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A method to increase the survival of probiotic bacteria *Lactobacillus brevis* at a lowered pH

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Abstract: *Lactobacillus brevis* PCM 2570 is a strain of lactic acid bacteria, i.e. probiotic bacteria whose major fermentation product is lactic acid. The efficiency of lactic acid production is limited by the value of ambient pH. This study aimed to increase the survival of this bacterial strain at a reduced pH (3.9), which would result in an increased yield of lactic acid fermentation. In our experiment the survival rate of probiotic bacteria *L. brevis* PCM 2570 was increased 1.2-fold to 6.96-fold due to the presence of Fe_3O_4 magnetic nanoparticles, as compared to the control. The minimum concentration of nanoparticles with a positive effect was 8 mg/ml, but the optimum concentration was 20 mg/ml.

Keywords: Fe_3O_4 nanoparticles, *Lactobacillus brevis* PCM 2570, probiotic bacteria, survival rate, low pH

INTRODUCTION

The major fermentation product of the investigated strain of probiotic bacteria *L. brevis* PCM 2570 (Polish Collection of Microorganisms, Wrocław, Poland) is lactic acid. This acid is important for the packaging industry, as a precursor of compounds (San-Martin et al. 1992), and also for the cosmetic (Gao et al. 2011) and pharmaceutical industry (Bai et al. 2004). Nevertheless, it is used most commonly in the food industry, for production of yogurt, kefir, soured milk, and cheese (Salminen et al. 1993).

Lactic acid, however, is also an important factor in environmental stress during the fermentation. The growth of lactic acid bacteria (LAB) depends upon the pH value of their environment. The minimum value of pH for *Lactobacillus* spp. is 3.8-4.4 (Piard & Desmazeaud 1991). An increase in LAB survival rate at the lower pH is of great importance in various industries, as it would result in more efficient lactic acid production.

Previous research has focused on the impact of various stress factors on LAB survival, including the influence of pH (de Angelis & Gobetti 2004). Other studies were concerned with investigation of microbial adaptation to low pH (Sánchez et al. 2007), tolerance to low pH (Senouci-Rezkallah et al. 2011) or clarification of the mechanism of intracellular pH (pH_i) homeostasis (Baker-Austin & Dopson 2007; Zhang el al. 2013; Kirsch 2014).

Our earlier research team (Jurkowski et al. 2015) demonstrated the positive effect of Fe_3O_4 nanoparticles on survival of *Lactobacillus acidophilus* PMC 2499 at a lowered pH. In the present study, Fe_3O_4 nanoparticles were used to improve the survival of *L. brevis* PCM 2570 at pH 3.9.

MATERIAL AND METHODS

Synthesis and characterization of Fe_3O_4

Fe_3O_4 nanoparticles were synthesized and investigated according to Jurkowski et al. (2015), with the use of transmission electron microscopy, atomic force microscopy, and X-ray diffraction.

Experiment A: effect of time of incubation with Fe_3O_4 nanoparticles

The influence of nanoparticles on survival was tested in *Lactobacillus brevis* PCM 2570 cultures at a lower pH. For 24 h a starter culture of *L. brevis* PCM 2570 was incubated in MRS broth (Merck) at pH 5.6. Next, 96% acetic acid (POCh, Poland) was used to lower the pH of fresh MRS broth to 3.9. Then, 0.1 g of Fe_3O_4 nanoparticles and 1 ml of the 24-h starter culture were added to tubes containing 4 ml of this medium. The samples were incubated at 35°C for 0.5, 1.0, 1.5, 2.0 or 2.5 h. For each time variant, a control culture without Fe_3O_4 nanoparticles was established. After incubation, diluted samples were transferred to MRS agar (pH 5,6) in Petri dishes and incubated at 37°C for 48 h. Finally, colonies were counted to estimate the number of colony forming units (CFU) per ml for each variant. The experiment was performed in triplicate. The results were analysed statistically using Student's *t*-test.

