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Screening of *Lactobacillus* strains for their ability to bind Benzo(a)pyrene and the mechanism of the process

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ABSTRACT

In order to investigate the binding ability of *Lactobacillus* strains to Benzo(a)pyrene (BaP), 15 strains were analysed. *L. plantarum* CICC 22135 and *L. pentosus* CICC 23163 exhibited high efficiency in removing BaP from aqueous medium; the binding rates were 66.76% and 64.31%, respectively. This process was affected by temperature, incubation time and pH, and cell viability was not necessary for the binding ability. Additionally, both strains, especially strain CICC 23163 showed high specificity in binding BaP. The cell-BaP complexes were stable in aqueous medium. The mechanism of binding was investigated by examining the binding ability of different components of the microorganism cells. The results revealed that peptidoglycans played an important role in binding BaP and its structural integrity was required. Consequently, we proposed that the mechanism of this process was a physisorption and peptidoglycan was the main binding site. These two strains may be used for dietary detoxification in human diet and animal feed.

1. Introduction

Carcinogens occurring in food have harmful effect on human and animal health. Many carcinogens were found in human diet over the past decades, such as mycotoxins, polycyclic aromatic hydrocarbons (PAHs), heavy metals, and nitrosamines. Some of them are from environment and some may be produced during the processing or cooking of foods. Benzo(a)pyrene (BaP) is a member of PAHs and recognized as one of the most potent carcinogens that can cause breast cancer (Kazerouni et al., 2001). BaP is usually used as a marker to reflect PAHs levels in environmental samples and is the most known and studied member of PAHs (Narayan et al., 2004). BaP is contained in a wide variety of foods, especially roasted, smoked and fried foods (Lijinsky, 1991).

Lactic acid bacteria (LAB) are the most important probiotic microorganisms and having beneficial health effect when ingested by humans. They are widely used in food industry. The binding ability of LAB has been well documented. It was found that supplementation with *Lactobacillus* could attenuate the induction of colon cancer caused by 2-amino-3-methyl-3H-imidazo[4,5-f]quinolone (Knasmüller et al., 2001). Specific strains of LAB have the ability to bind aflatoxins and other mycotoxins to their surface (Fuchs et al., 2008; Hernandez-Mendoza et al., 2009a; Pizzolitto et al., 2012). *Enterococcus faecium* can also be used to remove aflatoxin B_1 and patulin, and the removal of aflatoxin B_1 and patulin was highest at pH 7.0 and 4.0, respectively (Topcu et al., 2010). Halttunen et al. (2008) reported that several LAB strains together might be beneficial for the removing of several toxic compounds. Pizzolitto et al. (2012) stated that cell wall structural integrity of the microorganisms was required for fumonisin B_1 removal and cell envelope was the binding site.

So far, most of the reports are focused on the removal of mycotoxins. There are almost no investigations about the binding properties of LAB towards BaP. Therefore, the objectives of this study were: (i) to determine the removal of BaP by *Lactobacillus*; (ii) to investigate the nature of the mechanism involved in BaP–microorganism interaction.

2. Materials and methods

2.1. Bacterial strains, growth medium and cultural conditions

Lactobacillus strains used in this study were listed in Table 1. *Lactobacillus* strains were cultured in MRS (De Man, Rogosa, and Sharpe) medium for 18 h at 37 °C under anaerobic condition. Viability was confirmed by standard count methods using MRS agar at 37 °C for 48 h under anaerobic condition.

2.2. Benzo(a)pyrene binding assay

BaP (Sigma) was dissolved in dimethylsulfoxide (DMSO) to obtain a concentration of 1 mg/mL (stock solution). The working solution I (100 μg/mL) was obtained by dissolving 1 mL stock solution in 9 mL DMSO. Working solution II (10 μg/mL) was prepared by adding 1 mL working solution I into 9 mL sterile double-distilled

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Table 1
The binding ability of different strains.

