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INFLUENCE OF ZINC SULPHATE ON THE PROBIOTIC PROPERTIES OF *LACTOBACILLUS PLANTARUM* CCM 7102

Mudroňová, D., Gancarčíková, S., Nemcová, R.

Department of Microbiology and Immunology
University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, 041 81 Košice
Slovakia

dagmar.mudronova@uvlf.sk

ABSTRACT

The effects of zinc sulphate on selected properties of *L. plantarum* CCM 7102 were tested *in vitro*. The resistance of lactobacilli to higher concentrations of $ZnSO_4$ (up to 5000 mg $Zn^{2+}.l^{-1}$) in growth media was strain-dependent. Further studies were carried out on the most resistant strain of *L. plantarum* CCM 7102. While the addition of low concentrations of zinc sulphate into the growth media (< 100 mg $Zn^{2+}.l^{-1}$) did not influence the properties of *L. plantarum* CCM 7102, the concentrations of 100—500 mg $Zn^{2+}.l^{-1}$ stimulated: the growth rate, production of lactic acid, adhesion to porcine enterocytes and the inhibition of pathogens *E. coli* O8:K88^{+}ent^{+}, *S. enterica* and *S. Typhimurium*. Conversely, however, high concentrations > 500 mg $Zn^{2+}.l^{-1}$ inhibited these properties. The addition of zinc (250 mg $Zn^{2+}.l^{-1}$) did not affect the resistance to antimicrobials, low pH, and the resistance to bile salt was affected only weakly. Zinc-resistant probiotic *Lactobacillus* strains are suitable for use in feedstuffs with a higher content of zinc designed for the prevention of post weaning diarrhoea in pigs.

Key words: *Lactobacillus*; probiotic; resistance; zinc

INTRODUCTION

Post-weaning diarrhoea (PWD) is a serious health, breeding and economic problem for pig farms. After the ban on the use of growth-promoting antibiotics in the EU in 2006, PWD cannot be controlled by those means, and consequently other safer alternatives to feed antimicrobials have been sought. Ways must also be found to improve the healthiness and safety of animal products reaching the consumer, and therefore safe natural products (e. g. probiotics, plant extracts, etc.) are at the centre of interest in this field.

Any potentially successful probiotic bacteria designated for oral administration must fulfil some selection criteria. Bacteria must be able to survive and grow in the

gastrointestinal tract and to adhere to the mucosa of the gut. It is also necessary to respect the origin of the strain used and its ability to inhibit pathogens. The strain should be genetically stable, it should have good growth promoting properties *in vitro* and *in vivo*, and maintain its high viability at processing and when in storage. Depending on the desired outcome, a probiotic strain may need to have additional properties, such as anticarcinogenic or hypocholesterolemic effects, or the ability to improve lactose utilization [15, 31].

Despite intensive research in this field, up to the present time probiotics are not an adequate substitute for antibiotics. Therefore it is necessary to look for ways and means to increase the efficacy of probiotics. One such way would seem to be combining the probiotic microorganisms with synergistically acting components of natural origin (such as oligosaccharides, polyunsaturated fatty acids, organic acids, phytocomponents or trace elements) which intensify the mode of action of the probiotic microorganisms or extend the range of beneficial effects of a probiotic preparation on the host. Such combined preparations are called potentiated probiotics [6].

Zinc, as an essential microelement, plays an important role in bacterial metabolism. Zinc is a part of many microbial enzymes, such as alcohol dehydrogenase, zinc-dependent proteinase, DNA- and RNA-polymerases, phospholipase C, endopeptidases or aminopeptidases [11, 22, 25, 39]. Zinc deficiency in microorganisms manifests itself by metabolic disturbances and by growth depression [9]. Conversely, the antimicrobial effect of zinc is well-known, and therefore a microorganism must precisely control its adequate intracellular level [2]. There are significant differences in the susceptibility to zinc not only among different bacterial species but also among the bacterial strains. Some bacteria, such as *Brevibacterium* sp. (strain HZM-1) isolated from the soil of the abandoned zinc mine or some strains of *Bacillus* spp., are zinc-resistant and they can grow in the presence of high concentrations of zinc and/or accumulate the zinc into the biomass easily [2, 30]. A lot of other bacteria are inhibited by zinc. The inhibition of bacterial glycolysis by zinc ions in oral microbes (e. g. *Streptococcus salivarius*, *Strep. sobrinus*, *Strep. mutans*) is expected to moderate dental caries [7]. The ability of zinc to inhibit the growth of *E. coli* is used in the prevention and therapy of post-weaning diarrhoea [21]. Nayak et al. [27] have noted a significant reduction in the adhesion of *Salmonella Typhimurium* ($P < 0.01$) on poultry skin after the application of zinc chloride. The numbers of salmonellae on the skin were also reduced [27]. In addition to direct inhibition of pathogens, zinc has a positive influence on the immune system in an infected organism. The stimulating effect of zinc on cellular and humoral immune responses has been confirmed by many authors [3, 5, 18, 19, 23].

