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Investigating the interactive adhesion ability between *Limosilactobacillus reuteri* DSM17938 and HT-29 cells regulated by soybean proteins and soybean peptides

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ABSTRACT

In this study, digested soybean protein (dspr) and peptides (dspe) were found to have different effects on the adhesion ability of *Limosilactobacillus reuteri* DSM17938 and HT-29 cells. Results found that dspr and dspe could increase *L. reuteri* adhesion ability by affecting their auto-aggregation and hydrophobicity during their stationary phase. The moonlighting protein may play a dominant role affected by dspe in the adhesion process of *L. reuteri* from stationary phase. Moreover, dspr and dspe also enhanced the adhesion ability of HT-29 cells to *L. reuteri* DSM17938, and they might play different role at different stages of adhesion. dspr mainly promoted the expression of adhesion-related genes before *L. reuteri* adhesion. dspe increased them after the adhesion of *L. reuteri* from log phase and dspr mainly enhanced their expression after stationary phase *L. reuteri* adhesion. Soybean protein and peptides may therefore be considered as a potential effective modulator of *L. reuteri* adhesion to intestinal tract.

1. Introduction

Probiotics are live microorganisms that promote host health, which can prevent chronic diseases and regulate intestinal flora[1]. The ability of probiotics to adhere to the surface of the host's intestinal mucosal cells is a prerequisite for their role in the gut. Adhesion of probiotics to host cell surfaces is initially non-specific by surface properties of the cell (hydrophobicity, auto-aggregation) and then the second stage is specific adhesion mediated by adhesins[2]. After adherence, probiotics continue to bind to the host's intestinal epithelium to form a stable biofilm, which helps them to become predominant and perform numerous physiological functions. Li et al.[3] found that *Lactobacillus plantarum* Y42 showed enhanced probiotic functions after biofilm formation, including adherence to HT-29 cells and anti-adherence to *Listeria monocytogenes*. In addition, the secretion of mucus from intestinal epithelial cells is equally vital to gut health, which helps probiotics to adhere[4].

The genus *Lactobacillus* was one of the most widely used probiotics, with *Limosilactobacillus reuteri* DSM17938 naturally occurring in the human gastrointestinal tract and being a major member of the gut microflora in newborns and healthy adults[5]. *L. reuteri* DSM17938 has functions such as regulating gut microbiota, improving intestinal environment, and maintaining gut microbiota balance. Adhesion of *L. reuteri* in the gut also requires nutrient supply[6]. Oligosaccharides have been found to significantly improve the adhesion ability of various *Lactobacillus*[7-9]. Krausova et al.[10] also found that various prebiotics such as lactose, glucose, human milk oligosaccharides, could enhance the adhesion of *Bifidobacterium* BBM and BBV to Caco-2 cells and HT-29 MTX cells. In addition to oligosaccharides, proteins, as one of the essential nutrients for human life, have received more and more attention from researchers for their role in probiotics adhesion. Several reports have also demonstrated that soybean proteins and its derivatives are among the important nutrients affecting intestinal flora[11-12]. Studies have

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shown that soybean peptides increase the number of *Lactobacillus* and *Bifidobacterium* in the digestive tract, thereby altering the composition of the gut microbiota[13]. Our previous studies have shown that soybean protein isolates and soybean peptides significantly stimulated the proliferation and metabolism of various probiotics, such as *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *L. reuteri*, among which *L. reuteri* were promoted by soybean protein and soybean peptides at different culture periods[14-16]. However, it is necessary to ensure their long-term colonization in the intestine to exert its effects. It has been shown that *Lactobacillus* can adhere to intestinal cells through both non-specific and specific adhesion, and can also enhance their adhesion to intestinal cells through changes in their surface properties (auto-aggregation, hydrophobicity, biofilm-forming ability)[17-19]. Furthermore, there is still less information on whether proteins can influence the adhesive ability of *Lactobacillus* to intestinal tract and how they worked.

Therefore, this study assessed the effect of digested soybean protein (dspr) and peptides (dspe) on the adhesion capacity of *L. reuteri* DSM17938 and HT-29 cells. In addition, it was assessed whether their effects on the adhesion capacity of *L. reuteri* DSM17938 were related to cell surface hydrophobicity, auto-aggregation assay, and biofilm formation ability. The gene expresions of adhesive related proteins before and after the process of adhesion of *L. reuteri* DSM17938 to HT-29 cells was also elucidated.

2. Materials and methods

2.1 Materials and reagents

L. reuteri DSM17938 was obtained from Hebei vermont Biotechnology Co., Ltd. (Handan, China). Soybean protein isolates and peptides were kindly provided by Shandong Yuxin Bio-Tech Co., Ltd. (Qingdao, China) and Rushan Hualong Biotechnology Co., Ltd. (Weihai, China). Pancreatin was purchased from Tokyo Chemical Industry (Tokyo, Japan), and pepsin was acquired from Yuanye Biotechnology Co., Ltd. (Shanghai, China).

2.2 In vitro simulated gastrointestinal digestion

Soybean protein isolates and peptides were digested by modifying the method of Minekus et al.[20]. The sample was simulated for gastrointestinal digestion using pepsin (enzyme to substrate ratio of 1:35 (*m*/*m*)) and trypsin (enzyme to substrate ratio of 1:25 (*m*/*m*)). After enzyme inhibition, dry powder was obtained by freeze-drying. The composition of dspr and dspe were shown in our previous research[16,21].

2.3 Cell culture

Colon adenocarcinoma cell line HT-29 was obtained from the Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). The cells were grown in DMEM/F12 (1:1) medium (Gibco, MA, USA) supplemented with 5% fetal bovine serum (ExCell Bio, Shanghai, China), 100 U/mL penicillin and streptomycin (Gibco, MA, USA) at 37 °C in a 5% CO_2 atmosphere in a humidified incubator. For adhesion assays, HT-29 cells were seeded in 24-well culture plates at a concentration of 3×10^5 cells per well. The experiments were performed after 90% confluency was reached.

2.4 Adhesion assays of L. reuteri DSM17938 under different treatment groups to HT-29 cells

L. reuteri DSM17938 cultured to log and stationary phases under different treatment groups were centrifuged at 7 000 r/min for 3 min, the supernatant was discarded, and the organisms were washed 3 times with PBS and in antibiotic-free cell culture medium to adjust the concentration to 1×10^8 CFU/mL. HT-29 cells were rinsed 2 times per well with PBS, and accessed to 1 mL of the bacterial suspension, and were co-instrumented for 6 h. Subsequently, cells were washed 3 times with 1 mL PBS to remove unadhered bacteria, then lysed with 0.5 mL Tritonx-100 solution (1% (*V*/*V*) in PBS) for 10 min to release the bacteria, and gradient dilutions were applied to MRS agar.

