



Short Communication

The investigation of soybean protein isolates and soybean peptides assisting *Lactobacillus plantarum* K25 to inhibit *Escherichia coli*

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ABSTRACT

Soybean protein isolates and their hydrolysates are considered as one of the most high-quality proteins among plant proteins, and current research has shown that they have potential probiotic functions. The purpose of this study was to investigate the effects of digested soybean protein isolates (dSPI) and digested soybean peptides (dPEP) on *L. plantarum* K25 alone and the two bacteria when co-cultured with *E. coli*. It showed that dSPI and dPEP promoted the growth and metabolism of *L. plantarum* K25, and dSPI had a better effect. Besides, dSPI and dPEP still promoted the growth and organic acid secretion of *L. plantarum* K25 when co-cultured with *E. coli*, and the dPEP treatment was more effective than dSPI. Moreover, dSPI and dPEP reduced the survival rate of *E. coli* when co-cultured with *L. plantarum* K25. These results to some extent explained the cooperation of dSPI and dPEP with *L. plantarum* K25 to produce acid thereby weaken the growth of *E. coli*.

1. Introduction

Functional food was developed to improve human health, which often consists of highly nutritional valuable proteins. Therefore, a broad search of such nutrients is constantly conducted (Swiatecka et al., 2013). Soybean protein and peptides are excellence candidates containing eight essential amino acids and no cholesterol. Studying the functionality of soybean proteins and peptides is beneficial to human diet and health (Zhang et al., 2020). Dietary intake appears to be a major regulator of the structure and function of intestinal microbiota in the short and long term, strongly affecting the relationship between human hosts and their microbiota. Changes in intestinal flora have been proven to be associated with many human diseases, including obesity, diabetes, neurodegenerative and inflammatory bowel diseases (Vujkovic et al., 2020). Intestinal probiotics are active microorganisms that colonize the human body and alter the composition of the flora in a particular part of the body. Probiotics colonize the intestinal tract and can effectively improve the intestinal microenvironment and regulate intestinal homeostasis (Comess and Abad, 2023). *Lactobacillus plantarum* (*L. plantarum*) is one of the most widely used probiotics with great beneficial effects on human and animal health. Studies have shown that *L. plantarum* can

produce lactic acid and various metabolites during colonization, which can effectively inhibit pathogenic bacteria growth and modulate immune functions (Chen et al., 2021; Peter et al., 2015). Therefore, regulating intestinal flora through dietary will be crucial to the health of human hosts.

The research showed that soybean protein hydrolysate (SPH) has potential for modulation or balance of gut microbiota, and SPH increased the number of *lactobacilli* after 72 h fermentation. Our previous studies have also shown that soybean protein and peptides can promote the growth and metabolism of *Lactobacillus reuteri* LR08 (Zhang, Xia, et al., 2022; Zhu et al., 2022). Some studies have also explored the key genes and proteins regulated by soybean protein and peptides in *Lactobacillus* through transcriptome and proteome methods (Zhang, Zhu, et al., 2022). However, there was controversy on the role of proteins in different types of lactic acid bacteria or probiotics. Research on the effects of soybean protein and peptides on different types of lactic acid bacteria is still very limited.

In the present study, soybean protein isolates and peptides were simulated gastrointestinal digestion *in vitro*. The purpose of this research was to assess the influence of dSPI and dPEP on the proliferation and metabolism of *L. plantarum* K25 in mono-culture and co-culture with

Abbreviations: dSPI, Gastrointestinal digested soybean protein isolate; dPEP, Gastrointestinal digested soybean peptide; MRS, Man Rogosa Sharpe broth.