Despite many studies on the relationship between zinc and bacteria, the interactions between zinc compounds and probiotic lactobacilli have not been studied adequately. The present study was performed to evaluate the influence of zinc sulphate on several desirable properties of probiotic lactobacilli under *in vitro* conditions and to select the strain appropriate for potential use in the prevention of PWD.

MATERIALS AND METHODS

Microorganisms and culture conditions

Six strains of lactobacilli used for the study of their resistance to the addition of zinc in the growth media were isolated from the jejunum or ileum of one week-old piglets and were grown under anaerobic conditions in MRS broth agar (Merck, Germany) at 37 °C for 18—24 h or 48 h. The strain showing the highest resistance to zinc was used for further analyses and was characterized as *Lactobacillus plantarum* CCM 7102. Pig strain of *E. coli* O8:K88^{+}ent^{+} was obtained from the Institute of Microbiology (Czech Academy of Sciences, Prague, Czech republic). *Salmonella enterica* SE1 and *S. Typhimurium* were identified in the State Veterinary and Food Institute (Košice, Slovak Republic). Pathogenic bacteria were cultivated at 37 °C for 24 h in PYG broth (peptone bacteriological, 5 g; trypticase peptone, 5 g; yeast extract, 10 g; D(+) glucose, 10 g.1000 ml^{-1}, pH 7). In order to examine the influence of zinc sulphate ($ZnSO_4.7H_2O$, Lachema, Brno, Czech Republic), it was added in the growth media in the respective concentrations. The pH of the growth media were adjusted with 0.1 N NaOH for lactobacilli to 5.8—6.2 and for pathogens to 6.9—7.2.

Growth dynamics

The growth media with $ZnSO_4.7H_2O$ in concentrations of: 0, 100, 250, 500, 1000, 2500 mg $Zn^{2+}.l^{-1}$ were inoculated with 5 % of an overnight culture of *L. plantarum* CCM 7102, and incubated in a shaker water bath (Julabo SW 20C, Labor Technik GmbH Selbach, Germany), at 37 °C

and 150 rev.min^{-1}. Regarding the high turbidity of growing media with zinc concentrations higher than 250 mg Zn^{2+}.l^{-1}, it was not possible to measure absorption. For this reason the growth dynamics were monitored by the decrease in pH of the media (ION Activity Meter MS20, Laboratórní přístroje, Prague, Czech Republic). The statistical analyses were done on the basis of pH decrease after every 2 h (0—12 h) and after 12 h (12—24 h). After 24 h the viable counts of lactobacilli were noted.

Organic acids analysis

Organic acids in the bacterial cultures were determined by capillary isotachophoresis (ITP ZKI-01, Spišská Nová Ves, Slovak Republic). As conducting and finishing electrolytes, 0.001 mmol.l^{-1} hydrochloric capronic acid + 0.1 % methylhydroxyethyl cellulosic acid (MHEC) and 5 mmol.l^{-1} capronic acid were used.

Testing the resistance to low pH

The MRS broth was adjusted to pH 2 by an addition of sterile 1 N HCl. For testing the influence of zinc, sterile $ZnSO_4.7H_2O$ (250 mg Zn^{2+}.l^{-1}) was added to the MRS broth. Bacterial cells were collected by centrifugation (3000 × g, 10 min) at 4 °C, rinsed once with phosphate buffered saline (PBS, pH 7.2). Test tubes containing pH-adjusted MRS broth with and without zinc sulphate were inoculated with the bacterial suspension to achieve a final cell concentration of 10^8 cfu.ml^{-1}. All tubes were incubated at 37 °C. The numbers of bacteria were determined at 0, 2, 4 and 8 h on MRS agar plates incubated anaerobically at 37 °C for 48 h.

Testing the resistance to bile salts

For testing the resistance to bile salts 0.3 % Oxgall-Dehydrated Bile (BBL Microbiology Systems, Becton Dickinson, Cockeysville, USA) in MRS broth was added. MRS broth was supplemented with 0.3 % Oxgall together with $ZnSO_4.7H_2O$ (250 mg Zn^{2+}.l^{-1}). The cultures were performed in triplicate, inoculated with an overnight culture of lactobacilli (1 %) and incubated in a shaker water bath (Julabo SW 20C, Labor Technik GmbH Selbach, Germany), at 37 °C and 100 rev.min^{-1} for 24 h. The growth in each culture was monitored by measuring the pH and after 0, 4, 8, 12 and 24 h the samples were collected for counting of the numbers of bacteria.

