



Immunomodulatory and antitumor activities of the exopolysaccharide produced by potential probiotic *Lactobacillus plantarum* YW11 in a HT-29 tumor-burdened nude mouse model

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Abstract

In this study, the exopolysaccharides (EPSs)-producing *L. plantarum* YW11 was initially evaluated for its tolerance to simulated artificial gastric juice and bile salts, and cell surface hydrophobicity. Subsequent study using a HT-29 tumor-burdened nude mouse model showed that the EPS had potent immunomodulatory and antitumor activities. Administration of the EPS had no influence on body weight of the mice, and it could significantly prevent HT-29 tumor cells induced acute liver and kidney damages. The purified EPS at a dose of 5 mg/mL was able to enhance effectively the killing activities of splenic natural killer cells and cytotoxic T lymphocyte, and to stimulate the secretion of serum cytokines IL-2 and TNF- α . As evidenced by a cell culture assay, the EPS exhibited obvious inhibition on growth of colon cancer HT-29 cells treated with 600 μ g/mL of the EPS for 72 h with an inhibition rate of 58.02%. Administration of the EPS obviously inhibited growth of HT-29 xenograft tumor to an extent near that of the positive control (5-Fu, 25 mg/kg bw). Therefore, the results of this study indicated the important roles of the EPS in the potential probiotic functions of the strain.

Keywords: exopolysaccharide; *Lactobacillus plantarum*; Immunomodulatory; antitumor, HT-29 cells.

Practical Application: EPS in the potential probiotic functions of the strain.

1. Introduction

Lactic acid bacteria (LAB) are widely used in a variety of foods or medicines due to their potential benefits for human health (Shao et al., 2014; Zendeboodi et al., 2020). These microorganisms are able to produce EPSs that are either covalently associated with the cell surface in the form of capsule or secreted into the growth medium in the form of slime. EPSs produced by LAB have aroused increasing attention in recent years, not only for their unique physicochemical properties, but also for biological activities including antioxidant, antibacterial, immunomodulatory and antitumor activities (Guo et al., 2013; Zhang et al., 2013; Li et al., 2014a; Kumar et al., 2021).

As an important *Lactobacillus* species, *L. plantarum* has been frequently used in food fermentation (Crowley et al., 2012; Mirlohi et al., 2014). Some *L. plantarum* strains were shown to produce EPSs, of which a few were reported with bioactivities (Wang et al., 2014b; Chakraborty et al., 2020). The EPS produced by *L. plantarum* YW32 was found to possess scavenging abilities toward hydroxyl and superoxide radicals, inhibitory effect on biofilm formation by several pathogenic bacteria, and inhibitory activity against colon cancer HT-29 cells (Wang et al., 2015a). The EPS from *L. plantarum* R315 exhibited antibacterial ability

against pathogens, and scavenging activity against DPPH, hydroxyl, and superoxide radicals (Li et al., 2014b). Both the EPS and the sulfated EPS from *L. plantarum* ZDY2013 had radical scavenging activities, and the antioxidant activities increased after sulfonation (Zhang et al., 2016). The EPS from *L. plantarum* C88 was shown to be effective in scavenging reactive oxygen species *in vitro* (Zhang et al., 2013). However, the roles of the EPSs produced by *L. plantarum* strains in their probiotic functions were not well studied.

In this study, *L. plantarum* YW11 strain isolated from Tibet kefir grains was initially evaluated for its potential probiotic properties by *in vitro* gastrointestinal tolerance and bacterial surface hydrophobicity tests. The EPS produced by *L. plantarum* YW11, which was previously characterized with monosaccharide composition of glucose and galactose in a molar ratio of 2.71:1 and a molecular mass of 1.1×10^5 Da (Wang et al., 2015a), was further studied using a HT-29 tumor-burdened nude mouse model and the human colon cancer cell line HT-29 to evaluate its immunomodulatory and antitumor activities in order to understand the roles of the EPSs produced by *L. plantarum* strains in their probiotic functions.

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2. Materials and methods

2.1 Materials and chemicals

Aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN) and creatinine (Cr) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), IL-2 and TNF- α ELISA kits were purchased from R&D Systems Inc. (Minneapolis, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), vitamin C (Vc) and 5-fluorouracil (5-FU) were purchased from Sigma Co. (St. Louis, MO, USA). All the other reagents were of analytical grade.

2.2 Animals and cell lines

Human colon cancer cell line HT-29 was obtained from Chinese Academy of Medical Sciences Cancer Hospital (Beijing, China). Cells were grown in DMEM medium (high glucose) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μ g/mL streptomycin, and incubated at 37 °C in a humidified atmosphere with 5% CO₂. BALB/c nude mice (male, 6 weeks old) weighing 18-22 g were purchased from Beijing Vital River Laboratories Co., Ltd. (Beijing, China).

2.3 Bacterial cultures

L. plantarum YW11 isolated and identified from Tibet kefir grains, and *L. rhamnosus* GG (LGG) isolated from commercial yoghurt were stored in 30% (v/v) glycerol at -80 °C in the Dairy Laboratory of Beijing Technology and Business University, China. The strains were inoculated into MRS (Man Rogasa Sharpe) (Difco, Sparks, MD, USA) broth and grown at 37 °C for 12 h to obtain a viable count of log 8-9 CFU/mL for use in this study.

