Optimization of in situ exopolysaccharides production by Lactobacillus helveticus MB2-1 in yogurt and its vitro functional characteristics

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Abstract

In order to improve the *in situ* exopolysaccharides (EPS) production of *Lactobacillus helveticus* MB2-1 in Sayram ketteki yogurt and explore its functional characteristics, the production optimization, rheology and microstructure of fermented yogurt, and emulsifying properties, antioxidant, antibiofilm activities of the *in situ* EPS were investigated. Based on the results of response surface methodology (RSM), the fermentation temperature 37°C, fermentation time 8.0 h and incubation amount 7.0% (w/w) was the optimum condition for *L. helveticus* MB2-1 growth and yogurt formation, and the obtained production of *in situ* EPS could be up to 340 mg/L. Under the optimized fermentation conditions, the rheology analysis illustrated that the Sayram ketteki yogurt fermented by *L. helveticus* MB2-1 formed better gel structure than the control yogurt. Besides, the *in situ* EPS presented fine emulsifying properties, which could be comparable to other biological emulsifiers. The *in situ* EPS exhibited potent antioxidant activities as well. Moreover, the antibiofilm activities of the *in situ* EPS on foodborne pathogens biofilm formation were obvious. Taken together, the results illustrated that the EPS produced in situ by *L. helveticus* MB2-1 in Sayram ketteki yogurt has the potentiality to be considered as a beneficial substitute for functional food products.

1. Introduction

Yogurt, obtained from milk fermentation by lactic acid bacteria (LAB), has been consumed among the most common dairy products around the world. The exopolysaccharides (EPS)-producing LAB has presented increasing concern mainly owing to their “generally recognized as safe” (GRAS) status and the beneficial functions to human body. Many EPSs produced by LAB possessed excellent physical and chemical properties to be applied as a tackifier, stabilizer, gel-forming agent or emulsifier in dairy industries, like the EPS produced by *Leuconostoc citreum* BMS, *Leuconostoc mesenteroides* XR1, etc. (Abid, Azabou, Blecker, Gharsallaoui, & Attia, 2020). The EPS was high-molecular-weight polymers, located outside the cell wall, had no covalent bond with bacterial membrane, and was composed of one or different kinds of monosaccharides and possess marked beneficial biological activity to human body, like anti-infective, antioxidant, antibiofilm, anti-tumor, hypolipidemic and cholesterol lowering effects, and immunostimulatory, etc. (Devi, Kavitake, Jayamanohar, & Shetty, 2021).

Reactive oxygen species (ROS) are potent oxidants that enable to react with all biological molecules, leading to the promotion of disease and aging, genetic damage and the interference and manipulation of protein. Induced oxidative stress could mediate a variety of pathological effects, including cancer, inflammation, epilepsy, atherosclerosis and lung injury, etc. (Rempe, Hartz, & Bauer, 2016). Recently, many kinds of exogenous antioxidants have been frequently added into food to get the effect of preservation and nutrition enhancement. However, many synthetic antioxidants were added into foodstuff, like butylated hydroxytoluene, may exhibit cytotoxicity. Thus, the development and utilization of efficacious natural antioxidants to prevent the human body from free radicals, is important and meaningful (M. Zhang et al., 2022).
Biofilm is the main growth pattern of bacteria in industry, clinic and natural environment, its formation by bacterial pathogens enabled to enhance their ability to escape from the host immune system and tolerance to exogenous stress, which could cause food corruption, biological pollution, ineffective treatment of chronic and repeated infections and so on, even lead to bacterial contamination and may hinder industrial processing. Some strategies targeting at the component of biofilm have been proposed to inhibit or eradicate bacterial adherence, like adding antibacterial agents (Y. Zhang et al., 2020). The EPSs produced by LAB with potential probiotic functions recently have been revealed the ability to make negatively regulation of biofilm formation and used in a wide range of applications including food products, bio-flocculants and so on. Besides, the demand of consumers for processed food without chemicals addition kept on increasing, the use of natural additives with antibiofilm activity were regarded as an option, due to no residues were left in the final products (Sacco, Castellane, Polachini, Gertrudes, & Alves, 2019). And EPSs of LAB have displayed excellent activities in inhibiting biofilm formation and they could be considered a direct replacement for chemical agents, which caused the studies about the antibiofilm actions of EPS produced by LAB were investigated more and more (Rendueles, Kaplan, & Ghigo, 2012).