Adhesion to isolated porcine enterocytes

Isolation of the epithelial cells from the jejunum of a 7-day old piglet and the adhesion test were performed using the method of Evans et al. [16]. Adhesion was studied by light microscopy of Gram stained preparations, from which counts were made by arithmetical means ± standard deviation of numbers of bacteria adhering to 50 enterocytes.

Adhesion to crude intestinal mucin

Crude intestinal mucin was prepared from the small intestine of a weaned pig according to the method described by Štyriak et al. [36]. EIA/RIA microtitre 96-well strip plates (Corning-Costar Corporation, Cambridge, USA) were coated with crude mucin (100 µl) in a concentration of 100 µg of mucin protein per ml. The microtitre plates were subsequently incubated overnight at 4 °C. Mucin was then removed and plates washed 3 times with PBS (MP Biomedicals, France). Finally, bacterial suspensions (100 µl; 10^9 cfu.ml^{-1}) of *L. plantarum* CCM 7102 cultivated in MRS broth with 0, 100, 250, 500 and 1000 mg Zn^{2+}.l^{-1} were added and the plates were incubated on the orbital platform shaker for 2 h at 37 °C. All unbound bacteria were subsequently removed by washing the wells 3 times with PBS. Bacteria in the wells were then fixed at 60 °C for 20 minutes and stained with crystal violet. The excessive stain was removed with PBS. After adding citrate buffer (100 µl, pH 4.3) and 45 min incubation at room temperature, the absorbance values (A570nm) were determined in a microplate reader (BioTek, USA) and the averages of five absorbance values were calculated. The strains were classified as strongly adherent (A570nm > 0.3), weakly adherent (0.1 < A570nm < 0.3) or non-adherent (A570nm < 0.1).

Inhibition assay

L. plantarum CCM 7102 was tested for inhibition of *E. coli* O8:K88$^+$ent$^+$, *S. enterica* and *S. Typhimurium* by paper disc assay. Petri dishes containing 20 ml of MRS agar were prepared. Sterile paper discs (6 mm diameter; BBL Microbiology Systems, Becton Dickinson, Cockeysville, USA) were placed onto the surface of each plate and 10 µl of 24-h culture of *L. plantarum* CCM 7102 was spotted onto the disc. Plates were incubated under conditions described above for 48 h. *Lactobacilli* were grown in the MRS broth with $ZnSO_4.7H_2O$ in concentrations of: 0, 100, 250, 500, 1000 and 2500 mg Zn^{2+}.l^{-1}. After incuba-

tion, all discs were removed and the lactobacilli were killed by exposure to chloroform vapour for 30 min. The plates were then overlaid with 3 ml of 0.7 % PYG agar, which was seeded with 0.3 ml overnight culture of pathogen. After incubation for 24 h at 37 °C, the diameter of the inhibition zone around the disc was measured in mm. Three replicates were done for each zinc concentration.

Susceptibility to antimicrobials

The susceptibility to 21 antimicrobials were determined by a plate diffusion method using the antibiotic discs: streptomycin (30 μg.ml^{-1}), neomycin (30 μg.ml^{-1}), chloramphenicol (30 μg.ml^{-1}), erythromycin (15 μg.ml^{-1}), tetracycline (30 μg.ml^{-1}), penicillin (10 μg.ml^{-1}), ampicillin (10 μg.ml^{-1}), bacitracin (10 μg.ml^{-1}), oxacillin (10 μg.ml-$^{-1}$), colistin (10 μg.ml^{-1}), lincomycin (10 μg.ml^{-1}), spiramycin (20 μg.ml^{-1}), kanamycin (30 μg.ml^{-1}), vancomycin (30 μg.ml^{-1}), rifampicin (10 μg.ml^{-1}), nalidixic acid (20 μg.ml^{-1}), amoxycillin (10 μg.ml^{-1}), cloxacillin (5 μg.ml^{-1}), amoxycillin+clavulanic acid (20 μg.ml^{-1}), gentamycin (10 μg.ml^{-1}) and cefquinome (10 μg.ml^{-1}).

Zinc analysis

The zinc content in the bacterial supernatants was determined by atomic absorption spectrometry (A Analyst 100, Perkin Elmer-Elmer Co., Norwalk, USA).