2.4 Determination of bacterial tolerance in simulated artificial gastric juice

The bacterial culture of *L. plantarum* K25 was taken, and centrifuged at 6000 \times g 4 °C for 10 min. The cell pellets were collected, washed twice with sterile PBS buffer (pH 6.5) and added with 5 mL PBS buffer (pH 6.5) to make bacterial suspension. 1 mL of bacterial suspension was added to a test tube containing 9 mL of simulated gastric juice (0.2 g/100 mL NaCl, 0.35 g/100 mL pepsin, filtered and sterilized) at pH 3.0, and placed at 37 °C for anaerobic incubation for 3 h. Samples were taken at 0 h and 3 h after inoculation, and then diluted and spread on MRS agar plates. After 48 h culture at 37 °C, viable bacterial counting was performed (Vinderola & Reinheimer, 2003). The survival rate of the strain is calculated as follows Equation 1

$$\text{Survival rate (\%)} = \log \text{cfu } N_1 / \log \text{cfu } N_0 \times 100 \quad (1)$$

N_1 is the number of viable bacteria after gastric juice treatment for 3 h; N_0 is the number of viable bacteria after gastric juice treatment for 0 h

2.5 Determination of bacterial tolerance to bile salt

L. plantarum YW11 was inoculated at 3% (v/v) in MRS liquid medium containing 0.3, 0.5 and 1.0 g/100 mL of bovine

bile salt, respectively, and incubated at 37 °C for 3 h. Samples were taken at 0 h and 3 h after inoculation, and then diluted and spread on MRS agar plate. After incubation at 37 °C for 48 h, viable bacterial counting was performed. The survival rate of the strain is calculated as follows Equation 2:

$$\text{Survival rate (\%)} = \log \text{cfu } N_1 / \log \text{cfu } N_0 \times 100 \quad (2)$$

N_1 is the number of viable bacteria after gastric juice treatment for 3 h; N_0 is the number of viable bacteria after gastric juice treatment for 0 h

2.6 Determination of cell surface hydrophobicity

The *L. plantarum* YW11 culture was centrifuged at 6000 \times g 4 °C for 10 min. The bacterial pellets were collected and washed twice with sterile PBS buffer (pH 6.5). The bacterial concentration was adjusted with PBS to reach an absorbance of 1.0 at 560 nm. 3 mL of the adjusted bacterial suspension was added with 0.6 mL of xylene, chloroform and ethyl acetate, respectively. The mixture was oscillated by eddy. After static stratification, the lower aqueous phase was taken, and the absorbance was measured at 560 nm with blank set to zero by PBS. The hydrophobic rate was calculated as follows Equation 3:

$$\text{Hydrophobic rate (\%)} = (A_0 - A) / A \times 100 \quad (3)$$

A_0 is the absorbance before mixing the bacterial suspension and adsorbent; A is the absorbance of the bacterial suspension after mixing with the adsorbent.

2.7 Isolation and purification of EPS

The EPS produced by *L. plantarum* YW11 was isolated and purified from the growth culture of *L. plantarum* YW11 according to our previously reported method (Wang et al., 2015b). The purified form of the EPS was used for evaluation of bioactivities in this study.

2.8 Assay of antitumor and immunomodulatory activities of EPS

2.8.1 Establishment of HT-29 tumor-burdened nude mouse model

HT-29 cells were cultured in DMEM medium at 37 °C for 24 h. The concentration of the cells was adjusted to 1.0 \times 10⁷ cells/mL in PBS. Then 0.2 mL of HT-29 cells suspension was inoculated subcutaneously into the right axilla of each mice at the beginning of the experiment. After inoculation for 24 h, the tumor bearing mice were divided into five groups (ten mice per group): model control group (0.9% saline), positive control group (5-Fu, 25 mg/kg bw), low-dose EPS group (10 mg/kg bw), mid-dose EPS group (25 mg/kg bw), high-dose EPS group (50 mg/kg bw). Another ten mice without tumor inoculation and drug administration were used as normal control group (0.9% saline). Drugs were orally administered to mice after grouping. The designated amounts of drugs were applied in 0.2-mL volume once a day for 14 days. In the course of treatment, tumor volumes

and body weights were monitored every other day. The tumor volume was calculated using the following Equation 4:

$$V \text{ (mm}^3\text{)} = (L \times S^2) / 2 \quad (4)$$

L and S (mm) were the largest and smallest diameters of the tumor, respectively.

2.8.2 Determination of tumor inhibition rate and organ indices

24 h after last drug administration, all the mice were weighed and killed by dislocation of cervical vertebra and the tumors, spleens, livers, kidneys and hearts were collected and weighed. The inhibition rate of tumor growth was calculated according to the following equation: inhibition rate (%) = [(tumor weight of the mice in model control group – tumor weight of the mice in EPS treatment group) / tumor weight of the mice in model control group] × 100. Relative spleen/liver/kidney/heart weights were expressed as the ratio of the spleen/liver/kidney/heart to body weight (mg/g).

2.8.3 Hematological and serum biochemical parameters assays

24 h after last drug administration, the blood of each mouse was collected with the anticoagulant in polystyrene tubes. The hematological parameters such as hemoglobin (Hb) content, red blood cell count (RBC), white blood cell count (WBC), total leukocytes count and lymphocyte count were determined by using analytical hematology system Symex XE-2100 (Symex Cooperation, Japan). After that, the serum of each mouse was obtained by centrifugation (3000 × g, 10 min, 4 °C) and stored at -80 °C for further analysis. The clinical parameters, including AST, ALT, BUN, Cr, SOD and MDA levels in serum were all determined spectrophotometrically using commercially available reagent kits (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's instructions.

2.8.4 Assay of NK cell activity

The activity of NK cell was measured using the method described by Wang with slight modifications (Wang et al., 2014a). Briefly, colon cancer cell HT-29 (target cells) and splenocytes (effector cells) were mixed in 96-well U-bottom microtiter plate in a 50:1 ratio and incubated at 37 °C in 5% CO₂ atmosphere. After 24 h incubation, 50 μL of MTT solution (2 mg/mL) was added to each well and the plate was incubated for another 4 h and subjected to MTT assay. NK cell activity was calculated according to the following Equation 5:

$$NK \text{ activity (}\%) = [A_T - (A_S - A_E)] / A_T \times 100 \quad (5)$$

where A_T is the absorbance value of target cells control, A_S is the absorbance value of test samples, A_E is the absorbance value of effector cells control.

2.8.5 Assay of CTL activity

The CTL activity was analyzed using MTT method as described above. Colon cancer cell HT-29 (target cells) and splenocytes (effector cells) were mixed in a 50:1 ratio. CTL activity

was calculated according to the following equation: CTL activity (%) = $[A_T - (A_S - A_E)] / A_T \times 100$, where A_T is the absorbance value of target cells control, A_S is the absorbance value of test samples, A_E is the absorbance value of effector cells control.

2.8.6 Assay of serum cytokine levels

The level of interleukin-2 (IL-2) and tumor necrosis factor-alpha (TNF-α) in the serum were measured using commercially available kits from R&D systems Inc. (Minneapolis, USA), with a solidphase enzyme-linked immunoabsorbent assay (ELISA) as described by the manufacturers.

