



Impact of *in situ* produced exopolysaccharides on rheology and texture of fava bean protein concentrate



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ABSTRACT

The aim of this study was to investigate the impact of *in situ* produced exopolysaccharides (EPS) on the rheological and textural properties of fava bean protein concentrate (FPC). EPS (dextrans) were produced from sucrose by two lactic acid bacteria (LAB). The acidification, rheology, and texture of FPC pastes fermented with *Leuconostoc pseudomesenteroides* DSM 20193 and *Weissella confusa* VTT E-143403 (E3403) were compared. A clear improvement in rheological and textural parameters was observed in sucrose-added pastes after fermentation, especially with *W. confusa* VTT E3403. Only moderate proteolysis of fava bean protein during fermentation was observed. The microstructure of the protein in FPC pastes, as observed by confocal laser scanning microscopy, revealed a less continuous and denser structure in EPS-abundant pastes. The beneficial structure formed during EPS-producing fermentation could not be mimicked by simply mixing FPC, isolated dextran, lactic acid, and acetic acid with water. These results emphasize the benefits of *in situ* produced EPS in connection with the LAB fermentation of legume protein-rich foods. Fermentation with EPS-producing LAB is a cost-effective and clean-labeled technology to obtain tailored textures, and it can further enhance the usability of legumes in novel foods.

1. Introduction

Exopolysaccharides (EPS) are long-chain polysaccharides produced by microorganisms using various sugars as substrates (Galle & Arendt, 2014; Welman & Maddox, 2003). They are often composed of branched and repeating units of sugars or sugar derivatives (Welman & Maddox, 2003). EPS are either associated with cell surfaces, forming capsules, or secreted into the environment, forming slimes (Di Cagno et al., 2006). Depending on their chemical composition, EPS can be classified as hetero-exopolysaccharides, which consist of different types of sugar units (e.g., galactose and rhamnose), or homo-exopolysaccharides, which consist of only one type of sugar unit (e.g., glucose and fructose) (Galle & Arendt, 2014). Glucans (dextran, mutan, alternan, and reuteran) are homo-exopolysaccharides produced by extracellular glucanases, using sucrose as the substrate (Galle & Arendt, 2014). In the food industry, microbial EPS have the potential to replace plant polysaccharides, which are widely used as thickening, stabilizing, and gelling agents (Galle & Arendt, 2014). EPS can be synthesized by some food-grade microbes, e.g., lactic acid bacteria (LAB), propionibacteria,

and bifidobacteria (Di Cagno et al., 2006). EPS produced by LAB have been intensively studied in dairy and cereal foods (Di Cagno et al., 2006; Folkenberg, Dejmek, Skriver, Skov Guldager, & Ipsen, 2006; Hess, Roberts, & Ziegler, 1997; Wolter, Hager, Zannini, Czerny, & Arendt, 2014). Recently, research interest in legume proteins has been increasing due to their high nutritional value and potential in animal protein substitution (Boye, Zare, & Pletch, 2010).

Fava bean (*Vicia faba* L.), which is also known as broad bean, is a traditional legume for human consumption and animal feed in many countries (Duc, 1997). It has been the object of many studies due to its wide cultivation area and adaptability, especially in cold climates (Jiang et al., 2016). The main components in fava bean seeds are protein (ca. 29%) and starch (ca. 39%), with vitamins, minerals, and dietary fiber representing the remainder (Jezierny, Mosenthin, & Bauer, 2010). The good solubility, emulsifying, foaming, and gelling properties of fava bean protein make it a highly interesting raw material for multiple foods (Boye et al., 2010; Cai, Klamczynska, & Baik, 2001). However, despite the increasing global interest in developing sustainable plant protein-based foods, the utilization of fava bean protein in

Abbreviations: FPC, Fava bean protein concentrate; EPS, Exopolysaccharides; LAB, Lactic acid bacteria; FAN, Free amino nitrogen; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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the food industry remains minor (Boye et al., 2010). One reason for this is the non-optimal texture resulting from the addition of a high concentration of fava bean protein to foods (Petitot, Boyer, Minier, & Micard, 2010; Rosa-Sibakov et al., 2016). The EPS produced by LAB have been proven to be good texture modifiers and are used in yogurt, wheat bread, and gluten-free bread (Amatayakul, Halmos, Sherkat, & Shah, 2006; Folkenberg, Dejmeck, Skriver, & Ipsen, 2006; Galle et al., 2012; Katina et al., 2009). The positive effect of EPS on the texture of fava bean doughs was recently revealed (Xu, Coda, et al., 2017; Xu, Wang, et al., 2017). Furthermore, the *in situ* EPS production through LAB fermentation can meet the increasing consumers' demand for the decreased usage of food additives (Asioli et al., 2017).

Two LAB strains, *Leuconostoc pseudomesenteroides* DSM 20193 and *Weissella cibaria* Sj 1b, were identified in our previous study as highly effective EPS producers in fava bean doughs, with good performance in thickening and gel strengthening (Xu, Wang, et al., 2017). The dextrans produced by these two strains were further used to study the interactions between the purified dextran and fava bean protein isolate (Xu et al., 2018). The results suggested different rheological behaviors with different dextrans, indicating the possibility of texture tailoring by using different dextran producers (Xu et al., 2018). As the next step, in this study, we aimed to tailor the structure of a fava bean protein-based food model by using these two strains. Commercially available fava bean protein concentrate (FPC) was chosen as the matrix due to its high protein content (65%). However, *W. cibaria* Sj 1b behaved poorly in terms of thickening and gel strengthening in FPC pastes according to our initial experiment. Therefore, *W. confusa* VTT E-143403, which was recently isolated from fava bean, was selected for this study, along with *Ln. pseudomesenteroides* DSM 20193. The sugar profiles, acidification, rheology, and texture of FPC pastes fermented by these two strains were evaluated and compared. Proteolysis of fava bean protein was evaluated in order to assess its role in the rheology and texture of the FPC pastes. Furthermore, the effects of EPS production on the microstructure of fava bean protein were investigated for the first time, which may provide more information on the function of EPS in protein system. Overall, this study aims to combine LAB fermentation with texture modification in legume protein-rich system by *in situ* production of EPS, which can be applied also in other protein systems.

2. Material and methods

2.1. LAB and raw material

Leuconostoc pseudomesenteroides DSM 20193 (ATCC 12291) was purchased from Leibniz Institute DSMZ (Braunschweig, Germany). *Weissella confusa* VTT E-143403 (E3403) was obtained from the VTT Culture Collection (Espoo, Finland). These two strains were routinely propagated in De Man, Rogosa, and Sharpe (MRS) broth (LAB M Limited, UK) at 30 °C. The FPC was obtained from Vestkorn Milling AS (Norway). The composition of the protein concentrate, as indicated by the manufacturer, included protein (65% ± 2%), starch (7% - 11%), fat (3.5% - 4%), ash (5.5% - 7%), fiber (2.5% - 3%), and moisture (8% - 10%).