Statistical analysis

The data were analyzed by the statistical software Graph Pad PRISM version 3.00. After analysis of variance (ANOVA), Tukey's test was used to identify the differences between the groups. The level of significance was set to $P < 0.05$.

RESULTS

The growth of six porcine *Lactobacillus* strains in culture media with high concentrations of zinc sulphate were tested. The most resistant strain, *L. plantarum* CCM 7102, grew in MRS broth with 5 g $Zn^{2+}.l^{-1}$ in high counts–1.10^9 cfu.ml^{-1}. This strain was selected for subsequent analyses.

The viable counts of *L. plantarum* CCM 7102 after growth in MRS broth with 0—2500 mg $Zn^{2+}.l^{-1}$ are presented in Table 1. No significant differences in numbers of lactobacilli were found in comparison to the control.

The growth dynamic monitored on the basis of pH decrease is displayed in the Figure 1. The pH decrease after the first 2 hours of the growth was similar for all zinc concentrations ($P > 0.05$). During the next 2 hours (2nd—4th h) the highest pH decrease was noted in the medium with 250 mg $Zn^{2+}.l^{-1}$ ($P < 0.001$ in comparison to all other groups), whereas that for zinc concentrations 1000 and 2500 mg $Zn^{2+}.l^{-1}$ were the lowest. From 4th to 6th hour of growth the greatest decrease in pH ($P < 0.001$ in comparison to all other groups) was found in the medium with 500 mg $Zn^{2+}.l^{-1}$ and the lowest acid production was measured in the group with addition of 2500 mg $Zn^{2+}.l^{-1}$. Dur-

Table 1. The influence of various Zn^{2+} concentrations (mg.l^{-1} of growing medium) on the viable counts of *L. plantarum* CCM 7102 (n = 3)

Zn^{2+} conc.	log10 cfu.ml^{-1}
0	9.59 ± 0.022
100	9˝.54 ± 0.039
250	9.59 ± 0.016
500	9.63 ± 0.017*
1000	9.56 ± 0.028
2500	9.53 ± 0.029
ANOVA	$P < 0.05$

*—significantly different from concentration 2500 Zn^{2+} mg.l^{-1} ($P < 0.05$)

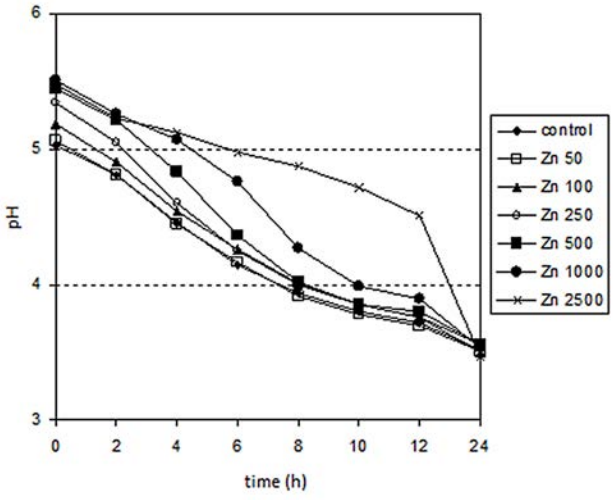

Fig. 1. Effect of addition of zinc sulphate (0, 50, 100, 250, 500, 1000, and 2500 mg $Zn^{2+}.l^{-1}$ of the PYG broth) on the growth dynamic of *L. plantarum* CCM 7102 (monitored by the decrease in pH of the growing media)

ing the next 4 hours (i. e. 6th—10th h) the fastest decrease of the pH was noted in the groups with 1000 and 500 mg $Zn^{2+}.l^{-1}$. Likewise, in this time period, the lowest decline in pH values was noted in the media with 2500 mg $Zn^{2+}.l^{-1}$. On the contrary, from 10th to 24th hour the pH decrease in this group was the most significant and after 24 hours the pH values in all media were very similar and ranged from 3.47 to 3.57. No significant differences in viable counts of lactobacilli were observed after 24 hours of growth.

The organic acid concentrations in each group were measured after 12 h of growth. The highest levels of lactic, acetic and acetoacetic acids were found in the media with 0—250 mg $Zn^{2+}.l^{-1}$ (Table 2). Based on the results described above, the most positive effect on the growth and acid production of *L. plantarum* CCM 7102 was observed when 250 mg $Zn^{2+}.l^{-1}$ were added into the medium and therefore some of the following tests were done only with this concentration.

