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In vitro assessment on effect of duodenal contents on the lead (Pb^{2+}) binding capacity of two probiotic bacterial strains

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ABSTRACT

In vitro Lead (Pb^{2+}) binding capacity of two probiotic bacteria strains, namely *Bifidobacterium longum*BB79 and *Lactobacillus pentosus*ITA23, was assessed following incubation with the intestinal contents (IC) of laying hens. Results of this study demonstrated that IC treatment significantly enhanced ($P < 0.01$) Pb^{2+} binding capacity of both bacterial strains. Fourier transform infrared analysis indicated that several functional groups (O-H or N-H, C-H, C=O, C-O, and C-O-C) on the bacteria cell wall involved in metal ion binding were altered after IC incubation, and new groups appeared between the 3700 cm^{-1} and 4000 cm^{-1}bands. Transmission electron microscopy demonstrated that after incubation with IC, unidentified IC components created new binding sites on the bacterial cell surface. These particles also changed the mechanism of Pb^{2+} binding of the two strains from intracellular accumulation to extracellular adsorption.

1. Introduction

Lead (Pb^{2+}) is a toxic heavy metal, and its contamination to the environment is mainly through anthropogenic sources, including metal mines (Florea et al., 2005; Zoghi et al., 2014). After entering the organisms, Pb^{2+} accumulates in the kidney and liver (EI-Sayed, 2013; Fu and Wang, 2011; Lei et al., 2014), disrupting basic cellular processes and adversely affecting the nervous system. The removal of toxic metals with bacteria has received increasing interest because it is safe and cost effective (Zoghi al., 2014). Several studies have investigated the Pb^{2+} binding capacity of probiotic bacteria and microbial products such as exopolysaccharides (Brown and Lester, 1982; Feng et al., 2012).

The biosorption mechanisms used by microorganisms to bind toxic metals were recently elucidated. In general, microbes remove metals with the following pathways: i) metal cations bind to cell wall surfaces; ii) metal ions are actively translocated in to the cells by metal binding proteins; iii) metal precipitation reacts with extracellular polymers or anions produced by microbes; and iv) metal volatilization occurs byenzyme-mediated biotransformation (Ahemad and Kibret, 2013; Giller et al., 2009). Several studies have reported enhancement of bacterial metal binding capacity by exploiting the above mentioned mechanisms (Kinoshita et al., 2013; Wu et al., 2016) and modification of bacteria to enhance metal ion binding capacity has received extensive attention in the heavy metal bioremediation. For example, Luo et al. (2014) grafted *Pseudomonas sp*. Lk9 with polyallylamine layer-by-layer (a process that likely increased the functional group sites) to enhance metal ion removal capacity. Heat or ethanol treatments have also been reported to increase the binding sites on bacterial cell walls (Göksungur et al., 2005;Zoghi et al., 2014). However, these anthropogenic treatments might not be the sole factors influencing bacterial cell metal binding capacity under natural conditions, because other factors, including pH and the concentration of biosorbents, can also influence or alter bacterial binding capacity (Ahemad and Kibret, 2013).

The application of probiotic bacteria to mitigate heavy metal toxicity in poultry production has gained increasing attention (Ebrahimi et al., 2015; Mustafa and Abdullah, 2009). Chickens are particularly susceptible to Pb^{2+} poisoning, even at trace levels (1.0 mg/kg diet) (Bakalli et al., 1995; Roegner et al., 2013). Furthermore, animal products such as eggs and milk are easily contaminated with $Pb^{2+}$from exposed animals (Roegner et al., 2013). Although the safe limit of heavy metals such as Pb^{2+} in animal feeds is regulated by most

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national feed standards, the implementation and monitoring of these regulations are often difficult or absent in many developing countries. Therefore, the use of probiotic bacteria, which are also known to promote gut health and nutrient absorption, might be an attractive option to the poultry producers.

As previously mentioned, the metal biosorption capacity of probiotic bacteria is influenced by many environmental factors. In poultry, the intestine, specifically the duodenum, is a major adsorption site for metal ions. However, we do not know of any study assessing the influence of intestinal contents (IC) on the metal binding capacity of probiotic bacteria. In this study, the effect of layer hen duodenal contents on *in vitro* Pb^{2+} binding capacity of two probiotic bacteria (*Bifidobacterium longum* BB79 and *Lactobacillus pentosus* ITA23) was tested. In addition, the possible mechanisms conferring Pb^{2+} binding capacity in the two bacterial strains, including identifying changes in the adhesion sites of functional groups on the cell surface, were elucidated.

2. Materials and methods

2.1. Bacterial strain preparation and culture conditions

The two probiotic bacteria strains, namely *Bifidobacterium longum* BB79 (American Type Culture Collection 51870, China Center of Industrial Culture Collection 6187) and *Lactobacillus pentosus* ITA23 (Ebrahimi et al., 2015; provided by the Institute of Tropical Agriculture and Food Security, University Putra Malaysia) were selected for this study because of their high PB^{2+} binding efficacy based on an earlier preliminary study. The two bacteria strains were cultured in De Man, Rogosa, Sharpe (MRS) broth (HuanKai Microbiology, Guangzhou, China) for 24 h at 37 °C in anaerobic jars containing AnaeroPack (Mitsubishi Gas Chemical Company, Inc. Tokyo, Japan). Before the experiment, the bacteria were washed three times with deionized water to remove any remaining culture medium (Teemu et al., 2008).

