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Modulation of soy flour bioactivity against enterotoxigenic *Escherichia coli* by fermentation with exopolysaccharides-producing lactic acid bacteria

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC)-mediated diarrhea can be mitigated by inhibiting bacterial adhesion to intestinal surface. Some lactic acid bacteria (LAB) produce exopolysaccharides (EPS) that can inhibit ETEC adhesion. In this study, we fermented soy flour-based dough (SoyD) with EPS-producing LAB strains *Pediococcus pentosaceus* TL (*Pp*TL), *Leuconostoc citreum* TR (*Lc*TR), *Leuconostoc mesenteroides* WA (*Lm*WA) and *L. mesenteroides* WN (*Lm*WN) to improve anti-adhesive activity of the dough against ETEC. The strains *Lc*TR, *Lm*WA and *Lm*WN produced EPS in SoyD fermentation with similar polysaccharide yields and compositions as when grown in liquid medium, whereas *Pp*TL was unable to produce EPS in SoyD. *Lc*TR produced high molecular weight (Mw) dextran (~900 kDa) while *Lm*WA and *Lm*WN produced dextran and levan with diverse Mw (~20–1000 kDa). SoyD fermentation by EPS-producing LAB increased the capability of the SoyD extracts to adhere to ETEC cells and block ETEC adhesion to porcine mucin. After Mw-based fractionation, all extract-fractions (>3 kDa) of *Lm*WA- and *Lm*WN-fermented SoyD retained their blocking activity indicating that various Mw populations of the EPS contributes to bioactivity against ETEC. This study shows the potential of EPS-producing LAB strains as fermenting microorganisms in the development of a functional food product with anti-diarrheal properties.

1. Introduction

Diarrhea is a common disease in humans and farm animals that can create economic and health burdens, especially in developing countries. The enterotoxigenic *Escherichia coli* (ETEC) is one example of a pathogenic bacterium that can cause diarrhea (Qadri et al., 2005). The cell surface of ETEC is decorated with fimbrial lectin that can specifically adhere to carbohydrate moieties present on the surface of mammalian intestinal epithelia (Jin & Zhao, 2000). This adhesion is a specific prerequisite in ETEC-mediated diarrhea that sets it apart from other diarrheagenic microorganisms. These fimbrial lectins vary between ETEC strains, allowing specific host-microbe interactions. For example, ETEC strain K88 produces lectin that is specifically adhere to porcine epithelial cell surface while ETEC strain H10407 makes colonization factor antigen I (CFA/I) that makes it specifically adhere to epithelial cells surface of human intestines (Crossman et al., 2010; Devriendt et al., 2010). The adhesion of ETEC to the gut epithelial surface is a prerequisite to the production of enterotoxins which can cause diarrhea in its host due to the disruption of epithelial permeability (Nagy & Fekete, 2005).

A possible approach to reduce the incidence of ETEC-mediated diarrhea is through the inhibition of ETEC adhesion to epithelial surface (González-Ortiz et al., 2014; Kiers et al., 2002). Some carbohydrate molecules can act as receptor analogues which will bind to ETEC fimbrial lectin, thereby blocking interaction of ETEC with the gut epithelial carbohydrate moiety (Sun & Wu, 2017). Such binding will allow the host body to either flush out ETEC or give time for the immune system to neutralize the pathogen. One such example of carbohydrates that can act as receptor analogues against ETEC adhesion to mammalian intestinal epithelial cells is exopolysaccharide (EPS) that is commonly present in fermented food products, such as fermented olive brine (González-Ortiz et al., 2014; Zhu et al., 2018).

EPS are polysaccharides synthesized by microorganisms and released into the environment (Cai et al., 2019). It has been reported that EPS presence shows potential health benefits to mammalian host body. For example, EPSs produced by several lactic acid bacteria (LAB) species can counteract the effect of certain bacterial toxins against mammalian cells *in vitro* (Ruas-Madiedo et al., 2010; Saadat et al., 2019).

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Four LAB strains have been previously isolated from the fermented soy product tempeh (Pramudito et al., 2024). The isolated LAB strains can produce two types of EPSs in diverse amounts: dextran (α-1,6-linked glucan) and levan (β-2,6-linked fructan) (Pramudito et al., 2024). These EPSs can bind to both human and pig-associated ETEC strains, which indicates their potential as receptor analogue against ETEC (Pramudito et al., 2024). Other than binding activity, the EPSs can also inhibit self-aggregation of ETEC cells. Self-aggregation allows ETEC cells to protect themselves against the host immune system (Clavijo et al., 2010) thus inhibition of such self-aggregation can help to reduce the incidence of diarrhea.

Considering the potential bioactivity of these EPS-producing LAB strains, we decided to explore their anti-diarrheal functionality when present in food products. Soy flour seems to be an ideal material for this approach since the LAB strains were isolated from soy-based products (Pramudito et al., 2024). Therefore, the strains are assumed to be able to use soy flour as fermentation substrate for EPS production. Abundant presence of sucrose is a common prerequisite for EPS production in most EPS-producing LAB (Bounaix et al., 2009) and soy flour contains around 5 % w/dry weight (dw) of sucrose (Bainy et al., 2008) which should be sufficient for EPS production (Xu, Coda, et al., 2017). In addition, soy flour contains members of raffinose family oligosaccharides (RFO), namely raffinose (0.6–1%w/dw), stachyose (4–4.7%w/dw) and verbascose (0.3–0.4%w/dw) (Huang et al., 2023). Raffinose in particularly can be used by the LAB strains along with sucrose (Pramudito et al., 2024). Xu, Coda, et al. (2017) reported that L. *mesenteroides* can grow on soy flour and produce EPSs such as dextran and fructan. However, it is not known if EPSs produced in soy fermentation still retain the same structure and give the same yield as EPSs produced in defined medium. It is hypothesized that tempeh-associated LAB strains can produce EPS when grown on soy flour as substrate and that these EPSs can improve the bioactivity of fermented soy flour towards ETEC. This research is aimed to characterize EPS produced during fermentation of soy flour by tempeh-associated LAB isolates and evaluate the activity of the corresponding fermented soy-extracts against ETEC adhesion capability.

2. Materials and methods

2.1. Materials

Full-fat untoasted soy flour (Panisoy) was kindly provided by Inveja (Twello, the Netherlands). Fructanase mix (containing *endo*-inulinase, *exo*-inulinase, and endo-levanase) was purchased from Megazyme (Wicklow, Ireland). Dextranase from *Chaetomium erraticum* was purchased from Sigma-Aldrich (Steinheim, Germany). Commercial levan from timothy grass (average molecular weight ~ 12.5 kDa) was purchased from Megazyme. Commercial dextran from *Leuconostoc mesenteroides* (average molecular weight of ~35 kDa) was purchased from Sigma-Aldrich. Verbascose and manninotriose standards were purchased from Biosynth Ltd. (Staad, Switzerland). Other carbohydrate standards were purchased from Sigma-Aldrich or Merck (Darmstadt, Germany).

2.2. Bacterial strains

Four EPS-producing LAB strains were previously isolated from tempeh-associated sources (Pramudito et al., 2024) and identified as *Pediococcus pentosaceus* TL (*Pp*TL), *Leuconostoc citreum* TR (*Lc*TR), *Leuconostoc mesenteroides* WA (*Lm*WA) and L. *mesenteroides* WN (*Lm*WN). Pig-associated ETEC K88 was kindly provided by Host-Microbe Interactomics, Wageningen University & Research. Human-associated ETEC H10407 was available from the culture collection of Food Microbiology, Wageningen University & Research. All strains were kept in 20 % (*v*/v) glycerol stock at −80 °C. Prior to use, LAB strains were grown on de Man, Rogosa, and Sharp agar (MRS) agar at 30 °C for 48 h and ETEC strains were grown on Luria Bertani agar (LA) at 37 °C for 24 h.