2.9 Assay of HT-29 cell proliferation as affected by EPS

The *in vitro* antitumor activity of the EPS was determined by a MTT assay using human colon cancer HT-29 cells. Briefly, HT-29 cells were incubated in 96-well microtiter plates (2.0 × 10⁴ cells per well) under 5% CO₂ at 37 °C for 24 h. Then the tumor cells were treated with various concentrations of the EPS (50, 100, 200, 400 and 600 μg/mL) and 50 μg/mL of 5-FU (positive control) for 24 and 72 h, respectively. At the end of each treatment, MTT reagent (5.0 mg/mL) was added to each well (10 μL per well). After 4 h, the liquid was removed, and 100 μL of DMSO was added to each well. After dissolving the formazan crystal that formed, the absorbance was measured by a Synergy TM 2 microplate reader (BioTek Instruments, Inc., Burlington, VT) at 570 nm. The inhibition ratio was expressed as follows Equation 6:

$$\text{inhibition ratio (}\%) = \left[1 - \left(\frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \right) \right] \times 100 \quad (6)$$

where A_{control} and A_{blank} were the absorbances of the system without the addition of the EPS and cells, respectively.

2.10 Statistical analysis

The results were presented as the mean ± standard deviation (SD) and were analyzed by one-way analysis of variance (ANOVA) using SPSS version 16.0. The values of $P < 0.05$ were used to identify statistically significant differences.

3. Results and discussion

3.1 *In vitro* gastrointestinal tolerance of *L. plantarum* YW11

The prerequisite for probiotics to exert their health beneficial function in human body is to ensure their survival in the acidic environment of gastric juice and the bile salt environment of intestinal tract. The pH value of human gastric juice is generally between 1.3 and 1.8 on a fasting stomach, which rises to about 3.0 or higher after eating, and the food stays in the stomach for about 3 h, bile salt content in normal human small intestine fluctuates between 0.03% and 0.3% (Kosmerl et al., 2021). In this study, the tolerance of *L. plantarum* YW11 was evaluated by simulating the low pH environment and high bile salt in human gastrointestinal tract by *in vitro* experiments. LGG, the well-known probiotic strain with clear probiotic characteristics, was used as the control strain. As shown in Table 1, *L. plantarum* YW11 showed good tolerance in simulated artificial gastric

juice environment. The survival rate reached 82.28%, but lower than that (93.80%) of LGG after 3 h of culture. Assay of the tolerance to bile salt at 0.3, 0.5 and 1.0 g/100 mL for 3 h showed that the survival rate of *L. plantarum* YW11 decreased with the increase of bile salt concentration. When bile salt was at 0.6 g/mL, the survival rate of *L. plantarum* YW11 was 95.35%, higher than that of LGG (91.16%). However, increasing bile salt to 1.0% decreased significantly the survival rate of *L. plantarum* YW11 to about 70%.

3.2 Determination of cell surface hydrophobicity of *L. plantarum* YW11

Probiotics are required to not only tolerate the environment of gastrointestinal fluid, but also adhere to intestinal epithelial cells to ensure their colonization to exert probiotic functions. The adhesion ability of a strain is positively correlated with the bacterial hydrophobicity, and high hydrophobicity is conducive to the colonization of the strain in the intestinal tract (Zeng et al., 2020). In this study, the hydrophobic capacity of *L. plantarum* YW11 in three organic solvents, including xylene, chloroform and ethyl acetate was determined. Xylene can be used as a nonpolar solvent to test the surface hydrophobicity of the strain. As shown in Table 2, *L. plantarum* YW11 showed high hydrophobicity at a rate >50%, indicating that *L. plantarum* YW11 had a hydrophobic cell surface.

The electron acceptor and electron donor on the bacterial surface of a strain can be determined by detecting the affinity of the strain to chloroform (unipolar, acidic organic solvent, electron acceptor) and ethyl acetate (unipolar, alkaline organic solvent, electron donor). As shown in Table 2, *L. plantarum* YW11 had a high hydrophobicity to chloroform at a hydrophobicity rate of 62.25%, indicating that *L. plantarum* YW11 was a strong electron donor. The hydrophobicity of *L. plantarum* YW11 to ethyl acetate was lower (hydrophobicity <30%), indicating that the strain was a weak electron acceptor.

3.3 Effect of the EPS from *L. plantarum* YW11 on body weights, organ indices, serum clinical and hematological parameters

During the whole process of the *in vivo* assay experiments of this study, the activities of all mice, their body weights and physiological parameters were checked. As shown in Table 3, the gain of body weight of the mice in all doses of EPS treatment groups had no significant difference from those in the model control group, indicating that the EPS from *L. plantarum* YW11 had no inhibitory effects on the growth of weight. However, treatment with 5-FU significantly decreased the gain of body weight of the mice. Compared with the model control group, the spleen indices of mice in all doses of EPS treatment groups were slightly elevated in different degrees ($P > 0.05$). Conversely, the spleen indices of the mice in positive control group were reduced,

Table 1. Tolerance of *L. plantarum* YW11 to simulated artificial gastric juice and bile salt ($\bar{x} \pm SD$, $n = 3$).

	gastric juice			bile salt		
	pH 3.0(0 h)	pH 3.0(3 h)	survival rate(%)	survival rate(%)		
				0.3 g/100 mL(3 h)	0.6 g/100 mL(3 h)	1.0 g/100 mL(3 h)
LGG	8.55 ± 0.38	8.02 ± 0.42	93.80 ± 6.15	95.42 ± 5.64 ^a	91.16 ± 2.38 ^a	80.15 ± 4.15 ^{ba}
<i>L. plantarum</i> YW11	8.58 ± 0.84 ^a	7.06 ± 0.35 ^b	82.28 ± 4.35	97.42 ± 5.15 ^a	95.35 ± 6.55 ^a	73.05 ± 3.61 ^{baB}

^{ab}Means in the same row followed by different superscripts are significantly different ($P < 0.05$); ^{AB} Means in the same column followed by different superscripts are significantly different ($P < 0.05$).