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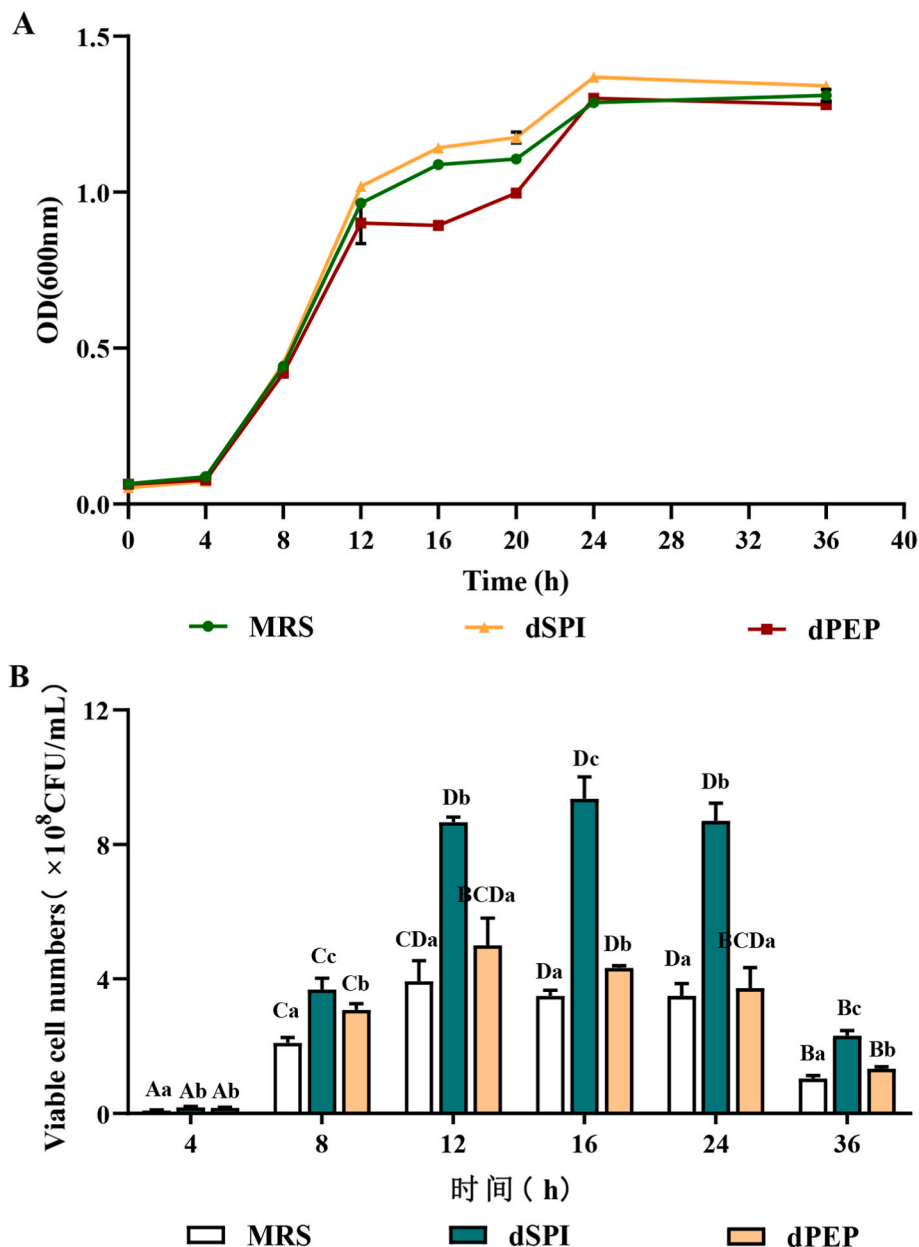


Fig. 1. Growth analysis of *L. plantarum* K25 incubated for 36 h in different treatment. (A) Growth curves determined by measuring the OD values at 600 nm. (B) Viable cell numbers determined by viable count method. Values represent the means of three independent experiments \pm standard deviation. For each tested, different small letters between different treatments and different capital letters between time points indicate significant differences between groups ($P < 0.05$).

E. coli, and whether soybean protein isolates and soybean peptides could cooperate with *L. plantarum* K25 to inhibit *E. coli* was also investigated.

2. Materials and methods

2.1. Materials and reagents

Soybean protein isolates and peptides are provided by Shandong Xinyu Biotechnology Co., Ltd and Rushan Hualong Food Co., Ltd. *L. plantarum* K25 were preserved by Dairy function laboratory of Beijing Technology and Business University. *Escherichia coli* (*E. coli*) was purchased from the China General Microbiological Culture Collection Center (Beijing, China). Beef extract, yeast extract, peptone and other culture media were purchased from Beijing Aoboxing Biotechnology Co., Ltd (Beijing, China). All other chemical reagents and solvents used in the experiments were of analytical reagent grade.