The EPS-producing *L. helveticus* MB2-1 was isolated from Chinese traditional Sayram ropy fermented milk in our laboratory, which can be independently used as a starter to ferment milk, named as Sayram ketteki yogurt. The obtained yogurt presented excellent viscosity, unique flavor and mellow taste. This could be related to the EPS produced from the *L. helveticus* MB2-1 metabolism and secretion in the fermentation process. In our previous study, the produced *in situ* EPS by *L. helveticus* MB2-1 in Sayram ketteki yogurt was determined to be a new heteropolysaccharide with the molecular weight of $9.34 \times 10^4$ Da, which was composed of mannose, rhamnose, glucuronic acid, glucose, galactose, arabinose and fucose at the ratio of 0.06:0.04:0.02:1.00:1.38:0.03:0.03 (Ge et al., 2022). Li et al. (2014) reported that the EPSs of *L. helveticus* MB2-1 with modified whey solution as basal medium presented evidently inhibition on the antibiofilm activities of three biofilm forming foodborne pathogenic bacteria and good potential probiotic capacity. However, the characterization and *in vitro* functional characteristics of the EPS produced *in situ* of *L. helveticus* MB2-1 in yogurt remains unclear. Therefore, the study chose sterilized milk as the medium and set *L. helveticus* MB2-1 as the starter to optimize the fermentation conditions, so as to obtain the optimum *in situ* EPS production. Then the rheology characteristics and microstructure of fermented yogurt, and emulsifying properties, antioxidant and antibiofilm activities of the *in situ* EPS produced in yogurt were all analyzed.

2. Materials and methods

2.1 Materials and reagents

The strain *L. helveticus* MB2-1 was isolated from Xinjiang traditional Sayram ropy fermented milk of China. It was identified in line with morphological characters and 16S rDNA sequences analysis (Li. W et al., 2014). The commercial starter was composed of *Streptococcus thermophilus* and *Lactobacillus*
*bulgaricus* at the ratio of 1:1 from Danisco France Co., Ltd. (Marseille, France). The *Pseudomonas aeruginosa* PA01, *Escherichia coli* K12 and *Staphylococcus aureus* ATCC 6538 were obtained from Collaborative Innovation Center for meat production and processing, Jiangsu province, China. These foodborne pathogens were cultured in Luria Bertani (LB) broth containing 0.5% (w/v) yeast extract, 1% (w/v) tryptone and 0.5% (w/v) NaCl at 37°C to determine biofilm formation.

1,1-diphenyl-2-picryl-hydrazyl (DPPH), Nicotinamide adenine dinucleotide (NADH), vitamin C (VC), phenazine metho-sulfate (PMS), nitro blue tetrazolium (NBT), ferrozine and 1,10-phenanthroline were obtained from McLean Biochemical Technology Co. Ltd. (Shanghai, China). The paraffin oil, olive oil, walnut oil, rice oil, sunflower oil, soybean oil, zanthoxylum oil, linseed oil, grape-kernel oil, corn germ oil, avocado oil and sesame oil were purchased from local supermarket. The hydrocarbon (cyclohexane, iso-octane, benzene and xylene) and other related reagents were all analytical pure level.

### 2.2 Analysis of response surface methodology (RSM)

#### 2.2.1 Activation and preparation of strains

The freeze-dried preserved *L. helveticus* MB2-1 and control starter were inoculated into sterilized fresh milk, cultured at 37 °C and repeatedly sub-cultured for three times to fully activate the strains, which was set as the working starter (10⁶ CFU/mL).

#### 2.2.2 Single factor test, titratable acidity (TA) determination and EPS extraction

The fermentation temperature (25, 30, 35, 40, 45°C), strain inoculation amount (1, 3, 5, 7, 9%) and fermentation time (4, 6, 8, 10, 12h), which are the factors affecting EPS production and TA of prepared yogurt were selected for single factor test.

The TA value of yogurt was determined by traditional titration (Alamo, Maquieira, Puchades, & Sagrado, 1993). The results were expressed in the unit of °T. Besides, the method of EPS extraction was on the basis of previous study (W. Li et al., 2014). Briefly, yogurts were diluted with appropriate amount of deionized water to reduce viscosity for centrifugation and bacterial separation. Then, the centrifuge, set at 4 °C, 12,000 × g for 15 min, was utilized to obtain the supernatant containing polysaccharides. The supernatant containing 4% (v/v) trichloroacetic acid (TCA) was prepared and set at 4°C for 6-8h to precipitate protein. Subsequently, the proteins were removed by centrifugation (12,000 ×g, 4°C, 30 min). Then, a 0.45µm membrane filter was used to filter the supernatant, and the EPS was isolated from the supernatant by adding three times volumes of 95% (v/v) ethanol at 4°C for overnight, and obtained by centrifugation (15,000 ×g, 15 min). The precipitates were dissolved in distilled water, put in dialysis bags, dialyzed against distilled water for 48 h at 4°C and then lyophilized.

#### 2.2.3 Optimization of response surface test
The single-factor experiment indicated that the suitable condition for yogurt fermentation by *L. helveticus* MB2-1 was 5% (w/w) of inoculation amount, 40°C of fermentation temperature and 6 h of fermentation time (Fig. 1). Then, the RSM test design was carried out with the fermentation temperature, inoculation amount and fermentation time as independent variables, and EPS production and titratable acidity (TA) as response values with Design Expert 8.0.6 version. According to the method of Li et al. (2020), each parameter was set to three levels (-1, 0, 1), and the Box-Behnken design was used to evaluate the optimal combination of fermentation parameters. The empirical model was obtained to visualize the relation between dependent and independent variables by second-order regression analysis, so as to derive the optimum conditions.