2.5 Determination of surface characteristics

2.5.1 Auto-aggregation analysis

L. reuteri DSM17938 in each treatment group was cultured to log and stationary stage respectively, and the suspension of each group was centrifuged at 4 000 r/min, 4 °C for 10 min, the supernatant was discarded, and the pellet was washed 3 times and re-suspended with PBS, and the absorbance was adjusted to 0.60 ± 0.02 (A_0) at 600 nm. Pipette 4 mL of suspension was left at 37 °C for 6 h. The absorbance was measured by taking the upper layer of suspension (A_1). The experiment was repeated for 3 measurements. The auto-aggregation percentage was expressed as $(A_0–A_1)/A_0 \times 100$.

2.5.2 Hydrophobicity analysis

Like auto-aggregation, the absorbance of *L. reuteri* DSM17938 for different phases was adjusted to 0.60 ± 0.02 (A_0) at 600 nm. Mix xylene with the resuspension solution 1:1 (*V*/*V*), vortex shock for 60 s, and leave for 30 min to separate the two phases, and measure the OD value of the aqueous phase (A_2). The hydrophobicity percentage was expressed as $(A_0–A_2)/A_0 \times 100$.

2.5.3 Biofilm formation analysis

L. reuteri DSM17938 was subjected to biofilm formation assay as described previously with some modifications[22]. For biofilm formation, wells of a 96-well plate were filled with 150 μL of 1×10^8 CFU/mL of testing bacteria and incubated without shaking for 72 h at 37 °C. The wells were washed 3 times with PBS, and the remaining attached bacteria were stained for 30 min with 150 μL 0.1% crystal violet in an isopropanol-methanol-PBS solution (1:1:18, *V*/*V*). Excess stain was washed with 200 μL water per well. Wells were air dried for 30 min, and the bounded dye was extracted with 150 μL ethanol-acetone (4:1). The OD of each well was measured at 595 nm using a microplate reader.

2.6 Adhesion assays of L. reuteri DSM17938 to HT-29 cells under different treatment groups

In this study, 1 mg/mL of dspe and dspr were co-incubated with HT-29 cells for 2 h at 37 °C in a 5% CO_2 atmosphere in a humidified incubator, and untreated probiotics were co-incubated with

HT-29 cells for 6 h, followed by the same steps as in Section 2.4, and gradient dilutions were applied to MRS agar.

2.7 Microscopic observation of the adhesion of *L. reuteri* DSM17938

HT-29 cells were inoculated into 24-well plates containing slides at 3×10^5 cells/mL, and other experimental steps were the same as those treated in Sections 2.4 and 2.6. After a total of 6 h of incubation, the slides were washed three times with sterile PBS buffer, and after removing the slides, the slides were fixed with methanol for 10 min. Gram staining was performed and the adhesion of *L. reuteri* DSM17938 was observed under a light microscope.

2.8 Gene expression and RT-qPCR analysis

Total RNA isolation was performed according to the manufacturer's instructions. The total RNA of *L. reuteri* DSM17938 and HT-29 cells was performed according to the kit instructions (Transgen, Beijing, China). RNA concentrations were determined at 260 nm using NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fischer Scientific, Wilmington, DE) and purity was assessed by the $A_{260\ nm}/A_{280\ nm}$ ratio. Reverse transcriptase (Transgen, Beijing, China) was used to synthesize cDNA for performing quantitative PCR. Thermal cycling conditions were as follows: 3 min denaturation at 95 °C, followed by 40 cycles at 95 °C for 10 s, 30 s at 55 °C. Data were collected and analyzed using the CFX Manager software (Bio-Rad, California, USA). PCR products were evaluated by melting curve analysis to confirm the specific amplification. As endogenous MRS, genes for *GAPDH* and Lpla16rsRNA were used for HT-29 cells and *L. reuteri* DSM17938, respectively. The $2^{-\Delta\Delta Ct}$ method was used to evaluate the fold change with relation to the MRS group of selected genes. All the gene primer sequences are listed in Table S1.

2.9 Statistical analysis

All the results are expressed as mean ± SD of 3 experiments. All data were analyzed with GraphPad Prism 9 software (GraphPad Software, Inc., San Diego, USA) and IBM SPSS Statistics 17 software (IBM, Chicago, IL, USA). Statistical significances were determined by One-way analysis of variance (ANOVA) and independent two-sample *t*-test. Significant difference was defined as $P < 0.05$.

3. Results and discussion

3.1 Effect of dspe and dspr on the adhesion capacity of *L. reuteri* DSM17938

The adhesion ability of *L. reuteri* DSM17938 to HT-29 cells was evaluated for each treatment group (Fig. 1). There was no significant difference in the degree of adhesion of *L. reuteri* DSM17938 from log phase (6 h) between dspe and MRS groups, whereas the rate of adhesion in the dspr group was significantly lower (Fig. 1B). While evaluating the adhesive ability of *L. reuteri* DSM17938 in the stationary phase (12 h), the adhesion rates of both the dspe and dspr groups exceeded those of the MRS group, with an increase of 14.37% and 48.68%, respectively. In addition, comparing the adhesion rates in log and stationary phases, it was found that the adhesion rate of the MRS group in the stationary phase decreased by 5.33% compared with that of the log phase. In contrast, the adhesion rates in the dspe and dspr groups increased by 7.64% and 51.73% compared with the log phase, indicating that both dspe and dspr improved the adhesion ability of *L. reuteri* DSM17938 to HT-29 cells with the increase of incubation time, and dspr showed a relatively stronger pro-adhesion ability. After staining, the morphology of both *L. reuteri* DSM17938 and HT-29 cells were clearly visible (Fig. 1A). Some *L. reuteri* DSM17938 adhered to the cell surface in clusters and the observations corresponding to the results of the adhesion rate (Fig. 1B).

3.2 Effect of dspe and dspr on the cell surface hydrophobicity, auto-aggregation assay, and biofilm formation ability of *L. reuteri* DSM17938

To investigate the patterns of dspr and dspe affecting the adhesion of *L. reuteri* DSM17938, their ability for *L. reuteri* DSM17938 auto-aggregation capacity, surface hydrophobicity and biofilm formation was explored (Fig. 2). Cellular auto-aggregation is the measure of self-clumping or the aggregating ability of cells of bacteria, which is usually related to its persistence and colonization in the intestine[23]. The cell auto-aggregation of *L. reuteri* DSM17938