Experiment B: minimum and optimum concentration of Fe_3O_4 nanoparticles

Like in the previous experiment, for 24 h a starter culture of *L. brevis* PCM 2570 was incubated in MRS broth at pH 5.6. Next, 96% acetic acid was used to lower the pH of fresh MRS broth to 3.9. Then, 0.18, 0.16, 0.14, 0.12, 0.1, 0.08, 0.06, 0.04, 0.02, and 0 g of Fe_3O_4 nanoparticles and 1 ml of starter culture were successively added to tubes containing 4 ml of this medium. After incubation at 37°C for 45 min, diluted samples were transferred to MRS agar (pH 5.6) in Petri dishes and incubated at 37°C for 48 h. Finally, colonies were counted to estimate CFU/ml for each variant. The experiment was performed in triplicate. The results were analysed statistically using Student's *t*-test.

RESULTS

Transmission electron microscopy and atomic force microscopy

Due to magnetic attraction between nanoparticles, agglomerates were observed. Their mean size was 17-20 nm. Several nanoparticles with different diameters were imaged and a few representatives were marked at a cross-section (diameter 10-35 nm, Fig. 1).

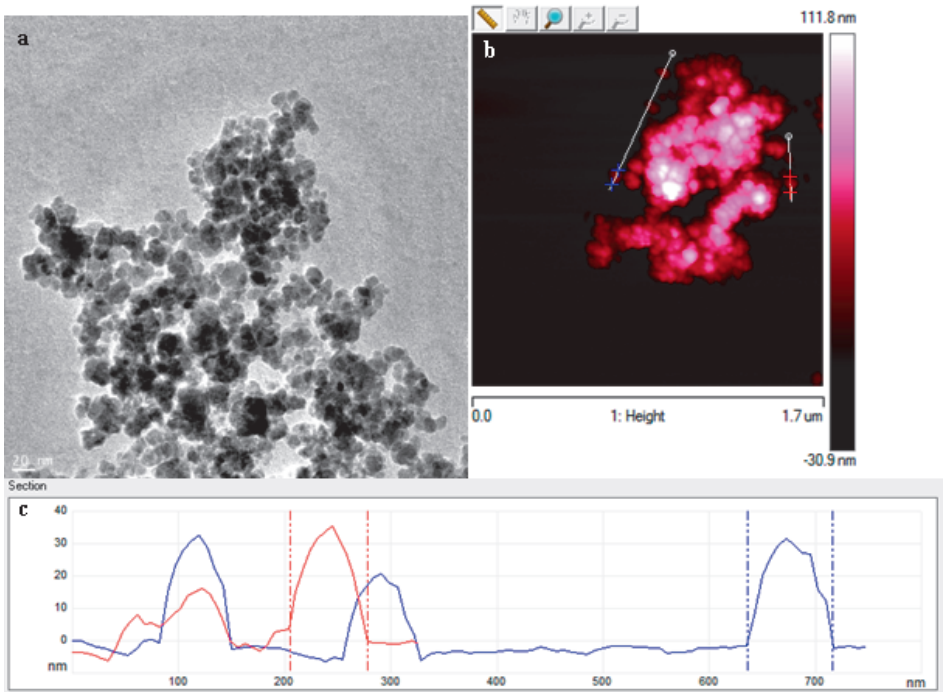

Fig. 1. Transmission electron microscopy (a) and atomic force microscopy (b and c) analyses of Fe_3O_4 nanoparticles: panel b presents a 2D height image of the nanoparticles dispersed on a mica surface within a scan area of 1.7 µm × 1.7 µm, while panel c shows height cross-sections for a few remarkable representatives (nanoparticle diameter 10-35 nm)

X-ray diffraction (XRD)

The XRD peaks correspond well to magnetite Fe_3O_4 (JCPDS file no. 00-011-0614), indicating that the sample has a cubic crystal system. The calculated mean diameter of obtained nanoparticles is 21 nm (Fig. 2).