2.2. Endogenous enzymatic activity

2.2.1. Proteolytic activity

The proteolytic activity in the FPC was measured using azogelatin as the substrate. One gram of FPC was mixed with 3 ml of 0.1 M sodium acetate buffer (pH 4.5), followed by incubation at 4 °C for 30 min and centrifugation (10,000 g × 10 min). The supernatant was then collected and treated according to a previously reported method (Loponen, Sontag-Strohm, Venäläinen, & Salovaara, 2007). Azogelatin was prepared based on an established method (Jones, Fontanini, Jarvinen, & Pekkarinen, 1998). The proteolytic activity was analyzed in triplicate at three pH values (5.0, 4.5, and 4.0), and the enzyme activity was defined

Table 1

Composition of fava bean protein concentrate (FPC) pastes with different starters.

Sample code ^a	FPC (g)	Sucrose (g)	Water (g)	Starter
20193C	20	0	75	<i>Leuconostoc pseudomesenteroides</i> DSM 20193
20193S	20	5	75	<i>Weissella confusa</i> VTT E-143403
3403C	20	0	75	
3403S	20	5	75	

^a C means control paste; S means sucrose-enriched paste.

according to Loponen et al. (2007).

2.2.2. α -Galactosidase activity

The FPC (1.0 g) was suspended in 5 ml of 0.1 M sodium citrate buffer (pH 5.0). After mixing, the suspension was centrifuged at 4 °C for 10 min (10,000 g). The supernatant was collected and used as the enzyme extract for α -galactosidase activity assay, using p-nitrophenyl galactopyranoside (9.9 mM) as a substrate according to Dey and Pridham (1969). Analysis was performed in triplicate.

2.3. Preparation of the FPC pastes

The FPC pastes were prepared according to Table 1. Sucrose (VWR international, Pennsylvania, USA, analysis grade) was added to two pastes, 20193S and 3403S, in order to enable the formation of EPS. Microbial cells were obtained as described perviously (Xu, Wang, et al., 2017). After mixing with distilled water, all pastes were inoculated with microbial cells at an initial cell density of 6.0 log cfu/g. All fermentations were carried out in triplicate at 30 °C for 24 h.

2.4. Preparation of chemically acidified FPC pastes

To provide a comparison with the fermented pastes (20193C and 3403C), two chemically acidified pastes (20193C* and 3403C*) were prepared. In detail, 10 g of FPC was mixed with 10 g of distilled water. Then, the pH of the paste was adjusted to the same value as the original value in 20193C and 3403C by a mixture of lactic and acetic acid at the same molar ratio as they were in the two fermented pastes. Cycloheximide (Sigma-Aldrich, Missouri, USA) and chloramphenicol (Sigma-Aldrich) were added at a concentration of 0.01% (w/w). The final weight (47.5 g) of the chemically acidified pastes was achieved by adding distilled water. After 24 h of incubation at 30 °C, the samples were stored at -80 °C for 24 h and then freeze-dried (15 °C shelf temperature, -80 °C condenser, and 1.0 mbar chamber pressure) for further analysis. Pastes were prepared in triplicate.

2.5. Preparation of mimicked FPC pastes

In order to mimic the fermented FPC pastes, dextran, FPC, lactic acid, acetic acid, and water were mixed together to prepare mimicked pastes. In detail, the dextrans produced by *Ln. pseudomesenteroides* DSM 20193 and *W. confusa* VTT E3403 were purified on a MRS agar plate according to a previously described method (Maina, Tenkanen, Maaheimo, Juvonen, & Virkki, 2008). Then, a certain amount of the purified dextran powder, the same as in the fermented pastes, was added to distilled water and dispersed overnight. Lactic acid and acetic acid were added based on the original amounts in the fermented pastes. After this, FPC was added according to Table 1. Cycloheximide (Sigma-Aldrich) and chloramphenicol (Sigma-Aldrich) were also added at a concentration of 0.01% (w/w) to inhibit the growth of microorganisms. The final weight (95 g or 100 g) was achieved by adding distilled water. After mixing, the mimicked pastes were incubated at 30 °C for 24 h, followed by rheological and textural analysis. Pastes were prepared in

triplicate.

2.6. LAB cell density, pH, and total titratable acidity (TTA)

The LAB cell densities in the FPC pastes before and after fermentation were analyzed according to the standard plate-count methods, as reported in our previous work (Xu, Wang, et al., 2017). The TTA and pH were measured using a Mettler Toledo EasyPlus Titrator (Schott, Germany) according to a previously described method (Xu, Wang, et al., 2017).

2.7. Analysis of sugars, mannitol, organic acids and dextran

Samples for chemical analysis were freeze-dried before analysis. Then, the sugars, mannitol, and organic acids in FPC pastes were analyzed by high performance liquid chromatography using different columns, eluents, and detectors as described previously (Xu, Wang, et al., 2017).

Dextran was analyzed using an enzyme mixture of dextranase (Sigma-Aldrich) and α -glucosidase (Megazyme, Ireland) according to Katina et al. (2009).

2.8. Assessment of proteolysis

2.8.1. Protein content

Freeze-dried samples (100 mg) were dissolved in 5 ml of 1% (w/v) sodium dodecyl sulfate (SDS). After thoroughly mixing, the insoluble substance was removed by centrifugation ($10,000 g \times 10$ min). The supernatant was then collected and diluted ten-fold with water. After this, 100 μ l of the diluted supernatant was added to 5 ml of Bradford reagent (Bio-Rad Laboratories, USA), and the absorbance at 595 nm was recorded after 5 min with a UV-1800 spectrophotometer (Shimadzu, Japan). Bovine serum albumin (Sigma-Aldrich) was used as the standard for quantification.

2.8.2. Amine content

Amine content was assessed via the specific reaction between o-Phthalaldehyde (OPA) and free primary amino groups in proteins, as described by Spotti et al. (2013). In brief, 10 mg of sample was dissolved in 1.6 ml of 0.1 M sodium tetraborate buffer (pH 9.0) with 200 μ l of 10% SDS and 200 μ l of 2-mercaptoethanol. After mixing, the samples were centrifuged ($10,000 g \times 10$ min). The supernatant (100 μ l) was added to 2 ml of the OPA reagent, which was composed of 80 mg of OPA, 2 ml of absolute ethanol, 5 ml of 10% SDS, 50 ml of 0.1 M sodium tetraborate buffer (pH 9.0), and 100 μ l of 2-mercaptoethanol. The absorbance at 340 nm was measured immediately after the reaction (2 min at 20 °C).