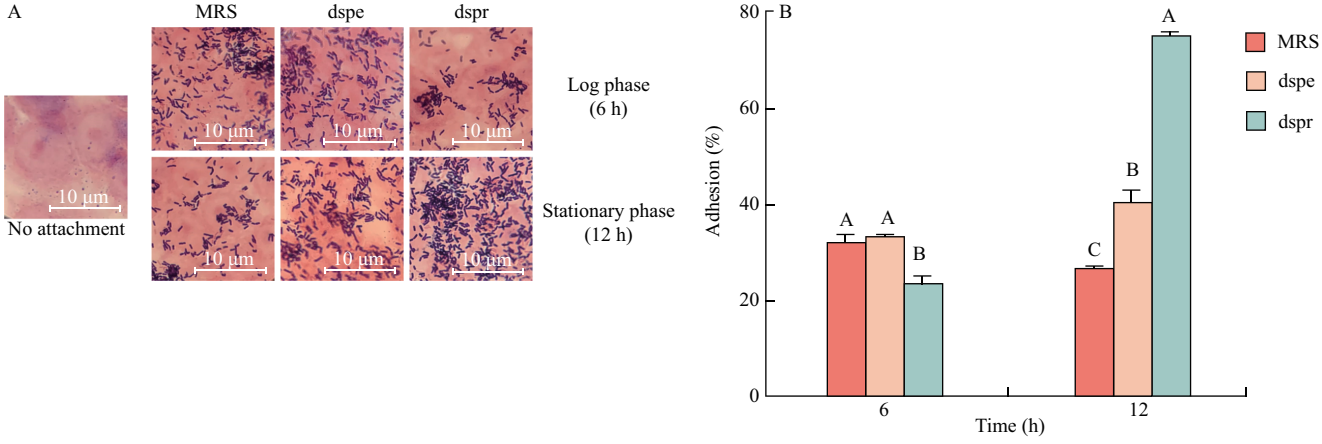

Fig. 1 Effect of dspe and dspr on the adhesion capacity of *L. reuteri* DSM17938 from log phase and stationary phase. (A) Microscopic observation images and (B) adhesion rate. Different uppercase letters indicate significant difference ($P < 0.05$).

under different treatment was presented in Fig. 2A. There was no significant difference was found between MRS and dspe groups for *L. reuteri* from log phase, and the rate of auto-aggregation in dspr group (34.09%) was significantly higher than that of the other 2 groups, suggesting that dspr could improve the auto-aggregation ability of *L. reuteri* DSM17938 at log phase. By the stationary phase, the auto-aggregation rates of both dspe and dspr groups were significantly higher than that of MRS group, and it was still the dspr group that had the highest rate of 52.37%, which indicated that both dspe and dspr increased the auto-aggregation ability of *L. reuteri* DSM17938 during the stationary phase. In addition, the auto-aggregation rate of the MRS group in the stationary phase was not significantly different from that of the log phase, but those of the dspe and dspr groups were significantly improved (6.84% and 18.28%), and the auto-aggregation rate of the dspr group was the highest in all the different incubation phases.

The adhesive ability of bacteria is usually related to cell surface hydrophobicity[24]. The surface hydrophobicity of *L. reuteri* DSM17938 was shown in Fig. 2B. At 6 h, the hydrophobicity of both the dspe and dspr groups was significantly lower than that of the MRS group. However, by 12 h, the dspr group had the highest hydrophobicity rate of 58.96%, followed by the dspe group with 36.41%, which were both significantly higher than that of the MRS group, suggesting that dspe and dspr were more effective in enhancing the hydrophobicity of *L. reuteri* DSM17938 during their stationary phase, and the dspr ability was stronger than that of dspe.

Biofilm formation ability in probiotics is considered a beneficial feature since the strain could colonize and maintain stability in the host, reducing colonization of pathogens[25]. Fig. 2C shows the biofilm formation capacity of *L. reuteri* DSM17938 in each group. The dspe group had the strongest biofilm formation ability at log phase ($OD_{595\ nm}$ = 0.62), but the dspr group showed significantly less biofilm formation ability than the MRS group. The results at 12 h were similar with those of the log phase, with the dspe group still having a greater ability to form biofilm ($OD_{595\ nm}$ = 0.86), which was 2.67 and 3.4 times greater than that of the MRS and dspr groups, respectively. The above results showed that dspe was able to increase the ability of *L. reuteri* DSM17938 biofilm formation during both the log and stationary phases of *L. reuteri* DSM17938 growth, whereas dspr inhibited biofilm formation of *L. reuteri* DSM17938, both in stationary and log phases.

In summary, dspe and dspr affected the adhesion characteristics of *L. reuteri* DSM17938 differently during different phases. Generally, they promoted adhesion of *L. reuteri* in the stationary phase more than the log phase. The effects of dspr and dspe on the auto-aggregation and hydrophobicity of *L. reuteri* DSM17938 during the stationary phase were consistent with the results of the adhesion rate. It was speculated that dspr might promote the adhesion ability of *L. reuteri* DSM17938 during the stationary phase by increasing its auto-aggregation and hydrophobicity. Non-specific adhesion was one of the important factors affecting the adhesion. Although Lee et al.[26] reported that adhesion in *Lactobacillus* occurs primarily through hydrophobic interactions that affect competition for specific receptors, these bacteria are also known to have surface adhesins that facilitate the immobilization process. In addition, dspe was able to enhance the biofilm formation of *L. reuteri* DSM17938 in all phases, while dspr group showed an inhibitory effect. However, the adhesion results showed that dspr played a significant role in promoting adhesion. It is speculated that dspr-regulated adhesion is not only related to the surface properties of the probiotics, but also may be regulated by gene expression of the surface proteins, or regulated by the adhesion of the intestinal cells. The difference caused by dspe and dspr may be related to their composition. Our previous studies have shown that soybean protein and soybean peptide have different effects on the growth and metabolism of *L. rhamnosus* due to differences in molecular mass and composition. Therefore, it was speculated that differences in the composition of soy proteins and soy peptides would also contribute to differences in their adhesion to *L. reuteri* DSM17938. Liu et al.[6] found that *Ilisha elongata* proteins, soybean proteins and whey proteins caused different adhesion results to LP45 due to differences in amino acid composition and structure. However, further research data are still needed to support the above speculations.

3.3 dspe and dspr regulated the adhesion-related genes expression before and after L. reuteri DSM17938 from log and stationary phases adhesion to HT-29 cells

Adhesive capacity could be influenced by the composition of the cell surface and the expression of specific adhesion related proteins, which can confer different degrees of adhesion capacity to the cell[26]. Moonlighting proteins are a class of multifunctional proteins that performs 2 or more physiological and biochemical functions. They have dual functions, in which they can either perform metabolic functions within the cell or be transported to the surface of the cell wall to promote biochemical functions such as adhesion[27]. In this study, 6 moonlighting proteins were measured to assess the effects of dspe and dspr on the adhesion capacity of *L. reuteri* DSM17938.