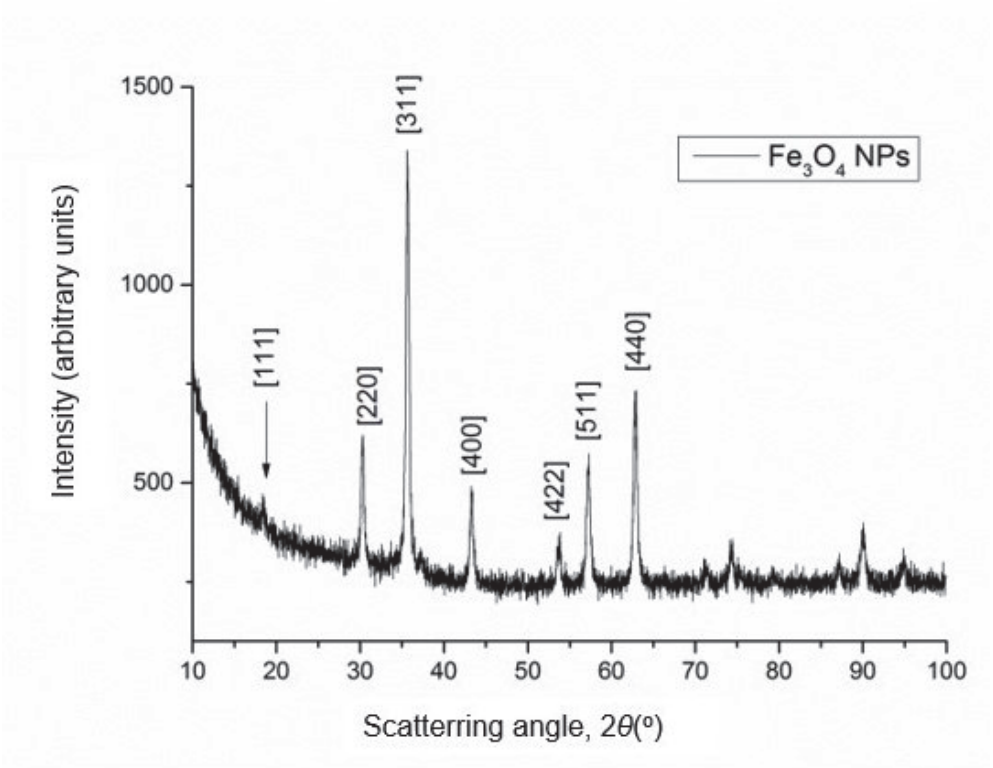

Fig. 2. The X-ray diffraction (XRD) pattern of a sample of Fe_3O_4 nanoparticles

Effect of time of incubation with Fe_3O_4 nanoparticles

Bacterial growth was observed for samples with and without Fe_3O_4 nanoparticles (Table 1). The survival rate of strain PCM 2570 decreased with time during 2.5 h of incubation both with and without Fe_3O_4 nanoparticles, but was markedly lower for the control samples (Fig. 3). Table 1 indicates the ratio of survival of bacteria with Fe_3O_4 nanoparticles to survival without nanoparticles in each time variant.

Minimum and optimum concentration of Fe_3O_4 nanoparticles

In the bacterial cultures with up to 40 mg Fe_3O_4/ml, colony growth was observed and the colonies were counted. The minimum concentration of nanoparticles with a significant positive effect was 8 mg/ml (Table 2) but the survival rate of strain PCM 2570 was the highest for cultures with 20 mg Fe_3O_4/ml (Fig. 4).