### 2.3 Rheological analysis

The Sayram ketteki yogurt and the control yogurt was fermented by 7% (w/w) of *L. helveticus* MB2-1 as starter and 7% (w/w) of commercial starter as starter, respectively, which were prepared under the optimal fermented condition (37°C, 8h) to compare and analyze their rheological properties. Based on the method of Meletharayil et al. (2016), the viscosity, oscillation and thixotropy assays were carried out using the Physica MCR101 rheometer system (Anton Par GmbH, Austria) with a plate geometry sensor (PP-50, 1.000 mm gap). The yogurt sample (3.00 g) were transferred carefully from the beaker to the platform of the rheometer, and the excessive samples were trimmed away.

As a function of shear stress or shear rates, viscosity was kept a lookout over at 5°C for yogurt samples. The shear stress and shear rate were set from 0.01 Pa to 100 Pa and from 0.01s⁻¹ to 1000 s⁻¹, respectively. Then, referred to the methods of Fu et al. (2018), temperature sweep was performed by oscillation pattern to keep tabs on the structural change of samples. During the test, temperature increased from 5°C to 40°C and all samples were treated under invariable deformation (1% strain, 1Hz). Damping factor (tanδ) and complex viscosity (η*) were obtained according to the value of G' and G". The anti-shear characteristics of samples were obtained by Three-interval thixotropy test (3ITT). The sample was prepared under 1% deformation and 1Hz until reaching stable status. Then, a high shear rate (1000 s⁻¹) was executed in 1 s. After G" and G' were recorded until the stable status restored (~ 400s), the temperature was linearly increased from 5°C to 40°C at a heating rate of 0.075 °C/s, and the modulus was continuously recorded.

### 2.4 Microstructure observation

The microstructure observation of the yogurts was based on previous method and performed by Hitachi SU8010 cryo-scanning electron microscope (Tokyo, Japan) (Hussain et al., 2017). The samples were placed on copper holders and plunging into liquid nitrogen slush at -207°C, which then were transferred into an attached preparation chamber and fractured with a cold scalpel blade. The specimen was coated with 300Å of sputtered gold and put onto the cold stage maintaining at -95°C under vacuum for observation.
2.5 Functional properties of the *in situ* EPS produced from Sayram ketteki yogurt

2.5.1 Emulsifying property determination

The emulsifying property of *in situ* EPS was investigated based on the method of Li et al. (2014) with slight modification. About 3mL prepared *in situ* EPS solution (10 mg/mL) was absorbed and mixed with 3.0 mL oil of different kinds (paraffin oil, olive oil, walnut oil, rice oil, sunflower oil, soybean oil, zanthoxylum oil, linseed oil, grape-kemel oil, corn germ oil, avocado oil and sesame oil) or hydrocarbon (cyclohexane, isoctane, benzene and xylene). The mixture was agitated violently for 2 min by vortex. The height of aqueous and emulsion layers was both measured after 24 h. The following formula was used to obtain emulsification index (EI<sub>24</sub>):

\[
EI_{24} = \left( \frac{V_{\text{emulsion layer}}}{V_{\text{total}}} \right) \times 100.
\]

All assays were carried out in triplicates.

2.5.2 Antioxidant activity analysis

The 1.0 mL sample solution (0.125-4.0 mg/mL) and 2.0 mL of deionized water were sequentially added into 0.2 mL ethanolic DPPH radical solution (0.4 mM). Then, the full mixed solutions were stood at room temperature in the dark for 30 min. The absorbance was read at 517 nm. The scavenging activity on DPPH radical (%) = \[(1 - (A_{\text{sample}} - A_0)/A_{\text{blank}}) \times 100\], where \(A_0\) represented the absorbance of the sample under identical condition as \(A_{\text{sample}}\) with water instead of DPPH radical solution, and deionized water and VC were used as the blank and positive control, respectively (W. Li et al., 2014; Tang et al., 2017). The lower the absorbance was, the stronger the scavenging activity was.

The hydroxyl radical was generated when 1 mL of 0.75 mM FeSO<sub>4</sub>, 1 mL of 0.75 mM 1,10-phenanthroline, 1.5 mL of 0.15 M sodium phosphate buffer (pH 7.4) and 1 mL of H<sub>2</sub>O<sub>2</sub> (0.01%, v/v) were mixed together. Then, the mixture was added with 1.0 mL sample solution (0.125-4.0 mg/mL) and incubated at 37°C for 30 min. The absorbance of the mixture was read at 536 nm. The hydroxyl radical scavenging activity (%) = \[(A_{\text{sample}} - A_{\text{blank}})/(A_0 - A_{\text{blank}})\] \times 100, where \(A_0\) represented the absorbance of the deionized water in the assay system excluding H<sub>2</sub>O<sub>2</sub> and sample, VC and deionized water were used as the blank and positive control, respectively (Tang et al., 2017).

Based on the method of Li et al. (2014), the superoxide radical scavenging activity was evaluated. When the 0.1 M sodium phosphate buffer (pH 7.4) containing 20 mM PMS, 156 mM NADH and 52 mM NBT was prepared, the superoxide radical was arised in the mixture. Then, the mixture was added with 1.0 mL sample solution (0.125-4.0 mg/mL) and incubated for 5 min at 25°C. The absorbance of the mixture was determined at 560 nm. The superoxide radical scavenging activity (%) = \[(1 - A_{\text{sample}}/A_{\text{blank}}) \times 100\]. VC and deionized water were used as positive control and the blank, respectively.