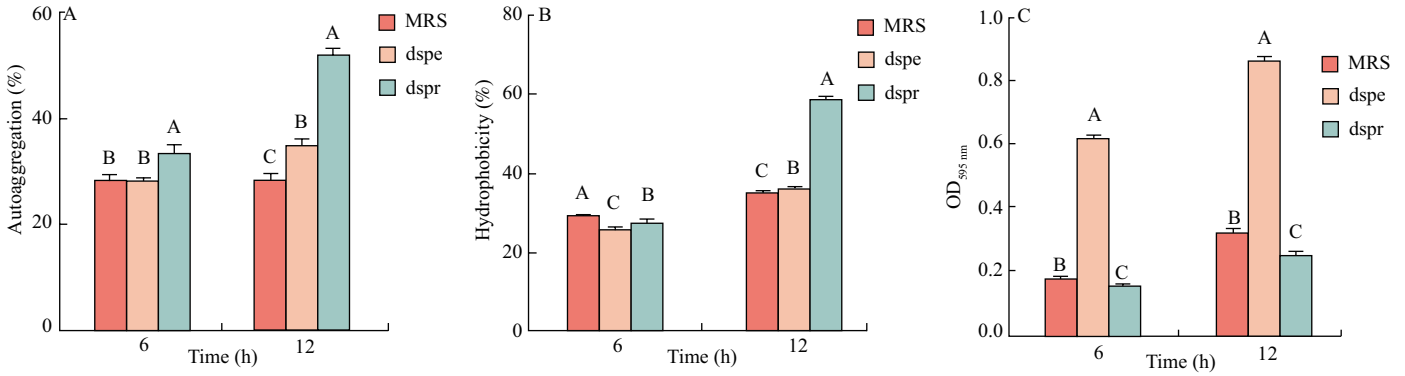

Fig. 2 Analysis of (A) auto-aggregation, (B) hydrophobicity, and (C) biofilm of *L. reuteri* DSM17938 from log phase and stationary phase under different treatment. Different uppercase letters indicate significant difference ($P < 0.05$).

The results of adhesion related genes before and after *L. reuteri* DSM17938 of log phase adhered to HT-29 cells were shown in Figs. 3A and C. Before adhesion (Fig. 3A), dspe increased the mRNA expression of the mucus adhesion-promoting protein *MapA*, which was a component of ABC transporter proteins and has also been shown to be a mucin and epithelial adhesion factor[28]. Enolase *EnoA1* gene expression was significantly up-regulated in the dspe group, which was 7.2 folds higher than the MRS group (Fig. 3A). The glycolytic enzyme α-enolase is expressed on some eukaryotic and prokaryotic cell surfaces, exerting moonlight functions[29], and *EnoA1* was already found to bind to collagen type I (CnI) and enhance the adhesion capacity of *L. plantarum* LM3[30]. The genes encoding the transcription elongation factor *GreA* and glutamine synthetase (*GS*) were significantly down-regulated in the dspe group compared with the MRS group, with a decrease of 7.09% and 6.87%, respectively. It was shown that *GreA* was highly expressed in *L. pentosus* CF2-20 N, which has a high adhesion rate, and could be used as one of the markers to characterize the adhesion capacity of *L. pentosus*[31]. GS could bind fibronectin, laminin, and type I collagen, and was also associated with nitrogen cycling[32]. Similarly, dspr significantly increased the expression of *MapA* and *EnoA1* by 1.6- and 4.32-fold, respectively, while *GreA* and *GS* were significantly reduced by 70.34% and 64.15%. Unlike the dspe group, PGM, a phosphoglycerate translocase, was downregulated by 17.05%. It was shown that PGM is a key enzyme in central metabolism and plays a key role in the adhesion of *Lactobacillus pentosus* to mucus[33-34]. PGM could participate in and positively regulate probiotic biofilm formation, which could be involved in stronger biofilm formation of *L. reuteri* DSM17938 in the dspe group in the log phase (Fig. 2C). Adhesion related gene expression was also analyzed after their adhesion to HT-29 cells (Fig. 3C). Compared with the MRS group, *EnoA1*, *GreA*, *PGM* and *GS* gene expressions were significantly up-regulated by 1.46-, 1.96-, 1.76- and 1.23-fold in the dspe group. Although dspe increased the expression of *L. reuteri* DSM17938 moonlighting protein-related genes during the adhesion process, there was no significant difference in the adhesion rate between the dspe group and the MRS group, suggesting that the moonlighting protein-related genes may not be the main factor regulated by dspe for the adhesion of *L. reuteri* DSM17938 when it was in the log phase.

Adhesion related gene expression before and after *L. reuteri* DSM17938 in the stationary phase adhered to HT-29 cells were shown in Figs. 3B and D. Before adhesion, compared with the MRS group, the dspe group showed a significant upregulation of *MapA* gene expression by 1.59-fold, while all other adhesion related genes decreased significantly (Fig. 3B). The trends of the dspr group were similar with those of the dspe group. It was speculated that moonlighting proteins mostly perform metabolic functions within cells before adhesion which was also not affected by soybean proteins and peptides. Interestingly, after adhesion (Fig. 3D), gene expression was overall up-regulated compared with pre-adhesion. The *EF-Tu* was significantly up-regulated in both the dspe and dspr groups compared with the MRS group. *EF-Tu* is a GTP-binding protein responsible for loading aminoacyl-tRNA into the receptor site of ribosomes during protein synthesis. It has been shown that EF-Tu on the bacterial surface is able to bind to membrane receptors and the extracellular matrix on the surface of enterocytes, mediating bacterial attachment to human enterocytes and mucins, and promoting probiotics colonization[35]. It suggested that during the adhesion process, the moonlighting protein-related genes begin to play adhesion-related roles, and dspe and dspr may be able to promote the adhesion ability of *L. reuteri* DSM17938 in stationary phase by increasing the expression of genes such as *EF-Tu*.

Probiotics adhesion could favor the promotion of mucin production in the cellular mucus layer, thus gene expression of mucins, glycosylation genes and trehalose factors in HT-29 cells after protein and peptide treated *L. reuteri* DSM17938 adhesion were determined. In log phase (Fig. 3E), *MUC2*, *MUC3* and *MUC11* gene expression was significantly up-regulated in the dspe group compared with the *MRS* group. It has been shown that *MUC2* and *MUC5AC* were secreted mucins and *MUC3* and *MUC11* were transmodel mucins[36]. These mucins were glycosylated proteins that are initiated by the addition of *N*-acetylgalactosamine (GalNAc) to Ser or Thr residues, followed by the gradual expansion of that first epitope into more complex and branched glycan chains[37]. The up-regulation of mucin expression in the dspe group did not result in an increase in the adhesion rate, probably since the enzyme responsible for initiating the *O*-glycosylation modification. *GALNT7* was significantly down-regulated by 37.66%, which prevented the process from being initiated and reaching the endoplasmic reticulum and mucus layer. Thus, still failing to promote *L. reuteri* DSM17938 adhesion to HT-29 cells. It was shown that fucosyltransferase 2 (*FUT2*), the only enzyme identified to catalyse the addition of terminal α-1,2-fucose residues to the Gal of the glycan, was up-regulated in the dspe group, and the high expression of Core 2 β-1,6-*N*-acetylglucosaminyltransferase-2 in colon tissue (*C2GnT2*) could catalyse the transfer of *GlcNAc* to the C6 carbon of the initial GalNAc. However, it was possible that the glycosylation modification process was not initiated or the initiation is inhibited leading to difficulties in the functioning of these 2 genes[38-40]. *GALNT7*, *MUC3*, *MUC5AC* and *MUC11* were significantly down-regulated in the dspr group, with *MUC5AC* being the most down-regulated at 46.08%, and the rest of the genes were not significantly different from those in the MRS group, which might bring about a lower adhesion rate than that in the dspe group (Fig. 3E).