2.3. Soy dough fermentation by LAB

Soy dough fermentation was carried out based on the method described by Xu, Coda, et al. (2017). About 40 g of soy flour was transferred into sterile Erlenmeyer flask, added with 60 mL sterile distilled water and mixed thoroughly with sterile spatula to form soy dough (SoyD). SoyD was heated in a water bath at 100 °C for 20 min and then cooled down to room temperature. LAB culture was prepared by inoculating a colony into MRS broth followed with incubation at 30 °C for 24 h without shaking. Bacterial culture was washed twice with sterile phosphate buffered saline pH 7.4 (PBS) and the cell concentration was adjusted to ~8 log CFU/mL. SoyD was inoculated with LAB suspension in the amount of ~6 log CFU/g. Fermentation was carried out at 30 °C for 48 h. SoyD inoculated with PBS was used as a control that was either immediately stored at -20 °C or incubated at 30 °C for 48 h (referred as SoyD 0 h and 48 h respectively).

LAB in fermented SoyD was quantified by homogenizing 10 g sample in 90 mL PBS using a stomacher (Seward 400 Circulator Lab Blender, Worthing, UK) for 1 min. The homogenized suspension was serially diluted in PBS and plated on MRS agar. Bacterial colonies were counted after 48 h incubation at 30 °C. For pH measurement, 10 g sample was homogenized in 90 mL sterile distilled water as described above and pH was measured using a pH meter. Soluble protein content was measured by suspending the sample in MilliQ water (10 mg/mL) and mixing for 10 min. The suspension was centrifuged (10.000 ×*g*, 10 min) and the supernatant was freeze-dried. Protein content of the freeze-dried samples was determined using Dumas method (Dumas FlashSmart, Thermo Fisher Scientific Inc., Waltham, MA, USA) based on conversion factor of measured nitrogen content (6.25) as described by Verhoef et al. (2002). The remaining samples were stored at −20 °C to stop the fermentation and keep for further analysis.

2.4. Measurement of organic acid, free sugar and mannitol content

2.4.1. Sample preparation

Fermented SoyD samples were prepared based on the method described by Xu, Wang, et al. (2017). For free sugar measurement, SoyD samples were freeze-dried and ground into powder. Afterwards, 100 mg of samples were suspended in 5 mL MilliQ water and boiled for 10 min with rigorous mixing in every 5 min. The suspensions were cooled down to room temperature and centrifuged at 20.000 ×*g* for 10 min. The supernatant (400 μL) was collected and filtered using Amicon Ultra-0.5 mL (cutoff 10 kDa) centrifugal filter units (Millipore, Billerica, MA) at 12.000 ×*g* for 10 min. The flowthrough was collected and diluted with MilliQ prior to analysis. For organic acid measurement, samples were not freeze-dried to avoid the loss of volatile organic acids. Samples were ground directly to break any lumping and processed through the same water extraction and centrifugal filtration as in preparation for free sugar measurement.

2.4.2. Organic acid

Organic acid content was determined using high performance liquid chromatography (HPLC). The samples (10 μL) were injected into Ultimate 3000 HPLC (Dionex, Sunnyvale, California, United States) equipped with a Shodex RI-101 detector (Showa Denko K.K., Tokyo, Japan) and a Vanquish™ Variable Wavelength Detector (Thermo Fisher Scientific), an autosampler and an ion-exclusion Aminex HPX – 87H column (7.8 × 300 mm) equipped with a guard column (Bio-Rad, Hercules, CA). The mobile phase consisted of 5 mM H_2SO_4 and the flow rate was 0.6 mL/min with the column oven set at 40 °C. Organic acids quantification was based on absorbance at UV 210 nm wavelength. Standards of ethanol and citric, pyruvic, lactic, formic, acetic and propionic acid (2–20 μmol/mL) were used for quantification. The data was analyzed using Chromeleon 7.3 (Thermo Fisher Scientific).

2.4.3. Free sugars

Free sugar content (including mono-, di-, and oligosaccharides) was determined using high performance anion exchange chromatography (HPAEC). Samples were diluted with MilliQ to 25 μg/mL before injected to an ISC6000 HPAEC system with a CarboPac PA-1 column (250 mm × 2 mm ID), a CarboPac PA guard column (25 mm × 2 mm ID) and a ICS6000 ED detector in pulsed amperometric detection (PAD) mode (Thermo Fisher Scientific). Mobile phases were kept under helium flushing and the column temperature was at 20 °C. A flow rate of 0.3 mL/min was used with the following gradient of 0.1 M sodium hydroxide (NaOH: A), 1.0 M sodium acetate (NaOAc) in 0.1 M NaOH (B) and Milli-Q water (C): 0–5 min 84 % C (16 % A); 5–33 min 84–0 % C (16–100 % A); 33–45 min 0–4 % B (100–96 % A); 45–50 min 100 % B; 50–55 min 100 % A; 55–65 min 84 % C (16 % A). Standards of glucose, galactose, fructose, sucrose, melibiose (α-1,6-linked Gal-Glc), manninotriose (Gal-Gal-Glc), raffinose, stachyose and verbascose (2.5–25 μg/mL) were used for quantification. To detect the presence of manninotetraose (Gal-Gal-Gal-Glc), verbascose (100 μg/mL) was subjected to mild (8 mM) TFA hydrolysis at 90 °C for 1 h (Pramudito et al., 2024) to remove the fructose moiety and subsequently injected into HPAEC-PAD. The data was analyzed using Chromeleon 7.3 (Thermo Fisher Scientific).

2.4.4. Mannitol

Mannitol was quantified using HPAEC-PAD with the same setup as described above. A flowrate of 0.3 mL/min was used with the following gradient: 0–30 min 85 % C (15 % A), 30–45 min 100 % B, 45–60 min 85 % C (15 % A). Mannitol (Thermo Scientific Chemicals, Geel, Belgium) was used as standard for quantification (0.5–5 μg/mL). The data was analyzed using Chromeleon 7.3 (Thermo Fisher Scientific).

2.5. Quantification of EPSs

EPSs in SoyD were detected and quantified based on the method described by Katina et al. (2009) for dextran and Shi et al. (2019) for levan. The amount of dextran was determined based on the amount of glucose and isomaltose released after treatment of SoyD with dextranase while the amount of levan was based on fructose released after treatment with fructanase mix that contained levanase. In the latter case, inulinase present in the fructanase mix should not pose a problem since inulin is neither present in soy flour nor could be synthesized by LAB.

Samples were freeze-dried and ground as described above. About 100 mg of samples were suspended in 5 mL ethanol 70 % (*v*/v) to remove small sugars. The suspensions were heated at 70 °C for 20 min with rigorous mixing every 10 min. After cooling, the suspensions were centrifuged at 5.000 ×*g* for 10 min. The supernatant was removed and the pellets were washed with ethanol 70 %. After another centrifugation, the pellets were air-dried. The pellet was suspended (10 mg/mL) in 20 mM sodium acetate buffer pH 5.0 with 5 mM $CaCl_2$ for dextranase treatment or 0.1 M sodium acetate buffer pH 4.5 for fructanase treatment. Enzymes were added (0.2 U/mg substrate) to the suspension followed with incubation at 40 °C for 24 h with constant mixing using a head-over-tail rotator. The suspensions were boiled for 10 min to deactivate the enzymes. After cooling down, the suspensions were centrifuged at 20.000 ×*g* for 10 min and the supernatant was diluted to 25 μg/mL with MilliQ. Released sugars were quantified with HPAEC-PAD as described above. Standards of glucose, isomaltose, isomaltotriose, fructose and sucrose (2.5–25 μg/mL) were used for quantification. Commercial dextran, commercial levan, and SoyD 0 h spiked with either commercial dextran or levan (each to the concentration of 1 % *w*/w dry base of SoyD 0 h) were used as controls and subjected to the washing steps as well to estimate EPS loss through the process. The data was analyzed using Chromeleon 7.3 (Thermo Fisher Scientific).