The chelating ability of metal ion was determined in line with the method reported by Li et al. (2014). Briefly, aspirating 2.75 mL deionized water, 0.2 mL 5 mM ferrozine solution, 0.05 mL 2 mM ferrous...
chloride (FeCl₂) solution and 1.0 mL sample solution (0.125-4 mg/mL) to mix together sufficiently and keep at room temperature for 10 min. The absorbance of the mixture was read at 562 nm. The ferrous ion chelating ability (%) = \[ \frac{A_{\text{blank}} - (A_{\text{sample}} - A_0)}{A_{\text{blank}}} \times 100 \], where \( A_0 \) represented the absorbance of the sample under identical conditions with water as \( A_{\text{sample}} \) instead of FeCl₂ solution, the EDTA-2Na and deionized water were used as the positive control and the blank, respectively.

### 2.5.3 Determination of antibiofilm activity

The antibiofilm activity of *in situ* EPS was analyzed based on the method described by Zhang et al. (2020). The activation of *P. aeruginosa* PA01, *E. coli* K12 and *S. aureus* ATCC 6538 strains with 1% inoculation were performed by LB broth. Different concentrations of *in situ* EPS samples (0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/mL) were prepared by LB broth and added into 96 wells plate. The LB broth without *in situ* EPS addition was set as negative control. Then, the plates were covered and incubated aerobically at 37°C for 24 h.

Then, the plate was gently rinsed with sterile normal saline to remove any bacteria that did not adhere. The remaining bacteria were fixed with 95% methanol, and the plates were dried naturally. Then, each well was stained with 0.2 mL 2% crystal violet for 5 min. The excessive dyes were removed by sterile distilled water to wash the plate for three times. After air drying naturally, 0.16 mL of 33% (v/v) glacial acetic acid was used to resolubilize the dyes bound to the adherent cells. The dissolved dye from each well (125 mL) was diluted and equivalently transferred to the separation well in the optically transparent 96-well plate, and the value of OD was determined at 595 nm. According to the formula: Inhibition rate of biofilm formation = \[ 1 - \frac{\text{OD}_{\text{assay}}}{\text{OD}_{\text{control}}} \] × 100%, each data point was obtained from the average of three repeated wells, and the standard deviation (SD) was calculated.

### 2.5.4 Visual observation of the biofilm of *S. aureus* ATCC 6538

The biofilm of *S. aureus* ATCC 6538 was observed based on the method of Yin et al. (2021) by the confocal laser scanning microscopy (CLSM, Ultra View VOX; PerkinElmer, USA). The LB medium was inoculated with 1% (v/v) activated *S. aureus* ATCC 6538 (\( \text{OD}_{600} \) nm = 0.4) to prepare the mixture supplemented with *in situ* EPS (4 mg/mL) or not. The two kinds of mixture were transferred 2 mL into a 6-well plate and a 35 mm confocal dish, respectively, which were incubated for 24 h at 37°C. Each well of the 6-well plate was contained a cover glass of 1 × 1 cm. After that, the cover glasses and the dishes were carefully washed and stained by DAPI (1µg/mL). The cover glasses were observed under 2D image in grey and color mode, and the dishes were observed under 3D image in color mode, respectively.

### 2.6 Statistical analysis

The one-way ANOVA was used to analyze the obtained data by the IBM SPSS Statistics 25.0 (IBM Corp., Armonk, N.Y., USA). Means of different experiments were compared by the least significant difference test at \( p < 0.05 \) and \( p < 0.01 \) level. In all experiments, three replicates were performed (\( n = 3 \)). The graphs were constructed by OriginPro 2019b (OriginLab Corporation, Massachusetts, USA).
3. Results and discussion

3.1 RSM

Based on the result of single-factor test, the assay was designed as Table 1. Under the same temperature, the TA value increased with the extension of fermentation time. Under the conditions of fermentation temperature of 37°C, inoculation amount 7% (w/w) and fermentation time 8 h, the in situ EPS production and TA value both reached the optimum values. According to these experimental data, the empirical model was established to further carry out the quadratic regression analysis to assess the relationship between the independent variables and the response variables. Then, two models were obtained and exhibited in Eqs. (1) and (2) as followed:
Table 1
Results for response surface analysis