Strains	Source[a]	Binding rate (%)	Binding after water washes	Binding after benzene washes
Lactobacillus salivarius	CICC 23182	11.24 ± 0.92	11.24 ± 1.26	4.79 ± 2.16
Lactobacillus casei	CICC 23185	26.47 ± 1.19	25.65 ± 0.83	7.85 ± 1.37
Lactobacillus plantarum	CICC 22135	66.76 ± 0.95**	66.72 ± 1.31	8.32 ± 1.22
Lactobacillus plantarum	CICC 23165	19.26 ± 0.86	19.22 ± 1.26	6.47 ± 0.93
Lactobacillus rhamnosus	ATCC 7469	35.22 ± 1.12	34.75 ± 1.65	7.98 ± 1.44
Lactobacillus acidophilus	ATCC 4356	39.93 ± 1.31	39.03 ± 2.12	6.91 ± 1.75
Lactobacillus murinus	CICC 23150	32.13 ± 0.91	32.13 ± 1.37	7.61 ± 1.44
Lactobacillus plantarum	CICC 22133	41.32 ± 1.35	41.08 ± 1.88	6.37 ± 2.17
Lactobacillus pentosus	CICC 22156	12.93 ± 0.31	12.93 ± 0.95	5.93 ± 1.21
Lactobacillus pentosus	CICC 22193	14.81 ± 0.76	14.81 ± 1.62	5.84 ± 1.39
Lactobacillus helveticus	CICC 22171	48.59 ± 0.18	47.83 ± 1.22	8.02 ± 1.53
Lactobacillus casei	CICC 23184	12.20 ± 0.97	12.00 ± 1.33	5.39 ± 0.73
Lactobacillus pentosus	CICC 23163	64.31 ± 1.42**	64.11 ± 1.87	7.83 ± 1.02
Lactobacillus salivarius	CICC 23175	18.56 ± 1.06	18.48 ± 1.45	5.52 ± 1.25
Lactobacillus rhamnosus	CICC 23119	22.81 ± 0.78	22.52 ± 1.47	5.79 ± 0.68

[a] ATCC, American type culture collection; CICC, China center of industrial culture collection.
** $P < 0.01$ compared with other strains. The concentration of BaP was 10 μg/mL.

H_2O. Cells of *Lactobacillus* strains were collected by centrifugation at 5000*g* for 15 min at 4 °C. The pellets were washed twice with sterile double-distilled H_2O. The cell concentration was determined using plate count methods. Then culture (approx. 5.0×10^9 CFU/mL) was suspended in 1.0 mL of working solution II contained BaP (10 μg/mL) and incubated for 4 h at 37 °C. The cells were removed by centrifugation and supernatants containing unbound BaP were collected and analyzed by high performance liquid chromatography (HPLC).

To evaluate the stability of the bacterial cell–BaP complex, the complex was suspended in 1 mL of sterile double-distilled H_2O or benzene and incubated for 30 min at 37 °C with shaking. Then, the bacterial cells were removed and the supernatant was collected for HPLC analysis. Positive (sterile H_2O + BaP) and negative (sterile H_2O + cells) controls were included for all experiments. The percentage of BaP bound to the bacteria was calculated using the equation:

$$\text{Binding rate } (\%) = 100 \times \left(1 - \frac{\text{BaP peak area of sample}}{\text{BaP peak area of positive control}}\right)$$

2.3. The effect of temperature, pH, time and cell viability on binding ability

To determine the effect of temperature on binding ability, cells and BaP were incubated for 4 h at 5 °C, 20 °C, 37 °C and 50 °C, respectively. Then samples were analyzed by HPLC.

The effect of pH on the binding ability was performed in 0.1 M PBS (phosphate-buffered saline) medium contained BaP. The pH of the PBS medium was adjusted to 3.0, 4.0, 5.0, 6.0 and 7.0 with 1.0 M HCl.

The effect of incubation time was determined during 48 h. Samples (1.0 mL) were collected by centrifugation after incubation for 1, 2, 4, 10, 24 and 48 h, and analyzed by HPLC.

The effect of cell viability on binding ability was conducted by heat-treatment (121 °C, 15 min) and acid-treatment (2.0 M HCl for 90 min) of the cells before incubation with BaP. After treated, the cells were washed three times with sterile double-distilled H_2O. Then binding assay was performed as given above (Section 2.2). Nonviable cells were monitored by plating in MRS agar.