Table 2. Surface hydrophobicity of *L. plantarum* YW11 in comparison with LGG ($\bar{x} \pm SD$, $n = 3$).

	Hydrophobicity rate(%)		
	Xylene	Chloroform	Ethyl acetate
LGG	22.35 ± 3.40 ^{aA}	30.10 ± 0.96 ^{ba}	16.82 ± 1.54 ^{aA}
<i>L. plantarum</i> YW11	53.54 ± 1.65 ^{aC}	62.25 ± 2.34 ^{bc}	25.02 ± 1.25 ^{cb}

^{abc} Means in the same row followed by different superscripts are significantly different ($P < 0.05$); ^{ABC} Means in the same column followed by different superscripts are significantly different ($P < 0.05$).

Table 3. Effects of the EPS from *L. plantarum* YW11 on body weights and organ indices in HT-29 tumor-burdened nude mice.

Groups	Dose (mg/kg bw)	Gain of body weight (g)	Spleen (mg/g)	Liver (mg/g)	Kidney (mg/g)	Heart (mg/g)
Normal		3.46 ± 0.38	6.16 ± 1.06	53.11 ± 3.11	16.53 ± 0.81	6.37 ± 0.64
Model control		2.80 ± 0.25	4.68 ± 0.58	51.90 ± 5.74	18.80 ± 1.99	7.48 ± 0.90
Positive control	25	1.91 ± 0.05 ^a	2.09 ± 0.20	53.78 ± 5.61	18.64 ± 1.27	7.84 ± 0.47
EPS	10	2.60 ± 0.14	5.44 ± 0.73 ^b	60.49 ± 4.10	18.76 ± 1.71	6.85 ± 0.99
	25	3.45 ± 0.26 ^{bb}	5.28 ± 1.01 ^b	57.24 ± 3.56	16.67 ± 0.82	6.80 ± 0.79
	50	2.84 ± 0.45 ^b	6.17 ± 1.83 ^{bb}	60.05 ± 4.09	17.37 ± 0.96	6.06 ± 0.73

Data were expressed as mean ± SD ($n = 10$ mice per groups); ^a $P < 0.05$, significantly different from model control group; ^b $P < 0.05$, significantly different from positive control group; ^{bb} $P < 0.01$, significantly different from positive control group. Indicate the letter 'D' in the table.

indicating that 5-FU caused a decline in immune function to a certain extent. In addition, there was no significant difference in liver, kidney and heart indices between the EPS treatment groups and the model control group. Bárbara F. Cordeiro reported that during colitis induction, mice that consumed the probiotic cheese exhibited reduced in the severity of colitis, with attenuated weight loss, lower disease activity index, limited shortening of the colon length, and reduced histopathological score (Cordeiro et al., 2021).

Serum clinical parameters including liver-related ALT and AST activities, and kidney-associated BUN and Cr levels, were measured to verify whether the EPS from *L. plantarum* YW11 would cause damage to the normal organs, especially liver and kidney. As shown in Figure 1A-1D, an obvious increase in ALT, AST, BUN and Cr was observed in HT-29 tumor-bearing mice as compared with those of the normal control

group. However, EPS treatments at different doses decreased significantly ($P < 0.01$) serum AST, ALT, BUN and Cr, and the activities of AST and BUN were reduced to the levels near those in the normal control group, indicating no damage to the body by EPS treatment. However, for the 5-FU treatment group, the serum ALT and AST activities increased, suggesting an adverse effect on liver function by 5-FU. Hematological parameters in HT-29 tumor-burdened nude mice on day 14 were shown in Table 4. All EPS-treated mice had significantly ($P < 0.01$) increased WBC and LYM counts as compared with those of the model control mice, confirming the immunomodulatory effect of the EPS. However, treatment with 5-FU caused declined immunity with significantly ($P < 0.05$) decreased WBC and LYM counts.

Inoculation of tumor cells in mice was found to cause hepatocellular and kidney cellular necrosis, characterized with formation of lipid peroxidation products and a decrease

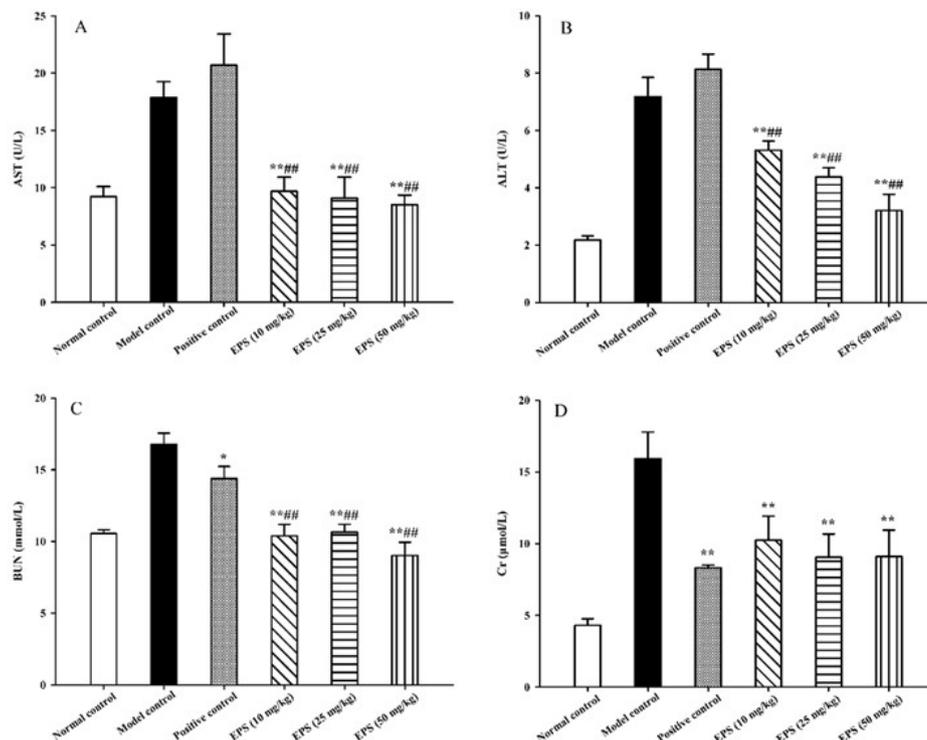


Figure 1. Effect of the EPS from *L. plantarum* YW11 on serum AST, ALT, BUN and Cr levels in HT-29 tumor-burdened nude mice. Data are expressed as mean \pm SD (n = 10 mice per groups), * $P < 0.05$, ** $P < 0.01$ compared with model control group; ### $P < 0.01$ compared with positive control group.