2.6. Characterization of EPS molecular weight

Water-soluble EPS was extracted from fermented SoyD by suspending 200 mg sample in 20 mL MilliQ water. The suspension was boiled for 10 min with rigorous mixing every 5 min. After cooling down, the suspension was centrifuged at 21.000 ×*g* for 10 min. The supernatant was collected and frozen at −80 °C followed with freeze-drying. The freeze-dried samples were stored at −20 °C. EPS produced by LAB strains in liquid medium was used as a reference. EPS-producing LAB strains were grown in modified MRS medium with sucrose and raffinose (each 5 % *w*/*v*) as sole carbon sources (MRS-SR) for 48 h and isolated with ethanol precipitation using the method previously described (Pramudito et al., 2024).

Molecular weight (Mw) distribution of EPS isolated from fermented liquid medium and SoyD were determined using high performance size exclusion chromatography with refractive index detection (HPSEC-RI) with the method described in our previous paper (Pramudito et al., 2024). The retention times of pullulan standards were used to estimate the Mw of samples. In order to identify peaks corresponding to dextran and levan EPSs, the EPS-containing extracts were treated with dextranase and levanase-containing fructanase mix with the method described above. The data was analyzed using Chromeleon 7.3 (Thermo Fisher Scientific).

2.7. Bioactivity assay

2.7.1. Sample and bacterial preparation for bioactivity assay

Freeze-dried samples were suspended in sterile PBS to the concentration of 10 mg/mL. The suspension was boiled for 10 min with rigorous mixing every 5 min. After cooling, the suspension was centrifuged at 21.000 ×*g* for 10 min. The resulting supernatant was used for bioassays. Suspensions of commercial levan and dextran were prepared in the same way to act as controls. Another set of controls were prepared by adding commercial dextran or levan into SoyD 0 h in the amount of 1 % (w/dw SoyD). The mixture was homogenized and suspended in PBS as described above. To identify which Mw fraction of fermented SoyD samples was responsible for bioactivity against ETEC, part of the supernatant was filtered using Amicon Ultra-15 mL (cutoff 3 kDa) centrifugal filter units (Millipore) at 4.000 ×*g* for 30 min. The flowthrough and retentate were collected and the volumes of both were re-adjusted with PBS to the starting volume before being used in blocking assay.

For ETEC cell preparation, a colony from LA plate was transferred to Luria Bertani broth (LB) and incubated overnight at 37 °C. The overnight liquid culture was centrifuged at 1.700 ×*g* for 10 min, 4 °C. The cell pellet was washed twice and re-suspended in sterile PBS. Cell density was adjusted to ~7–8 log CFU/mL for adhesion assay and ~ 5–6 log CFU/mL for blocking assay.

2.7.2. Adhesion assay

Adhesion assay measured the capability of samples to adhere to ETEC cells and was carried out according to the method described in our previous paper (Pramudito et al., 2024). Adhered bacteria was determined based on the time it took to reach OD_{600} of 0.05 ($t_{OD600} = 0.05$). Measurements were done in triplicates from biological duplicates.

2.7.3. Blocking assay

The blocking assay measured the capability of sample to inhibit ETEC adhesion to mucin layer and was carried out based on the method described by González-Ortiz et al. (2014). Porcine mucin type III (Sigma-Aldrich) was suspended in PBS to the concentration of 1 % (*w*/*v*) and autoclaved. A high-binding 96-well microplate (flat-bottom, Greiner Bio-One, Frickenhausen, Germany) was coated with 200 μL mucin suspension in each well and incubated overnight at 4 °C. Afterwards, wells were washed with PBS and coated with 1 % (w/v) bovine serum albumin (BSA) to exclude non-specific adhesion. The microplate was incubated for 2 h at 4 °C and BSA was removed by washing the wells twice with

PBS. Meanwhile, ETEC cells in PBS were mixed with sample suspension with the ratio of 1:1. For negative control, PBS was added into cell suspension instead of sample suspension. The mixture was incubated at 37 °C for 30 min with gentle shaking (300 rpm). ETEC cell-EPS mixture was transferred to the wells (200 μL) followed with incubation at room temperature for 30 min. The wells were washed three times with PBS and 250 μL of LB was added. Bacterial growth was measured based on $t_{OD600} = 0.05$ as described in Pramudito et al. (2024). Measurements were done in triplicates from biological duplicates. In this assay, mannose (1 % *w*/w) was used as a positive control for blocking bioactivity.

2.7.4. Data and statistical analysis

The value of $t_{OD600} = 0.05$ was converted into colony forming unit (CFU) using the same approach as previously reported (Pramudito et al., 2024). Results from adhesion and blocking assays were statistically analyzed to determine the significance of sample bioactivity against ETEC. In the adhesion test, the number of adhered ETEC cells was tested against treatment with SoyD 0 h while in the blocking test, it was tested against the mixture of ETEC cells and PBS. Blocking activity between two Mw fractions of SoyD extract were also statistically tested against one another. Data were analyzed with one-way analysis of variance (ANOVA) followed with Dunnett T3 test for unequal variances by using SPSS (IBM, USA). Difference was considered significant at $p < 0.05$ and very significant at $p < 0.01$. Values are presented as means ± standard deviation.

3. Results

3.1. LAB growth in fermented soy dough

The growth of lactic acid bacteria (LAB) in soy dough (SoyD) after 48 h of incubation resulted in an increase in viable plate counts of LAB (Table 1). LAB were present in the unfermented sample (SoyD 0 h) indicating that heating at 100 °C for 20 min was not enough to inactivate LAB to a level below the detection threshold. This resulted in a 5 log increase of LAB in the uninoculated sample after 48 h incubation at 30 °C (SoyD 48 h). Compared to the uninoculated sample, SoyD inoculated with LAB had around 1–2 log further increase of LAB indicating that LAB inoculation successfully influenced the LAB population in fermented samples. Highest LAB concentration was observed in SoyD-*Lm*WA and the lowest was SoyD-*Pp*TL.

Acidification of SoyD during fermentation also provided evidence for LAB growth (Table 1). All samples inoculated with LAB showed a pH decrease, albeit at a variable level. The pH of both SoyD 0 h and 48 h were around 6.7 indicating that background fermentation did not influence pH. The pH of SoyD fermented with *Pediococcus pentosaceus* TL (SoyD-*Pp*TL) only decreased to 6.3 while the pH of SoyD-*Lc*TR decreased to 5.6. The lowest pH of approximately 4.7 was observed in SoyD-*Lm*WA and SoyD-*Lm*WN.

Fermentation by LAB strains decreased the amount soluble protein in SoyD samples (Table 1). The concentration of soluble protein in SoyD 0 h and 48 h was unaltered at around 25 % w/dry weight (dw) of SoyD. SoyD-*Pp*TL had the highest soluble protein content among LAB-fermented samples at 21.3 ± 0.1%w/dw. The soluble protein content of SoyD-*Lc*TR was almost half to SoyD 0 h at 13.5 ± 0.7%w/dw. The lowest soluble protein content was observed in SoyD-*Lm*WA and SoyD-*Lm*WN at 10.0 ± 0.3 and 9.8 ± 0.6%w/dw, respectively. This indicates either that the added LAB consumed soluble protein in SoyD for bacterial growth during fermentation or decreased protein solubility due to lower pH of SoyD after fermentation.

Table 1
LAB concentration, pH and water-soluble protein content of SoyD fermented for 48 h by EPS-producing LAB strains.

Sample	LAB concentration[a] (log CFU/g dw)	pH	Water-soluble protein (%w/dw)
SoyD[b] 0 h	3.6 ± 0.4	6.7 ± 0.0	25.2 ± 3.2
SoyD 48 h	8.2 ± 0.0	6.7 ± 0.0	25.5 ± 1.6
SoyD-*Pp*TL[c]	9.6 ± 0.0	6.3 ± 0.0	21.3 ± 0.1
SoyD-*Lc*TR[d]	9.7 ± 0.1	5.6 ± 0.0	13.5 ± 0.7
SoyD-*Lm*WA[e]	10.0 ± 0.0	4.7 ± 0.0	10.0 ± 0.3
SoyD-*Lm*WN[f]	9.9 ± 0.0	4.8 ± 0.0	9.8 ± 0.6

[a] Detection threshold: 2.5 log CFU/g.
[b] SoyD: soy dough.
[c] *Pp*TL: *Pediococcus pentosaceus* TL.
[d] *Lc*TR: *Leuconostoc citreum* TR.
[e] *Lm*WA: *Leuconostoc mesenteroides* WA.
[f] *Lm*WN: *L. mesenteroides* WN.

