml^{-1} and 8.72 log CFU·ml^{-1}, respectively) on the surface of plate count agar led to prolongation of the lag phase by 40.3 h, 70.9 h and 101.1 h, respectively.

Keywords

Lactobacillus plantarum; growth competition; inhibition; food spoilage microorganism

In spite of application of modern technologies and safety concepts, such as hazard analysis and critical control point (HACCP) system, the reported number of food-borne illnesses and intoxications is still on increase [1]. Food products can be contaminated by a variety of pathogenic and spoilage microbiota, the former causing foodborne diseases and the latter causing a significant economic loss to the food industry. Thus, prevention or inhibition of microbial growth in foods is of utmost importance for the current globalized food production. Hence, there is a need for new processing methods to be used, either alone or in combination with the already existing ones, to reduce or eliminate foodborne pathogens and spoilage bacteria [2]. Current trends in the food industry are focusing on the use of natural compounds that are considered as safe alternatives and satisfy consumer preferences [3].

Lactic acid bacteria (LAB) are intentionally added as starter cultures to food products with a purpose to develop a new kind of foods, achieve their stability and safety with unique organoleptic characteristics [4]. *Lb. plantarum*, as a member of LAB, represents an important group of microorganisms taking part in fermentation of many plant products (silage, sauerkraut, pickles), sourdough, cheeses and fermented sausages [5]. Comparative studies between various LAB showed that *Lb. plantarum* strains had the broadest spectrum of antimicrobial activity [6, 7]. Because of their antagonistic abilities, some *Lb. plantarum* strains are used in food preservation to extend the shelf life and reduce or even replace chemical addi-

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tives, or they can be used as supporting therapeutic agents in the treatment of infections caused by susceptible microorganisms [8]. Despite the broad interest in the use of *Lb. plantarum* within food industry, due to the diversity of the strains it is complicated to perform an accurate control and realistic prediction of the shelf life of foods. Moreover, selection of suitable strains to ensure microbial safety of food products is a subject of great interest of food microbiologists and food technologists all over the world. Thus, this work deals with inhibitory effects of a human milk isolate *Lb. plantarum* HM1 and its culture cell-free supernatant against pathogenic and food spoilage bacteria, namely, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Bacillus cereus*, *Staphylococcus aureus*, and against fungi, namely, *Candida parapsilosis* and *Aspergillus niger*.

MATERIALS AND METHODS

Microorganisms

The used *Lactobacillus plantarum* HM1 was previously isolated from breast milk and identified in a study by LIPTÁKOVÁ et al. [9]. Further, *Escherichia coli* CCM 3988, *Pseudomonas aeruginosa* CCM 3955 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium CCM 4763 were used as model gram-negative bacteria. Strains *Bacillus cereus* CCM 2010 and *Staphylococcus aureus* CCM 3953 were used as representatives of gram-positive pathogenic bacteria. *Candida parapsilosis* ATCC 22019 was used as a representative of yeasts and *Aspergillus niger* CCM 8189 as a representative of micromycetes. The CCM strains were provided by the Czech Collection of Microorganisms (Brno, Czech Republic), the ATCC strain was obtained from the American Type Culture Collection.

Microorganism's preparation and storage

The isolate *Lb. plantarum* HM1 was maintained in de Man, Rogosa and Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France). Strains of *E. coli, P. aeruginosa, S.* Typhimurium, *B. cereus*, and *Staph. aureus* were maintained in Brain Heart Infusion (BHI) broth (Sigma Aldrich, St. Louis, Missouri, USA) and *A. niger* and *C. parapsilosis* were kept on Yeast Extract Glucose Chloramphenicol (YGC) agar slants (Biolife, Milan, Italy). All microorganisms were kept at (6 ± 0.5) °C and were subcultured monthly. The "24 h culture" of *Lb. plantarum* HM1 was obtained by overnight static incubation at (37 ± 0.5) °C, 5% CO_2 in MRS broth. The "24 h culture" of the pathogenic bacteria were obtained by overnight static incubation of each strain aerobically in BHI broth at the same temperature as *Lb. plantarum*. The standard suspension of *C. parapsilosis* was prepared from "72 h culture" grown on YGC agar at (37 ± 0.5) °C in tubes by standard rinsing with sterile saline solution of 8.5 $g \cdot l^{-1}$ NaCl and 0.1 $g \cdot l^{-1}$ peptone (Biolife). Spore suspension of *A. niger* was prepared from a culture grown for 120 h at (25 ± 0.5) °C on YGC agar by rinsing with sterile saline solution. To prepare the cell-free supernatant (CFS) of *Lb. plantarum*, the suspension was obtained by static incubation at (37 ± 0.5) °C, 5% CO_2 in MRS broth for 72 h.

