Department of Food and Nutrition

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Exopolysaccharides Produced by Lactic Acid Bacteria in Fava Bean Matrix

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Academic Dissertation

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Abstract

Fava bean is a good source of plant protein that is increasingly gaining attention due to its health benefits and sustainability. However, the addition of fava bean flour or protein concentrate at high concentrations to food products may result in an unsatisfying texture. Therefore, texture modification of the fava bean matrix is essential to improve its usability in various food systems. Microbial exopolysaccharides (EPS) are effective texture modifiers, with lactic acid bacteria (LAB) being widely used in the food industry to produce them. In this thesis, EPS were produced *in situ* from sucrose by LAB during the fermentation of fava bean flour or fava bean protein concentrate (FPC), and their texture modification effects on fava bean matrix were evaluated.

Leuconostoc pseudomesenteroides DSM 20193 and *Weissella confusa* VTT E-143403 were found to be good dextran producers in fava bean matrix. With the same starter, sucrose addition strongly increased paste viscosity after fermentation. By separately hydrolyzing the fermented paste with dextranase and levanase, this increase was demonstrated to be primarily driven by dextran. The gel-strengthening ability of EPS was revealed by dynamic oscillatory rheology analysis, with obvious elasticity increases in sucrose-enriched pastes after fermentation. Dextrans produced by *Ln. pseudomesenteroides* DSM 20193 and *W. cibaria* Sj 1b showed considerable gel-strengthening ability.

Two mechanisms of degradation for raffinose family oligosaccharides (RFO) were established in this thesis, involving plant-derived α-galactosidase and microbial levansucrase (LSR). In fava bean flour, RFO were preferentially degraded by endogenous α-galactosidase, producing galactose and sucrose that could be further used for EPS synthesis. In the absence of endogenous α-galactosidase, LSR could act on RFO, forming melibiose, manninotriose, and manninotetraose. A joint function of endogenous α-galactosidase and microbial LSR in RFO degradation was also observed.

Texture evaluation of fermented FPC pastes revealed higher firmness, consistency, cohesiveness, and index of viscosity in sucrose-enriched pastes. Proteolysis of fava bean protein was very weak after fermentation, thus contributing less to texture modification of FPC pastes than did EPS. Confocal laser scanning microscopy of fermented fava bean protein showed more concentrated protein aggregates in dextran-enriched pastes.

The study of fava bean protein-dextran interactions indicates the importance of intermolecular interactions between these two polymers in determining the rheological properties of the system. During acidification, dextran stabilized the network of fava bean protein through intermolecular

interactions. Conjugation of dextran to fava bean protein through the Maillard reaction decreased both viscosity and gel elasticity during protein gelation. The molar mass and conformation of dextrans affected their behavior in the protein system, especially during protein gelation.

This thesis shows for the first time the feasibility of connecting EPS production by LAB to the fermentation of fava bean matrix, and identifies two promising dextran producers for use in this matrix. Analysis of the microstructure of fava bean protein with dextran and fava bean protein–dextran interactions clearly showed the role of dextran in the protein network. Furthermore, the results indicate that different EPS producers may allow texture tailoring of the fava bean matrix, which may contribute to the development of novel plant protein-based food or meat substitutes.

Preface

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Helsinki, September 2018

Yan Xu

List of original publications

This thesis is based on the following papers:

I **Xu, Y.**, Coda, R., Shi, Q., Tuomainen, P., Katina, K., & Tenkanen, M. (2017). Exopolysaccharides production during the fermentation of soybean and fava Bean flours by *Leuconostoc mesenteroides* DSM 20343. *Journal of Agricultural and Food Chemistry*, 65 (13), 2805-2815.

II **Xu, Y.**, Wang, Y., Coda, R., Säde, E., Tuomainen, P., Tenkanen, M., & Katina, K. (2017). *In situ* synthesis of exopolysaccharides by *Leuconostoc* spp. and *Weissella* spp. and their rheological impacts in fava bean flour. *International Journal of Food Microbiology*, 248, 63-71.