3.2. Organic acid content of fermented SoyD

SoyD fermentation by LAB strains resulted in the production of organic acids, mainly lactic and acetic acid (Table 2). Generally, the amount of produced acids in samples followed the same trend as the increase of the viable plate count of LAB and pH decrease. SoyD-*Pp*TL with the lowest LAB concentration and acidification also showed the lowest concentration of lactic and acetic acid at 5.1 ± 0.5 and 8.5 ± 0.4 μmol/100 mg dw. SoyD-*Lm*WA has the highest lactic acid concentration at 23.6 ± 0.8 μmol/100 mg followed by SoyD-*Lm*WN at 20.0 ± 0.6 μmol/100 mg. The concentration of acetic acid in SoyD-*Lc*TR was the highest at 25.8 ± 1.4 μmol/100 mg. Citric acid was detected in both SoyD 0 h and 48 h and the concentration was decreased in samples fermented by LAB, with the exception of SoyD-*Pp*TL.

3.3. Monosaccharides, sucrose and mannitol content

Concentrations of monosaccharides, sucrose and mannitol in SoyD were affected by fermentation (Table 3). Galactose, glucose and fructose in SoyD 0 h were only present in small amounts ranging from 0.06 to 0.10%w/dw. This amount did not change much in SoyD control during 48 h of incubation and generally decreased in SoyD samples that were inoculated with LAB. SoyD 0 h contained 6.73 ± 0.28%w/dw sucrose, which should be sufficient to stimulate EPS production by LAB strains. Sucrose content in SoyD decreased after fermentation as seen in SoyD 48 h and SoyD-*Pp*TL. No sucrose was detected in the other three LAB-inoculated SoyD samples. Mannitol was present in SoyD 0 h and the amount did not change in SoyD 48 h. LAB-inoculated SoyD had an increase of mannitol content with the lowest observed in SoyD-*Pp*TL (0.16 ± 0.02%w/dw) and the highest was in SoyD-*Lm*WA (1.26 ± 0.03%w/dw).

3.4. Soy oligosaccharides and their degradation products

SoyD contains, next to especially sucrose, also raffinose, stachyose

Table 2
Concentrations of citric acid, lactic acid and acetic acid in fermented SoyD.

Sample	Lactic acid (μmol/100 mg dw)	Acetic acid	Citric acid
SoyD[b] 0 h	n.d.[a]	n.d.	8.1 ± 0.4
SoyD 48 h	n.d.	n.d.	8.0 ± 0.9
SoyD-PpTL[c]	5.1 ± 0.5	8.5 ± 0.4	8.6 ± 0.7
SoyD-LcTR[d]	8.2 ± 0.5	25.8 ± 1.4	2.2 ± 0.2
SoyD-LmWA[e]	23.6 ± 0.8	15.9 ± 1.0	7.7 ± 0.3
SoyD-LmWN[f]	20.0 ± 0.6	13.1 ± 0.4	7.5 ± 0.4

[a] n.d.: not detected.
[b] SoyD: soy dough.
[c] *Pp*TL: *Pediococcus pentosaceus* TL.
[d] *Lc*TR: *Leuconostoc citreum* TR.
[e] *Lm*WA: *Leuconostoc mesenteroides* WA.
[f] *Lm*WN: *L. mesenteroides* WN.

Table 3
Monosaccharides, sucrose and mannitol contents in LAB-fermented SoyD.

Sample	Gal[a] (%w/dw)	Glc[a]	Fru[a]	Suc[a]	Mannitol[a]
SoyD[c] 0 h	0.08 ± 0.00	0.06 ± 0.00	0.10 ± 0.01	6.73 ± 0.28	0.09 ± 0.02
SoyD 48 h	0.09 ± 0.00	0.07 ± 0.00	0.12 ± 0.00	5.99 ± 0.25	0.10 ± 0.02
SoyD-PpTL[d]	n.d.[b]	0.04 ± 0.02	0.07 ± 0.01	5.80 ± 0.35	0.16 ± 0.02
SoyD-LcTR[e]	n.d.	n.d.	n.d.	n.d.	0.41 ± 0.01
SoyD-LmWA[f]	0.06 ± 0.00	n.d.	0.08 ± 0.01	n.d.	1.26 ± 0.03
SoyD-LmWN[g]	0.03 ± 0.00	n.d.	0.06 ± 0.01	n.d.	1.20 ± 0.07

[a] Gal: galactose, Glc: glucose, Fru: fructose, Suc: sucrose.
[b] n.d.: not detected.
[c] SoyD: soy dough.
[d] *Pp*TL: *Pediococcus pentosaceus* TL.
[e] *Lc*TR: *Leuconostoc citreum* TR.
[f] *Lm*WA: *Leuconostoc mesenteroides* WA.
[g] *Lm*WN: *L. mesenteroides* WN.

Table 4
Raffinose family oligosaccharide levels in fermented SoyD.

Sample	Raf[a] (%w/dw)	Sta[a]	Ver[a]	Mel[a]	Manninotriose
SoyD[c] 0 h	0.44 ± 0.05	3.78 ± 0.20	0.13 ± 0.02	n.d.[b]	n.d.
SoyD 48 h	0.48 ± 0.05	4.07 ± 0.27	0.15 ± 0.01	n.d.	n.d.
SoyD-PpTL[d]	0.44 ± 0.02	3.50 ± 0.26	0.13 ± 0.01	0.02 ± 0.01	0.20 ± 0.07
SoyD-LcTR[e]	0.43 ± 0.02	3.46 ± 0.17	0.12 ± 0.01	n.d.	n.d.
SoyD-LmWA[f]	n.d.	n.d.	n.d.	0.10 ± 0.01	3.28 ± 0.12
SoyD-LmWN[g]	n.d.	1.17 ± 0.09	0.07 ± 0.01	0.03 ± 0.00	2.25 ± 0.18

[a] Raf: raffinose, Sta: stachyose, Ver: verbascose, Mel: melibiose.
[b] n.d.: not detected.
[c] SoyD: soy dough.
[d] *Pp*TL: *Pediococcus pentosaceus* TL.
[e] *Lc*TR: *Leuconostoc citreum* TR.
[f] *Lm*WA: *Leuconostoc mesenteroides* WA.
[g] *Lm*WN: *L. mesenteroides* WN.

and verbascose as shown in HPAEC chromatogram (Fig. 1). These oligosaccharides were variably consumed in LAB-treated SoyD (Table 4). Raffinose, stachyose and verbascose were completely consumed in SoyD-*Lm*WA. In SoyD-*Lm*WN, raffinose was fully consumed while stachyose and verbascose partially decreased. HPAEC of SoyD-*Lm*WA and SoyD-*Lm*WN showed the appearance of melibiose, manninotriose and manninotetraose (Fig. 1). Melibiose can be formed from raffinose after the release of fructose moiety, while manninotriose and manninotetraose can be formed accordingly from stachyose and verbascose respectively. The amount of manninotriose in both SoyD-*Lm*WA and SoyD-*Lm*WN was higher than melibiose which corresponds with the relative amount of stachyose and raffinose in SoyD 0 h, where the former is higher than the latter.