<table>
<thead>
<tr>
<th>Sequence Number</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>pH value</th>
<th>EPS production (mg/L)</th>
<th>TA value(°T)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Actual Value</td>
<td>Predicted Value</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.18</td>
<td>346.00</td>
<td>342.01</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.18</td>
<td>347.82</td>
<td>341.92</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>4.05</td>
<td>311.00</td>
<td>312.76</td>
</tr>
<tr>
<td>4</td>
<td>-1</td>
<td>0</td>
<td>-1</td>
<td>4.34</td>
<td>238.10</td>
<td>236.82</td>
</tr>
<tr>
<td>5</td>
<td>-1</td>
<td>-1</td>
<td>0</td>
<td>4.29</td>
<td>290.14</td>
<td>293.17</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>3.95</td>
<td>327.81</td>
<td>324.78</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.18</td>
<td>342.20</td>
<td>341.83</td>
</tr>
<tr>
<td>8</td>
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<td>0</td>
<td>-1</td>
<td>4.21</td>
<td>277.55</td>
<td>283.55</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>-1</td>
<td>1</td>
<td>4.30</td>
<td>310.13</td>
<td>313.10</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.20</td>
<td>330.00</td>
<td>341.78</td>
</tr>
<tr>
<td>11</td>
<td>-1</td>
<td>0</td>
<td>1</td>
<td>4.25</td>
<td>321.45</td>
<td>315.45</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>-1</td>
<td>0</td>
<td>4.28</td>
<td>293.32</td>
<td>289.08</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>3.84</td>
<td>282.02</td>
<td>283.30</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
<td>1</td>
<td>-1</td>
<td>4.08</td>
<td>301.20</td>
<td>298.23</td>
</tr>
<tr>
<td>15</td>
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<td>4.24</td>
<td>343.60</td>
<td>341.18</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>-1</td>
<td>-1</td>
<td>4.36</td>
<td>251.00</td>
<td>249.24</td>
</tr>
<tr>
<td>17</td>
<td>-1</td>
<td>1</td>
<td>0</td>
<td>4.06</td>
<td>301.88</td>
<td>306.12</td>
</tr>
</tbody>
</table>

Note: A, B, C represented fermentation temperature, fermentation time and inoculation amount, respectively.

\[ Y_1 = -5175.07 + 231.19 \times A + 42.75 \times B + 282.24 \times C + 0.94 \times A \times B + 3.28 \times A \times C - 3.08 \times B \times C - 2.89 \times A^2 - 3.13 \times B^2 - 9.01 \times C^2 \]  
\[ R^2 = 0.9793 \]
$Y_2 = -1175.96948 + 55.66417A + 39.29708B + 23.54667C - 0.60833AB - 0.34792AC + 0.32BC - 0.64875A^2 - 1.14281B^2 - 0.86719C^2 \quad (2)$

$R^2 = 0.9690$

In Eq. (1) and Eq. (2), the predicted EPS production (mg/L) and TA value (°T) of yogurt were represented by $Y_1$ and $Y_2$, respectively. The A, B and C represented fermentation temperature (°C), fermentation time (h) and inoculation amount (%), respectively. The analysis of variance (ANOVA) was used to analyze the two models (Supplementary Table 1 and Table 2). The results showed that the difference of Eq. (1) and Eq. (2) both reached a very significant level ($P < 0.01$), and no significant difference was found in the missing fitting terms ($P = 0.4653$, $P = 0.3790$), which fully reflected the actual relationship between the three parameters and the response value. The interaction term (B×C) made obvious effect on EPS production ($P < 0.05$), and the interaction term (A×B) played significant role in TA value of *L. helveticus* MB2-1 fermented yogurt ($P < 0.01$). Besides, the linear terms of fermentation time (B) and inoculation amount (C), interaction terms (A×C) and quadratic terms (A×A, B×B and C×C) all played the important role on EPS production and TA value of yogurt ($P < 0.05$). In addition, the determination coefficients ($R^2$) of the two models reached 0.9793 and 0.9690, respectively, indicating that the linear relationship between the dependent variable and the independent variable was good, and the coefficient of variation of the test was 19.386 and 14.999, respectively, demonstrating the high reliability of the test. Moreover, as can be seen from the response surface and contour plots of the effects of different interaction factors on EPS production (Fig. 2) or TA value of fermented yogurt (Fig. 3), which showed that the regression model has a maximum value.
Table 2
Emulsifying properties of the *in situ* EPS

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample</th>
<th>( \text{El}_{24}(%) ) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Xylene</td>
<td>53.62 ± 2.51 cd</td>
</tr>
<tr>
<td>2</td>
<td>Benzene</td>
<td>55.14 ± 2.39 cd</td>
</tr>
<tr>
<td>3</td>
<td>Isooctane</td>
<td>44.07 ± 7.07 e</td>
</tr>
<tr>
<td>4</td>
<td>Cyclohexane</td>
<td>48.77 ± 8.49 de</td>
</tr>
<tr>
<td>5</td>
<td>Paraffin oil</td>
<td>53.70 ± 1.16 cd</td>
</tr>
<tr>
<td>6</td>
<td>Zanthoxylum oil</td>
<td>60.56 ± 5.46 bc</td>
</tr>
<tr>
<td>7</td>
<td>Corn germ oil</td>
<td>51.45 ± 4.53 d</td>
</tr>
<tr>
<td>8</td>
<td>Olive oil</td>
<td>70.19 ± 1.08 ab</td>
</tr>
<tr>
<td>9</td>
<td>Sunflower oil</td>
<td>74.36 ± 3.74 a</td>
</tr>
<tr>
<td>10</td>
<td>Linseed oil</td>
<td>64.64 ± 4.71 bc</td>
</tr>
<tr>
<td>11</td>
<td>Rice oil</td>
<td>59.29 ± 1.89 c</td>
</tr>
<tr>
<td>12</td>
<td>Soybean oil</td>
<td>53.80 ± 5.01 cd</td>
</tr>
<tr>
<td>13</td>
<td>Avocado oil</td>
<td>66.67 ± 3.70 b</td>
</tr>
<tr>
<td>14</td>
<td>Walnut oil</td>
<td>61.73 ± 2.14 bc</td>
</tr>
<tr>
<td>15</td>
<td>Sesame oil</td>
<td>64.81 ± 2.62 bc</td>
</tr>
<tr>
<td>16</td>
<td>Grape-kernel oil</td>
<td>71.20 ± 3.31 ab</td>
</tr>
</tbody>
</table>

The statistically similar groups were denoted by the same letter and statistically different groups were denoted by different letters.