III **Xu, Y.**, Coda, R., Holopainen-Mantila, U., Laitila, A., Katina, K., & Tenkanen, M. (2018). Impact of *in situ* produced exopolysaccharides on rheology and texture of fava bean protein concentrate. *Food Research International*, in press. https://doi.org/10.1016/j.foodres.2018.08.054.

IV **Xu, Y.**, Pitkänen, L., Maina, N. H., Coda, R., Katina, K., & Tenkanen, M. (2018). Interactions between fava bean protein and dextrans produced by *Leuconostoc pseudomesenteroides* DSM 20193 and *Weissella cibaria* Sj 1b. *Carbohydrate Polymers*, 190, 315-323.

Contribution of the author to papers I through IV:

I Yan Xu planned the study together with the other authors, and carried out all the experiments. She was responsible for interpreting the results and was the corresponding author for the paper.

II Yan Xu participated in planning the study and conducted most of the experiments, excepting dough fermentation and bacterial counting. She had primary responsibility for interpreting the results and was the corresponding author for the paper.

III Yan Xu planned and performed most of the experiments, excepting the confocal laser scanning microscopy. She was responsible for result interpretation and was the corresponding author.

IV Yan Xu designed the experimental work and discussed the details with the other authors. She performed all experiments and had primary responsibility for result interpretation. She prepared the manuscript and was the corresponding author.

Other related publications by the author:

Coda R., **Xu Y.**, Moreno D.S., Mojzita D., Nionelli L., Rizzello C.G., Katina K. (2018). Performance of *Leuconostoc citreum* FDR241 during wheat flour sourdough type I propagation and transcriptional analysis of exopolysaccharides biosynthesis genes. *Food Microbiology*, 76, 164-172.

Abbreviations

ANF	anti-nutritional factors
ANOVA	analysis of variance
α	slope of the Mark-Houwink plot
CLSM	confocal laser scanning microscopy
c*	critical overlap concentration
cfu	colony forming unit
Da	Daltons
DMSO	dimethyl sulfoxide
DP	degree of polymerization
DSR	dextransucrase
DX	dextran
EPS	exopolysaccharides
FAN	free amino nitrogen
FOS	fructo-oligosaccharides
FPC	fava bean protein concentrate
FPI	fava bean protein isolate
FQ	fermentation quotient
FS	fructansucrases
GOS	gluco-oligosaccharides
GS	glucansucrases
HPAEC-PAD	high-performance anion exchange chromatography with pulse amperometric detection
LAB	lactic acid bacteria
LSR	levansucrase
NMR	nuclear magnetic resonance
PAS	periodic acid-Schiff
RFO	raffinose family oligosaccharides
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	size-exclusion chromatography
TFA	trifluoroacetic acid
TTA	total titratable acidity

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1 Introduction

Exopolysaccharides (EPS) are long-chain polysaccharides synthesized from different sugars by microorganisms (Galle & Arendt, 2014; Welman & Maddox, 2003). They are either associated with the cell surface in the form of capsules or secreted into the environment in the form of slimes (Di Cagno et al., 2006). Positive effects of EPS have been reported on the texture, mouth feel, taste perception, and stability of fermented food (Korakli, Gänzle, & Vogel, 2002; Korakli, Rossmann, Gänzle, & Vogel, 2001; Tieking & Gänzle, 2005). Due to these effects, EPS are regarded as potential alternatives to the plant polysaccharides that have been widely used as thickening, stabilizing, texturizing, and gelling agents in the food industry (Galle & Arendt, 2014).

Some food-grade microorganisms, such as lactic acid bacteria (LAB), propionibacteria, and bifidobacteria, can synthesize EPS (Di Cagno et al., 2006). LAB are preferable EPS producers due to their long history of safe use, and also provide a potential approach for the substitution of hydrocolloid additives (Katina et al., 2009; Wolter, Hager, Zannini, Czerny, & Arendt, 2014).