3.5. EPS content of fermented SoyD

The presence of EPS in fermented SoyD was confirmed by analyzing the release of monosaccharide building blocks of EPS after enzymatic hydrolysis of SoyD extract. It should be noted that the amount dextran could be an underestimation due to the linear commercial dextran giving 92 % recovery and the release of oligosaccharides when reference EPSs (which were produced in liquid medium) was treated with dextranase (Pramudito et al., 2024). EPSs (either dextran, levan or both) were present in SoyD after 48 h fermentation although SoyD-*Pp*TL and SoyD 48 h only contained trace amount of EPS (Table 5). SoyD-*Lc*TR contained only dextran (0.22 ± 0.02%w/dw) and no levan. Both levan and dextran were present in SoyD-*Lm*WA and SoyD-*Lm*WN but the relative amount of each EPS was different between the two samples. SoyD-*Lm*WA contained more levan (0.88 ± 0.01%w/dw) than dextran

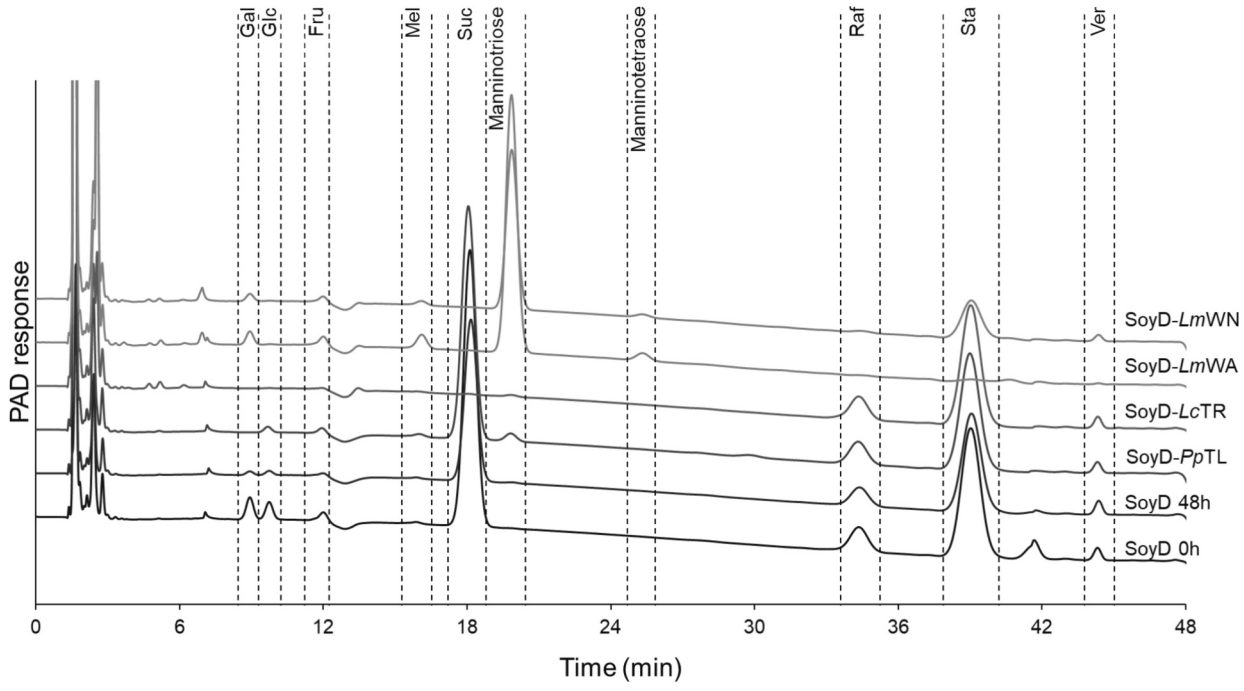

Fig. 1. HPAEC-PAD elution profiles of uninoculated SoyD before (SoyD 0 h) and after 48 h incubation at 30 °C (SoyD 48 h) and SoyD fermented by LAB strains. Gal: galactose, Glc: glucose, Fru: fructose, Mel: melibiose, Suc: sucrose, Raf: raffinose, Sta: stachyose, Ver: verbascose.

Table 5
Dextran and levan levels in fermented SoyD and total EPS yield when LAB strains were grown on SoyD and liquid medium.

Sample	Dextran (%w/dw)	Levan (%w/dw)	Total EPS in fermented SoyD (%w/dw)	EPS yield from liquid medium[a] (%*w/v*)
SoyD[c] 0 h	n.d.[b]	n.d.	–	–
SoyD 48 h	n.d.	0.09 ± 0.01	0.09	–
SoyD-PpTL[d]	0.02 ± 0.00	0.09 ± 0.00	0.11	0.26
SoyD-LcTR[e]	0.22 ± 0.02	n.d.	0.22	0.19
SoyD-LmWA[f]	0.26 ± 0.00	0.88 ± 0.01	1.14	1.08
SoyD-LmWN[g]	0.75 ± 0.02	0.34 ± 0.02	1.09	0.93

[a] Pramudito et al., 2024; modified MRS medium with sucrose and raffinose (each 5 % w/v) as sole carbon source.
[b] n.d.: not detected.
[c] SoyD: soy dough.
[d] *Pp*TL: *Pediococcus pentosaceus* TL.
[e] *Lc*TR: *Leuconostoc citreum* TR.
[f] *Lm*WA: *Leuconostoc mesenteroides* WA.
[g] *Lm*WN: *L. mesenteroides* WN.

(0.26 ± 0.00%w/dw) while it was the opposite for SoyD-*Lm*WN (0.75 ± 0.02 and 0.34 ± 0.02%w/dw of dextran and levan, respectively). Based on the sum of dextran and levan produced, the total EPS yield from fermented SoyD was rather similar to EPS yields for these LAB strains grown on liquid medium (Table 5). The exception to this was *Pp*TL, which EPS yield in SoyD was only around 50 % compared to yield from liquid medium.

3.6. Mw distribution of EPS in fermented SoyD extract

HPSEC of SoyD extracts showed the presence of high Mw compound and enzymatic treatments using dextranase and levanase indicated that these compounds corresponded to EPSs (Fig. 2). SoyD-*Pp*TL did not show any peak that was degraded by dextranase or levanase, indicating the sample did not contain EPS or that the produced EPS had low Mw, thus eluting together with smaller molecules. SoyD-*Lc*TR showed the presence of high Mw compound (~900 kDa) that was identified as dextran with same Mw as reference EPS (dashed line in Fig. 2). SoyD-*Lm*WA showed a high intensity peak corresponding to compound with Mw of ~900 kDa and a range of compounds corresponding of ~20–500 kDa that were likely to be broad Mw levan. Dextran was present in low amount (eluted at around 11 min) with Mw of ~20–40 kDa. SoyD-*Lm*WN showed the presence of high intensity (~200 to >1000 kDa) and low intensity (~20–200 kDa) compound populations that mostly consist of dextran. Levan was detected with the average Mw of ~600 kDa while high Mw dextrans were present with >1000 and ~700 kDa. In general, HPSEC analysis indicated that the Mw distributions of EPSs were more diverse when *Lm*WA and *Lm*WN were grown on SoyD, compared to growth in liquid medium.

