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SHORT COMMUNICATION

The Effect of Fe_3O_4 Nanoparticles on Survival of Probiotic Bacteria *Lactobacillus acidophilus* PCM2499 at Lower pH

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Abstract

This paper presents a description of an experiment in which the survival rate of the probiotic bacteria *Lactobacillus acidophilus* PCM2499 was increased only due to the presence of Fe_3O_4 magnetic nanoparticles. The survival rate increased from 1.3 to 10 times compare to the control. It has been shown that the minimum concentration of NPs with a positive effect equals 8 mg/ml and the maximum concentration of the NPs equals 24 mg/ml.

K e y w o r d s: *Lactobacillus acidophilus* PCM2499, Fe_3O_4 nanoparticles, probiotic bacteria, survival at low pH

Generally, lactic acid fermentation is used to produce a wide variety of food products such as: cottage cheese, yogurt, kefir, *etc*. The major fermentation product of the investigated probiotic bacterium *Lactobacillus acidophilus* PCM2499 (Polish Collection of Microorganisms, Wrocław, Poland) is lactic acid. Lactic acid is an important factor in environmental stress, occurring during the fermentation of foods and beverages. The growth of LAB depends upon the pH value of the environment and therefore also on the concentration of lactic acid in the environment. The minimum value of pH for *Lactobacillus* sp. amounts to 3.8–4.4 (Piard and Desmazeaud, 1991).

The increase of bacterial survival rate at lowered pH is of great importance in the food industry. The lactic acid produced by LAB reduces the pH thus inhibiting the activity of these bacteria and reducing the efficiency of the process. Therefore, the development of a method to increase the survival of LAB would result in a more efficient process of lactic fermentation.

Previous studies have focused primarily on the impact of various stress factors on the survival of microorganisms, including the influence of pH on the survival rate of LAB (de Angelis and Gobetti, 2004). The main topic of the research was also concerned with the investigation of microorganisms' adaptation processes to low pH (Sánchez *et al*., 2007) as well as the tolerance of low pH (Bang *et al*., 2000; Bang *et al*., 2002; Matsui and Cvitkovitch, 2010; Senouci-Rezkallah *et al*., 2011). Studies on the clarification of the mechanism of intracellular pH (pH_i) homeostasis were also conducted (Baker-Austin and Dopson, 2007; Hutkins and Nannen, 1993; Kirsch, 2014; Quinn *et al*., 2012; Zhang *el al*., 2013). A number of programs that model stress factor-dependent survival have been created, such as *e.g.* Pathogen Modeling Program Version (http://www.usda.gov).

Adding $CaCO_3$ or other alkaline salts into the bacterial environment is a widely used method of increasing the pH. However, Zapotoczny *et al*. (2013) demonstrated the nanobuffering property of Fe_3O_4 magnetic nanoparticles (NPs). The buffering properties of NPs are explained by the change in superficial charge on the oxide NPs. It has been shown, both theoretically and experimentally, that pH value for acidic solution is less acidic in the NPs environment (measured in nanoparticles suspension gathered by external magnet), less alkaline in alkaline environment and remains at the same level when the pH value is close to the point of zero charge (PZC). NPs react neither with acids nor bases and the buffering effect is limited around the nanoparticles. Since the pH near the NPs surface is more neutral than in bulk, the effect was named nanobuffering. Within our area of interest there is a critical pH value for *L. acidophilus* PCM2499 which, as mentioned

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above, ranges 3.8–4.4. In that pH range the nanobuffering shown by Zapotoczny *et al.* (2013) is $\Delta pH \approx 0.5$. The change of pH value allows to be efficient to notice the difference in survival of LAB in the presence and absence of Fe_3O_4 NPs. Therefore, Fe_3O_4 magnetic nanoparticles were used to improve the survival of *L. acidophilus* PCM2499 at a lower pH.

All chemicals were of analytical reagents grade (iron sulphate $FeSO_4$ (ACS reagent, ≥ 99.0% (Sigma-Aldrich), iron chloride $FeCl_3 \cdot 6H_2O$ (POCH) and 25 % ammonia NH_4OH (POCH)) and used directly without further purification.