Fava bean (*Vicia faba* L.) is a traditional legume used for food and animal feed in Europe, Africa, and Asia (Jezierny, Mosenthin, & Bauer, 2010; Li et al., 2009). It can tolerate the cold climate (Murray, Eser, Gusta, & Eteve, 1988) and grow in dryland with a high yield (Loss & Siddique, 1997). Fava bean seeds are rich sources of plant protein, vitamins, minerals, and dietary fiber (Jezierny et al., 2010). The food use potential of fava bean protein has been studied at the laboratory scale, showing good solubilizing, emulsifying, foaming, and gelling properties (Boye, Zare, & Pletch, 2010; Cai, Klamczynska, & Baik, 2001). However, despite these beneficial properties and the increasing demand for plant protein, fava bean still remains underutilized in the food industry.

One reason for this underutilization is that the addition of high quantities of fava bean flour or protein concentrate causes an unsatisfying texture in foods. Another is the anti-nutritional factors (ANF) in fava bean, such as raffinose family oligosaccharides (RFO), tannins, and protease inhibitors (Liener, 1990). These are present in raw seeds, and reduce the digestibility of other nutrients or even lead to pathological reactions (Gupta, 1987). Various processing methods such as soaking, extrusion, heat-treatment, and fermentation have been applied to eliminate the ANF (Alonso, Aguirre, & Marzo, 2000; Coda et al., 2015; Luo & Xie, 2013). In particular, fermentation with LAB has shown high efficiency in reducing the ANF content and in improving the nutritional value of fava bean protein (Coda et al., 2015; Rizzello et al., 2016). Furthermore, the EPS produced *in situ* by LAB during fermentation could also impart beneficial effects to the texture of the fava

bean matrix, while at the same time meeting consumers' increasing demands for the reduced usage of food additives.

In this thesis, the literature review gives an overall outline of EPS, including their classification, synthesis mechanism, structure, producers, rheological properties, and applications. Then, a general introduction on fava bean is provided, including its composition, nutritional value, utilization, and the elimination of ANF. Next, the advantages of combing food fermentation and EPS production by EPS-producing LAB are summarized, followed by the potential applications of this method in different food systems. The experimental part of the thesis mainly describes the preparation of fava bean pastes, the measurement of sucrose metabolites formed during LAB fermentation (e.g. lactic acid, acetic acid, glucan, and mannitol), and the rheological and textural analysis of fava bean pastes. The aim of the thesis was to evaluate the role of EPS in texture modification of fava bean matrix (Studies **I**-**III**), contributing to the development of fava bean protein-based foods and thus widening the applications for EPS and fava bean protein in the food industry. The thesis also attempted to understand the mechanisms underlying the capability of EPS to modify the texture of fava bean matrix by studying interactions between dextran and fava bean protein (Study **IV**).

2 Review of the literature

2.1 Exopolysaccharides (EPS)

2.1.1 Structure, composition, and classification of EPS

EPS are long-chain polysaccharides produced by microorganisms using different sugars as substrates (Galle & Arendt, 2014; Welman & Maddox, 2003). They form either slimes outside the microbial cells or capsules attached to the cells (Di Cagno et al., 2006; Donot, Fontana, Baccou, & Schorr-Galindo, 2012). Many EPS are composed of repeating units of sugars or sugar derivatives, such as glucose, fructose, galactose, and rhamnose (De Vuyst & Degeest, 1999a). EPS are classified according to the chemical composition of the repeating units, as either heteropolysaccharides consisting of two or more sugar units (e.g. gellan and xanthan) or homopolysaccharides composed of only one type of sugar (e.g. glucan and fructan) (Laws, Gu, & Marshall, 2001). The structures of homopolysaccharides vary, and can have either a single backbone or the combination of a backbone and a limited number of branches (Laws et al., 2001). In contrast, heteropolysaccharides are constructed from multiple copies of repeating units, each containing three to seven sugar residues (Laws et al., 2001). Generally, homopolysaccharides are produced at higher concentrations than heteropolysaccharides (De Vuyst & Degeest, 1999b). The focus of this thesis was given to homopolysaccharides.