Cell-free supernatant preparation

To prepare CFS of *Lb. plantarum*, the culture was centrifuged for 5 min at 3500 ×*g*. The supernatant was collected into sterile tubes and centrifuged again under the same conditions. After centrifugation, supernatant was passed through a sterile syringe microfilter of a pore size of 0.22 μm (Sarstedt, Nümbrecht, Germany) according to the procedure of MANČUŠKOVÁ et al. [10].

Antimicrobial activity and microorganism's quantification

The "24 h culture" of *Lb. plantarum* or its CFS were added to 100 ml of pre-tempered BHI broth to final concentration of 1%, 2% and 5% (v/v). The "24 h cultures" of pathogenic microorganisms were inoculated into pre-tempered BHI broth to achieve the initial concentration (N_0) of approximately 3 log $CFU \cdot ml^{-1}$. The inoculated samples were incubated aerobically in a thermostat cabinet (Pol-Eko Aparatura, Wodzisław Śląski, Poland) at (37 ± 0.5) °C for *E. coli*, *S.* Typhimurium, *P. aeruginosa, Staph. aureus*, *C. parapsilosis* and at (30 ± 0.5) °C for *B. cereus* and *A. niger* growth. The actual densities of microorganisms were determined at pre-defined time intervals with respect to the incubation temperature by serial 10-fold dilutions in saline-peptone solution. *E. coli* and *S.* Typhimurium were enumerated by the plate-count method on Chromocult agar (Merck, Darmstadt, Germany), *P. aeruginosa* on Violet Red Bile Lactose (VRBL) agar (Merck) after 24 h of incubation at 37 °C. *Staph. aureus* was enumerated on Baird-Parker agar (Merck) after 24 h incubation at 37 °C, *B. cereus* on Cereus selective agar (Merck) at 30 °C after 72 h. *C. parapsilosis* and *A. niger* were enumerated on YGC agar at 37 °C after 48 h and 30 °C after 120 h, respectively. Each experiment, comprising two parallel cultivations, was carried out under aerobic conditions without stirring. The results were compared to the control experiments where the antimicrobial agent was

replaced by sterile deionized water. At the same time of microbiological quantification of the samples, pH values were examined using the pH meter WTW 720 (Inolab, Weilheim, Germany).

Radial growth quantification

The growth dynamics of *A. niger* was also studied on the surface of Plate Count Agar (PCA; Biolife). In our study, the method of Koňuchová and Valík [11] and Hudecová et al. [12] was adapted. *A. niger* was grown for 120 h on the top layer of YGC slants at (25 ± 0.5) °C to reach heavy sporulation. Spores were then suspended in 5 ml of sterile saline solution by scraping gently the surface of the medium with a sterile pipette tip. Immediately after preparation, suspension was diluted to yield an inoculum count of approximately 3 log CFU·ml^{-1}. The standard PCA growth medium was autoclaved, cooled to approximately 45 °C and *Lb. plantarum* or CFS were added into each flask, to reach 1%, 2% and 5% (v/v), respectively. Medium in portions of 30 ml was poured into sterile Petri dishes (diameter 140 mm). After solidifying, 2 μl of diluted spore suspensions were used to inoculate the centre of the Petri dishes. The initial diameter of the inoculated spore suspension drop was 4.6 mm. For all experiments, zero time was defined as the time when the suspension was applied to the surface of the agar plate. After inoculation, the plates were sealed in polyethylene bags to prevent water loss and were stored under controlled conditions in programmable incubators set at (30 ± 0.5) °C under aerobic conditions. Growth experiments were carried out in triplicate. The diameters of developing colonies were measured at appropriate time intervals using a Vernier calliper (150 mm × 0.02 mm; Sinochem Jiangsu, Nanjing, China) in two orthogonal directions per plate, without opening the dishes. The final diameter of colonies was calculated as arithmetic mean. The measurements were taken from the early stages of growth in order to capture the lag phase.