Table 4. Effects of the EPS from *L. plantarum* YW11 on hematological parameters in HT-29 tumor-burdened nude mice.

Groups	Dose (mg/kg bw)	RBC (10^6 cells/ μ L)	WBC (10^3 cells/ μ L)	Hb (g/dL)	LYM (10^3 cells/ μ L)	PLT (10^5 cells/ μ L)
Normal		8.49 \pm 0.37	3.87 \pm 0.28	16.80 \pm 2.75	2.47 \pm 0.43	9.70 \pm 1.82
Model control		8.99 \pm 0.68	2.42 \pm 0.51 ^{cc}	17.62 \pm 0.97	1.13 \pm 0.38 ^c	10.19 \pm 1.39
Positive control	25	8.71 \pm 0.59	1.19 \pm 0.34 ^{acc}	18.00 \pm 1.46	0.52 \pm 0.08 ^{acc}	7.25 \pm 1.67
EPS	10	8.47 \pm 0.46	4.28 \pm 0.41 ^{aabb}	15.93 \pm 2.20	3.00 \pm 0.20 ^{aabb}	9.97 \pm 1.65
	25	8.76 \pm 0.46	4.82 \pm 0.28 ^{aabb}	17.21 \pm 2.23	3.02 \pm 0.82 ^{aabb}	10.39 \pm 1.62
	50	8.95 \pm 0.48	5.15 \pm 0.53 ^{aabbc}	17.12 \pm 2.16	3.19 \pm 0.51 ^{aabb}	9.87 \pm 2.05

Data were expressed as mean \pm SD (n = 10 mice per groups). ^a $P < 0.05$, significantly different from model control group; ^{aa} $P < 0.01$, highly significantly different from model control group; ^{ab} $P < 0.01$, highly significantly different from positive control group; ^c $P < 0.05$, significantly different from normal control group; ^{cc} $P < 0.01$, highly significantly different from normal control group.

in antioxidative enzymes (Wang et al., 2014b). Serum ALT and AST enzymatic activities were considered to be sensitive indicators of hepatocellular injury, which caused leakage of the enzymes from the hepatocytes. Serum BUN and Cr levels were good biomarkers of renal injury, and their high content often indicated malfunctions of kidney (Fu et al., 2012). In this study, treatment with the EPS from *L. plantarum* YW11 had no influence on body weight, and no toxicity to liver and kidney. The significantly ($P < 0.01$) decreased ALT and AST activities, and the levels of BUN and Cr in serum of HT-29 tumor-burdened nude mice indicated that treatment with the EPS from *L. plantarum* YW11 could significantly prevent HT-29 tumor cells induced acute liver and kidney damages.

3.4 Immunomodulatory activity of the EPS from *L. plantarum* YW11

The immunomodulatory activity of the EPS from *L. plantarum* YW11 was assayed by measuring the cytotoxic activities of NK cells and CTL of mice. As shown in Figure 2A, the EPS (25 and

50 mg/kg) significantly increased the splenic NK and CTL cytotoxic activities of HT-29 tumor-burdened nude mice when compared with those of the model control mice ($P < 0.05$ or $P < 0.01$). However, the NK cell and CTL activities of the model control mice were higher than those in 5-FU-treated mice.

EPS treatment of HT-29 tumor-burdened nude mice stimulated production of serum cytokines to different degrees when compared with the model control group, and significant ($P < 0.01$) increase of IL-10 and IL-2 levels was observed with EPS at 50 mg/kg (Figure 2B). The TNF- α level also increased significantly ($P < 0.05$ or $P < 0.01$) in all doses of EPS-treated mice. Conversely, there was no significant difference in the serum levels of IL-10, IL-2 and TNF- α between the 5-FU treatment group and the model control group.

The immuno-regulating activity of EPS was thought to be influenced by its molecular weight, monosaccharide composition, electric charges, chain conformation, and water-solubility (Zjawiony, 2004). Hidalgo-Cantabrana hypothesized that EPSSs having negative charges and/or low molecular weight were able to act as strong/mild stimulators of immune cells, whereas those neutral EPSSs with a high molecular weight exhibited a weak stimulation and even suppressive profile (Hidalgo-Cantabrana et al., 2012). This was in agreement with the study by López, that the acidic and lower-molecular-weight EPSSs produced by *Bifidobacterium* strains demonstrated stronger immune response stimulation than those of the neutral EPSSs with high molecular weight (Lopez et al., 2012). The acidic nature and relatively low molecular weight (1.1×10^5 Da) of the EPS from *L. plantarum* YW11 as reported earlier (Wang et al., 2015a), might be relevant to its strong immunomodulatory activity as shown in this study. However, the mechanism of how the EPS from *L. plantarum* YW11 affected the intracellular immune system of the HT-29 tumor-burdened nude mice needs to be further studied.

3.5 Antitumor activity of the EPS from *L. plantarum* YW11

The *in vitro* inhibitory activity of the EPS from *L. plantarum* YW11 on the growth of HT-29 cells was determined by a MTT assay of the viability of the cells treated with increasing concentrations of the EPS for 24 and 72 h. As shown in Figure 3A, C, the EPS exerted inhibitory activities on HT-29 cells in time- and concentration-dependent manners. Treatment of the cells for 24 h with the EPS (50-600 $\mu\text{g}/\text{mL}$) resulted in inhibition rates of 30.65-55.11%, which were higher than those of the chemotherapy drug 5-FU ($28.64 \pm 2.39\%$). When treated with 600 $\mu\text{g}/\text{mL}$ of the EPS for 72 h, the inhibition rate reached a maximum of $58.02 \pm 3.93\%$, which was also higher than that of the treatment with 5-FU ($50.30 \pm 3.14\%$) for 72 h.