2.2. In vitro simulated gastrointestinal digestion

Soybean protein isolates and peptides were digested by modifying the method of Minekus et al. (2014). Pepsin (enzyme to substrate ratio of 1:35 w/w) and one pancreatin (enzyme to substrate ratio of 1:25 w/w) were used to simulate digestion of the stomach and intestines *in vitro*, respectively. After proteolysis, the reaction was terminated by heating to 95 °C for 10 min, and the supernatant was freeze-dried for 48 h.

2.3. Determination of *L. plantarum* K25 growth characteristics

L. plantarum K25 was activated twice for 24 h in 5 mL of MRS, and 200 μ L was transferred into 50 mL of medium with three different treatments (Man Rogosa Sharpe broth (MRS), MRS with a nitrogen content reduced by half and added equal dPEP and dSPI) and then sampled every 2 h for 36 h in a 37 °C shaker under non-controlled pH

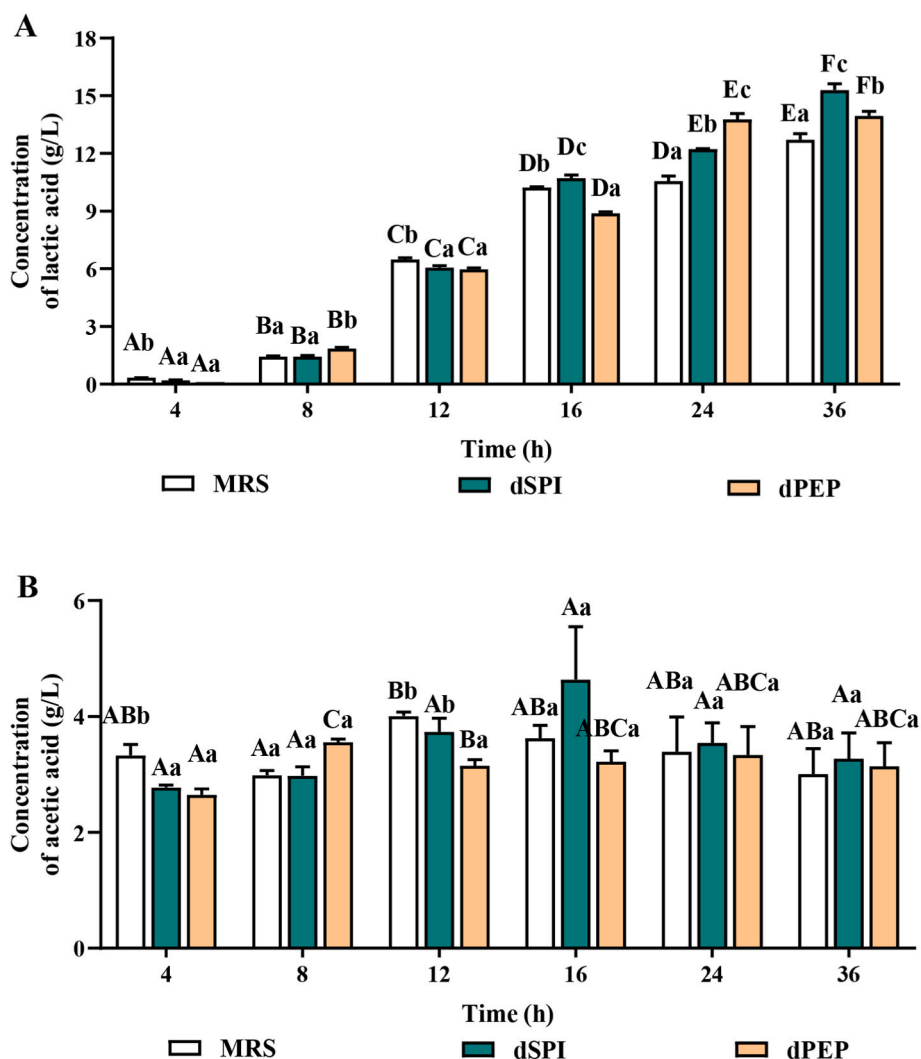


Fig. 2. Analysis of SCFAs produced by *L. plantarum* K25 in different treatment throughout 36 h incubation at 37 °C. (A) concentration of lactic acid (g/L), (B) concentration of lactic acid (g/L). Results are expressed as the mean \pm standard error of triplicates. For each bioactivity tested, different small letters between different treatments and different capital letters between time points indicate significant differences between groups ($P < 0.05$).

