EFFECT OF TIME AND INCUBATION TEMPERATURE ON ABILITY OF PROBIOTICS FOR REMOVAL OF POLYCYCLIC AROMATIC HYDROCARBON IN PHOSPHATE BUFFER SALINE

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ABSTRACT

The ability of *Lactobacillus acidophilus* LA-5, *Bifidobacterium lactis* BB-12, *Lactobacillus delbrueckii* subsp. *bulgaricus* PTCC 1737 and *Streptococcus thermophilus* PTCC 1738 in removing Benzo(a)pyrene (BaP), Benz(a)anthracene (BaA), Chrysene (Chr) and Benzo(b)fluoranthene (BbF) from contaminated aqueous solution with respect to strain types, time and temperature of incubation was studied. The results showed that BaA, Chr, BbF and BaP could be significantly removed from the phosphate buffer saline by the tested bacteria and this reduction significantly depends on strain types and incubation temperature and time. The reduction of four PAHs varied in the range from 50.94% to 74.51%, 54.48% to 77.98%, 47.35% to 68.84%, and 61.96% to 81.22% for BaA, Chr, BbF and BaP, respectively. The results illustrated that by increasing time and temperature of incubation, binding ratio increased and the highest binding ratio of four PAHs was achieved by *L. acidophilus* LA-5 at 37 °C. Furthermore, the FTIR analysis was performed for identifying the different functional groups that might participate in the binding of PAHs to bacterial cell wall. The FTIR revealed that cell wall has an important role in removal of BaA, Chr, BbF and BaP from phosphate buffer saline.

Keywords: Adsorption, Cell wall, FTIR, Lactic acid bacteria, Polycyclic aromatic hydrocarbon, Probiotics

INTRODUCTION

The presence and formation of carcinogenic and toxic compounds in food are one of the most important human concerns in the food industry in recent years. Heavy metals, mycotoxins, nitrosamines, heterocyclic aromatic amines (HCA), acrylamide and polycyclic aromatic hydrocarbons (PAHs) can be named as an example of these carcinogens that have been found in human diets **(Khorshidian et al., 2016)**. Some of these compounds come from the environment, water and contaminated raw materials, and some others are formed during food processing **(Zhao et al., 2013)**. Polycyclic aromatic hydrocarbons (PAHs) are a group of organic compounds that contain at least two fused aromatic rings of carbon. They are environmental pollutants and can enter the food chain through contamination of water, air and soil **(Amirdivani et al., 2019; Purcaro et al., 2013; Yousefi, Shemshadi, et al., 2018)**. Beside of incomplete combustion of fossil fuels, pyrolysis of organic matter and geochemical processes, PAHs can be formed through different food processing methods such as frying, barbecuing, smoking, roasting, baking and drying **(Bansal & Kim, 2015; Naseri et al., 2010; Yousefi, Shemshadi, et al., 2018)**. Therefore, contamination of various foodstuffs such as smoked food products, meat products, oil and fat with PAHs is inevitable.

Except for occupational and smoker individuals, the most important route of PAHs exposure takes place by dietary intake of food and water **(Bansal & Kim, 2015; Shi et al., 2016)**. It has been reported that PAHs have toxic, carcinogenic, mutagenic and teratogenic properties and therefore, due to their proven adverse health effect, there is a concern about the presence of PAHs in foods **(Mocek & Ciemniak, 2016)**. Therefore, different methods are used to significantly reduce contaminants amount to acceptable levels **(Dowaidar et al., 2007; Kawashima et al., 2009; Mandal & Das, 2018; Zeledon-Toruno et al., 2007)**. Today, attention has been drawn to the use of microorganisms, especially probiotics and lactic acid bacteria, in reducing toxic compounds and contaminants **(Eddine et al., 2018; Halttunen et al., 2008; Hamad et al., 2017; Hathout & Aly, 2014; Yousefi, Shariatifar, et al., 2018; Zou et al., 2015)**. Lactic acid bacteria (LAB), particularly, probiotics bring beneficial effects to the host when consumed in adequate quantity. Various health benefits attributed to the LAB and probiotics, among which antimutagenic properties have been considered by researchers. LABs and probiotics may prevent cancer through various mechanisms such as increasing activity of antioxidative enzymes, conversion of procarcinogens and carcinogens into less toxic metabolites, lowering the intestinal pH and binding the mutagens through the cell surface and peptidoglycans **(Gupta & Sharma, 2017; Ryu & Chang, 2013; Tumbarski et al., 2018; Yousefi et al., 2017; Yousefi et al., 2021)**. It seems that the binding of toxin to cell wall is one of the most important mechanisms that participates in removing toxin and carcinogens and many studies investigated the ability of LABs and probiotics in removing various toxins. However, there are few studies in removing PAHs by LABs and probiotics. Therefore, this study is focused on the effect of time and temperature of incubation on the PAHs adsorption of LABs and probiotics as well as determining interaction types.