2.8.3. Free amino nitrogen (FAN)

Freeze-dried samples (50 mg) were thoroughly dispersed in 5 ml of distilled water, followed by centrifugation ($10,000 g \times 10$ min). The supernatant was collected, and the FAN content was measured according to Lie (1973).

2.8.4. Protein electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to evaluate the proteolysis of fava bean protein after fermentation according to Laemmli (1970), with some modifications. Freeze-dried samples (10 mg) were dissolved in 1 ml of Laemmli sample buffer (Bio-Rad Laboratories), followed by heating in a boiling water bath for 5 min. The insoluble substance was removed by centrifugation ($10,000 g \times 10$ min). The samples were analyzed on a 12% resolving gel under a constant voltage of 150 V for ca. 50 min, with a loading volume of 10 μ l. Proteins were stained with Coomassie Brilliant Blue solution (0.1%) and destained with a mixture of methanol (20%) and glacial acetic acid (20%).

2.9. Rheological analysis

The shear viscosity was measured under various shear rates, from 2 to 100 1/s (up and down sweeps), by a HAAKE RheoStress rheometer (RS 50, HAAKE Rheometer, Germany). Samples were measured immediately after fermentation at 20 °C. The viscosity values at 100 1/s were taken for sample comparison. The hysteresis loop area between the upward and downward flow curves was calculated using the RheoWin Pro software.

The dynamic moduli (G' , G'') were recorded as a function of frequency from 0.1 to 10 Hz by a HAAKE RheoStress rheometer with a parallel plate system (2 mm gap) at 20 °C. Samples were rested for 5 min before analysis.

2.10. Texture analysis

Texture analysis was performed by a TA.XT 2i texture analyzer (Stable Micro Systems Ltd., England). After fermentation, the FPC pastes (100 g) were carefully poured into acrylic cylindrical containers (60 mm diameter \times 75 mm height) at room temperature and equilibrated for 15 min before texture analysis. A 35 mm diameter solid rod (A/BE35) was used for back extrusion to a sample depth of 30 mm at a speed of 1.0 mm/s downwards and upwards. The trigger force was 4.0 g. From the resulting force-time curves, the values for texture evaluation were obtained using the Exponent software (Stable Micro Systems Ltd.). Four parameters, firmness (peak positive force), consistency (positive area), cohesiveness (peak negative force), and index of viscosity (negative area), were used to evaluate the texture.

2.11. Microstructure

Samples were visualized using confocal laser scanning microscopy (CLSM) equipment consisting of a Zeiss LSM 710 (Zeiss, Jena, Germany) attached to a Zeiss Axio Imager.Z microscope. Protein was stained by adding 10 μ l of 0.2% (w/v) Rhodamine B (Merck, Darmstadt, Germany) to 1 ml of sample, followed by examination on a microscope slide as sealed preparates. A HeNe laser operating at 543 nm was used for the excitation of Rhodamine B, and emissions were collected at 550–650 nm. Images were assembled of the optical sections taken using a 20 \times objective (Zeiss EC Epiplan-Neofluar, numerical aperture of 0.50) to the depth of 14–22 μ m, with a 2.37 μ m z step and a resolution of 1024 \times 1024, using ZEN software (Zeiss). Representative images were selected for publication.

2.12. Statistical analysis

The data in this study were taken from three independent parallels and analyzed by one-way analysis of variance (ANOVA) using Origin 8.6 software (OriginLab Inc., USA). The means were compared using Tukey's test ($P < 0.05$).

3. Results

3.1. LAB growth and acidification

The LAB cell densities in all pastes were above 9.0 log cfu/g after 24 h of fermentation, with little variance (Table 2). The addition of sucrose increased the final cell density, but no significant difference was observed between sucrose-enriched and control pastes.

The initial pH values of the FPC pastes without starters were around 6.5 (Table 2). After fermentation, these values dropped by from 0.6 to 2.1 units, reaching values in the range of 4.4–5.9. With the same starter, sucrose-enriched pastes presented significantly lower pH values as compared with control pastes. The TTA values increased from 2.5 to 15.2 ml after fermentation, with the highest value in 20193S (19.2 ml) and the lowest in 3403C (6.8 ml). Compared with the pastes fermented

Table 2

LAB growth and acidification of FPC pastes with or without sucrose addition. The table shows LAB cell density, cell density increase, pH, pH drop, TTA, TTA increase, and concentrations of organic acids (citric acid, lactic acid, and acetic acid) in FPC pastes.

Sample code ^a	Cell density (log cfu/g)	$\Delta \log$ ^b	pH	ΔpH ^c	TTA (ml)	ΔTTA ^d (ml)	Citric acid (mmol/100 g)	Lactic acid (mmol/100 g)	Acetic acid (mmol/100 g)	FQ ^e
C_0h ^f	—	—	6.5 ± 0.0	—	4.3 ± 0.1	—	8.26 ± 0.16	nd ^g	nd	—
S_0h ^h	—	—	6.5 ± 0.0	—	4.0 ± 0.1	—	6.70 ± 0.14	nd	nd	—
20193C	9.6 ± 0.0 ^A	3.0	4.8 ± 0.0 ^A	1.7	12.9 ± 0.2 ^A	8.6	9.34 ± 0.08 ^A	43.11 ± 2.22 ^A	12.34 ± 0.16 ^A	3.49
20193S	9.7 ± 0.0 ^A	3.2	4.4 ± 0.1 ^B	2.1	19.2 ± 0.1 ^B	15.2	7.28 ± 0.03 ^B	43.37 ± 0.51 ^A	31.92 ± 0.26 ^B	1.36
3403C	9.5 ± 0.2 ^A	3.2	5.9 ± 0.0 ^C	0.6	6.8 ± 0.1 ^C	2.5	nd	25.01 ± 0.40 ^B	22.40 ± 0.09 ^C	1.12
3403S	9.7 ± 0.1 ^A	3.5	5.0 ± 0.0 ^A	1.5	10.9 ± 0.2 ^D	6.9	nd	38.09 ± 1.10 ^C	18.06 ± 0.65 ^D	2.11

^{A-F} Values in the same column with different letters are significantly different ($p < 0.05$).

^a Details about the sample code can be found in Table 1.

^b Cell density increase.

^c pH drop.

^d TTA increase.

^e Fermentation quotient.

^f Control paste without starter before fermentation.

^g Not detected.

^h Sucrose-enriched paste without starter before fermentation.

with *Ln. pseudomesenteroides* DSM 20193, the pastes fermented with *W. confusa* VTT E3403 were significantly less acidic (higher pH and lower TTA).