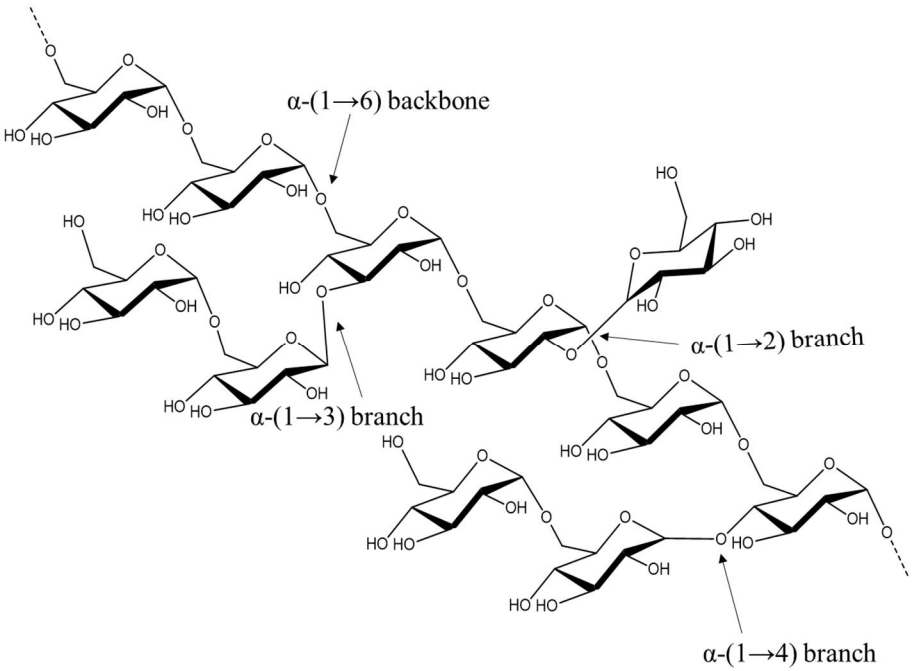

Figure 1. Possible structures for dextran with α-(1→2), α-(1→3), and α-(1→4) linked branches. The three different branches can coexist in one dextran molecule, or the molecule may have only one branch type.

Two groups of homopolysaccharides, α-glucan and β-fructan, have found potential applications in various food products (Di Cagno et al., 2006; Galle, Schwab, Arendt, & Gänzle, 2010; Han et al., 2014; Han, Xu, Gao, Liu, & Wu, 2016). These groups are further subdivided based on the type of glycosidic linkages involved. α-glucan can be divided into dextran, mutan, alternan, and reuteran (Leemhuis et al., 2013). Dextran mainly comprises α-(1→6) linkages and α-(1→2), α-(1→3), and α-(1→4) branches (Figure 1). Mutan contains mainly α-(1→3) linkages, while alternan contains alternating α-(1→6) and α-(1→3) linkages, and reuteran is composed of α-(1→4) linkages with some α-(1→6) branches (Bounaix et al., 2009; Naessens, Cerdobbel, Soetaert, & Vandamme, 2005). Similarly, β-fructan is classified as levan if it consists of β-(2→6) linkages and β-(2→1) branch linkages, or inulin if it consists mainly of β-(2→1) linkages (Figure 2) (Gupta et al., 2011; Zannini, Waters, Coffey, & Arendt, 2016).