MATERIAL AND METHODS

Bacterial strains and media

Strains of *Lactobacillus acidophilus* LA-5 and *Bifidobacterium lactis* BB-12 were obtained from Chr. Hansen (Denmark). *Lactobacillus delbrueckii* subsp. *bulgaricus* PTCC 1737 and *Streptococcus thermophilus* PTCC 1738 were purchased from the Iranian Research Organization for Science and Technology (IROST). The strains were kept at -18°C until they used. De Man-Rogosa-Sharpe (MRS) media, all chemical and solvents were provided from Merck (Darmstadt, Germany).

Bacterial growth condition

All strains except for *B. lactis* BB-12 were activated in MRS broth for 24 h at 37°C. *B. lactis* was cultured in the same medium plus 0.05% of L-cysteine at 37°C for 24 h in an anaerobic incubator. Then, 1 ml of 24-hold culture was added to 100 mL fresh MRS broth and incubated at 37°C for about 18-20 h until bacterial population reached to 1×10^{10} CFU/mL. Bacterial count was determined by plate counting method **(Niderkorn et al., 2006)**. After that, the bacterial pellet were obtained from MRS medium by centrifugation at 5000 g for 15 min at 4°C (Hettich, Tuttlingen, Germany). The obtained cells were washed with phosphate buffer saline (PBS, pH 7.2, 0.85% NaCl) and finally used in binding assays.

Preparation of BaP, BaA, Chr and BbF standard solutions

Four PAHs namely Benzo(a)pyrene (BaP), Benz(a)anthracene (BaA), Chrysene (Chr) and Benzo(b)fluoranthene (BbF) which obtained from Sigma–Aldrich (Steinheim, Germany) (purity of ≥ 99.5%) were dissolved in dichloromethane to obtain a stock solution with concentration of 5 mg/mL of each PAHs. The work solution I (0.5 mg/mL) was prepared by adding 1 mL of stock solution into 9 mL methanol. After that, working solution II with concentration of 10 µg/mL of each PAHs was obtained by dilution of working solution I with phosphate buffer saline (pH=5). Based on the previous study, it was found that the beast binding reaction was carried out in pH 5 (**Yousefi et al., 2019**).

Effect of temperature and time of incubation on PAH-binding ability of bacteria

Each bacterial pellet with specific concentration (1×10^{10} CFU/mL) that harvested by centrifuging at 5000 g for 15 min at 4°C was washed twice with phosphate buffer saline and suspended into l mL working solution II containing 10 µg/mL of BaP, BaA, Chr and BbF with pH 5. The pH of the working solution II was adjusted to 5.0 with 1.0 mol/l HCl. The mixture transferred into an Eppendorf vial, shaken thoroughly and finally placed in shaking incubator (100 rpm) at 4, 25 and 37°C for 10 h to evaluate the effect of temperature on the PAHs adsorption of tested bacteria. Moreover, in order to study the effect of incubation time, the mixture of bacterial pellet and PAHs were placed in shaking incubator for 24 h at the best temperature point that have been obtained in the previous step. After incubation, bacterial suspensions were centrifuged and the amount of free PAHs in cell-free supernatant was analyzed using GC-MS. PBS with PAHs and PBS with bacterial strains were used as positive and negative controls, respectively.