3.7. Capability of fermented SoyD extract to adhere with ETEC cells

Generally, extracts of SoyD fermented by LAB strains showed higher adhesion bioactivity against both ETEC K88 and H10407 compared to unfermented SoyD (Fig. 3). Both ETEC strains can adhere to SoyD 0 h, thus the number of ETEC adhered to SoyD 0 h extract was set as 100 % to determine the relative adhesion bioactivity of the other samples. SoyD 0 h spiked with commercial dextran or levan (1 % w/dw of SoyD) showed an increase of adhesion indicating that the presence of EPS could influence the bioactivity of SoyD extract. SoyD-*Lc*TR, SoyD-*Lm*WA and SoyD-*Lm*WN showed around 50 % increase of adhesion bioactivity

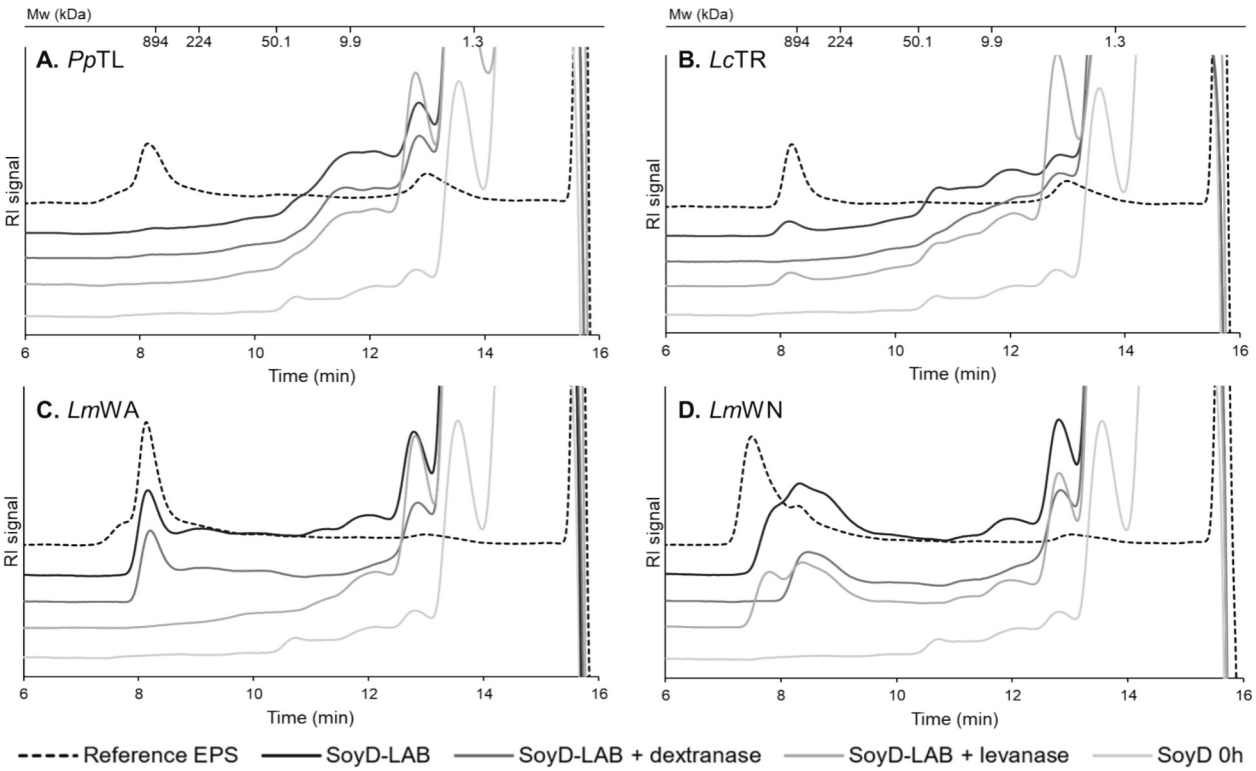

Fig. 2. HPSEC elution profiles of EPS extract from SoyD fermented with (A) *Pediococcus pentosaceus* TL, (B) *Leuconostoc citreum* TR, (C) *Leuconostoc mesenteroides* WA and (D) *L. mesenteroides* WN before and after enzymatic treatment with levanase or dextranase. Molecular weight (kDa) calibration was with pullulan standards. Reference EPSs (dashed lines) are EPS isolated from LAB grown in liquid medium.

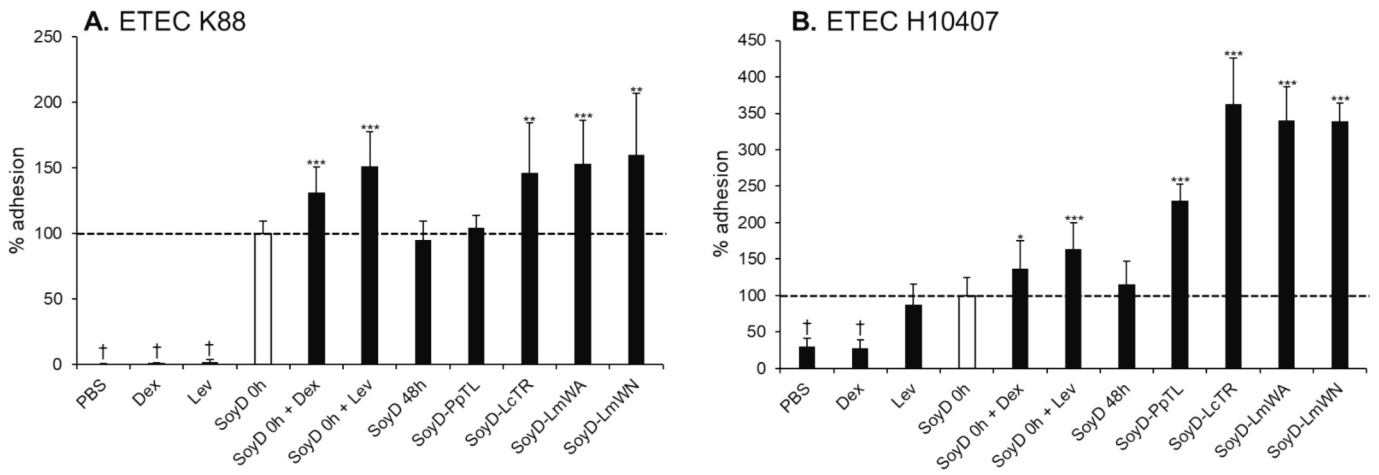

Fig. 3. ETEC adhesion to fermented soy dough (SoyD) extract (1 % *w/v*) using the strains (A) K88 and (B) H10407. Bars refer to averaged value from triplicates from biological duplicates. Adhesion bioactivity of unfermented and uninoculated soy dough (SoyD 0 h) was set at 100 % (corresponding to 2×10^6 and 2.5×10^4 CFU/well for ETEC K88 and H10407, respectively) and marked with horizontal dashed line. Asterisks above standard deviation bars indicate significantly higher (*: $p < 0.1$, **: $p < 0.05$, ***: $p < 0.01$) bioactivity compared to SoyD 0 h. Dagger signs (†) indicate significantly lower ($p < 0.01$) bioactivity from SoyD 0 h. PBS: phosphate buffered saline, Dex: commercial dextran, Lev: commercial levan, SoyD 0 h + Dex/Lev: unfermented SoyD spiked with commercial dextran or levan (1 % w/dw SoyD).

against ETEC K88 while SoyD-*Pp*TL did not show significant increase (Fig. 3A). All extracts of SoyD fermented with LAB strains showed a significant increase of adhesion bioactivity against ETEC H10407 compared to SoyD 0 h (Fig. 3B). The three samples (SoyD-*Lc*TR, SoyD-*Lm*WA and SoyD-*Lm*WN) that showed the highest increase in adhesion activity were from fermentations with strains that had the highest EPS yields and produced EPSs with high Mw (up to >100 kDa).

3.8. Blocking bioactivity of SoyD extract against ETEC adhesion to porcine mucin

Extracts from several LAB-fermented SoyD showed the capability to block adhesion of ETEC K88 to porcine mucin (Fig. 4A). SoyD-*Lc*TR, SoyD-*Lm*WA and SoyD-*Lm*WN extracts decreased ETEC adhesion by tenfold compared to PBS control. This decrease was on par with mannose as positive control for blocking bioactivity. Extracts of uninoculated SoyD (0 h and 48 h) and SoyD-*Pp*TL did not show significant change in ETEC adhesion, indicating the blocking bioactivity could be derived from significant presence of EPS.

Human-associated ETEC H10407 did not show lectin-mediated adhesion to porcine mucin (Fig. 4B). The number of adhered ETEC H10407 was not affected by mannose treatment indicating that either mannose did not bind to fimbrial lectin or ETEC cells adhered non-specifically to well surface. Commercial dextran and levan and uninoculated SoyD extract showed a significant increase of adhesion showing that non-specific adhesion of ETEC cells to mucin can be facilitated by carbohydrate compounds. None of the LAB-fermented SoyD samples showed a significant change in the adhesion of ETEC H10407 to porcine mucin.