Growth curve fitting

Growth parameters of *A. niger* on the surface of PCA were calculated using the mechanistic model DMFit by Baranyi and Roberts [13] that is incorporated in the DMFit tools kindly provided by Dr. J. Baranyi (University of Debrecen, Debrecen, Hungary). The growth function of Baranyi and Roberts expressed in the explicit form was applied as follows:

$$y(t) = y_0 + \mu_{\max} A(t) - a \quad (1)$$

where a is defined as

$$a = \frac{1}{m} \ln\left(1 + \frac{e^{m\mu_{\max} A(t)} - 1}{e^{m(y_{\max} - y_0)}}\right) \quad (2)$$

and where $y(t)$ is the natural logarithm of the cell concentration, y_0 is the natural logarithm of the cell concentration at $t = t_0$, t is time, t_0 is initial time of the growth, $\mu_{\max}$ is the maximum specific growth rate, $y_{\max}$ is the natural logarithm of the maximum cell concentration, m is the curvature parameter to characterize the transition from the exponential phase (suggested values m ranging from 1 to 10), $A(t)$ is the function that plays the role of a gradual delay in time:

$$A(t) = t + \frac{\ln(e^{-m\mu_{\max} t} + e^{-h_0} - e^{-\nu t - h_0})}{\mu_{\max}} \quad (3)$$

where t is time, h_0 is the dimensionless parameter quantifying the initial physiological state of the cells, ν is the rate determining the quickness of the transition from lag to the exponential phase and the lag time λ can be calculated as:

$$\lambda = \frac{h_0}{\mu_{\max}} \quad (4)$$

Statistical analysis

Each experiment was carried out in two separate trials. Statistical analyses were carried out using Microsoft Excel 2013 (Microsoft, Redmond, Washington, USA). Data were treated by Student's *t*-test with a least significant difference of 95 %.

The coefficient of variation (*CV*) shows the extent of variability in relation to the mean of the population and is reported as a percentage:

$$CV = \frac{SD}{\bar{x}} \times 100 \quad (5)$$

where *SD* is standard deviation and $\bar{x}$ is mean.

RESULTS AND DISCUSSION

The food industry is facing the challenge of reducing the use of chemical preservatives without compromising the microbiological safety and stability of final products [14]. The development of natural antimicrobial substances derived from LAB is of great importance for the food industry. Several studies focused on antimicrobial activity of metabolites produced by *Lactobacillus* spp. to prevent the growth of pathogenic bacteria. In a study by Ačai et al. [15], Fresco culture at a concentration as low as 3 log CFU·ml^{-1} was found to be a fast growing LAB culture able to suppress

E. coli and *Staph. aureus* in a stationary phase in milk. The study of SAVADOGO et al. [16] demonstrated the inhibitory effect of selected LAB from fermented milk against a wide range of pathogenic microorganisms (*Staph. aureus, E. coli* and *B. cereus*). Thus in this work, the growth competition of *Lb. plantarum* and effects of its CFS on selected pathogenic bacteria and fungi were tested. The concentrations of *Lb. plantarum* used at 1%, 2% and 5% (v/v) were (7.77 ± 0.26), (8.06 ± 0.21) and (8.49 ± 0.14) log CFU·ml^{-1}, respectively. Even though the concentrations of culturable *Lb. plantarum* within different inoculum concentrations were similar, varying only by 0.7 log CFU·ml^{-1}, the concentrations of metabolites were supposed to differ much more. Therefore, the effect of entire inoculum was important for consideration.

Effects against Gram-negative bacteria

The ability of *E. coli* to produce organic acids, the resistance to short-chain organic acids and the metabolic features evolved in *Escherichia* genus may help this organism to survive in the presence of LAB [10]. Our results (Tab. 1) are in agreement with the previous knowledge. The application of 1% and 2% (v/v) (7.36 log CFU·ml^{-1} and 7.98 log CFU·ml^{-1}, respectively) of *Lb. plantarum* had no significant effect on the growth of *E. coli* as its counts increased from initial (3.50 ± 0.26) log CFU·ml^{-1} (*CV* = 7.6 %) to final (9.33 ± 0.18) log CFU·ml^{-1} (*CV* = 2.0 %) after 12 h of incubation at 37 °C in BHI broth. However, increased addition of *Lb. plantarum* (5% (v/v); 8.31 log CFU·ml^{-1}) kept the counts of *E. coli* about 2 log lower in comparison to the control sample. Despite 98% inhibition during first 12 h of incubation, the counts of *E. coli* reached levels of 7–9 log CFU·ml^{-1} finally in stationary phase, which is not acceptable from microbial food safety point of view. In the study of LIPTÁKOVÁ et al. [17], the most significant inhibition of *E. coli* was observed in co-cultivation with *Lactococcus lactis* subsp. *lactis*, when counts were not above 4 log CFU·ml^{-1} after 72 h both at 18 °C and 21 °C. To discriminate the antimicrobial activity from that of organic acids in CFS of LAB, active acidity is usually adjusted by NaOH to pH 6.8–7.0. Nevertheless, several studies proved lower antimicrobial potential of pH-adjusted CFS compared to non-adjusted [10, 18, 19]. Therefore, the inhibitory activity of organic acids, as well as non-lactic acid molecules in non-pH adjusted culture supernatant of *Lb. plantarum* was further examined. In our experiments, growth competition of *Lb. plantarum* and *E. coli* was noted, but *E. coli* still reached counts higher than 8 log CFU·ml^{-1} after 24 h, even at 5% (v/v) of CFS. Supernatant of *Lb. plantarum* in a study of LASH et al. [20] exerted most notable inhibitory activity on *Listeria innocua* and *P. aeruginosa* with values higher than 96% compared to the control. Cell-free supernatants (pH 6.0) of *Lb. plantarum* strains in a study of YU et al. [21] proved a lower inhibitory activity against *E. coli* O157:H7 and *S.* Typhimurium.