The values are the means ± standard deviations (n = 3). For each oil and hydrocarbon sample, 10 mg/mL of *in situ* EPS was used to measure the emulsifying activity.

The TA value was one of the main parameters to assess the sensory evaluation of yogurt to ensure the quality and flavor. It was influenced by numerous elements, like fermentation time, fermentation temperature, fermentation degree, etc. You et al. (2020) reported that the LAB strains and fermentation time made effects on the yield of R-5-EPS based on the determination of titratable acidity. Through the model of Eq. (1) and Eq. (2), under an optimum fermentation condition of fermentation temperature 37°C, inoculation amount 7% (w/w) and fermentation time 8 h, the TA value of yogurt was predicted as
106.78 °T, while the polysaccharide production could be up to 342 mg/mL, which is in line with the quality index of high-quality yogurt. Under the optimum conditions, the verification test was carried out. The results showed that the EPS production was 340 mg/L and the TA value was 106.41 °T, which was basically consistent with the predicted value, indicating that the model established by the response surface test had high accuracy.

3.2 Rheological properties and microstructure observation

The rheological behavior of Sayram ketteki yogurt was compared and analyzed with the control yogurt prepared with commercial starter. The flow behavior for control yogurt and Sayram ketteki yogurt were determined with the increase of shear stress and shear rate (Fig. 4). With shear rate increasing, all samples were observed typical non-Newtonian shear thinning behavior. Before a certain shear stress was up to 10 Pa, the dynamic viscosity kept relative stable. After that, the viscosities started to decrease obviously (Fig. 4A). This could be explained that the expected behavior of the solid gelatinous structure requires sufficient destruction of the structure before appreciable flow could be achieved (Laiho, Williams, Poelman, Appelqvist, & Logan, 2017). The zero-shear viscosity was used to represent the initial viscosity under low shear stress. Sayram ketteki yogurt presented a significantly higher zero-shear viscosity than the control at 0.1 Pa and 1 Pa (p < 0.05). Moreover, many previous studies have reported that set yogurts need more shear stress before flowing, and the yield stress could be considered as an index to determine the initial firmness (Fu et al., 2018; Laiho et al., 2017). This indicated that Sayram ketteki yogurt displayed a higher yield stress, formed a firmer gel structure than control yogurt. The viscosity of the two yogurts exhibited a second rapid decrease at 100 Pa. As the shear stress kept in increase, the viscosity of two yogurts retained the tendency of decrease (Fig. 4A) and were statistically different from each other at 100 Pa (p < 0.05). This viscosity difference could be reflected as a function of shear rate as well (Fig. 4B). When the shear rate was 100 s⁻¹, the average viscosity of Sayram ketteki yogurt was 2.41 Pa*s, while that of control was 1.84 Pa*s. The second rapid decrease for the two yogurts maybe be related to the protein network gel structure of them. As the explain of previous studies, the breakdown of the weaker casein-WP (whey protein) mixed network could lead to the initial drop in viscosity, and the breakdown of the stronger WP-WP aggregation network under the higher shear stress resulted in the second drop in viscosity (Prasanna, Grandison, & Charalampopoulos, 2013).

Oscillation analysis illustrated that Sayram ketteki yogurt had better yogurt gel structure (Fig. 5A). The quotient of G” divided by G’ was damping factor (tanδ). As shown in Fig. 5B, the mean value of tanδ was 0.23 for control yogurt whereas it was 0.26 for Sayram ketteki yogurt. Besides, the values of tanδ presented more stable in Sayram ketteki yogurt than that in control yogurt during heating (5°C-40°C). Similar results were also obtained when salecan was added into yogurt (Fu et al., 2018). However, the viscous character represented by G” did not decrease proportionally with G’. This demonstrated that the interaction between yogurt protein molecules and EPS were existent (Xu et al., 2019).

Through thixotropy tests, the result illustrated the structure of yogurt fermented by *L. helveticus* MB2-1 strain was fortified (Fig. 6). The structure of Sayram ketteki yogurt presented more stable states than
control yogurt during heating after shear treatment. The recovery rates for G" and G' of control yogurt after high shear rate treatment were 47.34% and 13.71% (Fig. 6A), respectively, and the recovery rates were separately 48.16% and 14.64% for Sayram ketteki yogurt (Fig. 6B). The recovery rate of Sayram ketteki yogurt was higher than that of control yogurt, demonstrating that the yogurt structure could be strengthened by *L. helveticus* MB2-1 fermentation.