The *L. plantarum* CCM 7102 grown in the medium with 250 mg $Zn^{2+}.l^{-1}$ has produced significantly higher amounts of lactic acid after 2 h ($P < 0.001$) and also after 4 h ($P < 0.001$) as compared to the control without the addition of zinc (Table 3). From 2nd to 4th hour of the incubation no significant increase in lactic acid concentration in either the zinc or in the control group was noted.

The influence of zinc on the ability of the *L. plantarum* CCM 7102 to resist incubation by pH 2 is shown in Fig. 2. This strain can be characterized as an acid-resistant and 250 mg $Zn^{2+}.l^{-1}$ of the medium did not negatively influenced its acid-tolerance. After 4 hours of the incubation by pH 2 the reduction of viable cells was of 4.6 log in both groups. Lactobacilli were able to survive for 8 hours in numbers about 10^2 cfu.ml^{-1}.

Zinc sulphate (250 mg $Zn^{2+}.l^{-1}$) had only a weak negative effect on the resistance of strain CCM 7102 to bile salts (Fig. 3). The number of microorganisms at time 0 was al-

Table 2. Production of organic acids [mmol.l^{-1}] by *L. plantarum* CCM 7102 after 12 hours of the growth in PYG broth with various zinc concentrations

Zn^{2+} conc. mg.l^{-1}	Lactic acid	Acetic acid	Acetoacetic acid	Succinic acid	Formic acid
0	127.85	50.33	57.38	6.04	4.02
50	124.83	55.36	57.30	7.04	6.04
100	124.96	54.22	54.63	5.03	4.02
250	131.88	54.36	62.41	7.04	4.02
500	108.72	46.30	48.32	6.44	3.02
1000	81.54	33.22	36.24	5.03	6.04
2500	78.52	32.21	33.22	5.03	3.62

Table 3. Production of organic acids [mmol.l^{-1}] by *L. plantarum* CCM 7102 after 2 and 4 hours of the growth in PYG broth with 0 (control) and 250 Zn^{2+} (zinc) mg.l^{-1} (n = 3)

Organic acid mmol.l^{-1}	Control 2 h	Control 4 h	Zinc 2 h	Zinc 4 h
Lactic	17.80 ± 0.50	18.49 ± 0.55	20.90 ±0.23[a]	21.19 ± 0.23[b]
Acetic	18.07 ± 0.56	18.73 ± 0.66	18.33 ± 0.32	18.69 ± 0.65
Acetoacetic	9.19 ± 0.41	9.26 ± 0.07	9.39 ± 0.12	9.39 ± 0.06
Succinic	6.69 ± 0.25	4.38 ± 0.47	5.22 ± 0.60	4.43 ± 0.17
Formic	3.94 ± 0.16	4.38 ± 0.33	4.03 ± 0.11	4.74 ± 0.38
Valeric	3.76 ± 0.13	4.07 ± 0.23	2.52 ± 0.22	3.32 ± 0.57

a—significantly different from control after 2 hours of growth ($P < 0.001$)
b—significantly different from control after 4 hours of growth ($P < 0.001$)

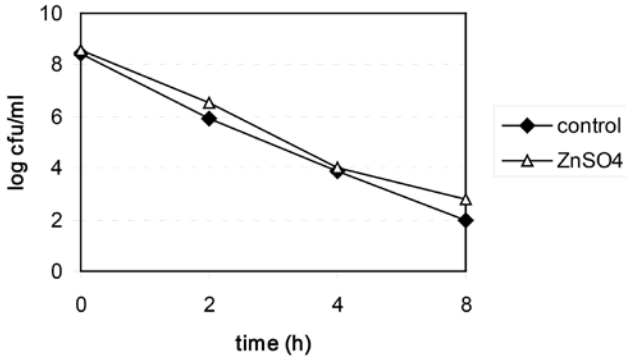

Fig. 2. Influence of zinc sulphate (250 Zn_{2+} mg.l^{-1}) on the survival of *L. plantarum* CCM 7102 in the presence of HCl (pH 2)

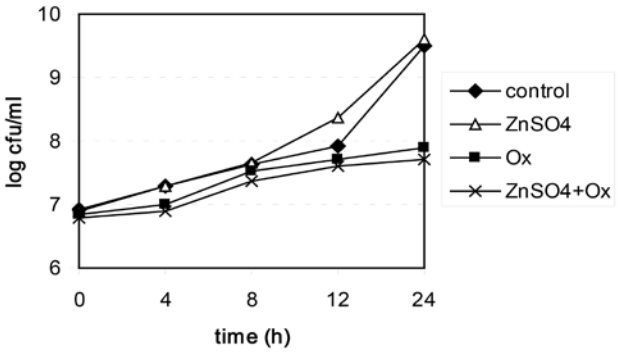

Fig. 3. Influence of Oxgall (0.3 %) and/or $ZnSO_4$ (250 mg Zn^{2+}.l^{-1}) on the growth of *L. plantarum* CCM 7102