The *in vivo* antitumor activity of the EPS from *L. plantarum* YW11 was determined by monitoring the tumor growth of the male BALB/c nude mice bearing HT-29 colon cancer tumors during treatment with different doses of the EPS and daily injection of 5-FU for 14 days. As shown in Figure 3B, the tumor volume of model control group increased rapidly, while the 5-FU group and all EPS-treated groups had significant tumor inhibition effects. On day 14 after the last treatment, the

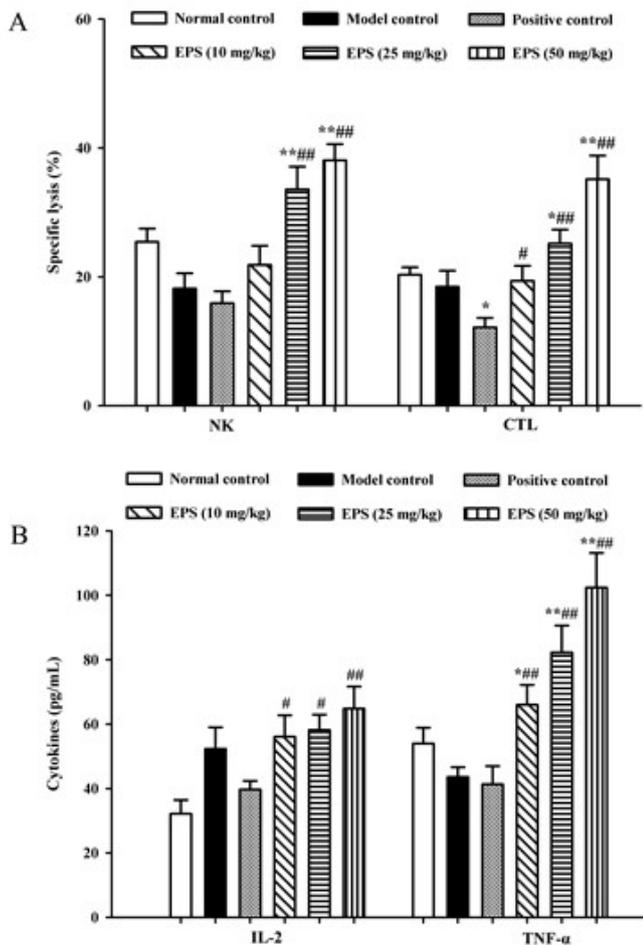


Figure 2. Effect of the EPS from *L. plantarum* YW11 on splenic NK cells and CTL cytotoxic activities (A), and on serum cytokines IL-2 and TNF- α levels in HT-29 tumor-burdened nude mice (B). Data are expressed as mean \pm SD ($n = 10$ mice per groups), * $P < 0.05$, ** $P < 0.01$ compared with model control group; # $P < 0.05$, ## $P < 0.01$ compared with positive control group.

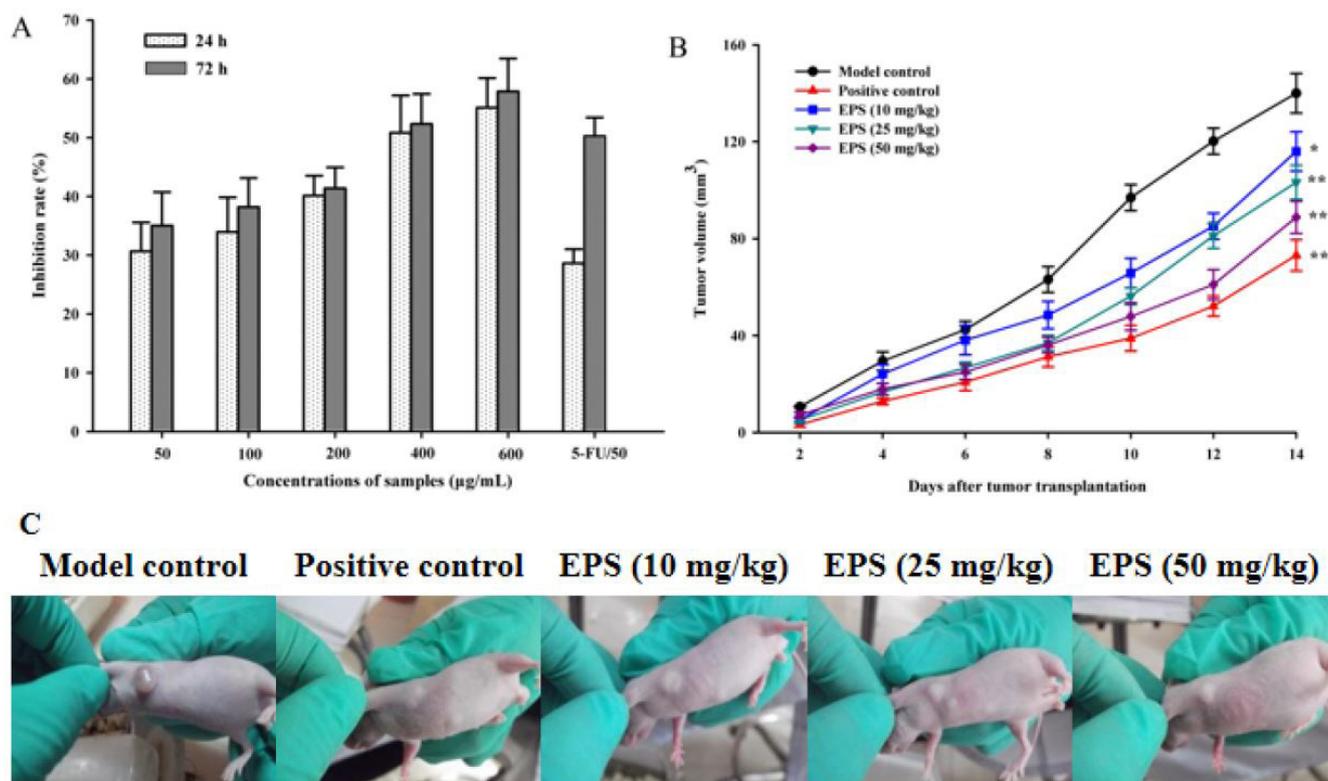


Figure 3. *In vitro* inhibitory activity of the EPS from *L. plantarum* YW11 against human colon cancer HT-29 cells at different concentrations (50, 100, 200, 400 and 600 µg/mL) for two periodic intervals, 24 and 72 h (A,C); data are presented as the mean ± SD of triplicates. *In vivo* inhibitory effect of the EPS from *L. plantarum* YW11 on tumor growth of the HT-29 tumor-burdened nude mice (B), and the picture clearly showed inhibition on the tumor growth by the EPS in a dose-dependent manner; data are expressed as mean ± SD (n = 10 mice per groups), **P* < 0.05, ***P* < 0.01 compared with model control group.