The results of rheological analysis fully suggested that *L. helveticus* MB2-1 strain made significant effects on strengthening the microstructure of yogurts to improve their anti-shear performance. This was verified by the comparison between the microstructure of the control yogurt (Fig. 7A) and Sayram ketteki yogurt (Fig. 7B) as well. The homogeneous fat globules in the two yogurts were both distributed in the network structure formed by casein micelles. The Sayram ketteki yogurt had a more compact structure with even porous compared to the microstructure the control. The EPS existed in the pores of casein micelles as filaments. Under different magnification, the EPS in Sayram ketteki yogurt can be obviously observed, whereas there was relatively little in the control yogurt. Taken together, the existence of produced EPS *in situ* made positive effects on the rheological properties and microstructure of Sayram ketteki yogurt.

### 3.3 Functional characteristics of *in situ* EPS *in vitro*

#### 3.3.1 Emulsifying properties of EPS produced *in situ*

Some vegetable and mineral oils, and hydrocarbons, as the hydrophobic substances, were used to evaluate the emulsifying property of EPS samples. From Table 2, the $E_{24}$ value of *in situ* EPS was significant in sunflower oil (74.36%), grape-kernel oil (71.20%), olive oil (70.19%), avocado oil (66.67%), sesame oil (64.81%), linseed oil (64.64%) and walnut oil (61.73%), whereas the emulsification activity of the *in situ* EPS upon zanthoxylum oil, rice oil, benzene, soybean oil, paraffin oil, xylene and corn germ oil was moderate, the $E_{24}$ value of *in situ* EPS were 60.56%, 59.29%, 55.14%, 53.80%, 53.70%, 53.62% and 51.45%, respectively. The other hydrocarbons tested, namely, isooctane and cyclohexane, having lower $E_{24}$ value (44.07%, 48.77%, respectively). The similar study were reported by Kannmani et al. (2011), which indicated that EPS of probiotic *Streptococcus phocae* PI80 produced in MRS broth had fine emulsifying abilities to stabilize various vegetable oil/water or hydrocarbon/water emulsions, which might be comparable to other biological emulsifiers. The results illustrated that the EPS produced *in situ* by *L. helveticus* MB2-1 had good potential of emulsion formation and stability for hydrophobic compounds. This providing a theoretical basis for its use as a soluble natural polymer in food texture optimization and the development of new emulsifiers or foods.

#### 3.3.2 Analysis of antioxidant activities of *in situ* EPS

The scavenging activities of *in situ* EPS on DPPH, hydroxyl and superoxide radicals, and chelating activity on ferrous ions were dependent on concentration, whereas the scavenging activities of them were all lightly lower than VC at the same concentration (Fig. 8A-C). The results had some similarity with the
results of Li et al. (2014). As for the scavenging ability of DPPH, when the concentration of VC was < 0.125 mg/mL, it reached the EC_{50} value. So did the \textit{in situ} EPS sample, with lower scavenging ability than that of VC. Besides, the scavenging activity of \textit{in situ} EPS on DPPH radicals reached 85.43% at 4 mg/mL. These results illustrated that \textit{in situ} EPS of \textit{L. helveticus} MB2-1 in yogurt was an effective scavenger of DPPH free radical, which could better inhibit oxidative damage and disease, slow down aging as well.

The highest scavenging activity of \textit{in situ} EPS on hydroxyl free radicals was 71.74% at the concentration of 4 mg/mL (Fig. 8B). The similar to Fig. 8A, when the concentrations of EPS and VC both were < 0.125 mg/mL, they reached the EC_{50} value, and the hydroxyl radical scavenging activity was 60.84% and 64.25%, respectively. Besides, the scavenging activity of EPS on superoxide anion was obviously weaker than that on DPPH radical and hydroxyl radical (Fig. 8C). Its scavenging capacity was always relative lower than VC under the same concentration. However, EC_{50} value could be reached as well when the concentration was < 0.125mg/mL. And the scavenging activity was 64.32% at the concentration of 4 mg/mL.

The chelating ability on metal ion of EPS was similar to that of EDTA-2Na ($p > 0.05$). At the concentration of 0.25 mg/mL, it reached EC_{50} value (Fig. 8D). The chelating ability of EPS was 50.50%, which was slightly lower than that of EDTA-2Na (52.21%). And the chelating ability of EPS on Fe^{2+} at 4.0 mg/mL was reached 79.71%. This demonstrated that the chelating activity of the EPS \textit{in situ} by \textit{L. helveticus} MB2-1 in yogurt on Fe^{2+} seemed to be higher than the EPS from the optimum whey medium (W. Li et al., 2014). As Yu et al. (2020) reported that acidic polysaccharides may include more negative charges, which caused the chelating activities of them were relatively high when metal ions were attacked.