conditions. Triplicate 200 μ L aliquots were transferred to a 96-well microplate (Corning, New York, USA), and cellular growth was determined by measuring the OD values at 600 nm using microplate reader (Tecan, Shanghai, China). Six time points (4 h, 8 h, 12 h, 16 h, 24 h, 36 h) were selected, and 100 μ L of sample was serially diluted with 0.9% sterile NaCl solution and plated in triplicate on MRS agar.

L. plantarum K25 and *E. coli* were each successively subcultured in MRS broth at 37 °C for 24 h. A total of 6×10^8 CFU of *L. plantarum* K25 and an equal amount of *E. coli* were transferred into 50 mL of medium with the different treatments mentioned above and sampled every 4 h for 16 h in a 37 °C shaker under non-controlled pH conditions, and then incubated for 48 h by dilution plate coating method.

2.4. Measurement of lactic acid and acetic acid

The lactic acid and acetic acid after various treatments were determined with the method of Haris et al. (2023). 10 μ L of each supernatant was injected into the HPLC-DAD system (Agilent, California, USA) equipped with an anion exchange Aminex HPX-87H column (300 mm \times 7.8 mm, Bio-Rad, Richmond CA, USA) maintained at 65 °C. The detection wavelength was 220 nm, and the flow rate was 0.8 mL/min. Concentrations were determined based on calibration curves previously prepared with appropriate chromatographic standards. The mobile

phase consisted of 13 mM sulfuric acid.

2.5. Statistical analysis

The normality of the data was verified and since the values were uniformly distributed, the parametric statistical tests were used in the assessment. Statistical analysis for biochemical results were performed with ANOVA ($P < 0.05$) using IBM SPSS Statistics 26 software (IBM, Chicago, IL, USA). Results were performed in triplicate and presented as a mean \pm standard deviation. Data analysis and drawing was carried out using GraphPad Prism Version 8 (GraphPad Software, Inc., San Diego, USA).

3. Results and discussion

3.1. Proliferation analysis of *L. plantarum* K25 treated with dSPI and dPEP

The growth curve and the number of viable bacteria of *L. plantarum* K25 in different treatment groups at different time points are shown in Fig. 1. The growth trend of *L. plantarum* K25 was similar in each group and all entered the stable period in the twenty-fourth hour. The OD value of dSPI group was higher than that of other groups in both