Fe_3O_4 NPs were synthesized by coprecipitation of iron salts in alkaline solution. Water solutions of 137 mM of $FeSO_4 \cdot 7\ H_2O$ and 274 mM of $FeCl_3 \cdot 6\ H_2O$ were placed in a beaker. Gaseous nitrogen was used for 30 minutes for degassing an aqueous solution and disposal of diluted oxygen. The solution was stirred mechanically at 1200 rpm and during that process 130 mM NH_4OH was added dropwise. The color of the solution changed from orange-brown to black indicating synthesis of Fe_3O_4 NPs. The final pH value exceeded 10. The stirring process was continued for 30 minutes. Then NPs were separated by external magnet and washed 5–7 times with distilled water until the pH value was lower than 7. Finally, the NPs were dried under vacuum for 500 minutes at 55°C.

The synthesized samples were investigated using different techniques. AFM (Bioscope Catalyst, Bruker) and TEM/EDX (Tecnai G2 F20 S Twin, Fei) were used to investigate the shape and size of synthesized nanoparticles. For AFM a small amount of dried NPs was dissolved in distilled water and sonicated for 1 hour. Then a single drop was put on a mica surface and air dried. The analysis of topography was conducted using a tapping mode. Crystallographic structure of NPs was investigated using X-ray diffraction (XRD) to determine the phase of synthesized iron oxides. The measurements were performed on a BRUKER D8 Advance diffractometer using Johansson monochromator ($\lambda Cu\ K_{\alpha 1} = 1{,}5406$Å). Additionally, Scherrer equation was used to calculate diameter of single crystals. The pH was measured with InLab Combination pH Micro Electrode (Mettler Toledo) on SevenExcellence™ pH/mV meter.

The influence of nanoparticles on survival was tested by culturing *L. acidophilus* PCM2499 at lower pH. The following experiment was performed. A 24 hour *L. acidophilus* PCM2499 starter culture in MRS broth (Merck) was employed. 96% acetic acid was used to lower the pH of the MRS broth to 3.901. The pH was measured with InLab Combination pH Micro Electrode (Mettler Toledo) on SevenExcellence™ pH/mV meter.

One hundred miligrams of Fe_3O_4 NPs and 1 ml of the 24 hour starter culture were added to the tubes containing 4 ml of this medium. The samples were incubated at 37°C in the following intervals of 0.5, 1.0, 1.5, 2.0, 2.5 hours. For each time variant a control culture without Fe_3O_4 NPs was set up. After incubation the samples were transferred to MRS agar in dilutions. Petri dishes were incubated at 37°C for 48 h. Colonies were counted to calculate the CFU/ml for each variant. The experiment was performed in triplicate. T-test analysis was performed. The starter culture was transferred to MRS agar in dilutions. Petri dishes were incubated at 37°C for 48 hours. Colonies were counted to calculate the CFU/ml.

A 24 hour *L. acidophilus* PCM2499 starter culture in MRS broth (Merck) was employed. 96% acetic acid was used to lower the pH of the MRS broth to 3.904. The pH was measured with InLab Combination pH Micro Electrode (Mettler Toledo) on SevenExcellence™ pH/mV meter. 0.16; 0.14; 0.12; 0.1; 0.08; 0.06; 0.04; 0.02 and 0 g of Fe_3O_4 NPs and 1 ml of starter culture were successively added to the tubes containing 4 ml of this medium. They were incubated at 37°C for 45 min. After the incubation they were transferred to MRS agar in dilutions. Petri dishes were incubated at 37°C for 48 h. Colonies were counted to calculate the CFU/ml for each variant. The experiment was performed in triplicate. T-test analysis was performed. The starter culture was transferred to MRS agar in dilutions. Petri dishes were incubated at 37°C for 48 hours. Colonies were counted to calculate the CFU/ml.

Due to magnetic attraction between NPs agglomerates were observed. Nevertheless single NPs were still easy to notice. The mean size equals 17–20 nm. Several NPs with different diameters were imaged and a few representatives were marked on a cross-section. The chosen NPs' diameter ranges from 10–35 nm, where the value of scan height was taken into consideration.