Citric acid, which has been detected in fava bean flour (Xu, Coda, et al., 2017), was also detected in FPC (Table 2). In pastes fermented with *Ln. pseudomesenteroides* DSM 20193 (20193C and 20193S), the citric acid concentration increased. In contrast, in pastes fermented with *W. confusa* VTT E3403 (3403C and 3403S), citric acid was no longer detected, indicating its involvement in microbial metabolism. The lactic acid concentration ranged from 25.0 to 43.4 mmol/100 g of paste, with the highest concentration in 20193S and the lowest in 3403C. The addition of sucrose showed different effects on the concentrations of lactic and acetic acid in the pastes fermented by the two strains. In 20193S, the added sucrose did not significantly affect the lactic acid concentration, but increased the acetic acid concentration, leading to a lower fermentation quotient (FQ, the molar ratio between lactic acid and acetic acid). However, in 3403S, the added sucrose increased the lactic acid concentration but decreased the acetic acid concentration, resulting in a higher FQ.

3.2. Sugars and sucrose metabolites

3.2.1. Sugars

Endogenous sucrose was detected in unfermented paste, together with stachyose, verbasco, glucose, and galactose (Table 3). No raffinose was detected in any of the samples. After fermentation, sucrose was totally utilized, and no glucose was detected. Galactose, which is a degradation product of stachyose and verbasco by endogenous or

microbial α -galactosidase (Xu, Coda, et al., 2017), was found in all pastes except 20193C. Galactose was used to various extents in all the samples since the detected contents were below the theoretical values released from verbasco. The sugar profile in 20193S was different from that in 20193C, with the detection of galactose, stachyose, and verbasco. In 3403S, the addition of sucrose slightly increased the degradation of stachyose and verbasco, and more galactose was detected.

As a hydrolysis product of sucrose, fructose was found only in sucrose-enriched pastes after fermentation (Table 3). A significantly lower fructose content was found in 20193S (2.66%) as compared with 3403S (8.90%) due to the utilization of the released fructose for mannitol production by *Ln. pseudomesenteroides* DSM 20193. No fructose was detected in control pastes after fermentation by *W. confusa* VTT E3403, indicating the utilization of the liberated fructose for microbial growth.

3.2.2. Dextran and mannitol

Dextran was produced in all control and sucrose-enriched pastes after fermentation, with concentrations varying from 0.3% to 10.0% (Table 3). The addition of sucrose strongly facilitated dextran formation. The highest dextran content was found in 3403S, in which almost all added sucrose was utilized for dextran production, indicating the high dextran-producing ability of *W. confusa* VTT E3403. This ability was also noticeable in the higher dextran content from endogenous sucrose in 3403C compared with that in 20193C.

Mannitol was detected only in pastes fermented with *Ln. pseudomesenteroides* DSM 20193 (Table 3). With the addition of sucrose, more mannitol was produced, reaching the highest content of 6.77%, which

Table 3

Sugars and sucrose metabolites (dextran and mannitol) in FPC pastes before and after fermentation.

Sample code ^a	Sugars (% w/w) ^b						Sucrose metabolites (% w/w) ^c	
	Suc	Glc	Fru	Gal	Sta	Ver	Dextran	Mannitol
C_0h	1.61 ± 0.06	0.20 ± 0.00	nd ^d	0.17 ± 0.01	1.13 ± 0.03	3.31 ± 0.22	—	—
S_0h	22.37 ± 0.21	0.20 ± 0.00	nd	0.17 ± 0.01	1.13 ± 0.03	3.31 ± 0.22	—	—
20193C	nd	nd	nd	nd	nd	nd	0.34 ± 0.01 ^A	0.14 ± 0.01 ^A
20193S	nd	nd	2.66 ± 0.17	0.42 ± 0.02 ^A	1.12 ± 0.05 ^A	1.45 ± 0.18 ^A	6.84 ± 0.41 ^B	6.77 ± 0.19 ^B
3403C	nd	nd	nd	0.21 ± 0.01 ^B	1.50 ± 0.13 ^B	2.25 ± 0.08 ^B	0.72 ± 0.03 ^A	nd
3403S	nd	nd	8.90 ± 0.10	0.42 ± 0.01 ^A	1.37 ± 0.12 ^{A, B}	2.06 ± 0.32 ^{A, B}	10.00 ± 0.26 ^C	nd

^{A-C} Values in the same column with different letters are significantly different ($p < 0.05$).

^a Details about the sample code can be found in Table 1.

^b Sugar content was calculated based on dry weight. Suc, sucrose; Glc, glucose; Fru, fructose; Gal, galactose; Sta, stachyose; Ver, verbasco.

^c Content was calculated based on dry weight.

^d Not detected.

Table 4
Protein, free primary amine, and free amino nitrogen (FAN) content in FPC doughs before and after fermentation.

Sample code ^a	Protein (mg/100 mg total protein)	Amines (mg/100 mg total protein)	FAN (mg/100 mg total protein)
C_0h	63.00 ± 1.22 ^{A, C}	3.57 ± 0.06 ^A	0.15 ± 0.03 ^A
S_0h	65.09 ± 2.41 ^A	3.49 ± 0.07 ^A	0.20 ± 0.02 ^{A, C}
20193C	53.75 ± 1.03 ^B	4.44 ± 0.03 ^B	0.17 ± 0.01 ^{A, C}
20193S	58.78 ± 1.97 ^{B, C}	3.82 ± 0.07 ^{A, C}	0.18 ± 0.00 ^{A, C}
3403C	57.32 ± 1.75 ^B	4.52 ± 0.25 ^B	0.15 ± 0.02 ^A
3403S	64.04 ± 0.97 ^{A, C}	4.09 ± 0.19 ^{B, C}	0.23 ± 0.00 ^{B, C}

^{A-D} Values in the same column with different letters are significantly different ($p < 0.05$).

^a Details about the sample code can be found in Table 1.

is corresponding to the highest acetic acid content in 20193S (Table 2). A low mannitol content, less than the theoretical value, was found in 20193C, indicating the involvement of sucrose-liberated fructose in microbial growth when only endogenous sucrose is available.

3.3. Proteolysis evaluation

3.3.1. Protein content

The protein content of the pastes before and after fermentation were measured to evaluate the proteolysis of fava bean protein. Compared with the protein content in unfermented pastes, a decrease in protein content was observed in fermented pastes (Table 4). Generally, slightly lower protein content was found in pastes fermented with *Ln. pseudomesenteroides* DSM 20193 than in the corresponding pastes fermented with *W. confusa* VTT E3403.