Table 1. Number of survived bacteria *Lactobacillus brevis* PCM 2570 at pH = 3.9 after incubation with and without Fe_3O_4 nanoparticles

Time of incubation (h)	Number of bacteria (CFU/ml)				*t*
	culture with Fe_3O_4		control		
	mean	SD	mean	SD	
0	$15.66 \cdot 10^8$	$2.31 \cdot 10^8$	$15.66 \cdot 10^8$	$2.31 \cdot 10^8$	0.00
0.5	$80.10 \cdot 10^7$	$14.00 \cdot 10^7$	$83.50 \cdot 10^7$	$2.18 \cdot 10^7$	0.21
1.0	$24.70 \cdot 10^7$	$2.74 \cdot 10^7$	$20.46 \cdot 10^7$	$0.32 \cdot 10^7$	1.38
1.5	$16.37 \cdot 10^7$	$2.46 \cdot 10^7$	$9.20 \cdot 10^7$	$0.61 \cdot 10^7$	2.33
2.0	$16.10 \cdot 10^7$	$1.27 \cdot 10^7$	$3.76 \cdot 10^7$	$0.25 \cdot 10^7$	8.07*
2.5	$16.43 \cdot 10^7$	$1.42 \cdot 10^7$	$2.36 \cdot 10^7$	$0.22 \cdot 10^7$	8.55*

CFU = colony-forming units; SD = standard deviation; * significance level $\alpha = 0.05$

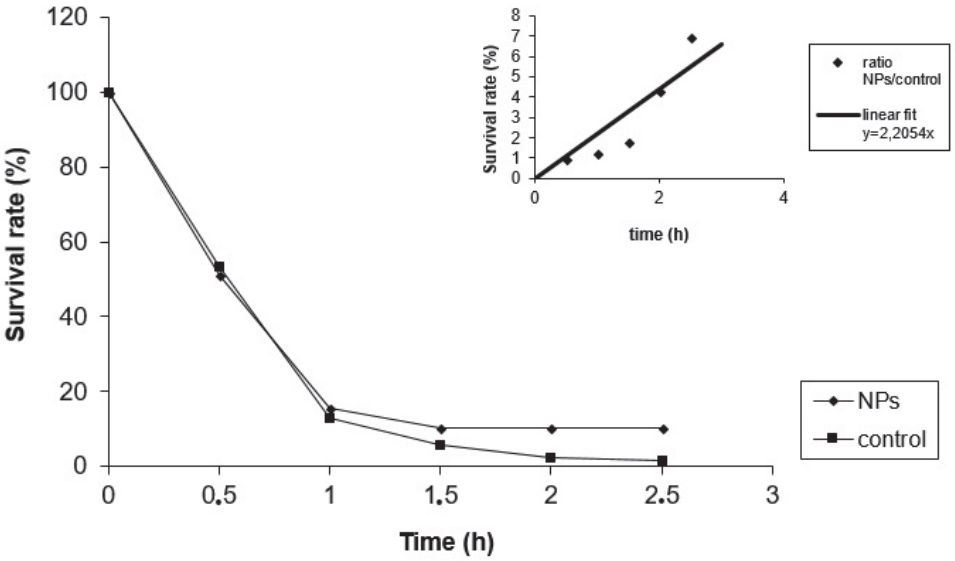

Fig. 3. The survival rate of *Lactobacillus brevis* PCM 2570 during incubation with and without Fe_3O_4 nanoparticles (NPs). The insert shows the survival rate ratio of samples with Fe_3O_4 nanoparticles to control samples and their linear fit