The XRD diffraction peaks correspond well to magnetite Fe_3O_4 (JCPDS file, No. 00-011-0614) indicating that the sample has a cubic crystal system. Also, we can see that no characteristic peaks of impurities were observed. To calculate the mean diameter of crystals of Fe_3O_4 NPs the Scherrer equation was used. The mean diameter is equal to:

$$D = \frac{\kappa \lambda}{\beta cos\ \theta}$$

where κ is shape factor and its value was assumed 0.94 for spheroidal shape of NPs, λ is X-ray wavelength (Cu 1,5406 Å), β is the line broadening at half the maximum intensity and θ is the Bragg angle. The value of β was calculated with the use of *Topas* software, where seven reflections were used to calculate the position and half-width values. Calculated mean diameter of obtained NPs is equal to 21 nm.

Bacterial growth was observed for samples with and without Fe_3O_4 NPs. Details of the number of bacteria

Table I
Number of survival bacteria *Lactobacillus acidophilus* PCM2499 in pH = 3.901 incubated with and without Fe_3O_4 NPs

Time of incubation [h]	Number of bacteria [CFU/ml]				t
	Culture with Fe_3O_4 NPs		Control without Fe_3O_4 NPs		
	Mean value	Standard deviation	Mean value	Standard deviation	
0	$6 \cdot 10^6$	0	$6 \cdot 10^6$	0	–
0.5	$2.19 \cdot 10^6$	$0.86 \cdot 10^6$	$1.69 \cdot 10^6$	$0.71 \cdot 10^6$	705717.3
1.0	$2.77 \cdot 10^5$	$1.76 \cdot 10^5$	$1.35 \cdot 10^5$	$0.86 \cdot 105$	85805.61
1.5	$3.92 \cdot 10^4$	$1.55 \cdot 10^4$	$0.6 \cdot 10^4$	$0.18 \cdot 10^4$	1769.82
2.0	$13 \cdot 10^3$	$9.56 \cdot 10^3$	$2.89 \cdot 10^3$	$0.22 \cdot 10^3$	220.56
2.5	$0.74 \cdot 10^3$	$0.72 \cdot 10^3$	$0.06 \cdot 10^3$	$0.05 \cdot 10^3$	48.29

and test t analysis are shown in Table I. Figure 1 shows the survival rate of *L. acidophilus* PCM2499 during 0–2.5 hours incubation with and without Fe_3O_4 NPs. Fig. 1 indicates the ratio of the survival of bacteria with Fe_3O_4 NPs to survival without NPs at each time variant.

In this experiment an investigation on the concentration of NPs below which there is no positive effect on survival rate was carried out. For all the variants (0.16; 0.14; 0.12; 0.1; 0.08; 0.06; 0.04; 0.02 and 0 g of Fe_3O_4 NPs in 5 ml of culture) colonies were obtained. The colonies were counted. T-test analysis was performed. Figure 2 shows the percentage of survival for different variants of the concentration of NPs.

Previous research using NPs was concerned with the antibacterial effect of gold and silver NPs (Jena *et al.*, 2014; Krishnaraj *et al.*, 2010; Nabikhan *et al.*, 2010; Mishra *et al.*, 2014; Puišo *et al.*, 2014). However no research was performed in order to overcome the harmful effects of stress factors.

The research conducted by our team has shown a positive effect of Fe_3O_4 NPs on the survival of *L. acidophilus* PCM2499 in conditions of low pH. During the cultivation of *L. acidophilus* under reduced pH we observed a higher survival rate of bacteria for all samples with Fe_3O_4 NPs than in the control group. T-test analysis shows that the results obtained for samples with Fe_3O_4 NPs are statistically significant. Graph 1 shows the ratio of the survival of the bacteria with NPs to survival without NPs. It can be noticed that the difference in bacterial survival increases along with increasing incubation time (trend line y = 1.25x). The survival rate increased from 1.3 to 10 times compared to the control group. T-test analysis shows that the minimum concentration of NPs having a positive effect