3.3.2. Amines and FAN

As an indication of proteolysis, the amine content was analyzed after fermentation. In all fermented pastes, an increase in free amine content was observed (Table 4), indicating the proteolysis of fava bean protein. The lowest free amine content was found in 20193S, and the highest in 3403C. Although pastes fermented with *Ln. pseudomesenteroides* DSM 20193 showed a higher degree of proteolysis (Table 4), the amine content was slightly lower in these pastes than in pastes fermented with *W. confusa* VTT E3403, without significant difference. This may indicate that *Ln. pseudomesenteroides* DSM 20193 can utilize more small peptides or amino acids for growth.

Since free amino acids were released after proteolysis, FAN content was measured to evaluate the proteolysis in the FPC pastes during LAB fermentation. No significant increases in FAN content were found after fermentation (Table 4), further indicating the utilization of free amino acids by LAB.

3.3.3. SDS-PAGE

SDS-PAGE was performed in order to confirm the proteolysis of fava bean protein that has been observed in our previous chemical analysis of protein, peptides, and amino acids (Table 4). According to Fig. 1, the unfermented sample (lane 1) and the two chemically acidified samples (lanes 2 and 5) showed no differences in protein profile, indicating a weak proteolytic activity in the FPC. This was further confirmed by the protease assay of FPC (0.95 AU, the unit was defined according to Loponen et al. (2007)). In contrast, the protein bands of the fermented samples faded to different extents depending on the starter, which was consistent with the lower protein content in these samples (Table 4). This also agrees with the fact that proteolytic systems are generally active in LAB, playing an important role in making protein and peptide nitrogen available for microbial growth (Law & Kolstad, 1983). The narrower protein bands of 20193C and 20193S may indicate a higher proteolytic activity of *Ln. pseudomesenteroides* DSM 20193 as compared

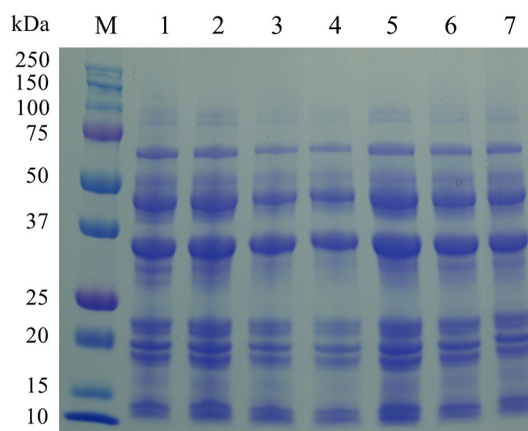


Fig. 1. SDS-PAGE of FPC pastes before and after fermentation. M: protein marker; 1: unfermented control (C_0h); 2: chemically acidified control prepared based on the control fermented with *Ln. pseudomesenteroides* DSM 20193 (20193C*); 3: control fermented with *Ln. pseudomesenteroides* DSM 20193 (20193C); 4: sucrose-enriched paste fermented with *Ln. pseudomesenteroides* DSM 20193 (20193S); 5: chemically acidified control prepared based on the control fermented with *W. confusa* E3403 (3403C*); 6: control fermented with *W. confusa* E3403 (3403C); 7: sucrose-enriched paste fermented with *W. confusa* E3403 (3403S).

to *W. confusa* VTT E3403. However, the influence of pH on proteolytic activity should be also considered, as the proteolytic enzymes in FPC showed a highest activity at a pH of 4.5. No major differences in protein profile were found between pastes fermented by the same starter.

3.4. Rheological analysis

3.4.1. Viscosity and hysteresis loop

The fermented pastes presented a typical shear-thinning behavior (pseudoplasticity) according to the viscosity flow curves (Figs. S1). Significantly higher viscosity values were found in the two sucrose-enriched pastes (20193S and 3403S), although all pastes had a similar starting viscosity (Table 5). Compared with 20193C, 3403C had a higher viscosity value, which was in agreement with its higher dextran content (Table 3). Similarly, the viscosity was higher in 3403S than in 20193S. Although containing the same amounts of dextran, organic acids, and water as the fermented pastes, the mimicked pastes showed significantly lower viscosity values.

Hysteresis loop was frequently observed in viscoelastic materials during the shear rate sweep and was assumed to be an index of structural reversibility (Purwandari, Shah, & Vasiljevic, 2007). In our study,

Table 5

Viscosity, hysteresis loop area, dynamic modulus (G'), and $\tan \delta$ of FPC pastes fermented with *Ln. pseudomesenteroides* DSM 20193 and *W. confusa* VTT E3403 and mimicked FPC pastes.

Sample code	Viscosity (Pa s)	Loop area (10^4 Pa/s)	G' ^b (Pa)	$\tan \delta$
20193C ^a	0.10 ± 0.04 ^{A, C}	0.06 ± 0.02 ^A	–	–
20193C_M ^c	0.03 ± 0.00 ^A	–	–	–
20193S ^a	1.76 ± 0.02 ^B	1.17 ± 0.02 ^B	860.32 ± 33.27 ^A	0.23
20193S_M ^c	0.14 ± 0.00 ^C	0.08 ± 0.00 ^A	–	–
3403C ^a	0.25 ± 0.02 ^D	0.06 ± 0.00 ^A	–	–
3403C_M ^c	0.03 ± 0.00 ^A	–	–	–
3403S ^a	2.27 ± 0.04 ^E	1.17 ± 0.02 ^B	127.80 ± 18.68 ^B	0.39
3403S_M ^c	0.18 ± 0.03 ^{C, D}	0.10 ± 0.02 ^A	–	–

^{A-F} Values in the same column with different letters are significantly different ($p < 0.05$).

^a Details about the sample code can be found in Table 1.

^b G' values were taken at 1.0 Hz.

^c M stands for mimicked FPC pastes.

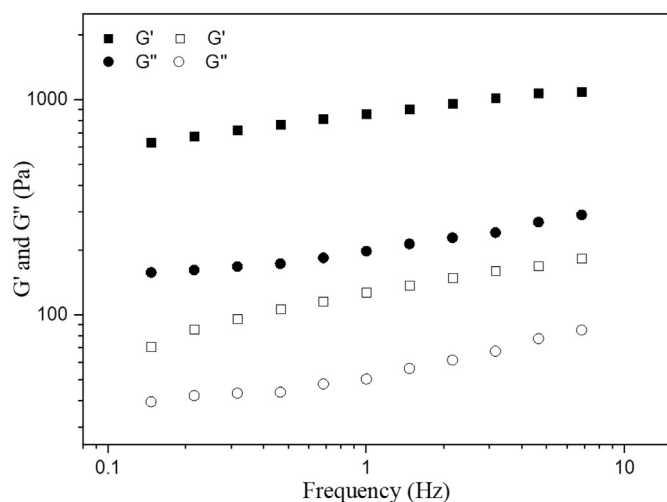


Fig. 2. Frequency sweeps of FPC pastes with *in situ* produced EPS. Squares (G') and circles (G'') in solid and hollow fill stand for pastes fermented with *Ln. pseudomesenteroides* DSM 20193 (20193S) and with *W. confusa* E3403 (3403S), separately.

a significantly higher hysteresis loop area was observed in 20193S and 3403S than in the control pastes due to the structure modification from dextran (Table 5). Among the mimicked pastes, only 20193S_M and 3403S_M formed hysteresis loops, and the loop areas were significantly lower than those of 20193S and 3403S, similar to the phenomenon observed in viscosity results.