Further experiments performed in our study with *S.* Typhimurium provided similar results regarding *E. coli* (Tab. 1). The maximum population density reached by *S.* Typhimurium (10.02 log CFU·ml^{-1}) decreased in co-culture with *Lb. plantarum* to the level of

Tab. 1. *Escherichia coli*, *Salmonella* Typhimurium and *Pseudomonas aeruginosa* in co-culture with *Lactobacillus plantarum* or its culture supernatant at 37 °C.

Lb. plantarum	Incubation time			
	0 h	6 h	12 h	24 h
	***E. coli* counts** [log CFU·ml^{-1}]			
0 %	3.11	8.10^{f}	9.55^{f}	9.72^{g}
1 %	3.79	8.11^{f}	9.42^{d}	9.22^{c}
2 %	3.21	7.37^{b}	9.13^{b}	9.12^{a}
5 %	3.37	6.37^{a}	7.65^{a}	9.18^{b}
1 % CFS	3.51	7.74^{e}	9.31^{c}	9.39^{f}
2 % CFS	3.63	7.60^{d}	9.50^{e}	9.37^{e}
5 % CFS	3.87	7.43^{c}	9.30^{c}	9.32^{d}
	***S.* Typhimurium counts** [log CFU·ml^{-1}]			
0 %	3.08	6.82^{f}	9.09^{g}	10.02^{f}
1 %	3.06	6.45^{b}	8.71^{f}	9.24^{d}
2 %	3.04	6.47^{c}	8.27^{c}	9.35^{e}
5 %	3.32	5.08^{a}	5.89^{a}	9.00^{c}
1 % CFS	3.24	6.58^{d}	8.23^{b}	8.96^{b}
2 % CFS	3.11	6.81^{f}	8.63^{e}	8.95^{b}
5 % CFS	3.13	6.73^{e}	8.48^{d}	8.23^{a}
	***P. aeruginosa* counts** [log CFU·ml^{-1}]			
0 %	3.59	7.99^{g}	9.46^{g}	9.82^{g}
1 %	2.76	6.93^{f}	8.95^{f}	8.89^{e}
2 %	2.65	6.30^{e}	8.73^{e}	8.93^{f}
5 %	2.60	5.04^{c}	5.95^{a}	8.86^{d}
1 % CFS	2.45	4.32^{a}	7.31^{b}	8.79^{c}
2 % CFS	2.30	4.60^{b}	7.38^{c}	8.56^{a}
5 % CFS	2.52	5.36^{d}	7.65^{d}	8.74^{b}

Concentration of *Lactobacillus plantarum* is expressed in volume percent.
1%, 2% and 5% (v/v) concentration of *L. plantarum* for *E. coli* represents 7.36 log CFU·ml^{-1}, 7.98 log CFU·ml^{-1} and 8.31 log CFU·ml^{-1}, respectively; for *S.* Typhimurium 7.65 log CFU·ml^{-1}, 7.99 log CFU·ml^{-1} and 8.41 log CFU·ml^{-1}, respectively and for *P. aeruginosa* 7.69 log CFU·ml^{-1}, 8.08 log CFU·ml^{-1} and 8.50 log CFU·ml^{-1}, respectively. Means within a column with different superscript letters are significantly different ($p < 0.05$).
CFS - cell-free supernatant.