Quantitation of unbound PAHs by GC-MS

Unbound BaP, BaA, Chr and BbF in cell free supernatants was determined by GC/MS. Prior to GC analysis, 1 mL of cyclohexane was added to the 1 mL of cell free supernatants and thoroughly mixed for about 1 min and centrifuged at 10000 g for 10 min. Then l µL of the upper phase was directly injected to GC/MS for determining the amount of free BaP, BaA, Chr and BbF. Agilent Technologies 7890A GC system with triple-axis detector (HP-5 MS capillary column, 30m × 250µm ID, 0.25µm film thickness) coupled with a 5975C inert MSD network mass selective detector was used to separate and quantify BaP, BaA, Chr and BbF. Helium was used as carrier gas at a constant flow rate of 1 mL/min. The injection port temperature was 290°C and almost 1 µm of the sample was injected into a split mode for analysis (split ratio 1:25). The column temperature was held at 220°C for 5 minutes. Then increased to 290°C at a rate of 3°C/min and held for 5 min. selected ion monitoring (SIM) mode was used for the quantitative and qualitative analysis of 4 PAHs. The percentage of bounded PAHs was calculated using the following equation:

$$\text{Binding ratio (\%)} = \left[1 - \left(\frac{\text{Peak area of each PAHs in sample}}{\text{Peak area of each PAHs in positive control}}\right)\right] \times 100$$

Fourier Transform Infra-Red (FTIR) spectroscopic analysis

FTIR analysis of bacterial pellet with and without PAHs were carried out using FTIR spectrophotometer (Fourier transform-infrared spectrometer, PerkinElmer Spectrum RX I, Waltham, MA, USA). Two strains that had the highest binding ability were selected for FTIR analysis. The sample pellets were prepared by mixing bacterial pellet with KBr (potassium bromide) and analyzed with a spectrophotometer at room temperature in the range of 4000-400 cm^{-1} at a resolution of 4 cm^{-1}.

Statistical Analysis

All experiments and analysis were carried out in triplicate and the date was expressed as means ± standard deviation (SD). One-way ANOVA and Tukey's test using the SPSS 21.0 software package program (SPSS Inc., Chicago, IL) were utilized for the statistical evaluation of data. Values of $P < 0.05$ were considered as significant.

RESULT AND DISCUSSION

Effect of incubation temperature on binding ability

The results of the PAHs adsorption to the tested bacteria at different temperatures (4, 25, 37°C) are shown in Table 1. As shown in Table 1, the tested bacteria can reduce BaA, Chr, BbF and BaP *in vitro* and this reduction depends on strain types and incubation temperatures. The results showed that reduction of four PAHs varied in the range from 50.94% to 74.51%, 54.48% to 77.98%, 47.35% to 68.84%, and 61.96% to 81.22% for BaA, Chr, BbF and BaP, respectively (Table 1). As seen in Table 1, in the all tested bacteria, binding ratio increased when temperature of incubation increased and the highest binding ratio of four PAHs was achieved by *L. acidophilus* LA-5 at 37 °C. Because of the fact that the reduction of various toxins by probiotics is carried out through physical interactions with cell wall, this phenomenon can therefore be influenced by various factors such as incubation temperature (**Khorshidian et al., 2016; Zhu et al., 2017**). The result of this study is similar to the results of **Zhao et al. (2013)** that evaluated the effect of incubation temperature on benzo (a) pyren adsorption of probiotics and they found that by increasing temperature from 4 to 40, benzo (a) pyren binding ability of *L. plantarum* CICC 22135 and *L. pentosus* CICC 23163 significantly increased (**Zhao et al., 2013**). It has been reported by **Hatab et al. (2012a)** that removal of patulin by LAB was dependent on temperature and the highest reduction was occurred at 37°C. Furthermore, they found that there were significant differences in patulin binding ratio either at 25°C or 37°C than at 4 ∘C (**Hatab et al., 2012a**). Similarly, **Zhu et al. (2017)** found that bisphenol A removal from PBS is temperature-dependent and the optimal temperatures for the removal of bisphenol A from the PBS solution were at 37 and 30°C (**Zhu et al., 2017**). Moreover, **Bovo et al. (2013)** reported that *L. bulgaricus* had greater ability to remove AFM1 at 37°C in comparison with 4°C (**Bovo et al., 2013**). According to the Table 1, it could be understood the all tested bacteria showed the highest ability in removing BaP, while the lowest adsorption was observed for BbF, indicating that this event is totally dependent on types of toxin and strains.

Effect of incubation time on binding ability

Based on the data presented in Table 2, it is observed that the binding ability is also dependent on time of incubation and by increasing incubation time the amount of free BaA, Chr, BbF and BaP decreased. The highest binding ratio for BaA (81.52%), Chr (80.14%) BbF (73.82%) and BaP (88.45%) were obtained by *L. acidophilus* LA-5 after 24 h incubation.