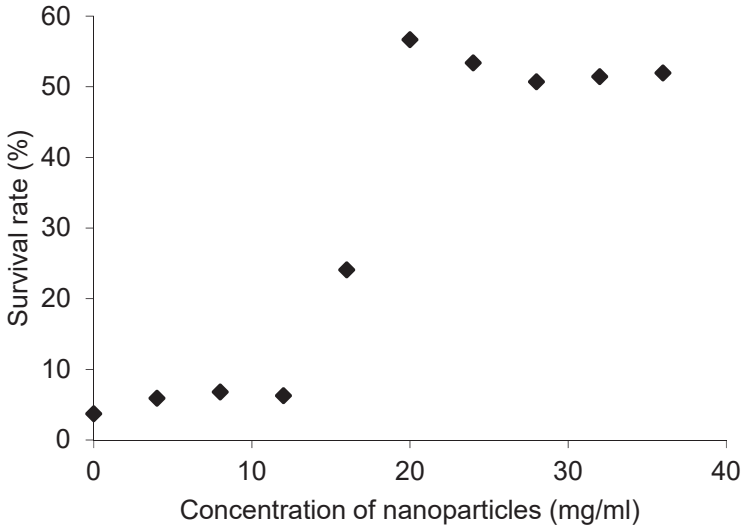

Fig. 4. The survival rate of *Lactobacillus brevis* PCM 2570 during incubation with various concentrations of Fe_3O_4 nanoparticles

Table 2. Number of survived bacteria *Lactobacillus brevis* PCM 2570 at pH 3.9 after incubation with various concentrations of Fe_3O_4 nanoparticles

Concentration of Fe_3O_4 (mg/ml)	Number of bacteria (CFU/ml)		*t*
	mean	SD	
36	$290.83 \cdot 10^6$	$21.53 \cdot 10^6$	6.02*
32	$287.93 \cdot 10^6$	$23.33 \cdot 10^6$	5.01*
28	$283.90 \cdot 10^6$	$17.41 \cdot 10^6$	6.47*
24	$298.83 \cdot 10^6$	$19.95 \cdot 10^6$	6.39*
20	$317.33 \cdot 10^6$	$64.85 \cdot 10^6$	4.57*
16	$134.83 \cdot 10^6$	$11.02 \cdot 10^6$	10.28*
12	$35.05 \cdot 10^6$	$28.90 \cdot 10^6$	4.82*
8	$38.00 \cdot 10^6$	$14.10 \cdot 10^6$	11.62*
4	$33.06 \cdot 10^6$	$109.00 \cdot 10^6$	1.12
0	$20.80 \cdot 10^6$	$0.70 \cdot 10^6$	N/A

CFU = colony-forming units; SD = standard deviation; N/A = not applicable; * significance level $\alpha = 0.05$

DISCUSSION

Studies using nanoparticles were commonly concerned with the antibacterial effect of gold and silver nanoparticles (Moritz & Geszke-Moritz 2013; Jena et al. 2014; Saravan et al. 2018). The first research performed to overcome the harmful effects of stress factors with the use of nanoparticles was conducted by our earlier research team (Jurkowski et al. 2015). We then observed a positive effect of Fe_3O_4 nanoparticles on the survival of *L. acidophilus* PCM 2499 at a low pH. During the cultivation of *L. acidophilus* under the reduced pH, a higher survival rate of bacteria was observed for all samples with Fe_3O_4 nanoparticles than in the control group.

This study shows a positive effect of Fe_3O_4 nanoparticles on the survival of another strain of probiotic bacteria, *L. brevis* PCM 2570, at a low pH. Bacterial survival increased with increasing incubation time (trend line $y = 2.2054\ x$), from 1.2 to 6.96-fold, as compared to the control group. Results of Student's *t* test show that the minimum concentration of nanoparticles having a positive effect was 8 mg/ml, while the optimum concentration of nanoparticles, above which no longer a positive increase was observed, equalled 20 mg/ml. The earlier study on Fe_3O_4 nanoparticles (Jurkowski et al. 2015) demonstrated for *L. acidophilus* PCM 2499 that survival rate increased from 1.3 to 10-fold, relative to the control, and the minimum concentration of nanoparticles having a positive effect was 4 mg/ml. Thus the results for *L. brevis* were less favourable than results for *L. acidophilus*, which suggests the necessity for further research with other probiotic bacteria.

In traditional technologies, the lactic acid produced during fermentation is neutralized with calcium carbonate or calcium hydroxide. Then calcium lactate is crystallized and then hydrolysed with sulfuric acid to isolate lactic acid from the post-fermentation solution (Datta & Henry 2006). The use of Fe_3O_4 nanoparticles during lactic acid fermentation instead of calcium carbonate or calcium hydroxide may facilitate and increase the efficiency of the process. However, lactic acid productivity of *L. brevis* PCM 2570 growth by Fe_3O_4 nanoparticles needs to be confirmed in further studies.