(9.19 ± 0.15) log CFU·ml^{-1} (CV = 1.6 %) after 24 h, representing 84% inhibition on average. Thus, the inoculum concentration of 1%, 2% and 5% (v/v) (7.65 log CFU·ml^{-1}, 7.99 log CFU·ml^{-1} and 8.41 log CFU·ml^{-1}) of *Lb. plantarum* reduced the counts of *S*. Typhimurium by about 1 log order. Also ACCURCIO et al. [22] recorded a moderate inhibitory activity of *Lb. plantarum* B7 on *S*. Typhimurium growth in BHI broth (inhibition by 0.2 log CFU·ml^{-1} compared to the control). When CFS was present, a decrease in concentration of *S*. Typhimurium by approximately 2 log orders was observed (94% inhibition on average). In a study of FAYOL-MESSAOUDI et al. [18], growth of *S*. Typhimurium in the presence of the supernatant of *Lb. johnsonii* La1, *Lb. casei* Shirota, *Lb. casei*, *Lb. rhamnosus* GR1 or *Lb. sakei* strains occurred at pH 6.5 in MRS broth. Antimicrobial activity of *Lb. plantarum* CFS in a study of LASH et al. [20] was lost when the pH was adjusted to values higher than 5.0 and lower than 4.0, suggesting that the inhibitory compounds were active only in the selected pH range.

The growth of *P. aeruginosa* at increasing concentrations of *Lb. plantarum* was studied as well and was found to be similar to our previous cases. In control trials, *P. aeruginosa* reached a concentration of 9.82 log CFU·ml^{-1} through the incubation period of 24 h. When in co-culture with strain HM1, initial concentration of *P. aeruginosa* ((2.69 ± 0.39) log CFU·ml^{-1}, CV = 14 %) increased to the final (8.6–8.9) log CFU·ml^{-1} after 24 h (Tab. 1). After 6 h, the level of *P. aeruginosa* was significantly the lowest in the medium inoculated with 5% (v/v) (8.50 log CFU·ml^{-1}) of *Lb. plantarum*. Growth inhibition by tested concentrations of *Lb. plantarum* CFS was almost the same in all cases and varied in the range of 90.8–94.5 % after 24 h. YESILIK et al. [23] showed the antibacterial effect of a "probiotic" yoghurt on viability of *S*. Typhimurium, *E. coli* and *P. aeruginosa* by using disc diffusion method. In their study, the most sensitive to the metabolites produced by LAB was *S*. Typhimurium, while the least sensitive was *P. aeruginosa*.

Despite the growth of gram-negative potentially pathogenic bacteria in our experiments, counts of *Lb. plantarum* after 24 h were on average (8.99 ± 0.24) log CFU·ml^{-1} (CV = 2.7 %) (data not shown).

Tab. 2. *Bacillus cereus* and *Staphylococcus aureus* in co-cultivation with *Lactobacillus plantarum* or its culture supernatant at 30 °C and 37 °C, respectively.

Lb. plantarum	Incubation time			
	0 h	6 h	12 h	24 h
	***B. cereus* counts** [log CFU·ml^{-1}]			
0 %	3.66	7.99^{g}	8.49^{f}	7.76^{d}
1 %	2.75	6.93^{f}	7.24^{c}	7.22^{b}
2 %	2.61	6.30^{e}	7.04^{b}	7.34^{c}
5 %	2.68	5.04^{c}	4.87^{a}	4.02^{a}
1 % CFS	3.75	4.32^{a}	8.31^{e}	7.96^{e}
2 % CFS	3.75	4.90^{b}	8.31^{e}	8.19^{f}
5 % CFS	3.73	5.36^{d}	8.13^{d}	8.20^{f}
	***S. aureus* counts** [log CFU·ml^{-1}]			
0 %	4.02	7.84^{e}	10.35^{g}	8.95^{d}
1 %	3.34	6.50^{c}	7.83^{c}	8.25^{c}
2 %	3.23	6.08^{b}	6.77^{b}	8.03^{b}
5 %	3.27	4.96^{a}	6.26^{a}	6.53^{a}
1 % CFS	3.55	7.70^{d}	9.44^{d}	9.71^{g}
2 % CFS	3.74	7.94^{f}	9.52^{f}	9.26^{e}
5 % CFS	3.71	8.01^{g}	9.48^{e}	9.46^{f}

Concentration of *Lactobacillus plantarum* is expressed in volume percent.
1%, 2% and 5% (v/v) concentration of *Lb. plantarum* for *B. cereus* represents 7.72 log CFU·ml^{-1}, 8.26 log CFU·ml^{-1} and 8.40 log CFU·ml^{-1}, respectively, for *Staph. aureus* 7.81 log CFU·ml^{-1}, 8.17 log CFU·ml^{-1} and 8.57 log CFU·ml^{-1}, respectively. Means within a column with different superscript letters are significantly different ($p < 0.05$).
CFS – cell-free supernatant,