### 3.3.3 Determination of antibiofilm activities of \textit{in situ} EPS

The inhibitory activities of the EPS on biofilm formation by the three foodborne pathogens were dependent on concentration (Fig. 9). Among these pathogens, the inhibition rate of \textit{in situ} EPS (4 mg/mL) on the biofilm formation of \textit{S. aureus} ATCC 6538, \textit{E. coli} K12 and \textit{P. aeruginosa} PA01 were obviously reached 75.34%, 72.31% and 69.88%, respectively. The results illustrated that the \textit{in situ} EPS of \textit{L. helveticus} MB2-1 from yogurt had antibiofilm activity with broad spectra, which is in line with the result of Li et al. (2014). Based on previous studies, Sacco et al. (2019) reported that the inhibition of Burkholderia cepacia biofilm formation was clearly detected in EPS MS5. Sarikaya et al. (2016) found that the inhibition rate of \textit{L. fermentum} LB-69 I-EPS (1 mg/mL) on the biofilm formation of \textit{B. cereus} RSKK 863, \textit{P. aeruginosa} ATCC 72853 and \textit{L. mono-cytogenes} ATCC 7644 were high up to 90%, 88% and 88%, respectively. These results have some similarity to the investigation of Kim et al. (2009).

Based on the best antibiofilm effect of \textit{in situ} EPS on \textit{S. aureus} ATCC 6538, the biofilm inhibition of which was observed visually. The result showed that the \textit{in situ} EPS significantly inhibited \textit{S. aureus} ATCC 6538 biofilm formation with decreasing the viable counts (Fig. 10). Besides, the confocal 3D images presented that the biofilm thickness of \textit{S. aureus} ATCC 6538 in the control group was 66.32 ± 1.85 μm, whereas the biofilm thicknesses dropped to 16.83 ± 1.81μm after being treated with 4mg/mL \textit{in situ} EPS, which
indicated that the in situ EPS mainly achieved the inhibitory effect by inhibiting the growth of S. aureus ATCC 6538 and destroying its biofilm structure formation.

4. Conclusion

The fermentation conditions for the high production of in situ EPS produced by probiotic L. helveticus MB2-1 in milk medium were optimized. Under the premise of guaranteeing yogurt quality, the optimal fermentation conditions for L. helveticus MB2-1 to produce EPS were as follows: fermentation temperature 37 ℃, fermentation time 8 h, inoculation amount 7%, corresponding EPS production 340 mg/L, TA value of fermented yogurt 106.41 °T. The texture and rheology analysis illustrated that the Sayram ketteki yogurt formed a firmer and better gel structure than traditional commercial yogurt. Besides, the in situ EPS exhibited excellent emulsifying properties, which could be comparable to other biological emulsifiers. Moreover, the vitro antioxidant experiments demonstrated that the antioxidant properties of EPS were strong, especially the scavenging abilities of DPPH radicals and chelating ability on metal ions. The inhibitory activities of the in situ EPS on biofilm forming were much obvious as well. Taken together, the results demonstrated that EPS of L. helveticus MB2-1 produced in situ in Sayram ketteki yogurt could be regarded as a prebiotic alternative source for nutrition and health food products.

Declarations

Declaration of competing interest

There is no conflict of interest to declare.

Authorship Contribution Statement


Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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References


**Figures**

![Figure 1](image1)

**Figure 1**

Effects of fermentation temperature (A), fermentation time (B) and inoculation amount (C) on *in situ* EPS production and titratable acidity of fermented yogurt.
Figure 2

Response surface and contour of different interaction factors on the production of exopolysaccharides.
Figure 3

Response surface and contour of different interaction factors on TA value of fermented yogurt.
Figure 4

The average viscosity (Pa*s) of set yogurts fermented by different starters as a function of (A) shear stress (Pa) and (B) shear rate (1 s⁻¹). Sayram ketteki yogurt, set yogurt fermented by *L. helveticus* MB2-1 strain; Control yogurt, set yogurt fermented by commercial starter, including *S. thermophilus* and *L. bulgaricus*. 
Figure 5

Temperature dependent oscillation graphs of Sayram ketteki yogurt and control yogurt. Experiments were carried out at 1 Hz, 1% strain.

Figure 6

Thixotropy test graphs of control yogurt (A) and Sayram ketteki yogurt (B). (Experimental conditions were set at 5°C, 1Hz, and 1% strain at the first stage. As shown by arrows in the figure, in the final stage, the temperature increased linearly from 5°C to 40°C with a heating rate of 0.075°C/s.)
Figure 7

Cryo-scanning electron micrographs of control yogurt (A) and Sayram ketteki yogurt (B).

Figure 8

Scavenging activities on DPPH radical (A), hydroxyl radical (B), superoxide radical (C) and metal ion chelating activity (D) of *in situ* EPS and positive control.
Data are presented as means ± SD (n=3).

**Figure 9**

Antibiofilm activities of EPS from *L. helveticus* MB2-1 against *P. aeruginosa* PAO1, *E. coli* K12 and *S. aureus* ATCC 6538. Data are presented as means ± SD (n=3).

**Figure 10**
CLSM micrographs of biofilm formation by *S.aureus* ATCC 6538 in the absence of *in situ* EPS (Control) and the presence of *in situ* EPS (Treatment). (A) 2D image in grey mode at the middle of the biofilm, (B) 2D image in color mode at the middle of the biofilm, and (C) 3D image of the biofilm.

**Supplementary Files**

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