During the stationary phase (Fig. 3F), the glycosylation genes *ST3Gal3* and *GALNT7*, the mucin gene *MUC3*, and the trefoil factor TFF1 were significantly up-regulated by 1.48-, 1.55-, 1.54-, and 1.92-fold, respectively in the dspe group compared with the MRS group, whereas the mucin gene *MUC11* was significantly down-regulated by 54.89%. In the dspr group, the *MUC3* gene was significantly down-regulated by 2.95%, while there was no difference in the expression changes of the *MUC5AC* and *MUC11* genes, and the expression of all the other genes was significantly up-regulated, with the most pronounced effect on the *TFF1* gene, which was up-regulated by 7.55-fold. Thus, at stationary state, mucus glycosylation was significantly increased after the dspe and dspr treated *L. reuteri* adhesion compared to the MRS group, and showed a better effect in the dspr group. dspe treated *L. reuteri* increased the expression of the membrane mucin MUC3 in HT-29 cells, whereas dspr treated *L. reuteri* increased the co-expression of MUC2 and TFF1. It has been shown that TFF1 and TFF3 interact strongly with mucins and were able to enhance mucosal barrier protection by increasing the viscosity of mucins[41-42]. This enabled both dspe and dspr to increase the expression of *L. reuteri* DSM17938 on HT-29 cellular mucins, and dspr was more effective than dspe, which agreed with the results of adhesion rate in the stationary phase.

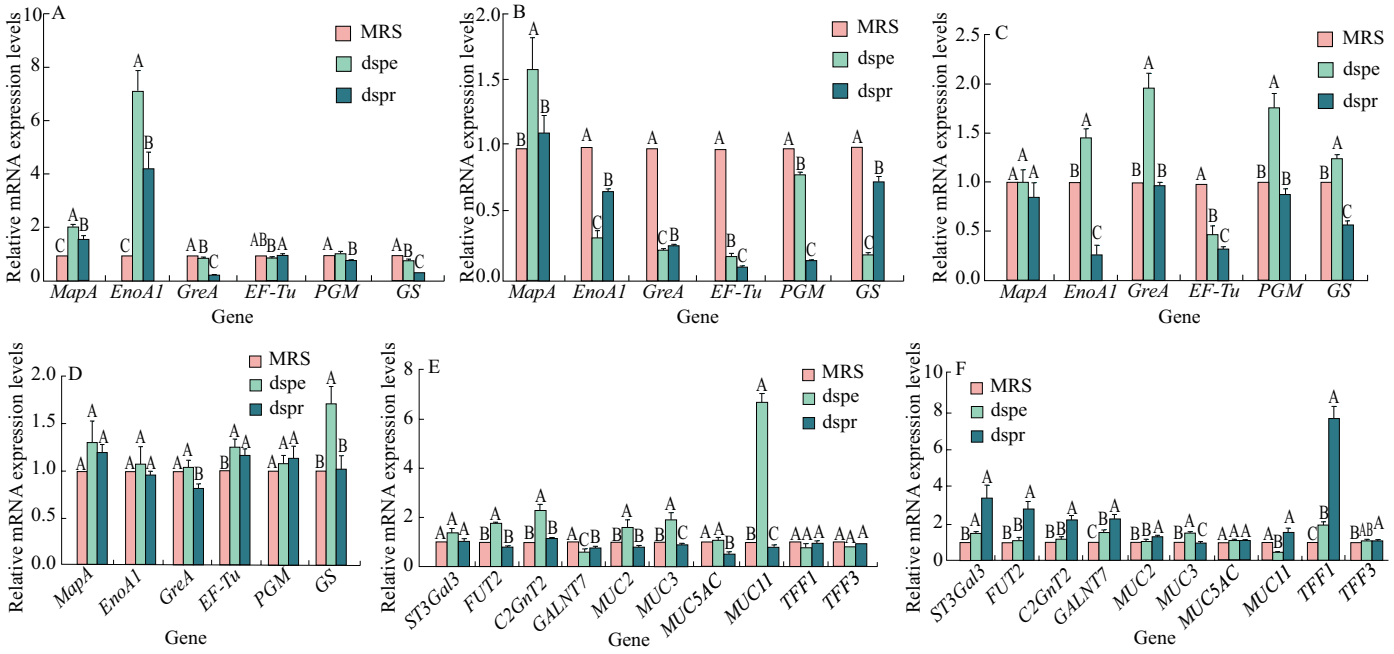

Fig. 3 Expression analysis of relevant adhesion genes under different treatment groups. Gene expression of *L. reuteri* DSM17938 from (A) log phase and (B) stable phase before adhesion to HT-29 cells, and *L. reuteri* DSM17938 from (C) log phase and (D) stable phase after adhesion to HT-29 cells. Gene expression of HT-29 cells after *L. reuteri* DSM17938 adhesion from (E) log phase and (F) stable phase. Different uppercase letters indicate significant difference ($P < 0.05$).

3.4 Effect of dspe and dspr on the adhesion capacity of HT-29 cells

To investigate whether dspe and dspr could also influence the HT-29 cells adhesion to *L. reuteri* DSM17938 at different growth phases, adhesion rates were determined by treating HT-29 cells with dspe and dspr for 2 h. Fig. 4 shows that both dspe and dspr significantly improved the ability of HT-29 adhered to *L. reuteri* DSM17938 by 5.17% and 5.81% in log phase, respectively. Similarly, both dspe and dspr also promoted the adhesive ability while *L. reuteri* DSM17938 was in the stationary phase. dspe promoted more effectively, increasing the adhesion rate by 9.01%. The above results indicated that both dspe and dspr can improve the adhesion ability of HT-29 cells to *L. reuteri* DSM17938 in all phases.

3.5 Analysis of adhesion-related gene expression of HT-29 cells affected by dspr and dspe

To explore the ways in which dspe and dspr affect the adhesion capacity of HT-29 cells, we examined HT-29 adhesion protein-related genes under different treatment groups (Fig. 5A). Compared with the MRS group, dspe group showed significant up-regulation of all glycosylation genes and the trefoil factor *TFF3*. *MUC5AC* and *MUC11* were significant down-regulated. The results of the dspr group were like those of the dspe group, except that there was no difference in the expression of the glycosylation gene *FUT2* and the mucin gene *MUC5AC*. The expression of all the other genes was significantly up-regulated, and the multiplicity of up-regulation was higher than that of the dspe group, suggesting that soybean peptides