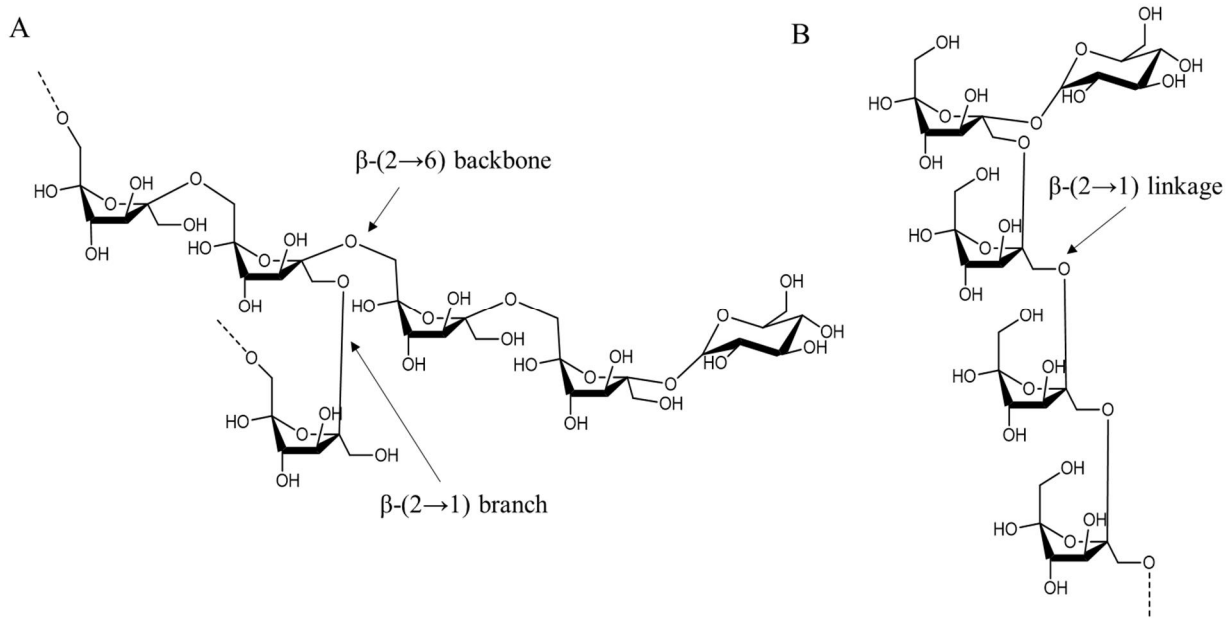

Figure 2. The structure of levan (A) and inulin (B).

2.1.2 Synthesis of EPS

2.1.2.1 Synthesis of dextran

Dextran is composed of glucosyl units with α-(1→6) linkages and α-(1→2), α-(1→3), and α-(1→4) branches (Figure 1). The proportion of α-(1→6) linkages in a dextran molecule can vary from 50% to 100% of the total glycosidic linkages (Amari et al., 2015; Rankin & Jeanes, 1954). It is synthesized by extracellular glucansucrase (GS) enzymes, also called glucosyltransferases. These enzymes belong to the glycoside hydrolase family 70 (GH 70) and are named according to the product they synthesize, e.g. dextransucrase (EC 2.4.1.5) and alternansucrase (EC 2.4.1.140)

(Leemhuis et al., 2013). Dextransucrase (DSR) can catalyze three reactions (Figure 3) depending on the acceptor molecule: (1) hydrolysis of sucrose, when water acts as the acceptor; (2) acceptor reaction, when some sugars such as maltose or isomaltose act as the acceptor, forming gluco-oligosccharides (GOS); (3) polymerization, when the growing dextran chain is the acceptor, producing dextran (Fu & Robyt, 1990; Monchois, Willemot, & Monsan, 1999).