Table 1 Effect of incubation temperature on binding ability

Bacterial strains	Temperature (°C)	Percentage of binding (%)			
		BaA	Chr	BbF	BaP
Bifidobacterium lactis BB-12	4	50.94 ± 1.13^{gC}	54.48 ± 1.51^{fB}	47.35 ± 0.70^{gC}	61.96 ± 1.36^{fA}
	25	56.70 ± 3.30^{efg}	61.58 ± 1.23^{deAB}	51.08 ± 0.94^{fg}	65.58 ± 0.81^{efA}
	37	60.54 ± 2.03^{cdeC}	64.95 ± 1.94^{cdB}	57.23 ± 1.16^{deCD}	70.38 ± 2.48^{cdeA}
Lactobacillus acidophilus LA-5	4	64.15 ± 0.96^{bcC}	67.48 ± 1.86^{cAB}	61.42 ± 1.54^{bcdCD}	69.87 ± 1.663^{cdeA}
	25	67.15 ± 2.77^{bC}	72.91 ± 1.59^{bB}	65.04 ± 1.82^{abCD}	76.49 ± 3.05^{abA}
	37	74.51 ± 2.87^{aB}	77.98 ± 1.65^{aAB}	68.84 ± 1.47^{aC}	81.22 ± 2.37^{aA}
Lactobacillus bulgaricus PTCC 1737	4	57.69 ± 1.71^{deB}	57.01 ± 1.28^{fB}	52.04 ± 1.07^{fC}	68.20 ± 0.85^{deA}
	25	64.54 ± 1.31^{bcB}	61.54 ± 1.68^{deBC}	57.97 ± 1.65^{deCD}	73.11 ± 2.06^{bcdA}
	37	67.32 ± 2.11^{bBC}	67.54 ± 0.81^{cBC}	63.78 ± 0.66^{bcC}	76.12 ± 0.93^{abA}
Streptococcus thermophilus PTCC 1738	4	51.45 ± 2.09^{fg}	58.05 ± 1.23^{efB}	53.97 ± 1.58^{efCB}	65.28 ± 1.19^{efA}
	25	57.29 ± 0.87^{efC}	65.24 ± 24^{cdA}	59.78 ± 2.46^{cdBC}	69.63 ± 2.55^{cdeA}
	37	63.48 ± 1.17^{bcdC}	67.55 ± 1.38^{cB}	64.18 ± 1.19^{bC}	75.04 ± 2.19^{bcA}

The phosphate buffer solution (pH: 5) contained 1×10^{10} CFU/mL and 10 µg /mL of each PAHs incubated for 10 h.
Values expressed as mean ± standard deviation for triplicate samples
In each column, different small letters represent statistical difference (P≤0.05)
In each row, different capital letters represent statistical difference (P≤0.05)

The results also revealed that the lowest ability of bacteria in removing PAHs over 24 h incubation at 37°C was related to *B. lactis* BB-12. Moreover, nearly in the most trials there were no significant differences among *L. delbrueckii* subsp. *bulgaricus* PTCC 1737 and *S. thermophilus* PTCC 1738 in reducing PAHs during incubation at 37°C for 24 h. It has been reported by **zhao et al. (2013)** that BaP binding ratio was reached to the maximum amount after 24 h incubation at 37°C and the extra time (24h) has no more effect on binding ratio **(Zhao et al., 2013)**.

On the other hand **Zhu et al. (2017)** revealed that the highest level of bisphenol A was obtained after 15 min of incubation time **(Zhu et al., 2017)**. Moreover, **Serrano-Niño et al. (2015)** pointed out that binding of acrylamide and AFB1 by *Lactobacillus* strains was relevant to incubation time. They also reported that the amount of free mycotoxin considerably increased when the incubation time enhanced to 4 and 12 h **(Serrano-Niño et al., 2015)**.