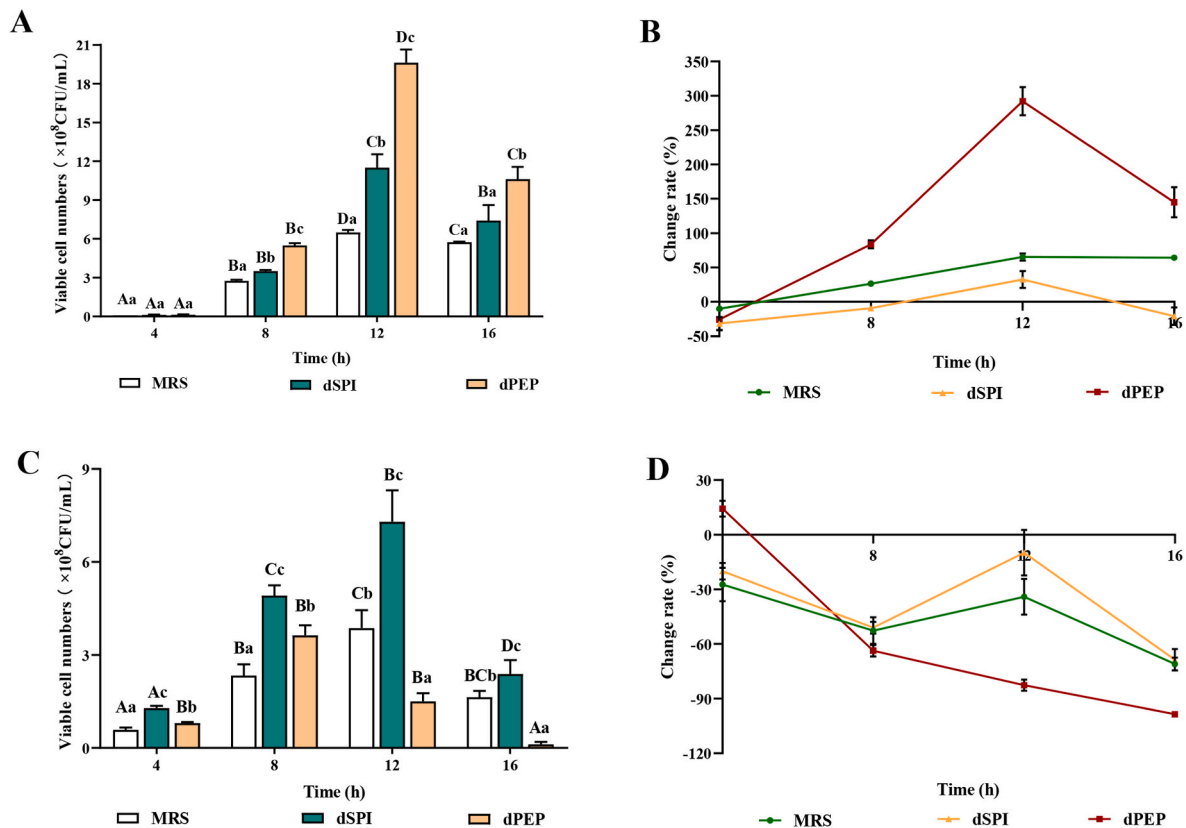


Fig. 3. The effect of soybean protein and soybean peptide on the viable counts of *L. plantarum* K25 and *E. coli* under co-culture conditions. (A) Number of viable *L. plantarum* K25 in co culture (B) Change rate of *L. plantarum* K25 in co-culture relative to monoculture (C) Number of viable *E. coli* in co culture (D) Change rate of *E. coli* in co-culture relative to monoculture. Results are expressed as the mean \pm standard error of triplicates. For each bioactivity tested, different small letters between different treatments and different capital letters between time points indicate significant differences between groups ($P < 0.05$).

logarithmic and stable periods. The OD value of dPEP group was lower than that of the MRS group during 12 h–20 h incubation, but after 24 h, it was the same as that of the MRS group. Fig. 1B showed that the number of live bacteria of *L. plantarum* K25 increased first and then decreased. At 4 h, the number of viable bacteria in dPEP group and dSPI group was significantly higher than that in MRS group. After incubating for 8 h, the number of viable bacteria in both groups increased significantly compared with 4 h, and the number of viable bacteria in dPEP group (3.09×10^8 CFU/mL) and dSPI group (3.69×10^8 CFU/mL) was still significantly higher than that in MRS group, which was in accordance with the growth curve. At 12 h, the number of viable bacteria in dPEP group and dSPI group continued to increase, which was 1.27 and 2.2 times of that in MRS group, respectively. However, the number of viable cells in each group at 16 h did not increase significantly compared with that at 12 h. At 24 h, the number of viable bacteria in dSPI group was still significantly higher than that in other groups, which was similar to our previous research results on *L. rhamnosus* Lra05 (Zhang et al., 2021). When cultured for 36 h, *L. plantarum* K25 entered a decline period, and the number of viable cells in each group decreased significantly. However, in dSPI group, it was still significantly higher than that in MRS group, suggesting that dSPI might slow down the decline of *L. plantarum* K25. Soybean protein isolates and soybean peptides could significantly promote the proliferation of *L. plantarum* K25 from logarithmic phase to decline phase. Especially, soybean protein isolates performed more effectively. The number of live bacteria in its group, which ranges from 12 h to 24 h, was twice more than that of MRS.