It should be noted that the blocking bioactivity of fermented SoyD against ETEC K88 as shown in Fig. 4 was based on total water extracted material, thus raising the possibility that low Mw molecules could also be responsible for the bioactivity. Therefore, blocking bioactivity of the SoyD samples against ETEC K88 was also determined after sample fractionation based on Mw (Fig. 5). Individual fractions (Mw cutoff 3 kDa) from uninoculated SoyD 0 h and 48 h samples showed an increase of ETEC adhesion, indicating that semi-purified components of soy flour stimulated adhesion. There was no significant difference between >3

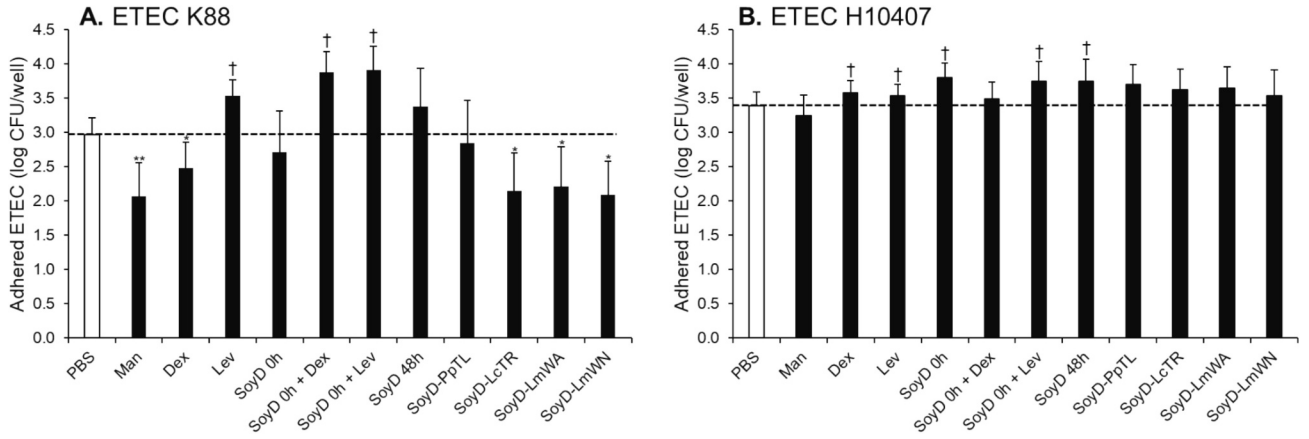

Fig. 4. Blocking-bioactivity of fermented soy dough (SoyD) extract (1 % *w/v*) against adhesion of ETEC strain (A) K88 and (B) H10407 towards porcine mucin. Bars refer to averaged value from triplicates from two biological duplicates. Blocking bioactivity of the samples were compared to phosphate buffered saline (PBS) and the level was marked with horizontal dashed line. Asterisks above standard deviation bars indicate significantly lower (*: $p < 0.05$, **: $p < 0.01$) bioactivity from PBS. Dagger signs (†) indicate significantly higher ($p < 0.01$) bioactivity from PBS. Man: mannose, Dex: commercial dextran, Lev: commercial levan, SoyD 0 h + Dex/Lev: unfermented SoyD spiked with commercial dextran or levan (1 % w/dw SoyD).

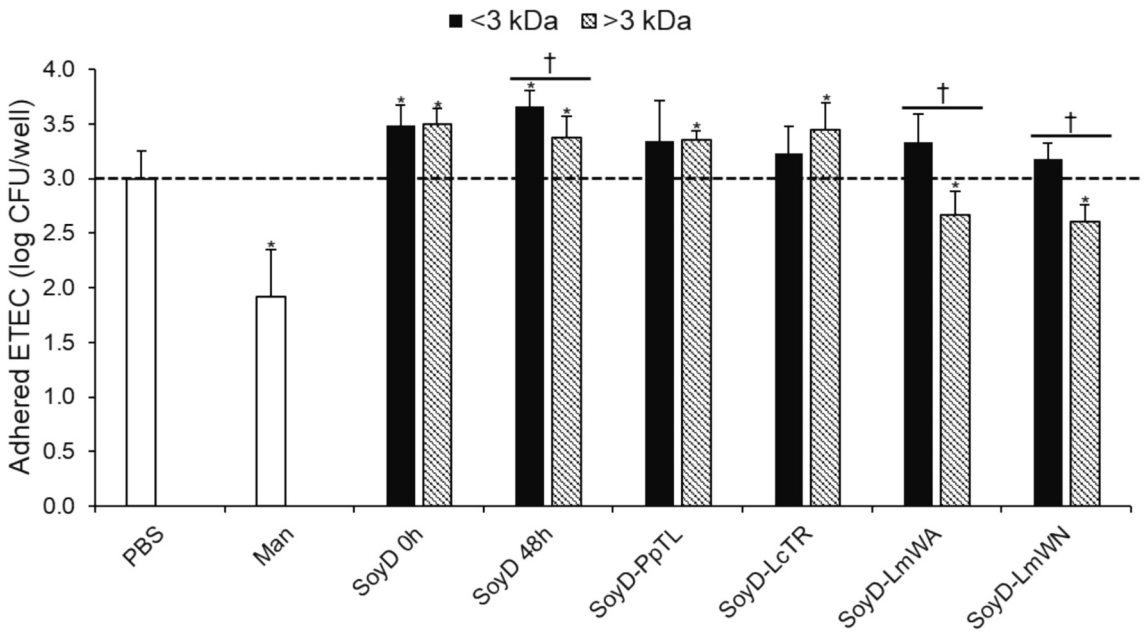

Fig. 5. Blocking bioactivity of fermented soy dough (SoyD) extract (1 % w/v) fractionated based on Mw (cutoff: 3 kDa) against ETEC strain K88 towards porcine mucin. Bars refer to averaged value from triplicates from two biological duplicates. Blocking bioactivity of the samples were compared to phosphate buffered saline (PBS) and the level was marked with horizontal dotted line. Asterisks (*) above standard deviation bars indicate significantly different ($p < 0.01$) bioactivity from PBS. Dagger signs (†) indicate significantly different ($p < 0.01$) bioactivity between two Mw fractions. Man: mannose.

kDa and <3 kDa fractions for SoyD-*Pp*TL and SoyD-*Lc*TR. Surprisingly, none of SoyD-*Lc*TR fractions showed blocking bioactivity. In contrast, we found a significant difference of blocking bioactivity between samples of fractions derived from SoyD-*Lm*WA and SoyD-*Lm*WN where blocking bioactivity was only observed in the larger sized fraction (>3 kDa). This indicated that blocking bioactivity in SoyD-*Lm*WA and SoyD-*Lm*WN extracts was driven by high Mw EPS.

4. Discussion

The strains *Lc*TR, *Lm*WA and *Lm*WN were shown to have robust EPS-producing capability regardless of substrate complexity and water availability. This is indicated by the total *in situ* EPS yield and composition in SoyD fermented by the three strains being similar to EPS from pure cultures grown on semi-defined liquid medium (Table 5). *Lc*TR only produced dextran, *Lm*WA produced dextran and levan at a ratio of ~1:4 and *Lm*WN produced more dextran than levan (at ratio of ~4:1). Moreover, HPAEC of fermented SoyD treated with dextranase or fructan-degrading enzymes showed very similar degradation patterns (data not shown) to reference EPS treated with the same enzymes (Pramudito et al., 2024), indicating highly similar carbohydrate structure of the EPS. *Pp*TL was the only strain that had a lower EPS-producing capability when grown on SoyD substrate compared to liquid medium (Table 5).