Table 2 Effect of incubation time on binding ability

Bacterial strains	Incubation time (h)	Percentage of binding (%)			
		BaA	Chr	BbF	BaP
Bifidobacterium lactis BB-12	4	48.21 ± 0.21[aB]	54.19 ± 0.17[aC]	46.58 ± 0.52[aA]	58.40 ± 0.30[aD]
	8	59.26 ± 0.18[cB]	65.69 ± 0.22[bkC]	53.55 ± 0.48[cA]	66.94 ± 0.92[dC]
	16	64.12 ± 0.12[eB]	72.44 ± 0.32[edC]	57.40 ± 0.23[dA]	73.20 ± 0.23[fC]
	24	66.25 ± 0.22[fB]	73.62 ± 0.45[eC]	56.09 ± 0.93[cdA]	74.38 ± 0.54[fC]
Lactobacillus acidophilus LA-5	4	61.58 ± 0.52[dB]	64.38 ± 0.31[bC]	59.58 ± 0.40[eA]	70.57 ± 0.39[eD]
	8	72.37 ± 0.33[hB]	73.37 ± 0.45[eB]	68.58 ± 0.52[iA]	78.33 ± 0.81[gC]
	16	75.87 ± 1.37[iA]	78.87 ± 0.37[fB]	74.73 ± 0.25[jA]	85.96 ± 0.30[iC]
	24	81.52 ± 0.50[jB]	80.14 ± 0.80[fB]	73.82 ± 0.74[jA]	88.45 ± 0.51[kC]
Lactobacillus bulgaricus PTCC 1737	4	55.43 ± 0.62[bB]	54.86 ± 0.35[aB]	51.40 ± 0.37[bA]	63.59 ± 0.15[cC]
	8	66.12 ± 0.83[fB]	67.20 ± 2.05[cB]	62.29 ± 0.26[fA]	73.23 ± 0.23[fC]
	16	72.33 ± 0.12[hB]	71.19 ± 0.17[dB]	66.24 ± 0.24[hA]	80.24 ± 0.15[iC]
	24	73.29 ± 0.25[hC]	65.25 ± 0.66[bA]	68.26 ± 1.41[iB]	83.58 ± 0.52[hD]
Streptococcus thermophilus PTCC 1738	4	55.12 ± 0.97[bA]	55.26 ± 0.23[aA]	55.13 ± 0.12[cA]	60.29 ± 0.26[bB]
	8	63.43 ± 0.62[eA]	65.32 ± 0.38[bcB]	64.23 ± 0.11[gAB]	69.64 ± 0.36[eC]
	16	66.87 ± 0.18[fA]	68.00 ± 1.00[cA]	68.49 ± 0.50[iA]	73.21 ± 0.24[fC]
	24	70.22 ± 0.23[gA]	74.69 ± 0.63[eB]	69.71 ± 0.22[iA]	73.27 ± 0.33[fB]

The phosphate buffer solution (pH: 5) contained 1×10^10 CFU/mL and 10 µg/mL of each PAHs incubated at 37°C
Values represented as mean ± standard deviation for triplicate samples
In each column, different small letters represent statistical difference (P≤0.05)
In each row, different capital letters represent statistical difference (P≤0.05)

FTIR analyses

FTIR spectra were an important tool for identifying the different functional groups that might participate in the binding of various components to bacterial cell wall **(Hatab et al., 2012b)**. FTIR bands and detailed wavenumber of *L. acidophilus* and *L. bulgaricus* before and after PAHs uptake are shown in Table 3. Furthermore, The FTIR spectrum of PAHs – bacteria complex and bacteria without PAHs in the wavenumber range of 400 to 4000 cm^{-1} are illustrated in Figure 1. Although the FTIR spectra of PAHs – bacteria complex had some changes in comparison with the unexposed bacterial pellet, however, the shapes are almost the same for each strain (exposed and unexposed). The FTIR analysis showed that the wavenumbers 2965 and 1498 cm^{-1} resulting from the C–H stretching mode proposing the presence of aliphatic chain. The peak at 3420, 1637 and 1550 could be attributed to the O-H/N-H stretching, amide I and amide II, indicating the presence of peptide bonds. Furthermore the wavenumber of 1070 is related to polysaccharides (C–O)

(Guo et al., 2012; S Hatab et al., 2012b). The peak at 960-1150 cm^{-1} could be ascribed to the asymmetric and symmetric stretching of PO2 – and P(OH)2 in phosphate; vibrations of C-OH and C-C bonds in polysaccharides and alcohol **(Jiang et al., 2004)**. As it can be seen in Figure 1, by addition of PAHs in bacteria, the C-H stretching band and C-O polysaccharides band has changed to upper wavenumber. Furthermore, for *L. acidophilus* LA-5+ PAHs, the intensity of the peak at 1637 has been increased when compared to *L. acidophilus* LA-5. According to the FTIR spectra, it can be understood that changed absorption bands at 3440, 3420 and 2990 relating to O-H stretching, N–H of the amine groups and C–H stretching might be the functional groups that participated in binding of PAHs to bacterial cell wall. Furthermore, for the PAHs exposed bacteria in comparison to unexposed bacteria, the absence of peak at 1564 and 1548 (N-H amide II) might justify the binding of PAHs to the bacterial cell wall.