3.2. The evaluation of the organic acid secretion of *L. plantarum* K25

The acid production ability of probiotic is one of their important

growth characteristics, and the proliferation of probiotics produced large amounts of organic acid and lowered the pH, which could inhibit the growth of various bacteria. The lactic acid production of *L. plantarum* K25 accumulated gradually and remained stable at 24 h (Fig. 2). By 8 h of incubation, the lactic acid content in dPEP group was significantly higher than that in dSPI and MRS groups. However, the number of live bacteria in dPEP group was not the highest during the same period, indicating that the addition of soybean peptides can significantly promote the production of lactic acid by *L. plantarum* K25. At 16 h, the content of lactic acid in dSPI group was significantly higher than that in MRS and dPEP groups, which was consistent with the highest viable count of *L. plantarum* K25 in dSPI group at 16 h. When incubated to 24 h, the lactic acid accumulation in the soybean peptide and soybean protein groups was 13.77 and 12.23 g/L, respectively, which was significantly higher than that in the MRS group. These were consistent with the results of viable bacterial count at this stage. Other research also demonstrated their promoting effect on the production of lactic acid of *Lactobacillus acidophilus* and *Bifidobacterium animalis* (Li et al., 2020, 2021), which was similar to our results.

It can be seen the acetic acid production in Fig. 2B. The increasing acetic acid content was probably due to the rapid metabolism in the early stage. The downtrend could be the consumption of acetic acid which was required for later growth of *L. plantarum* K25. There was no significant difference in the accumulation of acetic acid among different time periods, and it is speculated that soybean protein and peptides did not promote acetic acid production by *L. plantarum* K25, which is different from our previous research on *L. rhamnosus* Lra05 (Zhang et al., 2020). Rios et al. (2016) concluded that acetic acid was the most abundant organic acid followed by lactic acid, but our results showed that the formation of acetic acid was not as significant as lactic acid,