*Lm*WA and *Lm*WN produced EPS with different Mw distributions when grown on SoyD compared to in liquid medium (Fig. 2C and D). When grown in liquid medium, the two strains produced EPSs with narrow Mw distribution of around 1200 kDa for dextran and 700 kDa for levan (Pramudito et al., 2024). However, EPSs produced by the two strains that were detected in fermented SoyD had a wide Mw distribution, ranging from 20 to 1000 kDa (Fig. 2). On the other hand, the dextran present in *Lc*TR-fermented SoyD showed similar narrow Mw distribution at around 900 kDa just like when the strain was grown in liquid medium. Xu et al. (2020) reported that LAB species such as L. *mesenteroides* tend to produce EPS, particularly dextran, with polydisperse Mw distribution when grown on solid substrate. This could be caused by the osmolarity during SoyD fermentation. The low water content in SoyD compared to liquid medium means that the strains have to cope with more relatively higher osmotic pressure in the environment. Han et al. (2021) reported lower polymerization activity of dextransucrase and levansucrase activities at high osmotic pressure. Prechtl et al. (2018) also found that the polydispersity of dextran produced by *Latilactobacillus sakei* increased in a condition with higher osmolarity.

Several parameters such as mannitol and organic acid production and free sugar utilization could be linked to EPS production as well as the type of EPS (Xu, Coda, et al., 2017; Xu, Wang, et al., 2017). Sucrose is known to be the main sugar that is commonly enzymatically used by LAB in EPS synthesis (Bounaix et al., 2009) and we observed complete consumption of sucrose in SoyD by *Lc*TR, *Lm*WA and *Lm*WN (Fig. 3) indicating EPS production capability. We found that mannitol content follows a similar trend as the total EPS production for each strain (Tables 3 and 5). Mannitol and acetic acid concentrations have been reported to be linked to dextran production (Xu, Coda, et al., 2017; Zhang et al., 2015) and we observed that the concentration of both compounds increased in SoyD treated with the dextran-producing strains. The enzyme dextransucrase hydrolyzes sucrose into glucose and fructose and uses the released glucose in dextran production (Demuth et al., 2002). Accordingly, this results in an increase of fructose concentration in the environment. The excess fructose can then be consumed by LAB and converted into mannitol or fermented into acetic acid (Zhang et al., 2015). Interestingly, we observed a difference of preference in fructose utilization between L. *citreum* and L. *mesenteroides* where L. *citreum* prefers to convert fructose into acetic acid instead of reducing it into mannitol. This is shown by the higher concentrations of mannitol detected in SoyD-*Lm*WA and SoyD-*Lm*WN, while the highest concentration of acetic acid was observed in SoyD-*Lc*TR. This preference was also reported by Kajala et al. (2016) in *Weisella confusa*.

Levan production in fermented SoyD can be described by the consumption of soy oligosaccharides and formation of some novel oligosaccharides. Levan is synthesized by the enzyme levansucrase that removes fructose residues from fructose-containing sugars (Sutherland, 2007). The activity of levansucrase in SoyD-*Lm*WA and SoyD-*Lm*WN is confirmed not only through a decrease in sucrose but also through a decrease in RFOs, namely raffinose, stachyose and verbascose (Table 4).

Levansucrase removes fructose from the RFOs converting them into melibiose, manninotriose and manninotetraose (Xu, Coda, et al., 2017). All these compounds accumulated in fermented SoyD-*Lm*WA and SoyD-*Lm*WN (Table 4).

It is found that EPS as present in the water extract from fermented SoyD had the capability to adhere to ETEC cells and block ETEC K88 adhesion to porcine mucin. This confirmed our previous finding on the adhesion capability of EPS from the isolated strains (Pramudito et al., 2024) and reinforced it by showing the capability to inhibit ETEC adhesion, which is a prerequisite for diarrhea-causing toxin production (González-Ortiz et al., 2014). SoyD fermented with EPS-producing strains (*Lc*TR, *Lm*WA and *Lm*WN) showed an increase in adhesion capability to ETEC K88 but not for SoyD 48 h and SoyD-*Pp*TL. Interestingly, increase in adhesion activity against ETEC H10407 was observed in all for SoyD samples fermented with LAB strains, including SoyD-*Pp*TL. This indicates that ETEC H10407 is more sensitive against fermentation products from SoyD than ETEC K88, regardless of the presence of EPS. Sarabia-Sainz et al. (2013) reported that ETEC H10407 can adhere to galacto-oligosaccharides (GOS), mainly consisting of trimers and tetramers. We found that SoyD-*Pp*TL contained a small amount of manninotriose (Table 4) which can be categorically considered as α-linked-GOS (Panwar et al., 2020). This might contribute to an increase of adhesion bioactivity of extract from SoyD-*Pp*TL against ETEC H10407.

ETEC K88 can bind to porcine mucin through specific interaction between the K88 antigenic receptor in fimbriae with glycoprotein and glycolipid in mucin (Sauvaitre et al., 2022). After adhering to mucin, ETEC K88 produces mucolytic enzymes to degrade the mucin structure, allowing the bacteria to access the epithelial surface which may result in diarrhea (Qadri et al., 2005; Sauvaitre et al., 2022). Therefore, inhibition of adhesion to the mucin layer is a feasible strategy for diarrhea prevention. Three SoyD samples containing bacterial EPS showed a significant reduction of ETEC K88 adhesion to mucin and this reduction was not observed in uninoculated SoyD spiked with commercial dextran or levan (Fig. 4A). On the contrary, ETEC adhesion increased in the presence of these spiked samples. We hypothesize that blocking bioactivity of LAB-fermented SoyD could be due to the combination of unique structural features of EPS produced by the LAB strains and the presence of soy polysaccharide in dough matrix. For example, both commercial dextran and levan have the Mw of <100 kDa while the EPS in fermented SoyD can reach up to 1000 kDa (Fig. 2). Roubos-van den Hil et al. (2010) reported that blocking bioactivity in fermented soybeans was linked to larger polysaccharides with Mw >30 kDa. The high Mw EPS in combination with partially degraded soy polysaccharides could result in more optimal blocking bioactivity.

Broader Mw distribution of EPS could also contribute to blocking bioactivity, such as seen in SoyD-*Lm*WA and SoyD-*Lm*WN (Fig. 5). Cai et al. (2019) found that there is an optimal Mw (around 8 kDa) for blocking bioactivity of EPS where too high Mw can result in a bridging between ETEC and mucin. This could especially be the case when looking at the narrowly high Mw fraction of SoyD-*Lc*TR that increased ETEC adhesion (Fig. 5) indicating that the blocking bioactivity of SoyD extract was resulted from the presence of polydisperse Mw distribution. On the contrary, diverse Mw distribution of EPS, such as seen in SoyD-*Lm*WA and SoyD-*Lm*WN, could provide a shotgun approach that can increase the probability of EPS binding optimally to fimbrial lectin, thus blocking the adhesion capability of ETEC cells to mucin.

5. Conclusion

Three LAB strains (*Lc*TR, *Lm*WA, and *Lm*WN) were found to be promising candidates for application in soy-based fermentation due to their capabilities to produce EPS. The three strains have robust EPS production capability in the sense that they can produce EPS in SoyD substrate with the same yield and composition as when they were grown in liquid medium, disregarding substrate complexity and water availability. Therefore, the strains can potentially be applied in staple soy-based fermented food products such as tempeh especially to add EPS-related functionality. *Lm*WA and *Lm*WN in particular produced EPSs with more diverse range of Mw when grown on SoyD instead of liquid medium. Water extracts from SoyD fermented by the three strains also showed an increase blocking bioactivities against ETEC K88 adhesion to pig mucin compared to unfermented SoyD. The diverse Mw distribution of carbohydrates in the extract due to EPS presence could be the main driver of this bioactivity. This study shows that EPS production by LAB in soy fermentation products could be a viable strategy for the development of functional food product with anti-diarrheal properties.

CRediT authorship contribution statement

Theodorus Eko Pramudito: Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization. **Cynthia Klostermann:** Writing – review & editing, Conceptualization. **Eddy J. Smid:** Writing – review & editing, Supervision. **Henk A. Schols:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

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