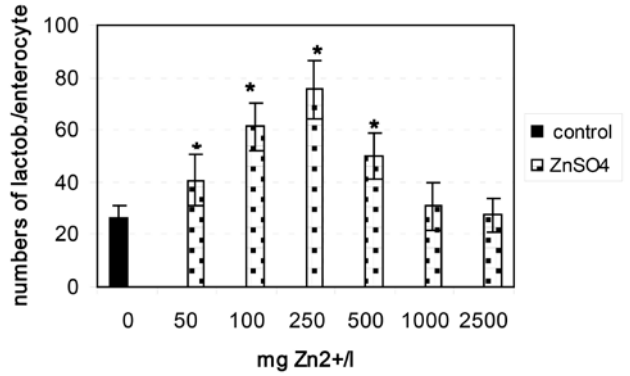

Fig. 4. The influence of various Zn^{2+} concentrations (mg.l^{-1} of growing medium) on the adherence of *L. plantarum* CCM 7102 to the isolated porcine enterocytes (n = 50)

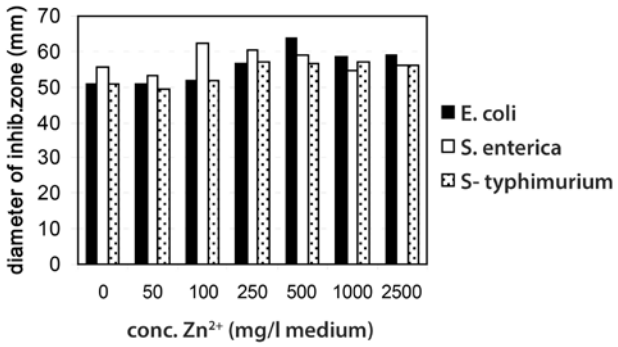

Fig. 5. The influence of various $ZnSO_4$ concentrations (mg.l^{-1} Zn^{2+} of growth medium) on the inhibition of pathogens by *L. plantarum* CCM 7102 (n = 5)

most the same in all groups. After 4 hours the viable counts in the both groups with Oxgall (0.3 %) were of 0.3—0.4 log lower than in the control group. After 8 and 12 hours it was of 0.1—0.3 log. After 24 hours the numbers of lactobacilli in the groups with Oxgall, and Oxgall plus $ZnSO_4$ were $7.9.10^7$ and 5.10^7 cfu.ml^{-1} respectively, whereas that for control was $3.2.10^9$ cfu.ml^{-1}.

$ZnSO_4$ in concentrations of: 50—500 mg Zn^{2+}.l^{-1} were found to significantly ($P < 0.001$ in all cases) increase the adhesion of *L. plantarum* CCM 7102 to isolated porcine enterocytes (Fig. 4). The highest numbers of adhering lactobacilli (75.4 ± 11.1; $P < 0.001$ in comparison to all other groups) were noted when 250 mg Zn^{2+}.l^{-1} was added.

This strain was classified as weakly adherent to porcine intestinal mucin and zinc had not influenced its adhesion (Table 4).

The addition of zinc to the growing media for *L. plantarum* CCM 7102 had increased its inhibitory efficiency towards pathogens (Fig. 5). The growth of *E. coli* O8:K88$^+$ent$^+$ ($P < 0.05$—0.001) and *S. Typhimurium* ($P < 0.01$) were significantly strongly inhibited in the presence of 250—2500 mg Zn^{2+}.l^{-1} as compared to the control without the addition of zinc. Antibacterial activity of this strain against *S. enterica* was the highest at the concentrations of 100—500 mg Zn^{2+}.l^{-1} ($P < 0.05$—0.001).

Table 4. The influence of various Zn^{2+} concentrations (mg.l^{-1} of growing medium) on the adherence of *L. plantarum* CCM 7102 to the crude intestinal mucin (n = 5)

Zn2+ conc. mg.l^{-1}	Binding to mucin A570 nm (x ± sd)
0	0.176 ± 0.035
100	0.156 ± 0.030
250	0.172 ± 0.033
500	0.174 ± 0.039
1000	0.192 ± 0.074
ANOVA	P > 0.05

Based on measurements by atomic absorption spectrometry it was found that *L. plantarum* CCM 7102 was not able to concentrate zinc into the biomass from the culture media, when $ZnSO_4$ was added in concentrations 0—2500 mg $Zn^{2+}.l^{-1}$.