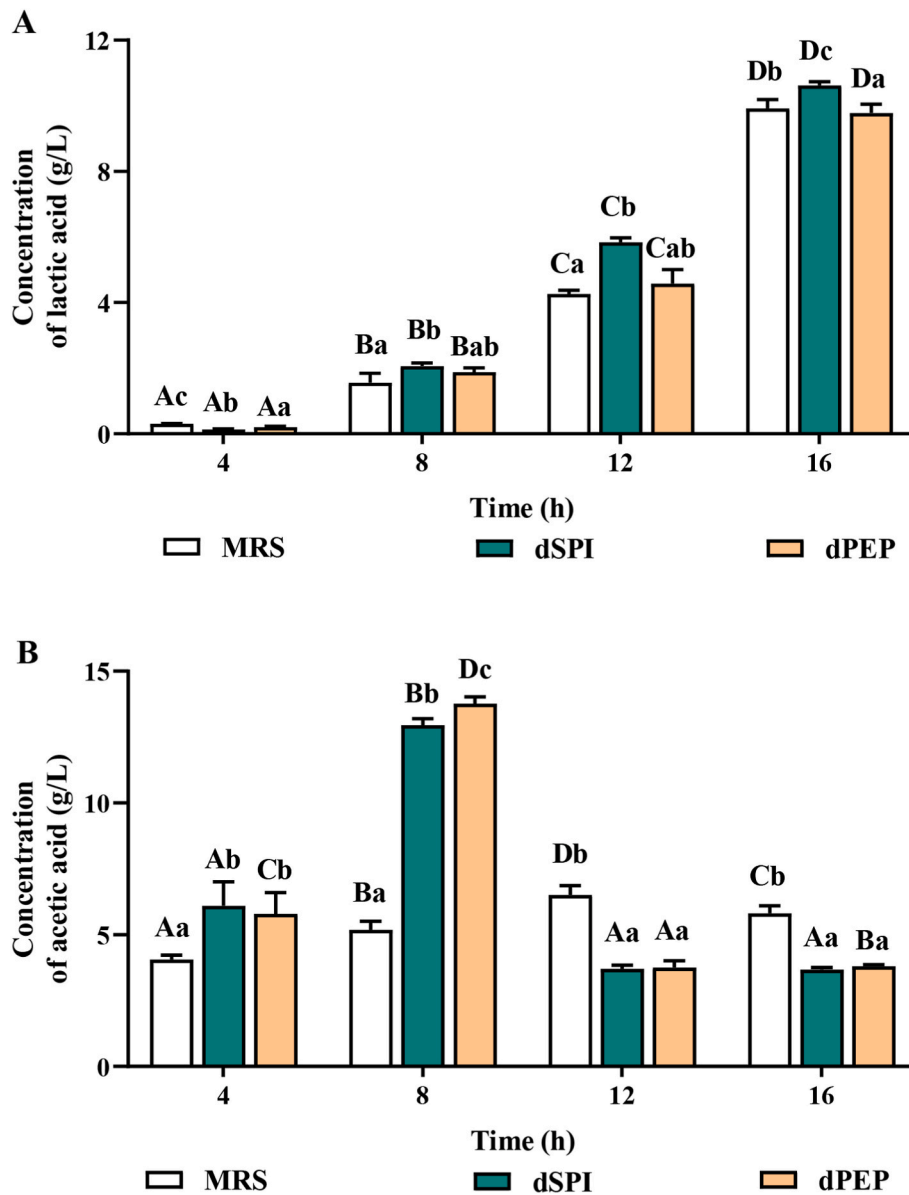


Fig. 4. Concentration of (A) lactic acid and (B) acetic acid for co-culture of *L. plantarum* K25 and *E. coli* under six groups. Results are expressed as the mean \pm SD of triplicates. For each tested, different small letters between different treatments in mono-culture and different capital letters in co-culture between time points indicate significant differences between groups ($P < 0.05$).

which may be due to the diversity of strains. Taken together, soybean protein isolates and soybean peptides can promote the accumulation of lactic acid production in *L. plantarum* K25 after 24 h of cultivation, but there is no significant increase in its acetic acid release.

3.3. The growth of *L. plantarum* K25 and *E. coli* under the co-culture condition

The utilization of co-culture conditions can not only reveal the growth situation of *L. plantarum* K25 in coexistence with pathogens, but also compare the nutrient utilization ability of these two bacteria in different populations and the effects of soybean peptides and proteins on them. Fig. 3 showed the number of viable bacteria and change rate of *L. plantarum* K25 and *E. coli* in co-culture. At 4 h, viable counts of *E. coli* in each group were significantly higher than that of *L. plantarum* K25, probably since the ability of *E. coli* to utilize nutrients was stronger in the initial period of co-culture. The change rate of *E. coli* in dPEP group was positive, indicating that the viable counts were higher than those of

mono-culture. The change rate of *L. plantarum* K25 in co-culture was negative in the three treatment groups, indicating that viable counts of *L. plantarum* decreased after co-cultured with *E. coli*.

The results at 8 h were completely different from that at 4 h. The *E. coli* in dSPI group still had the highest viable count (1.25×10^9 CFU/mL), which was significantly higher than MRS and dPEP groups. But the number of viable bacteria of *E. coli* in all groups in co-culture was lower than that in mono-culture, which was the result of competition between the two bacteria. The number of viable bacteria in MRS group was reduced by 52.7% compared with that in mono-culture, but that in dPEP group was reduced by 63.67%, indicating that soybean protein reduced the ability of *E. coli* to compete with *L. plantarum* K25. However, *L. plantarum* K25 in the dPEP group increased by 83.95% in the co-culture, which was consistent with the result of Luis Ruiz-Barba et al. (2010). They showed that the growth and survival of *L. plantarum* NC8 were greatly enhanced in olive fermentations upon co-culture with two specific bacteriocin production-inducing strains, *Enterococcus faecium* 6T1a-20 and *Pediococcus pentosaceus* FBB63. This may be due to the

recognition of the presence of specific bacteria by *Lactobacillus plantarum* NC8 as environmental stimuli that initiate specific adaptive responses. Similarly, Man et al. (2012) also suggested that viable count of *L. plantarum* KLDS1.0391 in co-culture with *L. helveticus* KLDS1.9207, *E. faecium* KLDS4.0352, *L. reuteri* KLDS1.0737 or *E. faecalis* KLDS4.0313 were higher than those shown by *L. plantarum* KLDS1.0391 in mono-culture.