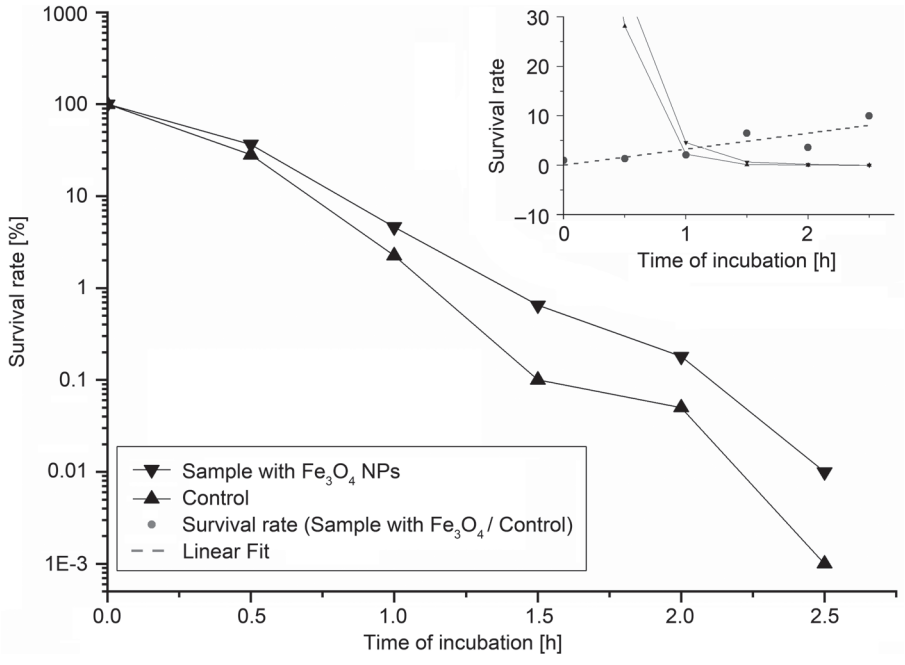

Fig. 1. Survival rate of *L. acidophilus* PCM2499 during incubation with and without Fe_3O_4 NPs. Insert shows survival rate (sample with Fe_3O_4 NPs to control ratio) and linear fit.

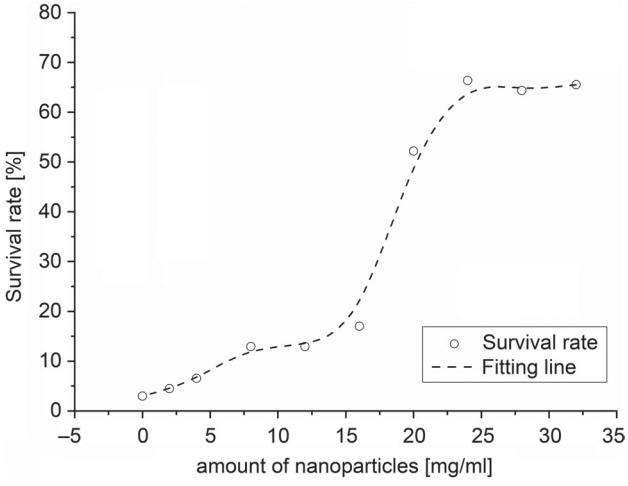

Fig. 2. Survival rate of *L. acidophilus* PCM2499 during incubation with Fe_3O_4 NPs. The fitting line is just a visual guide.

equals 8 mg/ml. In contrast, the maximum concentration of the NPs, above which no longer positive increase was observed equals 24 mg/ml. Along with the raise in the concentration of NPs an increase in the survival of the bacteria was also noted. From the obtained results it can be seen that in the range of 4–16 mg/ml the survival increases slightly (from 4.49% to 17%), while in the range of 20–24 mg/ml, a significant increase in survival was observed (52.17–66.37%). A further increase above 24 mg/ml no longer causes a further increase in the survival. The insignificant influence on the survival with small NPs concentration ranges (up to 16 mg/ml) may be due to the fact that such amount of NPs is able to change pH in a relatively small volume of culture resulting in an insignificant influence on survival increase. In contrast, the concentration of Fe_3O_4 NPs in the range of 20–24 mg/ml contains a sufficient amount of NPs to change the corresponding culture volume, and thus significantly affect the survival.

In conventional technologies the lactic acid created in fermentation is neutralized with calcium carbonate or calcium hydroxide. Then, to isolate the lactic acid from the post-fermentation solution calcium lactate is crystallized and then hydrolyzed with sulfuric acid. The use of Fe_3O_4 NPs during the lactic acid fermentation instead of calcium carbonate or calcium hydroxide could facilitate and increase the efficiency of the process. Lactic acid productivity growth by NPs needs to be confirmed in further studies.

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