This strain is resistant to streptomycin, neomycin, oxacillin, colistin, kanamycin, vancomycin, nalidixic acid, and gentamycin and it is susceptible to other tested antimicrobials. The addition of 0—2500 mg $Zn^{2+}.l^{-1}$ into the culture media did not affect the susceptibility of strain CCM 7102 to the tested antimicrobials.

DISCUSSION

The fundamental prerequisite for potential efficacy of a probiotic preparation for oral application is the selection of appropriate bacterial strains with good gastrointestinal colonization abilities, antimicrobial activity, tolerance of conditions in the gastrointestinal tract, resistance to different antimicrobial agents, survival during processing and storage, and autochthonous origin, eventually with other required properties [34].

Tests of different *Lactobacillus* strains have showed that resistance to higher concentrations of zinc in growth media is strain-dependent. In our previous studies 16 poultry *Lactobacillus* strains were tested in media with a high concentration of zinc. Big differences were found among the strains tested, where only one strain of *L. fermentum* was resistant to high concentrations (5000 mg $Zn^{2+}.l^{-1}$) of zinc [26]. Højberg et al. [21] noted a lowered number of lactobacilli, especially *L. reuteri* and *L. amylovorus*, in postweaning pigs receiving high ZnO doses (2500 ppm), whereas coliforms were increased. Similar results were received in studies performed by Broom et al. [8].

As indicated by the results of this study, the influence of zinc sulphate on the tested properties of lactobacilli was dependent on its concentration in the growth media. Low concentrations of zinc (< 100 mg $Zn^{2+}.l^{-1}$) did not influence growth and probiotic properties of the lactobacilli, whereas concentrations of 250 and 500 mg $Zn^{2+}.l^{-1}$ had accelerated the start of growth and concentrations 1000 and 2500 mg $Zn^{2+}.l^{-1}$ have retarded their growth. The growth dynamic was monitored on the basis of pH decrease because of the high turbidity of media after the addition of higher concentrations of zinc sulphate (> 200 mg $Zn^{2+}.l^{-1}$), where $Zn(OH)_2$ was formed. Zinc hydroxide was dissolved when organic acids were produced by lactobacilli and therefore the turbidity of such media gradually decreased. The decrease of pH was connected to organic acid production, above all lactic acid and that was reflected in certain extended fermentating activities and the growth dynamic of lactobacilli. These organic acids are weak acids and therefore a part of them is undissociated in the solution. For these reasons the concentration of H^+ ions does not reflect exactly the true content of acids in the media [4].

The production of organic acids by lactobacilli is very important for the regulation of gut microflora growth and composition. The inhibitive effect of organic acids is based on the reduction of gut content pH to values where the growth of unfavourable microbiota is depressed or stopped [34, 35]. In addition to lactic acid, which is produced in the highest concentrations, heterofermentative lactobacilli also produce other types of organic acids, primarily acetic and acetoacetic acid. The *L. plantarum* CCM 7102 produces, in addition to lactic acid, relatively high concentrations of acetic and acetoacetic acids which serves as a good prerequisite for the efficacy of a probiotic strain. Adams and Hall [1] have confirmed the synergistic effect of lactate and acetate in the inhibition of pathogens. Lactic acid has decreased pH, whereby the toxicity of the undissociated form of lactate was increased. During the first hours of growth the levels of lactate and acetate produced by *L. plantarum* CCM 7102 were similar, however during the exponential phase the concentration of lactate increased sevenfold while the concentration of acetate only increased threefold. The addition of 250 mg $Zn^{2+}.l^{-1}$ had a positive influence on the production of lactic acid after 2 and 4 hours of growth which could be a result of a faster growth start. Initially, concentrations of acetate and acetoacetate were not affected. Lower concentrations of organic acids were produced by the strain, when 500, 1000 and 2500 mg $Zn^{2+}.l^{-1}$ was added to the growth media. It can be assumed that an excess of zinc ions inhibits acidogenesis, but the reason could also be attributed to H^+ ion consumption by zinc hydroxide dissolving or the delayed growth of lactobacilli. Strong inhibition of acidogenesis in oral streptococci was noted when 1 mM Zn^{2+} in the form of $ZnCl_2$ was added to the medium. However acid production by *L. casei* was inhibited only weakly [20]. The decrease of succinic acid concentration between 2nd and 4th hour of growth can be caused by its metabolization—decarboxylation to propionic acid.