The bacterial cells were dyed with 5(6)-carboxy fluorescein diacetate succinimidyl ester (CFDA-SE, Life Technologies Corporation, New York, USA) to conveniently differentiate the cells from the IC. Briefly, CFDA-SE was suspended in dimethyl sulfoxide (DMSO) to obtain a final stock solution of 1000 μmol/L. Then, the solution was sterilized by filtering through a 0.22 μm membrane. The bacterial pellets were suspended in phosphate buffered saline (PBS, pH 7.0) to a final concentration of 1×10^9 CFU/mL, and the final concentration of CFDA-SE was 20 μmol/L. After lucifuge incubation for 20 min at 37 °C, the bacterial pellets were washed three times with PBS to remove any remaining dye. The dyed bacteria were immediately incubated in an IC suspension for 1 h at 41 °C as reported by Menkeand Steingass (1988) with minor modifications (1 g IC was diluted with 3 mL PBS). The IC was collected from the duodenum of 200-ᴅ-old laying hens; briefly, 50 laying hens were slaughtered by bleeding in the morning before feeding, and the duodenum was immediately collected to transfer the IC into a sterile tube. All of the laying hens came from a local farm (Guang Zhou, China). The dyed bacteria for the control groups were incubated in PBS solution without IC. The pH of each suspension was adjusted to and maintained at5.7 with 1 M NaOH or HNO_3. After incubation, the bacterial cells were sorted using flow cytometry (FACS Aria II, BD, USA)to evaluate their Pb^{2+} binding capacity.

2.2. Lead binding

To investigate the influence of IC on bacterial Pb^{2+} binding capacity, both the probiotic bacterial strains treated with IC and the respective controls (non-IC treated bacteria) were cultured in Pb^{2+} solutions. The experiment was performed in triplicate.

The concentration of the Pb^{2+} [$Pb(NO_3)_2$, Aladdin, China]in aqueous solution was 50 mg/L, and the final pH was adjusted to 5.0 using1M NaOH or HNO_3. The two IC-treated bacterial strains and the control groups (3.0×10^7 bacterial cells in each group) were incubated with 3 mL Pb^{2+} solution for 1 h at room temperature. After the binding treatment, each replicate was centrifuged (16000 g, 5 min, 4 °C), and 2 mL of the supernatant was preserved with 200 μL ultrapure HNO_3 for Pb^{2+}concentration analysis. The bacterial pellets were collected to characterize the functional groups and Pb^{2+} binding process using Fourier transform infrared (FTIR)and transmission electron microscopy (TEM). The concentration of $Pb^{2+}$was determined using an inductive coupled plasma mass spectrometer(7700 series ICP-MS, Agilent Technologies, Australia). The Pb^{2+} binding capacity of probiotic bacteria was calculated using the following equation:

$$Q = \frac{Ci - Cf}{N},$$

in which Q is the equilibrium Pb^{2+} concentration (mg/10^{10} CFU), Ci is the initial Pb^{2+} concentration (mg/L), Cf is Pb^{2+} final concentration, and N is the concentration of bacteria cells (1.5×10^{10} CFU/L).

2.3. Characterization of functional groups

Bacterial pellets incubated with and without IC were collected to investigate effect of IC on changes to the functional groups on the cell wall surface, which might affect Pb^{2+}binding. The IC-treated bacteria after Pb^{2+} binding (Section 2.2) were also collected to investigate changes to several major functional group sites. All samples were lyophilized before FTIR analysis, and the spectra were collected with an FTIR spectrophotometer (VERTEX33, Bruker, Germany) to characterize various functional groups. The frequency range of the FTIR measurements was 4000–500 cm^{-1}.

2.4. Transmission electron microscopy

After the Pb^{2+} binding experiment, all experimental bacterial cells and native bacterial cells (not incubated with Pb^{2+}or IC) were fixed with 5% glutaraldehyde in 0.16 M s-collidine buffer (Teemu et al., 2008). Before the bacterial pellets were dehydrated with an ethanol of various concentrations (30%, 50%, 70%, 90% and 100%), 1% osmium tetroxide was used to fix the bacterial pellets for 2 h. Dried bacterial cells were embedded in epoxy resins and cut into 4 μm sections. The slices were stained with uranyl acetate and observed with TEM (Tecnai G2 20, FEI, Hong Kong).

2.5. Statistical analysis

All experiments were performed in triplicate. Data were analyzed with analysis of variance (ANOVA)using Statistical Package for the Social Sciences (SPSS) software, version 17.0. Significant differences between the means were determined by Tukey's test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Lead binding capacity

The Pb^{2+} binding capacity of the probiotic bacterial strains with or without IC treatment is shown in Fig. 1. The final Pb^{2+}concentrations in the supernatant of the two IC-treated strains were lower than those of the untreated control groups, suggesting that IC treatment enhanced Pb^{2+}binding capacity of the two bacterial strains (*B. longum* BB79, $P = 0.04$; *L. pentosus* ITA23, $P < 0.01$).

3.2. FTIR analysis of functional groups

To elucidate how IC enhances Pb^{2+} binding capacity, the major functional groups involved with Pb^{2+} binding were identified with