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REFERENCES

Bai D., Zhao X., Li X., Xu S. 2004. Strain improvement of *Rhizopus oryzae* for over-production of L(+)-lactic acid and metabolic flux analysis of mutants. Biochem. Eng. J. 18: 41–48.

Baker-Austin C., Dopson M. 2007. Life in acid: pH homeostasis in acidophiles. Trends Microbiol. 15: 165–171.

Datta R., Henry M. 2006. Lactic acid: recent advances in products, processes and technologies – a review. J. Chem. Technol. Biotechnol. 81: 1119–1129.

De Angelis M., Gobetti M. 2004. Environmental stress responses in *Lactobacillus*: A review. Proteomics 4: 106–122.

Gao C., Ma C., Xu P. 2011. Biotechnological routes based on lactic acid production from biomass. Biotechnol. Adv. 29: 930–939.

Jena J., Pradhan N., Dash B. P., Panda P. K., Mishra B. K. 2014. Pigment mediated biogenic synthesis of silver nanoparticles using diatom *Amphora* sp. and its bactericidal and antimicrobial activity. J. Saudi Chemi. Soc. 19: 661–666.

Jurkowski A., Zapotoczny B., Kozioł J. J., Dudek M. R. 2015. The effect of Fe_3O_4 nanoparticles on survival of probiotic bacteria *Lactobacillus acidophilus* PCM2499 in lower pH. Pol. J. Microbiol. 64: 307–310.

Kirsch K. M. 2014. *The impact of CO_2 on inorganic carbon supply and pH homeostasis in Corynebacterium glutamicum.* PhD thesis, University of Cologne, Germany.

Moritz M., Geszke-Moritz M. 2013. The newest achievements in synthesis, immobilization and practical applications of antibacterial nanoparticles. Chem. Eng. J. 228: 596–613.

Piard J. C., Desmazeaud M. 1991. Inhibiting factors produced by lactic acid bacteria. 1. Oxygen metabolites and catabolism end-products. Lait 71: 525–541.

Salminen S., Ouwehand A., Wright A. V., Daly C. 1993. Future aspects of research and product development of lactic acid bacteria. In: Lactic acid bacteria microbiological and functional aspects (Salminen S., von Wright A., Ouwehand A., Eds.), pp. 429–432, , Marcel Dekker, Inc. New York.

Sánchez B., Champomier-Vergès M-Ch., del Carmen Collado M., Anglade P., Baraige F., Sanz Y., de los Reyes-Gavilán C. G., Margolles A., Zagorec M. 2007. Low-pH Adaptation and the Acid Tolerance Response of *Bifidobacterium longum* Biotype *longum*. Appl. Environ. Microbiol. 73: 6450–6459.

San-Martin M., Pazos C., Coca J. 1992. Reactive extraction of lactic acid with alamine 336 in the presence of salts and lactose. J. Chem. Technol. Biotechnol. 54: 1–6.

Saravanan M., Barik S.K., Ali D. M., Prakash P., Pugazhendhi A. 2018. Synthesis of silver nanoparticles from Bacillus brevis (NCIM 2533) and their antibacterial activity against pathogenic bacteria. Microb. Pathogenesis 116: 221–226.

Senouci-Rezkallah K., Schmitt P., Jobin M. P. 2011. Amino acids improve acid tolerance and internal pH maintenance in *Bacillus cereus* ATCC14579 strain. Food Microbiol. 28: 364–372.

Zhang W., Wang Y., Song Y., Wang T., Xu S., Peng Z., Lin X., Zhang L., Shen X. 2013. A type VI secretion system regulated by *OmpR* in *Yersinia pseudotuberculosis* functions to maintain intracellular pH homeostasis. Environ. Microbiol. 15: 557–569.