3.4.2. Dynamic oscillatory rheology

The storage modulus (G') and loss modulus (G'') of 20193S and 3403S both increased with the rising frequency (Fig. 2). Although containing less dextran than 3403S, 20193S showed higher G' values at any frequency, indicating a more elastic structure in this paste. A similar dependence of G' on frequency was observed in these two pastes, suggesting a similar gel stability, as the dependence of the storage modulus on frequency provides information regarding gel structure (Stading & Hermansson, 1990). Only the G' values of 20193S and 3403S at 1.0 Hz are shown (Table 5) because other fermented pastes presented a liquid-like structure due to the lack of dextran that could interact with proteins and further strengthen the gel structure (Spotti et al., 2013; Spotti, Santiago, Rubiolo, & Carrara, 2012). The value of $\tan \delta$ (G''/G'), which is an index of the relative viscoelasticity, also indicated the more rigid character of the gel formed in 20193S (Table 5).

3.5. Texture analysis

The textural properties of all fermented and mimicked doughs were evaluated in order to study the function of dextran in texture modification. A clear difference in texture profile was observed for the two sucrose-enriched pastes (Fig. 3). When comparing pastes fermented with the same starter, the sucrose-enriched paste had a considerably improved textural parameters due to the modifying effect of EPS. Among all the pastes, 3403S had the highest firmness, consistency, cohesiveness, and index of viscosity (Table 6), corresponding to its highest dextran content (Table 3). No significant differences were found in firmness, consistency, cohesiveness, and index of viscosity between the control pastes. The mimicked pastes, including the pastes with high amounts of dextran (20193S_M and 3403S_M), showed very similar textural properties to the fermented control pastes (Table 6).

3.6. Microstructure

Fava bean protein with or without *in situ* EPS showed different

microstructures, as revealed by CLSM images (Fig. 4). The protein phase formed clusters in the unfermented paste (Fig. 4A), and in fermented pastes, protein aggregates of various sizes appeared. When fermented with the same starter, the protein aggregates in the control pastes were located in a more scattered pattern compared to the corresponding sucrose-enriched pastes. Smaller protein aggregates were observed in pastes fermented with *Ln. pseudomesenteroides* DSM 20193 compared to pastes fermented with *W. confusa* VTT E3403.

4. Discussion

The fermentation of FPC with EPS-positive LAB enabled the texture modification of fava bean protein with *in situ* produced EPS. The inoculated LAB dominated in all samples after 24 h of fermentation, with no obvious promoting effect from sucrose addition, indicating that the FPC is a good medium for the growth of the two strains used. The growth of LAB leads to the production of lactic and acetic acid, lowering the pH and increasing the TTA of the fermented samples. The lower acidity of the pastes fermented with *W. confusa* compared to *Ln. pseudomesenteroides* is consistent with the results reported by us and others when *Weissella* spp. and *Leuconostoc* spp. were used to ferment carrot puree and wheat sourdough (Juvonen et al., 2015; Katina et al., 2009; Xu, Wang, et al., 2017).

Sucrose addition strongly facilitated the production of acetic acid in the paste fermented with *Ln. pseudomesenteroides* DSM 20193, with no effect on acetic acid production in the paste fermented with *W. confusa* VTT E3403. This phenomenon is in agreement with what we observed in our previous study, in which fava bean flour was fermented with both *Leuconostoc* and *Weissella* spp. (Xu, Wang, et al., 2017). The different metabolic pathways of the fructose released from sucrose during dextran synthesis probably explain this difference. It has been reported that *Leuconostoc* spp. are able to reduce the released fructose to mannitol, contributing to acetic acid formation (Wisselink, Weusthuis, Eggink, Hugenholtz, & Grobden, 2002), while *Weissella* spp. typically do not reduce fructose to mannitol, resulting in low acetic acid production (Galle, Schwab, Arendt, & Gänzle, 2010; Kajala et al., 2015). However, the high acetic acid content found in pastes fermented with *W. confusa* may be due to the use of citric acid as an electron acceptor (Corsetti & Settanni, 2007), which was also suggested by the disappearance of citric acid. The high fructose content in 3403S and the absence of mannitol in this sample represent additional evidence for the different metabolic pathways for fructose.

The absence of galactose and stachyose in 20193C may be due to the ability of *Ln. pseudomesenteroides* DSM 20193 to degrade stachyose and ferment galactose (Farrow, Facklam, & Collins, 1989). Because active endogenous α -galactosidase was detected in FPC (data not shown), verbascose could be degraded to stachyose and further used by this strain. The added sucrose strongly decreased the hydrolysis of stachyose and verbascose and the consumption of galactose, probably because sucrose or its hydrolysate was preferable carbon sources for this strain. The higher galactose content in 3403S as compared to 3403C may also indicate the preference of *W. confusa* E3403 in using sucrose or its hydrolysate. In this study, with the endogenous α -galactosidase, *Ln. pseudomesenteroides* DSM 20193 and *W. confusa* VTT E3403 showed different abilities to utilize raffinose family oligosaccharides, which has been observed among different LAB strains (Xu, Wang, et al., 2017).

Sucrose is typically utilized for microbial growth during the exponential phase and then for dextran production during the stationary phase (Han et al., 2014; Plante & Shriver, 1998). Fructose could be released during these two stages and further used for microbial growth or reduced to mannitol by mannitol dehydrogenase in *Leuconostoc* spp. (Wisselink et al., 2002). Theoretically, the sum of fructose and mannitol content should be roughly equal to the dextran content and half of the total initial sucrose content, when assumed that the released fructose was not utilized in microbial growth. However, in this study, the