Effects against Gram-positive bacteria

Although there was no inhibitory effect of antimicrobial substances produced by *Lb. plantarum* on the growth of gram-negative bacteria, further experiments performed in our study gave promising results. Since the first detection of *B. cereus* in milk in 1916, this microorganism has been recognized as a common contaminant of raw milk and some dairy products [24, 25]. To determine whether the growth of *B. cereus* might also be inhibited by *Lb. plantarum* during the food fermentation process, a co-incubation study was performed in sterile BHI broth. For *B. cereus* growth in the control experiment, pH remained above 6.15 after 24 h and its counts increased gradually (N_0 = (3.28 ± 0.52) log CFU·ml^{-1}; CV = 15.8 %) with statistically significant differences ($p < 0.05$) found at 12 h and 24 h (Tab. 2). In stationary phase, *B. cereus* reached concentrations higher than 7 log CFU·ml^{-1} within 1% and 2% (v/v) addition of *Lb. plantarum* HM1 (7.72 log CFU·ml^{-1} and 8.26 log CFU·ml^{-1}, respectively). The application of *Lb. plantarum* in a concentration of 5% (v/v) (8.41 log CFU·ml^{-1}) led to a decrease in *B. cereus* concentration to 4 log CFU·ml^{-1} after 24 h. In this case, changes of pH (1.5 unit) affected the counts of *B. cereus* co-cultivated with *Lb. plantarum* counts of which remained > 8 log CFU·ml^{-1}, thus contributing to a continuous reduction in active acidity. Our results are in agreement with those of

ZHANG et al. [26]. Those authors reported that the concentration of *B. cereus* began to decrease when pH was reduced to 5.0. ROSSLAND et al. [27] also reported that pH ranging from 4.9 to 5.0 is a critical value for inhibiting the growth of *B. cereus*. Thus, pH is considered as a critical control parameter for safe production of fermented dairy products regarding *B. cereus*. Other experiments performed in our study also confirmed the above statement. CFS of *Lb. plantarum* even stimulated *B. cereus* counts that were higher by approximately 0.2–0.4 log CFU·ml^{-1} compared to the control after 24 h (pH value was 6.28–6.50).

The results regarding *Staph. aureus* growth provided results similar to those regarding *B. cereus*. In pure culture, the level reached by *Staph. aureus* after 12 h was significantly the highest (10.4 log CFU·ml^{-1}), while 1% (v/v) (7.81 log CFU·ml^{-1}) of *Lb. plantarum* decreased the counts by approximately 1.3 log compared to the control. Application of *Lb. plantarum* at increased inoculum sizes (2% or 5% (v/v) (8.17 log CFU·ml^{-1} and 8.57 log CFU·ml^{-1}, respectively) reduced the counts of *Staph. aureus* by approximately 3.6 log CFU·ml^{-1} and 4.1 log CFU·ml^{-1}, respectively. In a study of MEDVEĎOVÁ et al. [28], the minimal Fresco culture concentration of 5 log CFU·ml^{-1} was assumed to ensure *Staph. aureus* increase by less than 1 log CFU·ml^{-1}. In our study, pH changes (by 1.3–1.7 units) caused by the metabolic activity of *Lb. plantarum* contributed to the reduction of counts of *Staph. aureus*. The inhibition in *Staph. aureus* ascribed to lowering of pH in skimmed milk was also reported in a study of RADOVANIC and KATIC [29] when counts decreased by approximately 2 log orders after 24 h compared to the control in a co-culture with selected LAB. As in our previous experiments, CFS of *Lb. plantarum* also stimulated *Staph. aureus* counts increase by approximately 0.3–0.5 log CFU·ml^{-1} compared to the control sample after 24 h. Results from the literature show that *Staph. aureus* is able to grow under quite harsh conditions. SUTHERLAND et al. [30] showed that *Staph. aureus* is able to grow in liquid media at pH 5.25 at 15 °C or at pH 4.48 at 30 °C. ALOMAR et al. [31] observed growth of *Staph. aureus* at 30 °C in milk adjusted to pH 5.6.