When cultured for 12 h, the viable count of *E. coli* in dSPI group in co-culture was still the highest and significantly higher than the other two groups. Co-culture method would cause competition between the two bacteria, so the change rate of viable *E. coli* in co culture was negative. The reduction rate in the dPEP group (82.69%) was still significantly higher than that in the MRS group (34.09%), which was consistent with the 8 h results. The viable number of *L. plantarum* in dPEP group and dSPI group was 3.02 times and 1.77 times higher than that in MRS group, respectively.

After 16 h of cultivation, the reduction rate of viable *E. coli* in MRS group, dPEP group and dSPI group in co-culture exceeded 50%, which may be related to the accumulation of acid production by *L. plantarum* K25. The reduction rate in dPEP group was 98.51%, which was significantly higher than that in MRS and dSPI groups. In co-culture, the viable count of *L. plantarum* in dPEP group increased by 145%, which was in accordance with the results of 8 h and 12 h. These indicated that soybean protein isolates and peptides could still promote the growth of *L. plantarum* K25 when co-cultured with *E. coli*, and cooperate with *L. plantarum* K25 to inhibit the proliferation of *E. coli*.

3.4. The lactic acid and acetic acid production in *L. plantarum* K25 and *E. coli* co-cultures

The comparison of lactic acid produced by *L. plantarum* K25 in co-culture with *E. coli* was shown in Fig. 4A. For 4 h cultivation, the lactic acid contents in dSPI group were higher than that in dPEP group, and lasting until 16 h, indicating that soybean protein isolates were more effective in promoting lactic acid production by *L. plantarum* K25 co-cultured with *E. coli*. Interestingly, the dPEP group had the highest viable cell numbers in all stages of co-culture. Moreover, the lactic acid content in dSPI group was higher than that in mono-culture, which may be caused by the stress reaction of *L. plantarum* K25 (Serrazanetti et al., 2009). After 8 h of incubation, the lactic acid accumulation of *L. plantarum* K25 in MRS group was significantly lower than that in dSPI group, which was consistent with the results of live bacterial count in co-culture.

The acetic acid content showed a trend of first increasing and then decreasing, which was opposite to the trend of *Lactobacillus rhamnosus* producing acetic acid (Zhang et al., 2021), possibly due to the metabolism of different strains. At 4 h, the acetic acid content in dSPI and dPEP groups was significantly higher than that in the MRS group, which may be related to the promotion of *L. plantarum* K25 metabolism by soybean protein isolates and soybean peptides. The acetic acid content of 8 h was significantly increased compared to 4 h. But after 12 h, the acetic acid content in dSPI and dPEP groups significantly decreased, which may be related to the consumption of sodium acetate by *L. plantarum* K25. When cultured 16 h, the acetic acid content in MRS group also significantly decreased, but the accumulation was significantly higher than that in dSPI and dPEP groups, which may be due to the better growth of *L. plantarum* K25.

4. Conclusion

In conclusion, this study revealed that the dSPI and dPEP can significantly promote the proliferation and metabolism of *L. plantarum* K25, especially in the soybean protein isolates group. In addition, dSPI and dPEP appeared to improve the competitive capacity of *L. plantarum* K25 and weaken the competitiveness of *E. coli* in a co-cultivation environment. The way in which soybean protein isolates and soybean

peptides collaborate *L. plantarum* K25 in inhibiting *E. coli* may be mainly involved in promoting organic acid production. However, the proof of mechanisms by which soybean protein isolates and soybean peptides collaborate with *L. plantarum* K25 to inhibit *E. coli* has not been fully explored, and other inhibiting modes of their effects will be needed. In addition, the effects of soybean proteins on more *Lactobacillus* from different sources and strains were supposed to investigate in subsequent research.

CRedit authorship contribution statement

Yinxiao Zhang: Data curation, Investigation, Validation, Visualization, Writing – review & editing. **Chi Zhang:** Data curation, Investigation, Validation. **Jingyi Wang:** Investigation, Visualization. **Yanchao Wen:** Investigation, Visualization. **He Li:** Validation, Visualization, Investigation. **Xinqi Liu:** Conceptualization, Visualization, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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