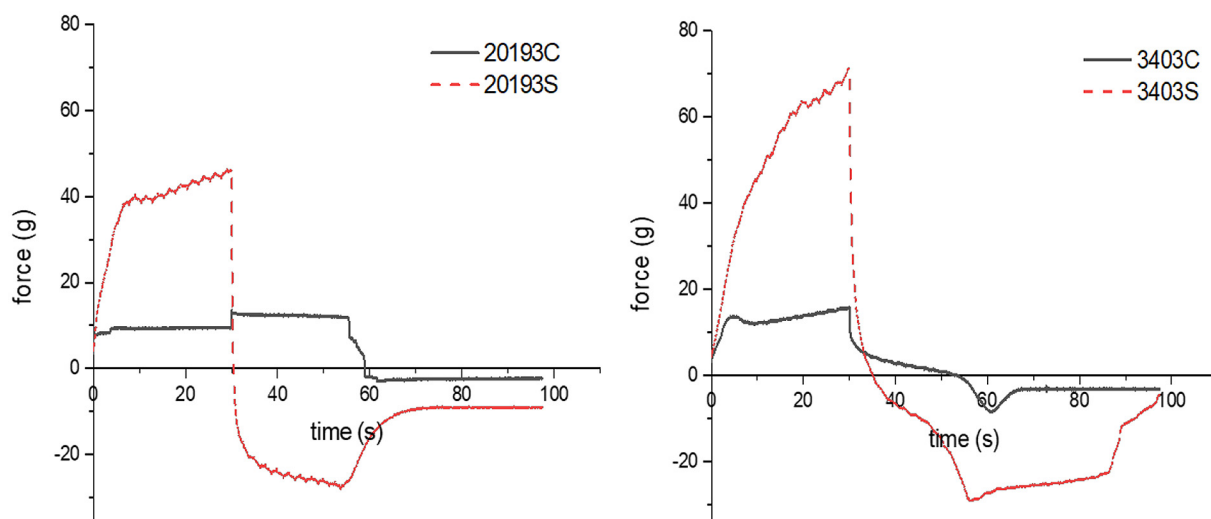


Fig. 3. Back-extrusion test for FPC pastes fermented with *Ln. pseudomesenteroides* DSM 20193 (A) and *W. confusa* E3403 (B). C stands for control paste. S stands for sucrose-enriched paste.

dextran content in the sucrose-enriched pastes was both below the theoretical values due to the usage of glucose for microbial growth, acid production, and gluco-oligosaccharide formation. Similarly, due to the possible involvement of fructose in microbial growth, the total contents of residual fructose and mannitol in the sucrose-enriched pastes were also below the theoretical values for the fructose released from the sum of the original and the added sucrose. This result is consistent with other studies reporting difficulties achieving a complete sugar balance in complex food systems (Juvonen et al., 2015; Xu, Wang, et al., 2017).

The proteolysis in the FPC pastes was weak, considering the contents of protein, free primary amines, and free amino nitrogen (FAN). The SDS-PAGE result also reveals this weak proteolysis, and no significant differences in protein profile were found between the control and the sucrose-enriched pastes. Therefore, fava bean protein was not responsible for the rheological or textural behavior changes observed in the FPC pastes after fermentation.

In this study, the production of EPS played a major role in viscosity increase. The significantly higher hysteresis loop area in the sucrose-enriched pastes was also attributable to the produced EPS. The lower gel stiffness in 3403S may suggest a weak correlation between dextran concentration and gel stiffness, which was also observed in our previous study (Xu, Wang, et al., 2017). The molar mass and spatial conformation of dextran in this sample may differ from those in 20193S, since these molecular properties could also affect gel stiffness (Xu et al., 2018). Considering the viscosity values, hysteresis loop area, and elastic modulus of all the mimicked pastes, we can conclude that the structure formed in the fermented pastes cannot be mimicked by simply mixing purified dextran, FPC, and organic acids together with water. This

strongly reveals the advantage of applying *in situ* synthesized EPS from sucrose in texture modification, which is more efficient and economical, since sucrose is much cheaper than commercial dextran.

The considerably improved texture in sucrose-enriched pastes confirmed the texture modification effect of EPS. It has been reported that EPS produced by LAB interact with proteins in gel structures, resulting in a more stable texture for shear (De Vuyst & Degeest, 1999). According to the dynamic oscillatory analysis, 20193S showed a more rigid character compared with 3403S (Table 5). However, this sample did not show higher firmness or consistency, correspondingly (Table 6). One possible explanation is that textural analysis evaluated the macro-properties, unlike rheological analysis, which evaluated the flow behavior and the response to the deformation in a small sample volume (2 ml). Agreeing with the phenomenon observed in rheological properties, the texture of the fermented pastes cannot be mimicked. This further revealed the speciality of the structure formed in FPC pastes with *in situ* produced EPS through LAB fermentation.

In the present study, we first observed the differences in the rheological and textural properties of FPC pastes with or without sucrose addition after fermentation. In order to understand these differences, the microstructure of fava bean protein in unfermented and fermented pastes was studied, which revealed a clear difference. The difference in protein microstructure between pastes with high or low EPS content was mainly attributable to the effects of EPS, which fill in the protein network pores (Hassan, Frank, & Qvist, 2002; Hassan, Ipsen, Janzen, & Qvist, 2003), altering the protein arrangement. This is also suggested by the denser structure in the samples with high amount of EPS (Fig. 4). The produced EPS interact with fava bean proteins, resulting in a less

Table 6

Texture analysis of FPC pastes fermented with *Ln. pseudomesenteroides* DSM 20193 and *W. confusa* E3403 and mimicked FPC pastes.

Sample code	Firmness (g)	Consistency (g s)	Cohesiveness (g)	Index of viscosity (g s)
20193C ^a	12.18 ± 1.51 ^A	594.09 ± 70.79 ^A	4.77 ± 2.25 ^A	24.27 ± 13.41 ^A
20193C_M ^b	10.66 ± 0.05 ^A	472.30 ± 2.96 ^A	5.84 ± 0.02 ^A	20.88 ± 0.28 ^A
20193S ^a	45.57 ± 0.63 ^B	1131.14 ± 15.16 ^B	27.85 ± 0.19 ^B	766.40 ± 9.10 ^B
20193S_M ^b	10.95 ± 0.08 ^A	467.96 ± 0.97 ^A	6.46 ± 0.01 ^A	31.01 ± 0.98 ^A
3403C ^a	13.16 ± 0.40 ^A	491.57 ± 12.56 ^A	7.08 ± 0.55 ^A	39.32 ± 2.61 ^A
3403C_M ^b	10.77 ± 0.10 ^A	475.07 ± 4.35 ^A	5.70 ± 0.12 ^A	20.15 ± 1.60 ^A
3403S ^a	77.35 ± 5.42 ^C	1734.77 ± 126.93 ^C	34.37 ± 3.79 ^C	1441.36 ± 200.23 ^C
3403S_M ^b	11.31 ± 0.13 ^A	473.77 ± 3.47 ^A	6.60 ± 0.09 ^A	33.17 ± 2.56 ^A

^{A-D} Values in the same column with different letters are significantly different ($p < 0.05$).

^a Details about the sample code can be found in Table 1.