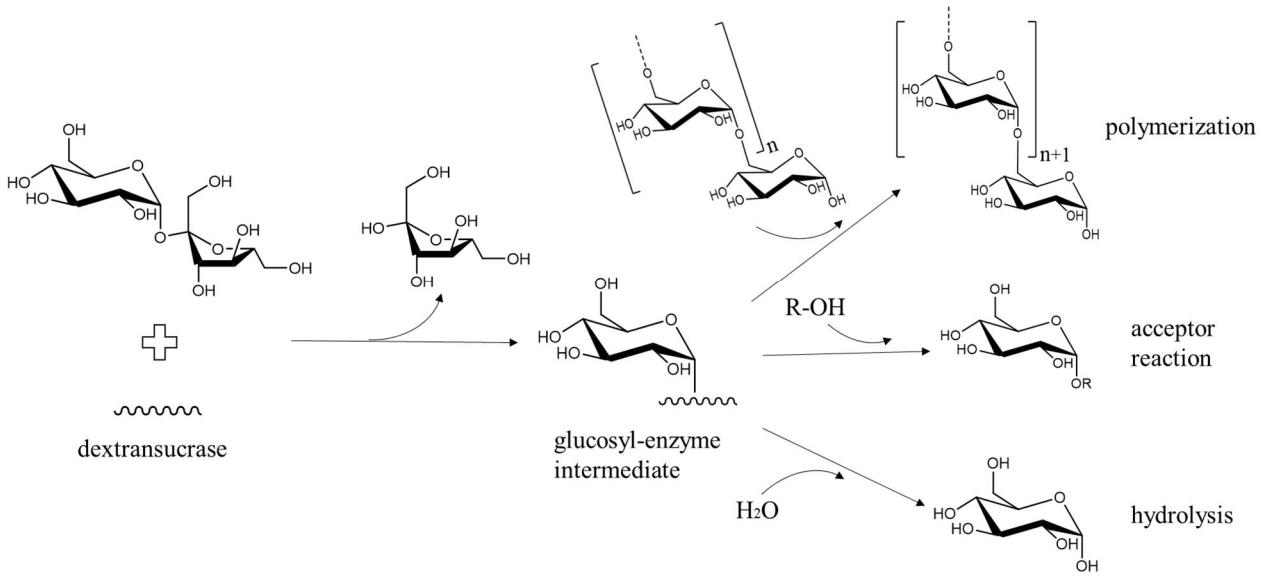

Figure 3. The three different reactions catalyzed by dextransucrase (DSR).

Based on resolved three-dimensional structures, DSRs contain five domains (A, B, C, IV, and V) and are characterized by a U-shaped fold (Brison et al., 2012; Pijning, Vujicic-Zagar, Kralj, Dijkhuizen, & W Dijkstra, 2012; Vujicic-Zagar et al., 2010). In this fold, domain C (residues 1,238–1,376) forms the bottom of the "U", while the other domains are built up from two discontiguous segments of the polypeptide chain, as illustrated by the schematic presentation of GS from *Lactobacillus reuteri* 180 (Figure 4). Generally, domains A, B, and C form the catalytic core of the DSRs; these resemble the domains in enzymes from the GH13 family (Leemhuis et al., 2013). Domain A contains a typical $(\beta/\alpha)_8$-barrel (Figure 4). Domains IV and V are unique in GH70 enzymes, elongating the overall structure (Meng et al., 2015). The function of domain IV is presently unknown. Ito et al. (2011) propose that it provides a hinge, bringing glucans bound on domain V towards or away from the catalytic site. Domain V is part of the glucan binding domain and important for polysaccharide synthesis (Meng et al., 2015).

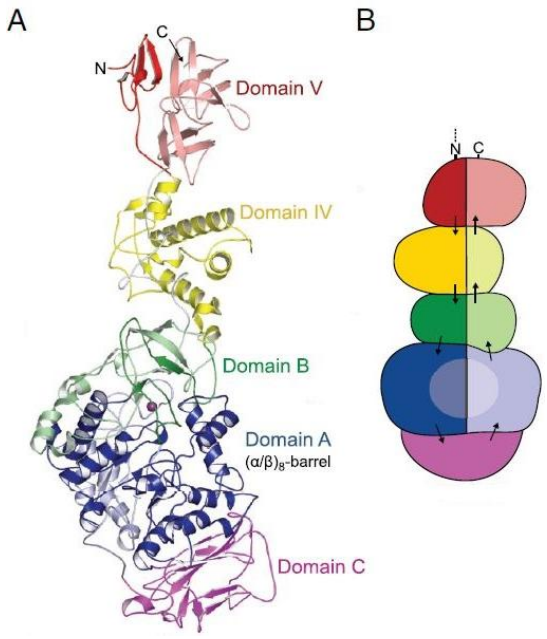

Figure 4. Crystal structure (A) and schematic presentation (B) of a truncated DSR from *Lactobacillus reuteri* 180 (Adapted from Vujicic-Zagar et al., 2010).