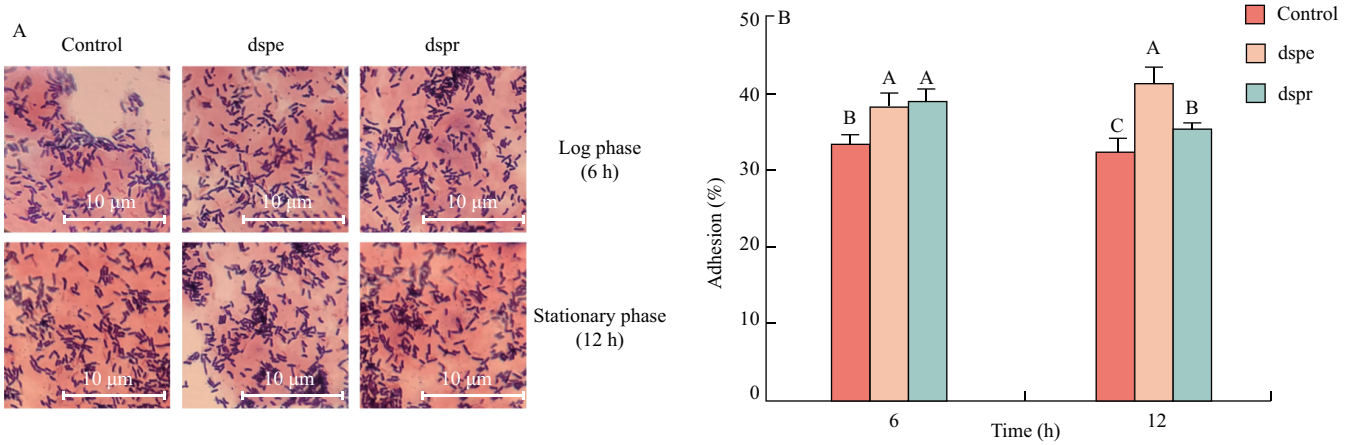

Fig.4 Effect of dspe and dspr on the adhesion capacity of HT-29 cells. (A) Microscopic observation images and (B) adhesion rate. Different uppercase letters indicate significant difference ($P < 0.05$).

and soybean proteins promote the adhesion of HT-29 cells in relation to their regulation of the expression of glycosylated mucins.

Gene expressions of HT-29 cells after adhered by *L. reuteri* DSM17938 from log phase were shown in Fig. 5B. Compared with the MRS group, the expression of glycosylation genes *ST3Gal3* and *GALNT7*, all mucin genes and trichothecene factor *TFF3* was significantly upregulated in the dspe group, whereas in the dspr group, only the expression of genes *ST3Gal3*, *MUC5AC*, and *TFF1* was upregulated by 1.63-, 1.24- and 1.27-fold, respectively. However, the adhesion rate results showed similar adhesion rates in the dspe and dspr groups. In contrast, the expression of adhesion genes in HT-29 cells showed a different trend after adhesion of *L. reuteri* DSM17938 from the stationary phase (Figs. 5C and 6). *C2GnT2*, *MUC2* and *TFF1* gene expression were significantly higher in the dspe group compared to the MRS group. None of the glycosylation genes were significantly changed in the dspr group. But there was a significant increase in *MUC3* and *MUC5AC* and a significant decrease in *MUC2* expression. The results showed that the co-expression of *MUC2* and *TFF1* was upregulated in the dspe group, which might be involved in promoting the thickness and viscosity of the mucus layer of HT-29 cells. The effect was still less than that of *MUC2* despite an increase in the expression of *MUC3* and *MUC5AC* in the dspr group. In addition, compared with *L. reuteri* DSM17938 from log phase, only the expression of *EnoA1* gene was increased by 8.66-fold in stationary phase, and all other genes were significantly decreased, among which the *GS* gene was the most severely down-regulated by 87.86%. That might be one of the reasons for different effects of *L. reuteri* DSM17938 on the expression of HT-29 cell adhesion genes in different phases.

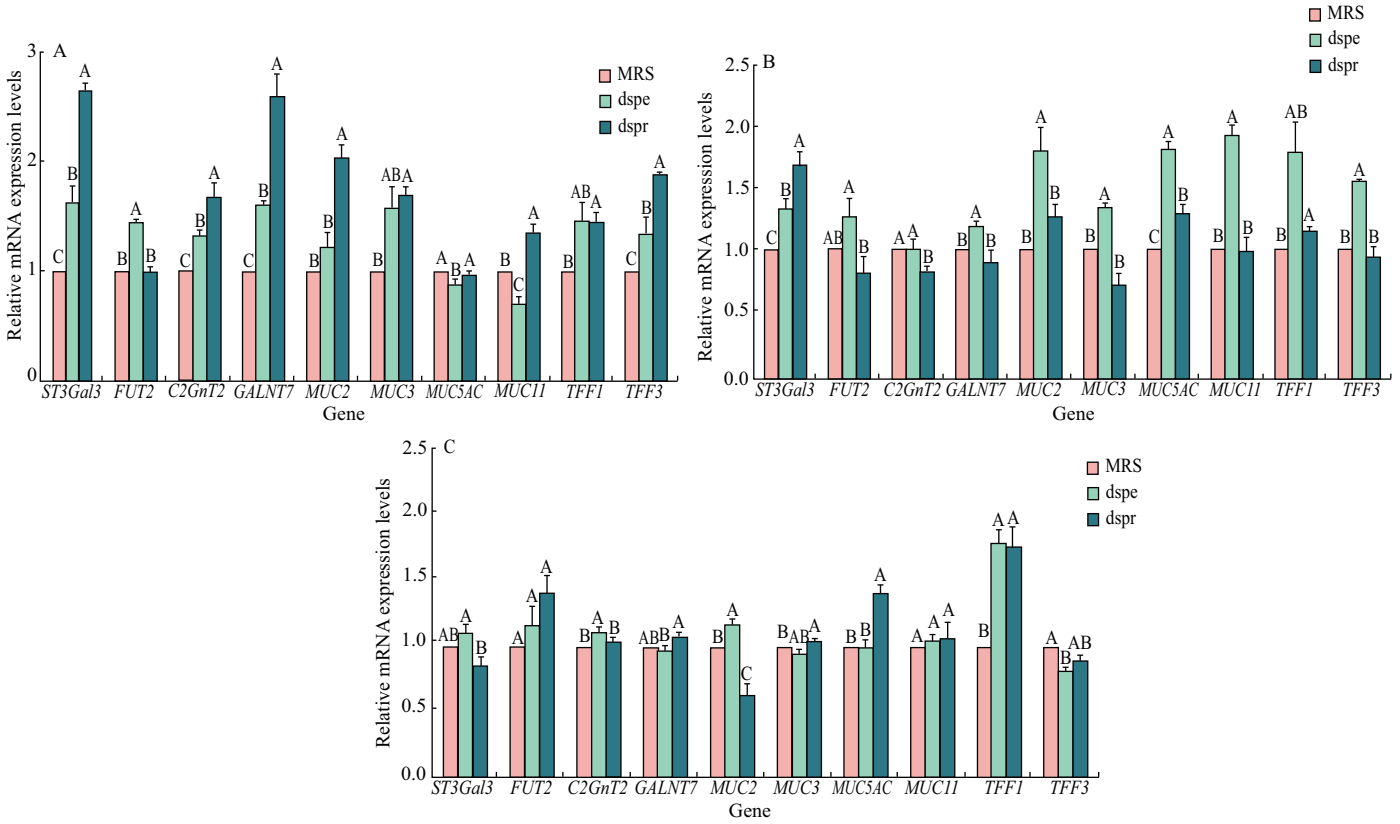

Fig. 5 Analysis of adhesion gene expression after dspe and dspr treatment of HT-29 cells. Expression of adhesion related genes of HT-29 cell (A) before and (B) after the adhesion of *L. reuteri* DSM17938 from log phase and (C) from stable phase adhesion to HT-29 cells. Different uppercase letters indicate significant difference ($P < 0.05$).