Fungi

While the most prevalent yeast strains frequently isolated from dairy products as contaminants are representatives of genera *Kluyveromyces*, *Debaryomyces, Yarrowia* and *Candida* [32], the most common contaminants of mould genera are *Penicillium*, *Fusarium*, *Mucor*, *Rhizopus*, *Aspergillus*, *Cladosporium* and *Alternaria* [33]. *C. parapsilosis* as an opportunistic fungal pathogen of humans [34] was targeted as a model microorganism to study the anti-yeast activity of selected LAB. SHARMA and SRIVASTAVA [35] proved in their study not only anti-candida activity of spent culture filtrate of *Lb. plantarum*, but also significant reduction in biofilm formation by *Candida* spp.

Tab. 3. *Candida parapsilosis* and *Aspergillus niger* in co-culture with *Lactobacillus plantarum* or its culture supernatant at 30 °C and 37 °C, respectively.

Lb. plantarum	Incubation time				
	0 h	6 h	12 h	24 h	72 h
	***C. parapsilosis* counts** [log CFU·ml^{-1}]				
0 %	3.08	3.20^{b}	4.18^{g}	6.10^{f}	–
1 %	3.10	3.34^{d}	3.33^{c}	3.11^{c}	–
2 %	3.02	3.01^{a}	3.06^{b}	3.02^{b}	–
5 %	3.04	3.00^{a}	2.92^{a}	2.98^{a}	–
1 % CFS	3.08	3.47^{e}	4.06^{f}	6.15^{g}	–
2 % CFS	2.98	3.35^{d}	4.00^{e}	5.60^{e}	–
5 % CFS	3.07	3.26^{c}	3.45^{d}	4.70^{d}	–
	***A. niger* counts** [log CFU·ml^{-1}]				
0 %	3.58	3.53^{e}	3.49^{c}	3.20^{c}	3.98^{f}
1 %	3.45	3.45^{c}	3.38^{a}	3.09^{b}	3.31^{c}
2 %	3.32	3.39^{b}	3.38^{a}	3.29^{d}	3.10^{b}
5 %	3.57	3.54^{e}	3.59^{e}	2.56^{a}	2.02^{a}
1 % CFS	3.58	3.35^{a}	3.56^{d}	3.56^{g}	3.60^{d}
2 % CFS	3.49	3.48^{d}	3.44^{b}	3.50^{f}	3.97^{f}
5 % CFS	3.53	3.48^{d}	3.50^{c}	3.43^{e}	3.89^{e}

Concentration of *Lactobacillus plantarum* is expressed in volume percent.
1%, 2% and 5% (v/v) concentration of *Lb. plantarum* for *C. parapsilosis* represents 8.22 log CFU·ml^{-1}, 8.37 log CFU·ml^{-1} and 8.72 log CFU·ml^{-1}, respectively, for *A. niger* 7.93 log CFU·ml^{-1}, 8.09 log CFU·ml^{-1} and 8.72 log CFU·ml^{-1}, respectively. Means within a column with different superscript letters are significantly different ($p < 0.05$).
CFS – cell-free supernatant.

Data on *C. parapsilosis* growth in a single culture as well as the effect of *Lb. plantarum* in co-culture, in order to get the yeast growth under control, are presented in Tab. 3. Counts of *C. parapsilosis* in BHI broth gradually increased from initial (3.48 ± 0.08) log CFU·ml^{-1} (CV = 2.2 %) to 4.2 log CFU·ml^{-1} and 6.1 log CFU·ml^{-1} after 12 h and 24 h, respectively. For *C. parapsilosis* growth alone, pH remained the same as at the beginning of the experiment during 24 h (pH 7.23; data not shown). When in co-culture (with 1%, 2% and 5% (v/v) of *Lb. plantarum*, representing 8.43 log CFU·ml^{-1} on average), 99.9% inhibition was reported after 24 h of incubation with a final concentration of *C. parapsilosis* being (3.04 ± 0.05) log CFU·ml^{-1}. This result can be ex-

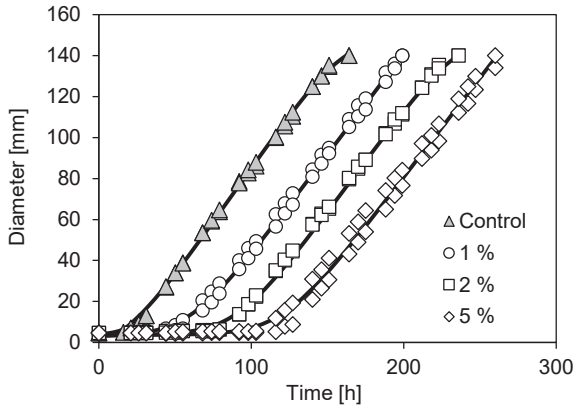

Fig. 1. Radial growth curves of *A. niger* at increasing concentrations of *Lb. plantarum* on agar plates.