average tumor volumes of 5-FU, low-dose (10 mg/kg), mid-dose (25 mg/kg) and high-dose EPS (50 mg/kg) groups were $52.20 \pm 3.21\%$, $82.85 \pm 8.24\%$, $73.74 \pm 6.38\%$ and $63.42 \pm 5.43\%$ of that of model control group, respectively. Therefore, the EPS treatment obviously inhibited the growth of HT-29 xenograft tumor in a concentration dependent manner and the maximal inhibition rate ($36.58 \pm 3.34\%$) achieved was approaching that of the treatment with 5-FU. Previously, EPSs from LAB were also reported to exert protective effects against cancer (Choi et al., 2006). The antitumor activity of polysaccharides was dependent on their chemical composition, molecular weight, monosaccharide composition, chain conformation and even charge characteristics (Wu et al., 2014). The immuno-regulating activity of the EPS from *L. plantarum* YW11 as shown in this study played a role in its antitumor activity, but the underlying mechanism needs to be further studied.

4. Conclusion

L. plantarum YW11 showed good tolerance to simulated artificial gastric juice and bile salts, and better adhesion ability based on assay of cell surface hydrophobicity, when compared with the well known probiotic strain LGG. Studies on the bioactivities of the EPS produced by *L. plantarum* YW11 using a HT-29 tumor-burdened nude mouse model indicated potent immunomodulatory and antitumor activities of the EPS. Administration of the EPS

significantly prevented HT-29 tumor cells induced acute liver and kidney damages, and obviously inhibited growth of HT-29 xenograft tumor in the mice to an extent near that of the positive control (5-Fu, 25 mg/kg bw). The antitumor activity of the EPS was related to its ability to activate immune response by increasing cytotoxic activities of NK cell and CTL in spleen, and promoting secretion of cytokines IL-2 and TNF-α in mice. The results of this study indicate the important roles of the EPS in the potential probiotic activities of *L. plantarum*, but the underlying mechanism needs to be further studied.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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References

- Chakraborty, P., Bhattacharya, B., Shivhare, U., & Basu, S. (2020). Investigation of heat-acid induced coagulation behaviour of whole milk systems employing front-face fluorescence spectroscopy.