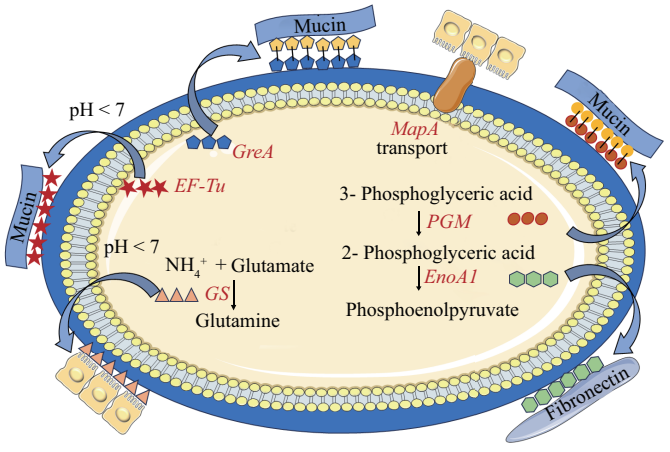

Fig. 6 Adhesion-related proteins of mucins from intestinal epithelial cells, and moonlighting proteins from *Lactobacillus* cells.

4. Conclusion

dpsr and dspe plays an important role in the adhesion between intestinal cells and probiotics. It was found that dspe and dspr were able to significantly increase the adhesion rate of *L. reuteri* DSM17938 from stationary phase to HT-29 cells, and the improvement of auto-aggregation and hydrophobicity were related to their adhesion. The moonlighting protein may be responsible for the adhesion process of *L. reuteri* from stationary phase. In addition, when HT-29 cells were directly treated with dspe and dspr, the adhesion of *L. reuteri* DSM17938 to HT-29 cells was also enhanced which might be associated with the upregulated expression of mucin genes, trefoil factors and glycosylation genes. After the adhesion of *L. reuteri* from log phase, dspe increased the expression of *ST3Gal3*, *GALNT7*, *MUC* and *TFF* genes of HT-29 cells, and *FUT2*, *MUC*, *TFF* genes were enhanced by dspr after the adhesion of *L. reuteri* from log phase to HT-29 cells.

Declaration of competing interest

There are no conflicts of interest to declare.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://doi.org/10.26599/FSHW.2024.9250428.

References

[1] C. Hill, F. Guarner, G. Reid, et al., The International Scientific Association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic, Nat. Rev. Gastro. Hepat. 11(8) (2014) 506-514. https://doi.org/10.1038/nrgastro.2014.66.

[2] N. Haddaji, A.K. Mahdhi, B. Krifi, et al., Change in cell surface properties of *Lactobacillus casei* under heat shock treatment, FEMS Microbiol. Lett. 362(9) (2015) fnv047. https://doi.org/10.1093/femsle/fnv047.

[3] J. Li, G. Mu, Y. Tuo, Phenotypic traits and probiotic functions of *Lactiplantibacillus plantarum* Y42 in planktonic and biofilm forms, Foods 12(7) (2023) 1516. https://doi.org/10.3390/foods12071516.

[4] L. Qi, X. Lu, H. Mao, et al., *Clostridium butyricum* induces the production and glycosylation of mucins in HT-29 cells, Front. Cell. Infect. Microbiol. 11 (2021) 668766. https://doi.org/10.3389/fcimb.2021.668766.

[5] P. Angela Di, B. Valentina, G. Cristina, et al., Protective effect of probiotic *Limosilactobacillus reuteri* DSM17938 against Western diet-induced obesity and associated metabolic alterations, J. Funct. Foods 109 (2023) 105805. https://doi.org/10.1016/j.jff.2023.105805.

[6] G. Liu, M. Chu, P. Xu, et al., Effects of *Ilisha elongata* proteins on proliferation and adhesion of *Lactobacillus plantarum*, Food Chem.: X 13 (2022) 100206. https://doi.org/10.1016/j.fochx.2022.100206.

[7] M. Liu, Z. Sun, C. Shi, et al., How do *Lycium barbarum* polysaccharides promote the adhesion of *Lactobacillus* to Caco-2 cells? J. Funct. Foods 89 (2022) 104929. https://doi.org/10.1016/j.jff.2021.104929.

[8] K. Salli, E. Soderling, J. Hirvonen, et al., Influence of 2′-fucosyllactose and galacto-oligosaccharides on the growth and adhesion of *Streptococcus mutans*, Br. J. Nutr. 124(8) (2020) 824-831. https://doi.org/10.1017/S0007114520001956.

[9] X.X. Liu, D.W. Chen, Q.M. Li, et al., Effect of complex prebiotics on the intestinal colonization ability of *Limosilactobacillus fermentum* DALI02, Fermentation 9(1) (2022) 25. https://doi.org/10.3390/fermentation9010025.

[10] G. Krausova, I. Hynstova, R. Svejstil, et al., Identification of synbiotics conducive to probiotics adherence to intestinal mucosa using an *in vitro* Caco-2 and HT29-MTX cell model, Processes 9(4) (2021) 569. https://doi.org/10.3390/pr9040569.

[11] S. Inoguchi, Y. Ohashi, A. Narai-Kanayama, et al., Effects of non-fermented and fermented soybean milk intake on faecal microbiota and faecal metabolites in humans, Int. J. Food Sci. Nutr. 63(4) (2012) 402-410. https://doi.org/10.3109/09637486.2011.630992.

[12] T. Li, X.S. Lu, X.B. Yang, Stachyose-enriched *α*-galacto-oligosaccharides regulate gut microbiota and relieve constipation in mice, Agric. Food Chem. 61(48) (2013) 11825-11831. https://doi.org/10.1021/jf404160e.

[13] T.J. Ashaolu, Soy bioactive peptides and the gut microbiota modulation, Appl. Microbiol. Biotechnol. 104(21) (2020) 9009-9017. https://doi.org/10.1007/s00253-020-10799-2.

[14] W. Li, H. Li, Y. Zhang, et al., Different effects of soybean protein and its derived peptides on the growth and metabolism of *Bifidobacterium animalis* subsp. *animalis* JCM 1190, Food. Funct. 12(13) (2021) 5731-5744. https://doi.org/10.1039/d1fo00480h.

[15] C. Zhang, S. Xia, Y. Zhang, et al., Identification of soybean peptides and their effect on the growth and metabolism of *Limosilactobacillus reuteri* LR08, Food. Chem. 369 (2022) 130923. https://doi.org/10.1016/j.foodchem.2021.130923.

[16] C. Zhang, Y. Zhang, G. Liu, et al., Effects of soybean protein isolates and peptides on the growth and metabolism of *Lactobacillus rhamnosus*, J. Funct. Foods 77 (2021) 104335. https://doi.org/10.1016/j.jff.2020.104335.