plained by the metabolic activity of *Lb. plantarum* (at a concentration of > 9 log CFU·ml^{-1}) resulting in a decrease in pH on average by 1.7 unit. The anti-yeast activity of *Lb. paracasei* in a study of Atanassova et al. [36] was reported as stable at pH 8, while maximum anti-yeast activity was observed at pH 6. Liptáková et al. [32] in their study proved the decrease in pH value as crucial for the antagonistic relationship between *Lb. rhamnosus* VT1 and *C. maltosa* YP1. Experiments performed with CFS confirmed the above statements, when 1% (v/v) of CFS even stimulated the growth of *C. parapsilosis*. Increasing the CFS concentration of *Lb. plantarum* HM1 led to a reduction in yeast counts by approximately 33.3 % and 81.3 %, respectively (pH values were 7.14–7.22 after 24 h). Thus, the experimental results of our study suggest that *C. parapsilosis* is inhibited by the active acidity due to the presence of *Lb. plantarum* in a concentration of > 9 log CFU·ml^{-1}.

Further, antifungal properties of *Lb. plantarum* on *A. niger* growth were characterized and the results are presented in Tab. 3. The actively growing cultures of *Lb. plantarum* showed a more pronounced antifungal activity compared to CFS added to broth. The maximum population density reached by *A. niger* (3.98 log CFU·ml^{-1}) after 72 h decreased in co-culture, whereas *Lb. plantarum* at concentrations of 2% (v/v) (8.09 log CFU·ml^{-1}) and 5% (v/v) (8.72 log CFU·ml^{-1}) reduced the counts by approximately 90.2 % and 98.0 %, respectively. The results obtained suggested that the antifungal activity was due to the production of organic acids (pH decreased by approximately 0.8–1.3 unit within 72 h). This hypothesis was confirmed when CFS was present and only minimal inhibition (18 %) was observed after 72 h. Luz et al. [37] reported that CFS of *Lb. plantarum* had antifungal activity against *Penicillium expansum* and *A. parasiticus*. *Lb. plantarum* in a study of Gerez et al. [38] was shown to be active against spoilage filamentous fungi typically found in baked goods, such as *Penicillum* spp. and *Fusarium* spp. Most (approximately 75 %) of *Lb. plantarum* strains analysed in a study of Russo et al. [39] exerted a strong inhibition against *Pe. chrysogenum*, *Pe. expansum* and *F. culmorum*.

Finally, the radial growth of *A. niger* in co-culture with *Lb. plantarum* at various concentrations was studied on the surface of PCA plates and the results were fitted using the Baranyi model [13]. Radial growth rates described the colony diameter as a function of cultivation time. The growth of fungi on the surface of the agar medium followed a typical growth curve with a lag phase, as can be seen in Fig. 1. The apparent absence of stationary phase was caused by limitation of the growth in Petri dishes (diameter 140 mm). Referring to the effects of LAB, the expected effects on lag phase prolongation were observed as the concentration of co-cultured *Lb. plantarum* increased. In a control sample, *A. niger* started to grow after 19.5 h of lag phase with the radial growth rate of 25.2 mm·d^{-1}, which was almost the same in all experiments performed. *Lb. plantarum* at 1%, 2% and 5% (v/v) (7.93 log CFU·ml^{-1}; 8.09 log CFU·ml^{-1}; 8.72 log CFU·ml^{-1}) led to the prolongation of lag phase by approximately 40.3 h, 70.9 h and 101.1 h compared to the control experiment, respectively. Similar to our previous experiments, no inhibition was observed when CFS was added.

CONCLUSIONS

Live LAB alone or with special adjunct strains, when present in adequate amounts in food, are known to positively affect the shelf life and safety of final products. In this study, data on parallel growth of *Lb. plantarum* and *E. coli*, *S.* Typhimurium, *P. aeruginosa, Staph. aureus, C. parapsilosis, B. cereus* as well as *A. niger* were collected. *Lb. plantarum* most effectively suppressed growth of *C. parapsilosis* (99.9% inhibition after 24 h). Increased addition of *Lb. plantarum* (1%, 2% and 5% (v/v)) led to the prolongation of lag phase of *A. niger* by approximately 40.3 h, 70.9 h and 101.1 h compared to the control experiment, respectively. In our experiments, *Lb. plantarum* was present at a level of > 8 log CFU·ml^{-1} after 24 h.

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