Successful colonization of the gut is dependent upon the survival of high counts of microorganisms in the conditions of the gastrointestinal tract—low pH in the stomach, the presence of bile and gut secretions, and adhesion to the gut mucosa [12, 38]. By oral administration of probiotics, their acid-resistance has a marked effect on their numbers and viability during passage through the stomach. Conway and Kjelleberg [14] reported that the antibacterial effect on lactic acid bacteria is evident by pH lower than 2.5. Neumann and Ferreira [29] have studied the influence of artificial gastric fluid (pH 2) in 3 strains of *L. acidophilus*, whereby the numbers of bacteria were reduced by 2—2.5 log after 2 h, and by 3.5—4 log after 4 h. In this study all 3 strains were classified as acid-resistant. The numbers of our strain were reduced (pH 2) by 2—2.5 after 2 h, by 4.5 after 4 h, and after 8 h this strain survived in numbers of 10^2 cfu.ml^{-1}. In the presence of $ZnSO_4$ the pH decrease was slightly lower in comparison to the control media (after 8 h by 5.8 log compared to 6.5 log in control). It is possible to suppose that a part of H^+ ions can react with precipitated zinc hydroxide and therefore pH could be slightly increased. The viability of microorganisms in the stomach under *in vivo* conditions is also influenced by the presence of food which decreases the impact of gastric fluids. Therefore, the higher viability *in vivo* can be assumed as compared to results received *in vitro* [29].

The resistance to bile salts differs considerably among lactobacilli strains. However the growth delay and reduced volume of bacterial biomass was observed in all strains of *Lactobacillus* cultured in media with the addition of bile salts [17, 38]. In our experiment growth delay was also noted, though it was statistically significant only from the beginning of the exponential phase (after 4th h). Zinc sulphate moderately augmented the inhibitive effect of 0.3 % Oxgall, which was expressed as lower numbers of lactobacilli (0.1—0.2 log) and higher final pH values (approx. 0.4).

The adhesion of *L. plantarum* CCM 7102 to isolated porcine enterocytes was significantly increased by the addition of zinc sulphate. The enhancement of this adhesive ability of lactobacilli by the addition of zinc can be explained by the formation of donor-acceptor bonds between the bacterial surface and enterocytes. Thus two- and three- valent cations can affect the adherence. Kleeman and Klaenhammer [24] noted increased adhesion of lactobacilli to human foetal enterocytes in the presence of calcium cations. Conway and Kjelleberg [14] have confirmed the participation of two-valent cations in the adhesion of *L. fermentum* to mouse stomach epithelial cells. The contribution of calcium cations to adherence of some *Lactobacillus* strains to Caco-2 cells was also observed by Chauviére et al. [10]. Kleeman and Klaenhammer [24] considered the mechanism of cation-influenced adhesion to be nonspecific and different from adhesion running without cation presence. It can be expected that zinc ions in the intestine can positively affect the adhesive abilities of the strains. Nemcová et al. [28] tested adherence of three *Lactobacillus* strains including *L. plantarum* CCM 7102 in gnotobiotic piglets. All three strains, showing very good adhesion ability *in vitro*, adhered to the jejunal and ileal mucosa in high numbers (10^4—10^5 cfu. cm^{-2}). Moreover, Zarate et al. [40] have observed the similar adhesive capability of propionibacteria under *in vitro* and *in vivo* conditions. On the other hand, Pedersen and Tannock [32] found that the adhesion of lactobacilli to porcine enterocytes *in vitro* did not correspond with their ability to colonize the gut *in vivo*.

The inhibition of tested pathogens (*E. coli, S. enterica, S. Typhimurium*) by *L. plantarum* CCM 7102 was increased after the addition of $ZnSO_4$ in concentrations of 100—2500 mg Zn^{2+}.l^{-1}. The increased antibacterial effect is probably a result of antimicrobial activity of zinc ions as well as improved probiotic properties of *Lactobacillus* strain (higher production of lactic acid, faster growth start, etc.) caused by the presence of zinc in the growth media. The antibacterial effect of zinc on different pathogens including *E. coli* and salmonellae was noted by other authors [13, 21, 27].

Even though some bacteria (e. g. *Bacillus* spp., *Microcystis* spp., some rumen bacteria) are able to bind zinc from their environment, our strain did not have this property [20, 33, 37].

CONCLUSIONS

Based on our results it is possible to consider using zinc sulphate (in concentrations 100—500 mg Zn^{2+}.l^{-1}) as an efficient enhancement of *L. plantarum* CCM 7102. Besides the positive effect on growth, production of lactic acid, adhesion to enterocytes and inhibition of pathogens, this strain does not influence resistance to antimicrobials, or low pH and also the resistance to bile salts is affected only weakly.

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