[17] A.L. Servin, M.H. Coconnier, Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens, Best Pract. Res. Cl. Ga. 17 **(**2003) 741-754. https://doi.org/10.1016/S1521-6918(03)00052-0.

[18] S.V. Poimenidou, A. Skarveli, G. Saxami, et al., Inhibition of *Listeria monocytogenes* growth, adherence and invasion in Caco-2 cells by potential probiotic, Microorganisms 11 (2023) 363. https://doi.org/10.3390/microorganisms11020363.

[19] B. Sophatha, R. Teanpaisan, Factors relating to adhesion and aggregation of *Lactobacillus paracasei*, *Lactobacillus rhamnosus* strains, Microbiology, 90 (2021) 793-800. https://doi.org/ 10.1134/S0026261721060151.

[20] M. Minekus, M. Alminger, P. Alvito, et al., A standardised static *in vitro* digestion method suitable for food - an international consensus, Food Funct. 5(6) (2014) 1113-1124. https://doi.org/10.1039/c3fo60702j.

[21] Y. Zhang, C. Zhang, S. Zhu, et al., Identification and characterization of soybean peptides and their fractions used by *Lacticaseibacillus rhamnosus* Lra05, Food. Chem. 401 (2023) 134195. https://doi.org/10.1016/j.foodchem.2022.134195.

[22] C.M.B. Pinilla, A. Brandelli, F. Galland, et al., Improved functional properties of the potential probiotic *Lacticaseibacillus paracasei* ItalPN16 growing in cheese whey, Lett. Appl. Microbiol. 76(7) (2023) ovad075. https://doi.org/10.1093/lambio/ovad075.

[23] R.C. Reuben, P.C. Roy, S.L. Sarkar, et al., Characterization and evaluation of lactic acid bacteria from indigenous raw milk for potential probiotic properties, Dairy. Sci. 103(2) (2020) 1223-1237. https://doi.org/10.3168/jds.2019-17092.

[24] A. Tyfa, A. Kunicka-Styczynska, J. Zabielska, Evaluation of hydrophobicity and quantitative analysis of biofilm formation by *Alicyclobacillus* sp, Acta Biochim. Pol. 62(4) (2015) 785-790.

[25] M. Jose Salas-Jara, A. Ilabaca, M. Vega, et al., Biofilm forming *Lactobacillus*: new challenges for the development of probiotics, Microorganisms 4(3) (2016) 35. https://doi.org/10.18388/abp.2015_1133.

[26] Y.K. Lee, K.Y. Puong, Competition for adhesion between probiotics and human gastrointestinal pathogens in the presence of carbohydrate, Brit. J. Nutr. 88 (2002) S101-S108. https://doi.org/10.1079/bjn2002635.

[27] G. Wang, Y. Xia, J. Cui, et al., The roles of moonlighting proteins in bacteria, Curr. Issues Mol. Biol. 16 (2014) 15-22.

[28] K. Nishiyama, M. Sugiyama, T. Mukai, Adhesion properties of lactic acid bacteria on intestinal mucin, Microorganisms 4(3) (2016) 34. https://doi.org/10.3390/microorganisms4030034.

[29] V. Vastano, U. Capri, M. Candela, et al., Identification of binding sites of *Lactobacillus plantarum* enolase involved in the interaction with human plasminogen, Microbiol. Res. 168(2) (2013) 65-72. https://doi.org/10.1016/j.micres.2012.10.001.

[30] M. Salzillo, V. Vastano, U. Capri, et al., Pyruvate dehydrogenase subunit β of *Lactobacillus plantarum* is a collagen adhesin involved in biofilm formation, Basic. Microbiol. 57(4) (2017) 353-357. https://doi.org/10.1002/jobm.201600575.

[31] B. Perez Montoro, N. Benomar, N. Caballero Gomez, et al., Proteomic analysis of *Lactobacillus pentosus* for the identification of potential markers of adhesion and other probiotic features, Food Res. Int. 111 (2018) 58-66. https://doi.org/10.1016/j.fm.2017.11.006.

[32] P. Bolay, M. Isabel Muro-Pastor, F.J. Florencio, et al., The distinctive regulation of cyanobacterial glutamine synthetase, Life 8(4) (2018) 52. https://doi.org/10.3390/life8040052.

[33] H. Abriouel, B. Perez Montoro, C.S. Casimiro-Soriguer, et al., Insight into potential probiotic markers predicted in *Lactobacillus pentosus* MP-10 genome sequence, Front. Microbiol. 8 (2017) 891. https://doi.org/10.3389/fmicb.2017.00891.

[34] M. Candela, S. Bergmann, M. Vici, et al., Binding of human plasminogen to *Bifidobacterium*, J. Bacteriol. 189(16) (2007) 5929-5936. https://doi.org/10.1128/jb.00159-07.

[35] D. Granato, G.E. Bergonzelli, R.D. Pridmore, et al., Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533 (La1) to human intestinal cells and mucins, Infect. Immun. 72(4) (2004) 2160-2169. https://doi.org/10.1128/iai.72.4.2160-2169.2004.

[36] K.E. Cox, S. Liu, T.M. Lwin, et al., The mucin family of proteins: candidates as potential biomarkers for colon cancer, Cancers 15(5) (2023) 1491. https://doi.org/10.3390/cancers15051491.

[37] T. Pelaseyed, G.C. Hansson, Membrane mucins of the intestine at a glance, J. Cell. Scie. 133(5) (2020) 240929. https://doi.org/10.1242/jcs.240929.

[38] L. Arike, J. Holmen-Larsson, G.C. Hansson, Intestinal Muc2 mucin *O*-glycosylation is affected by microbiota and regulated by differential expression of glycosyltranferases, Glycobiology 27(4) (2017) 318-328. https://doi.org/10.1093/glycob/cww134.

[39] A.S. Carvalho, A. Harduin-Lepers, A. Magalhaes, et al., Differential expression of α-2,3-sialyltransferases and α-1,3/4-fucosyltransferases regulates the levels of sialyl Lewis a and sialyl Lewis x in gastrointestinal carcinoma cells, Int. J. Biochem. Cell Biol. 42(1) (2010) 80-89. https://doi.org/10.1016/j.biocel.2009.09.010.

[40] S.H. Lee, S.Y. Yu, J. Nakayama, et al., Core2 *O*-glycan structure is essential for the cell surface expression of sucrase isomaltase and dipeptidyl peptidase-IV during intestinal cell differentiation, J. Biol. Chem. 285(48) (2010) 37683-37692. https://doi.org/10.1074/jbc.M110.162735.

[41] A. Dignass, K. Lynch-Devaney, H. Kindon, et al., Trefoil peptides promote epithelial migration through a transforming growth factor beta-independent pathway, J. clin. invest. 94(1) (1994) 376-383. https://doi.org/10.1172/jci117332.

[42] H. Kindon, C. Pothoulakis, L. Thim, et al., Trefoil peptide protection of intestinal epithelial barrier function: cooperative interaction with mucin glycoprotein, Gastroenterology 109(2) (1995) 516-23. https://doi.org/10.1016/0016-5085(95)90340-2.