Table 3 FTIR bands observed for bacterial strains before and after PAHs exposing

Functional groups	Wavenumber (cm1)			
	L. acidophilus LA-5+PAHs	*L. acidophilus* LA-5	*L. bulgaricus* PTCC 1737+ PAHs	*L. bulgaricus* PTCC 1737
O-H stretching	3445.14	3454.24	3498.50	3464.23
N–H amines	3419.74	3422.43	3417	3415
C–H stretching	2995.45	2969.20	2994.22	2969.5
C=O amide I	1637.59	1637.44	1632.35	1635.65
N-H amide II	-[a]	1564.3	-[a]	1548.50
CH 2 bending	1498	1499	1485.93	1493.60
C-O polysaccharides	1076.50	1061.50	1077.50	1061.26
PO$_2$	987.43	963.50	959.60	979.84

[a] Band not observed.
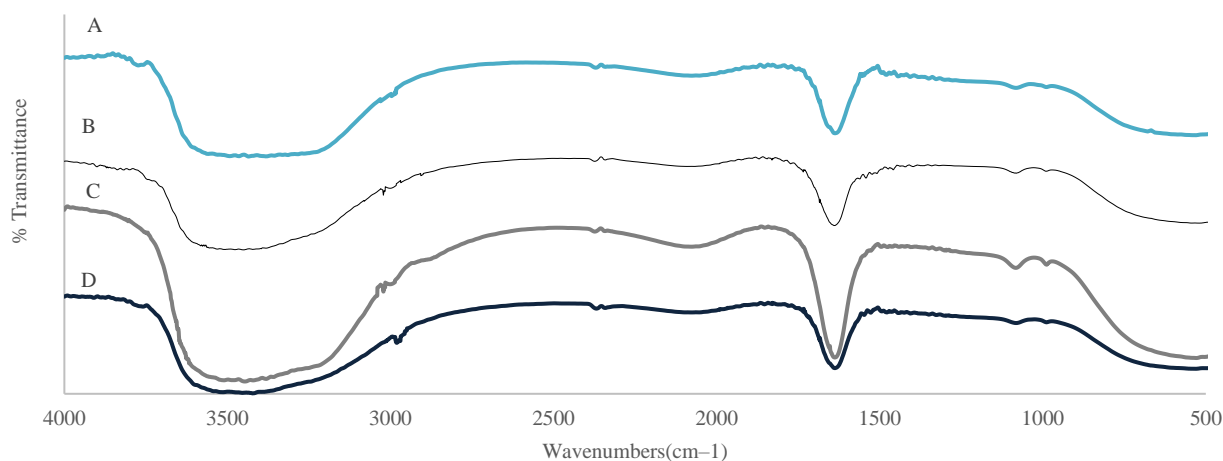
Figure 1 FTIR spectrum of L. *bulgaricus* PTCC 1737 (A: after PAHs uptake, B: before PAHs uptake) and *Lactobacillus acidophilus* LA-5 (C: after PAHs uptake, D: before PAHs uptake)

CONCLUSION

This study demonstrated that remove of BaA, Chr, BbF and BaP from phosphate buffer saline was carried out by the tested bacteria. The FTIR analysis showed that bacterial cell wall plays an important role in removal of BaA, Chr, BbF and BaP liquid medium. Therefore, as seen in this study, this reduction significantly affected by strains of bacteria, type of toxin, time and temperature of incubation. The highest and the lowest removal were related to BaP and BbF, respectively, indicating that this event is toxin-specific. The findings of this study showed that the binding capacity of bacteria increased when time and temperature of incubation increased and the highest binding ratio of four PAHs was achieved by *L. acidophilus* LA-5 at 37 °C. Generally it could be concluded that PAH-binding ability of LABs and probiotics might be considered as a new strategies for decontamination of PAHs from foods.

Conflict of interest: The authors declare there are no conflicts of interest.

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