The synthesis of dextran starts with the cleavage of sucrose by DSR, followed by the formation of a covalent glucosyl-enzyme intermediate and the release of fructose (Leemhuis et al., 2013; Mooser & Iwaoka, 1989). With the energy obtained from the cleavage of the glycosidic bond, the glucosyl residue is transferred to the acceptor; there are two different theories on how dextran synthesis proceeds (Moulis et al., 2006; Robyt, Yoon, & Mukerjea, 2008). Moulis et al. (2006) proposed that sucrose and glucose produced by hydrolysis act as initiators for polymerization, with the latter being preferred when produced in sufficient quantity. Then, elongation occurs by transferring the glucosyl moiety to the non-reducing end of the initially formed product (Moulis et al., 2006). However, according to Robyt et al. (2008), neither sucrose nor glucose from sucrose can be the initiating primer for dextran synthesis, since sucrose was not found to be present at the reducing end of dextran. They hold that the glucosyl moiety is successively transferred to the reducing end of a growing dextran chain, and the reaction is highly processive with the formation of glucosyl- and dextranyl-covalent enzyme complexes (Robyt et al., 2008). In this model, the elongation of dextran chains involves a two-site insertion mechanism (Robyt & Eklund, 1982; Robyt et al., 2008) and is terminated by the acceptor reaction (Robyt & Eklund, 1982, 1983).

The branching of dextran is primarily accomplished by DSR itself (Kim, Robyt, Lee, Lee, & Kim, 2003). According to Robyt and Taniguchi (1976), dextran is branched through an acceptor reaction, in which a dextran chain binds to the acceptor binding-site of DSR and acts as the acceptor. Then,

the C-3-OH group in the dextran chain makes a nucleophilic attack to C-1 of the glucopyranosyl or dextranyl group that is covalently linked at the active site, releasing the attacked group. Finally, an α-(1→3) linked branch is formed with the D-glucose or dextran chain. Previously, the formation of α-(1→2) branches was attributed to the action of a separate branching enzyme (Sidebotham, 1974). However, a DSR from *Leuconostoc mesenteroides* NRRL B-1299 with two catalytic domains was shown to be sufficient to synthesize α-(1→2) branches by itself (Fabre et al., 2005). Generally, the degree and nature of the branch points depends on the origin of the enzyme (Jeanes et al., 1954), and the degree of branching is dependent on enzyme conformation (Zannini et al., 2016). For instance, Torino, Font de Valdez, and Mozzi (2015) reported alteration of the ratio of α-(1→6) and α-(1→3) linkage by a mutation of the gene encoding for DSR. The possibility of using site-directed mutagenesis of DSR to produce dextrans with different branches has been reported as well (Wang, Zhang, Li, Hu, & Li, 2017).

2.1.2.2 Factors affecting dextran synthesis

Several factors can affect the synthesis of dextran by affecting the activity of DSR, such as pH, temperature, sucrose concentration, the presence of acceptors, and metal ions. For instance, DSR from *Wessella confusa* Cab3 is stable at temperatures ranging from 0 °C to 40 °C, and loses activity at 60 °C (Shukla et al., 2014). The optimum temperature for DSR from *Leuconostoc* spp. is 30 °C, and its optimum pH is from 5.0 to 5.5 (Kaboli & Reilly, 1980; Naessens et al., 2005). In *Weissella* spp., higher DSR activity was found in lower temperature (down to 6 °C) (Hu & Gänzle, 2018). It has been reported that temperature affects branching degree, with higher branched dextran produced at higher temperatures (Kim et al., 2003). High sucrose concentration promotes dextran synthesis (Hehre, 1946; Santos, Teixeira, & Rodrigues, 2000; Tsuchiya et al., 1952); however, too high sucrose concentration may also suppress dextran production (substrate suppression). For example, in liquid medium, dextran concentration starts to decrease when sucrose concentration is above 5% (Shukla & Goyal, 2011). Furthermore, sucrose concentration can affect the molar mass of dextran, with high molar mass dextran (> 10^6 Da) being produced at low sucrose concentrations (Kim et al., 2003). Branching degree is also affected by sucrose concentration, with high branching degree resulting from high sucrose concentration (Kim et al., 2003).