2.4. Determination of the binding specificity

In order to determine the specificity of binding process, folic acid, riboflavin, caffeine and vitamin B_{12} were chosen to mix with BaP (Turbic et al., 2002). The concentration of each substance was 10 μg/mL. Binding assays were performed as described above (37 °C, pH 6.0 for 4 h). Same parameters were used following binding assays. The quantifications of folic acid, riboflavin, caffeine and vitamin B_{12} were also carried out by HPLC analysis.

2.5. The binding ability of different cellular structures

In this experiment, some cell components which may involve in binding process were obtained to determine the binding ability. Same amount of cell (approx. 5.0×10^9 CFU/mL) was used. The details were as follows:

2.5.1. Spheroplasts

Spheroplast is a cell from which the cell wall has been removed. The preparation of spheroplasts was conducted according to previous report (Pizzolitto et al., 2012). Briefly, *Lactobacillus* was cultivated in 10 mL of MRS broth at 37 °C for 16 h. Then, cells were kept in ice water bath for 30 min. Pellet was obtained by centrifugation (5000*g*, 15 min, 4 °C). After washing twice with 0.02 M PBS (pH 7.0), the cells were suspended in 10 mL of TMS buffer (0.01 M Tris–HCl, 0.02 M $MgCl_2$, 0.5 M Sucrose). Lysozyme (Sigma) was then added to the suspension and incubated for 120 min. Following, spheroplasts were collected at 2000*g* for 5 min and washed twice with TMS buffer. Finally, spheroplasts were resuspended in 10 mL of TMS buffer for binding analysis.

2.5.2. The effect after removing exopolysaccharides and S-layer protein

The removal of exopolysaccharides (EPS) and S-layer protein (SLP) was carried out according to the reports by Hernandez-Mendoza et al. (2009a) and Syvie et al. (1992), respectively. After removing the two components, cells were collected by centrifugation. Then, binding assays were performed.

2.5.3. Cell wall

The cell walls were purified as described by Gopal and Reilly (1995). The cell suspension was disintegrated by ultrasonic vibrations using a sonifier (Xinzhi, Ningbo, China). The whole cells were removed by centrifugation (3000*g*, 10 min, 4 °C). Cell walls were obtained by centrifugation at 15,000*g* for 15 min at 4 °C, and then treated with 8.0 g/L of sodium dodecyl sulfate (SDS) at 90 °C for 1 h. Finally purified cell walls were obtained by removing SDS and resuspended in same amount of sterile water. 1.0 mL cell walls suspension was incubated with BaP (10 μg/mL) for 4 h at 37 °C.

2.5.4. Teichoic acids and peptidoglycans

The teichoic acids were purified as report by Yu et al. (2009). Intact structural peptidoglycans were obtained according to previous report (Song et al., 2005). Then, a part of peptidoglycans was disintegrated by ultrasonic vibrations using a sonifier (Xinzhi, Ningbo, China). Finally, teichoic acids, intact structural peptidoglycans and disintegrated peptidoglycans were used to evaluate binding ability.

2.6. Analytical methods

Benzo(a)pyrene was determined by HPLC (Agilent 1200) according to the report by Lei and Shi (2007). Supernatants were treated by reduced pressure distillation until the volume was 150 μL. Then supernatants were extracted by chloroform (500 μL) and the samples were obtained after removing aqueous phase. Samples were filtered immediately through a 0.45 μm membrane and frozen (−20 °C) in 2-mL vial replicates until analysis. The separation was performed on a Phenomenex C18 column (5 μm, 4.6 mm × 250 mm) at room temperature. The mobile phase was methanol, and the flow rate was 1.0 mL/min. The detection was performed at 299 nm and the sample injection volume was 10 μL.

Folic acid, riboflavin, caffeine and vitamin B_{12} were also determined by HPLC according to the reports by Turbic et al. (2002) and Akhtar et al. (2000).

2.7. Statistics

All experiments and analyses were carried out in triplicate and the values represented as the mean values. Microsoft Office Excel 2010 and SPSS (17.0) system software were used to data analysis. One-way ANOVA and independent-samples *T*-Test were used for statistical analysis.