^b M stands for mimicked FPC pastes.

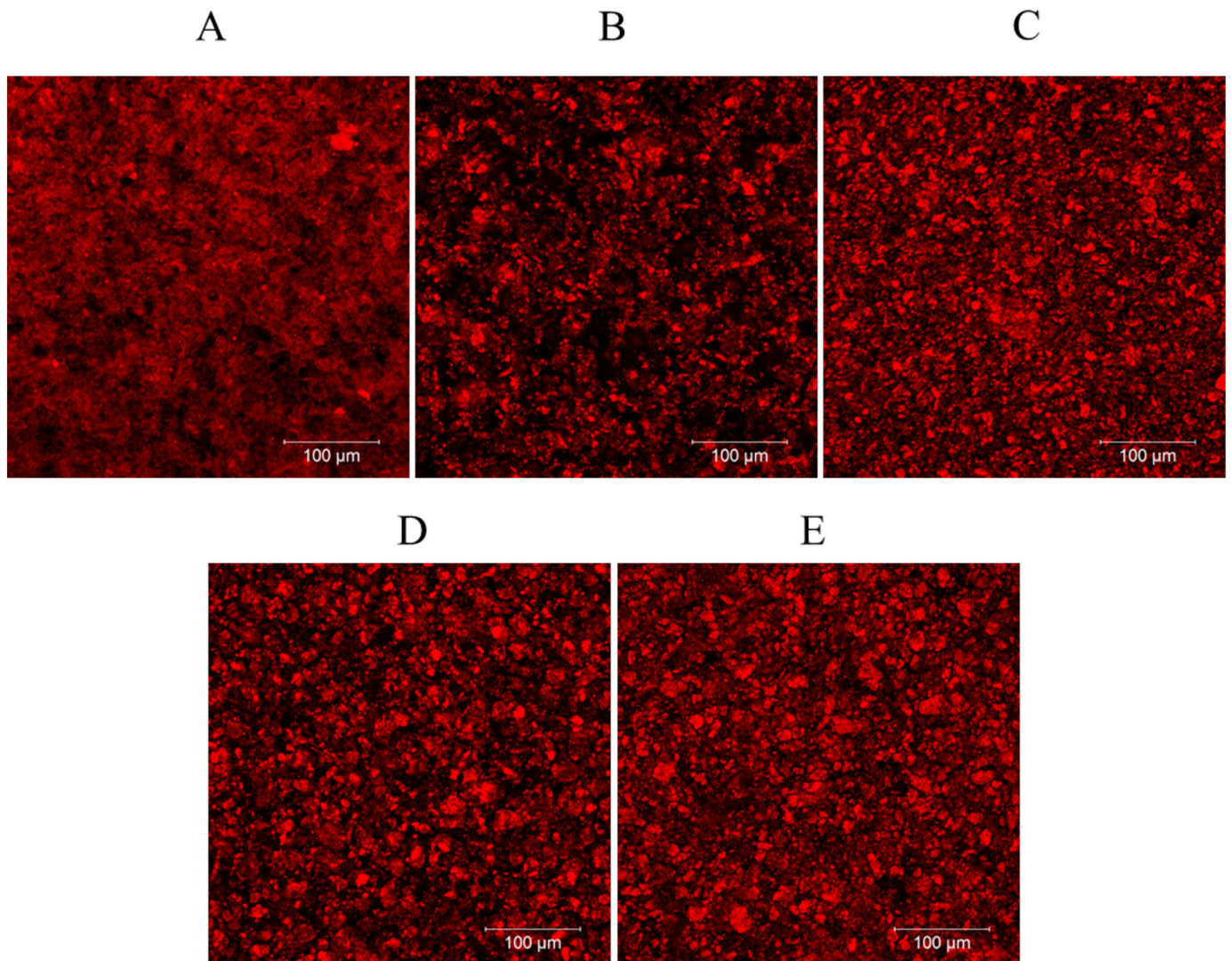


Fig. 4. CLSM images of FPC pastes without fermentation (A), fermented with *Ln. pseudomesenteroides* DSM 20193 without (B) or with (C) sucrose addition, and fermented with *W. confusa* E3403 without (D) or with (E) sucrose addition.

homogenous protein structure as a result of the exclusion effect, further leading to the phase separation that is frequently observed in polysaccharide/protein systems (Doublier, Garnier, Renard, & Sanchez, 2000; Montesinos-Herrero, Cottell, Dolores O'Riordan, & O'Sullivan, 2006; Mounsey & O'Riordan, 2008a, 2008b). This phase separation affects the viscoelastic behavior of the polysaccharide/protein systems, leading to differences in the rheological and textural properties of the systems (Döring, Nuber, Stukenborg, Jekle, & Becker, 2015; Hassan et al., 2003; Sołowiej et al., 2015; Upadhyay, Ghosal, & Mehra, 2012). Furthermore, the lower pH values observed in fermented pastes also affected the protein network, forming protein aggregates (Arogundade, Tshay, Shumey, & Manazie, 2006), as revealed by the particles appeared in the control pastes (Fig. 4B and D). However, the larger protein aggregates found in the pastes fermented with *W. confusa* VTT E3403 were not only due to the low pH, since the pH of the pastes fermented with *Ln. pseudomesenteroides* DSM 20193 were closer to the isoelectric point of fava bean protein (Arogundade et al., 2006). The milder proteolysis observed in the pastes fermented with *W. confusa* VTT E3403 may explain a bit the larger aggregates, and the EPS formed in these samples may affect the protein network differently. In order to confirm this speculation, more work needs to be done on the molecular properties of *in situ* produced EPS and the effect of these properties on EPS-protein interactions. In this study, the differences in rheology and

texture between the EPS-abundant and the EPS-insufficient pastes were also revealed by their microstructures. More work needs to be done on the relationship between protein microstructures and their rheological and textural behaviors.

5. Conclusions

The fermentation of FPC with EPS-producing LAB induces texture modification, and the structure formed in these FPC pastes cannot be mimicked by simply mixing the same amount of EPS, FPC, and organic acids with water. The added sucrose facilitated EPS formation, resulting in improved rheological and textural properties. Proteolysis contributed little to the rheology and texture of all pastes. Fermentation leads to the formation of protein aggregates, and the *in situ* produced EPS changed the arrangement of these aggregates. *W. confusa* VTT E3403 showed a potential application in foods due to its high dextran-producing ability and less acidified nature, which makes it more acceptable to consumers. The texture modification effect of EPS on fava bean protein emphasized the benefits of *in situ* produced EPS in connection with the LAB fermentation of legume protein-rich foods. Fermentation with EPS-producing LAB is cost-effective and clean-labeled and can increase the usability of legume matrixes.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2018.08.054>.

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