- International Journal of Dairy Technology*, 73(4), 674-682. <http://dx.doi.org/10.1111/1471-0307.12726>.
- Choi, S. S., Kim, Y., Han, K. S., You, S., Oh, S., & Kim, S. H. (2006). Effects of lactobacillus strains on cancer cell proliferation and oxidative stress in vitro. *Letters in Applied Microbiology*, 42(5), 452-458. <http://dx.doi.org/10.1111/j.1472-765X.2006.01913.x>. PMID:16620202.
- Cordeiro, B. F., Alves, J. L., Belo, G. A., Oliveira, E. R., Braga, M. P., Da Silva, S. H., Lemos, L., Guimarães, J. T., Silva, R., Rocha, R. S., Jan, G., Le Loir, Y., Silva, M. C., Freitas, M. Q., Esmerino, E. A., Gala-García, A., Ferreira, E., Faria, A. M. C., Cruz, A. G., Azevedo, V., & do Carmo, F. L. R. (2021). Therapeutic effects of probiotic minas frescal cheese on the attenuation of ulcerative colitis in a murine model. *Frontiers in Microbiology*, 12, 623920. <http://dx.doi.org/10.3389/fmicb.2021.623920>. PMID:33737918.
- Crowley, S., Mahony, J., & van Sinderen, D. (2012). Comparative analysis of two antifungal *Lactobacillus plantarum* isolates and their application as bioprotectants in refrigerated foods. *Journal of Applied Microbiology*, 113(6), 1417-1427. <http://dx.doi.org/10.1111/jam.12012>. PMID:22978783.
- Fu, J., Fu, J., Yuan, J., Zhang, N., Gao, B., Fu, G., Tu, Y., & Zhang, Y. (2012). Anti-diabetic activities of acanthopanax senticosus polysaccharide (asp) in combination with metformin. *International Journal of Biological Macromolecules*, 50(3), 619-623. <http://dx.doi.org/10.1016/j.ijbiomac.2012.01.034>. PMID:22326821.
- Guo, Y., Pan, D., Li, H., Sun, Y., Zeng, X., & Yan, B. (2013). Antioxidant and immunomodulatory activity of selenium exopolysaccharide produced by *Lactococcus lactis* subsp. *lactis*. *Food Chemistry*, 138(1), 84-89. <http://dx.doi.org/10.1016/j.foodchem.2012.10.029>. PMID:23265459.
- Hidalgo-Cantabrana, C., López, P., Gueimonde, M., de Los Reyes-Gavilán, C. G., Suárez, A., Margolles, A., & Ruas-Madiedo, P. (2012). Immune modulation capability of exopolysaccharides synthesised by lactic acid bacteria and bifidobacteria. *Probiotics and Antimicrobial Proteins*, 4(4), 227-237. <http://dx.doi.org/10.1007/s12602-012-9110-2>. PMID:26782182.
- Kosmerl, E., Rocha-Mendoza, D., Ortega-Anaya, J., Jiménez-Flores, R., & García-Cano, I. (2021). Improving human health with milk fat globule membrane, lactic acid bacteria, and bifidobacteria. *Microorganisms*, 9(2), 341. <http://dx.doi.org/10.3390/microorganisms9020341>. PMID:33572211.
- Kumar, R., Mada, S. B., Reddi, S., Kaur, T., Kapila, R., & Kapila, S. (2021). Comparative evaluation of the protective effects of cow, buffalo and goat milk in glucocorticoid-induced bone alterations in mice. *International Journal of Dairy Technology*, 74(2), 316-323. <http://dx.doi.org/10.1111/1471-0307.12770>.
- Li, S., Huang, R., Shah, N. P., Tao, X., Xiong, Y., & Wei, H. (2014a). Antioxidant and antibacterial activities of exopolysaccharides from bifidobacterium bifidum wbino3 and *Lactobacillus plantarum* r315. *Journal of Dairy Science*, 97(12), 7334-7343. <http://dx.doi.org/10.3168/jds.2014-7912>. PMID:25282420.
- Li, W., Ji, J., Chen, X., Jiang, M., Rui, X., & Dong, M. (2014b). Structural elucidation and antioxidant activities of exopolysaccharides from *Lactobacillus helveticus* mb2-1. *Carbohydrate Polymers*, 102, 351-359. <http://dx.doi.org/10.1016/j.carbpol.2013.11.053>. PMID:24507291.
- Lopez, P., Monteserin, D. C., Gueimonde, M., de Los Reyes-Gavilán, C. G., Margolles, A., Suarez, A., & Ruas-Madiedo, P. (2012). Exopolysaccharide-producing bifidobacterium strains elicit different in vitro responses upon interaction with human cells. *Food Research International*, 46(1), 99-107. <http://dx.doi.org/10.1016/j.foodres.2011.11.020>.
- Mirlohi, M., Soleimanian-Zad, S., Dokhani, S., & Sheikh-Zeinodin, M. (2014). Microbial and physicochemical changes in yoghurts containing different lactobacillus delbrueckii subsp. *Bulgaricus* strains in association with *Lactobacillus plantarum* as an adjunct culture. *International Journal of Dairy Technology*, 67(2), 246-254. <http://dx.doi.org/10.1111/1471-0307.12122>.
- Shao, L., Wu, Z., Zhang, H., Chen, W., Ai, L., & Guo, B. (2014). Partial characterization and immunostimulatory activity of exopolysaccharides from *Lactobacillus rhamnosus* kf5. *Carbohydrate Polymers*, 107, 51-56. <http://dx.doi.org/10.1016/j.carbpol.2014.02.037>. PMID:24702917.
- Vinderola, C. G., & Reinheimer, J. A. (2003). Lactic acid starter and probiotic bacteria: a comparative "in vitro" study of probiotic characteristics and biological barrier resistance. *Food Research International*, 36(9-10), 895-904. [http://dx.doi.org/10.1016/S0963-9969\(03\)00098-X](http://dx.doi.org/10.1016/S0963-9969(03)00098-X).
- Wang, D., Sun, S., Wu, W., Yang, S., & Tan, J. (2014a). Characterization of a water-soluble polysaccharide from boletus edulis and its antitumor and immunomodulatory activities on renal cancer in mice. *Carbohydrate Polymers*, 105, 127-134. <http://dx.doi.org/10.1016/j.carbpol.2013.12.085>. PMID:24708961.
- Wang, K., Li, W., Rui, X., Chen, X., Jiang, M., & Dong, M. (2014b). Characterization of a novel exopolysaccharide with antitumor activity from *Lactobacillus plantarum* 70810. *International Journal of Biological Macromolecules*, 63, 133-139. <http://dx.doi.org/10.1016/j.ijbiomac.2013.10.036>. PMID:24189393.
- Wang, J., Zhao, X., Tian, Z., Yang, Y., & Yang, Z. (2015a). Characterization of an exopolysaccharide produced by *Lactobacillus plantarum* yw11 isolated from tibet kefir. *Carbohydrate Polymers*, 125, 16-25. <http://dx.doi.org/10.1016/j.carbpol.2015.03.003>. PMID:25857955.
- Wang, J., Zhao, X., Yang, Y., Zhao, A., & Yang, Z. (2015b). Characterization and bioactivities of an exopolysaccharide produced by *Lactobacillus plantarum* yw32. *International Journal of Biological Macromolecules*, 74, 119-126. <http://dx.doi.org/10.1016/j.ijbiomac.2014.12.006>. PMID:25532782.
- Wu, Z., Lu, J., Wang, X., Hu, B., Ye, H., Fan, J., Abid, M., & Zeng, X. (2014). Optimization for production of exopolysaccharides with antitumor activity in vitro from *Paecilomyces hepiali*. *Carbohydrate Polymers*, 99, 226-234. <http://dx.doi.org/10.1016/j.carbpol.2013.08.010>. PMID:24274500.
- Zendeboodi, F., Khorshidian, N., Mortazavian, A. M., & Da Cruz, A. G. (2020). Probiotic: conceptualization from a new approach. *Current Opinion in Food Science*, 32, 103-123. <http://dx.doi.org/10.1016/j.cofs.2020.03.009>.
- Zeng, Y., Li, Y., Wu, Q. P., Zhang, J. M., Xie, X. Q., Ding, Y., Cai, S. Z., Ye, Q. H., Chen, M. T., Xue, L., Wu, S., Zeng, H. Y. (2020). Evaluation of the antibacterial activity and probiotic potential of *Lactobacillus plantarum* isolated from chinese homemade pickles. *Canadian Journal of Infectious Diseases & Medical Microbiology*, 2020, 8818989. <http://dx.doi.org/10.1155/2020/8818989>.
- Zhang, L., Liu, C., Li, D., Zhao, Y., Zhang, X., Zeng, X., Yang, Z., & Li, S. (2013). Antioxidant activity of an exopolysaccharide isolated from *Lactobacillus plantarum* c88. *International Journal of Biological Macromolecules*, 54, 270-275. <http://dx.doi.org/10.1016/j.ijbiomac.2012.12.037>. PMID:23274679.
- Zhang, Z., Liu, Z., Tao, X., & Wei, H. (2016). Characterization and sulfated modification of an exopolysaccharide from *Lactobacillus plantarum* zdy2013 and its biological activities. *Carbohydrate Polymers*, 153, 25-33. <http://dx.doi.org/10.1016/j.carbpol.2016.07.084>. PMID:27561468.
- Zjawiony, J. K. (2004). Biologically active compounds from aphylophorales (polypore) fungi. *Journal of Natural Products*, 67(2), 300-310. <http://dx.doi.org/10.1021/np030372w>. PMID:14987072.