The presence of competing acceptors decreases dextran synthesis due to the formation of GOS (Fu & Robyt, 1990; Pereira, Costa, Rodrigues, & Maugeri, 1998; Robyt & Eklund, 1983; Robyt & Walseth, 1978). Many sugars can act as acceptors, and they have been classified according to their capacities to form GOS (Fu & Robyt, 1990; Pereira et al., 1998; Robyt & Corrigan, 1977; Robyt & Eklund, 1983; Su & Robyt, 1993). Maltose and isomaltose have been demonstrated to be the most

effective acceptors (Robyt & Corrigan, 1977; Robyt & Eklund, 1983). Certain metal ions, such as Co^{2+} and Ca^{2+}, show positive effects on DSR activity (Shukla et al., 2014), and low levels of Ca^{2+} (0.005%) are necessary for optimal activity (Robyt & Walseth, 1979).

2.1.2.3 Synthesis of levan

Levan consists of fructosyl units linked mainly by β-(2→6) linkages (Figure 2A). Fructansucrases (FS), also called fructosyltransferases, are extracellular enzymes responsible for the synthesis of both high molecular fructans and low molecular fructo-oligosaccharides (FOS) (Korakli & Vogel, 2006). FS are divided into two groups according to their synthesis products: levansucrase (EC 2.4.1.10) and inulosucrase (EC 2.4.1.9) (van Hijum, Kralj, Ozimek, Dijkhuizen, & van Geel-Schutten, 2006). Similar to DSR, levansucrase (LSR) can also catalyze three reactions depending on the acceptor molecule: (1) hydrolysis of sucrose, when water is used as the acceptor; (2) acceptor reaction, when some sugars such as sucrose and kestose are used as the acceptor, leading to the formation of FOS; (3) polymerization, when the growing levan chain is used as the acceptor, producing levan (Galle & Arendt, 2014).

The synthesis of levan starts with the cleavage of the substrate sucrose by LSR, releasing glucose. Then, the fructosyl residue is transferred to the initial acceptor, also sucrose, using the energy obtained from cleavage of the glycosidic bond (Tanaka, Oi, & Yamamoto, 1980). Elongation occurs when fructosyl residues are successively transferred to the growing levan chain. That sucrose is used as a donor as well as an acceptor during levan synthesis is confirmed by the appearance of one mole glucosyl unit as the terminal sugar residue in one mole levan molecule (Tanaka et al., 1980). LSR can also use raffinose as the substrate for levan production (Yamamoto, Iizuka, Tanaka, & Yamamoto, 1985). This synthesis process starts with cleavage of the donor, raffinose, forming fructosyl residue and melibiose. The fructosyl residue is then transferred to the acceptor, also raffinose, followed by chain elongation with the growing levan chain as the acceptor. This process is verified by the appearance of a raffinose moiety at the end of levan molecule (Yamamoto et al., 1985). It has also been reported that stachyose can be used as a substrate for levan synthesis (Yamamoto et al., 1985).

2.1.2.4 Factors affecting levan synthesis

Similar to dextran synthesis, many factors can affect levan synthesis, e.g. temperature, substrate concentration, the presence of acceptors, and the concentration of acceptors. In detail, levan is more effectively synthesized at low temperatures than at room temperature or above (Tanaka, Oi, Iizuka, & Yamamoto, 1978). Temperature also affects the molar mass of levan. It has been reported that

high temperature facilitates the formation of levan with high molar mass (6.12×10^5), while low temperature decreases the molar mass (Nakapong, Pichyangkura, Ito, Iizuka, & Pongsawasdi, 2013). The substrate used for levan synthesis may also affect its molar mass, since levan synthesized from raffinose shows higher molar mass than that synthesized from sucrose (Malang, Maina, Schwab, Tenkanen, & Lacroix, 2015). The degree of polymerization (DP) of levan is regulated by the ionic strength of the environment. Under high salt concentration, only levan with low DP is formed (Tanaka, Oi, & Yamamoto, 1979). Levan synthesis can be improved effectively by increasing sucrose concentration (Korakli, Pavlovic, Gänzle, & Vogel, 2003; Tanaka et al., 1979). The presence of certain acceptors (e.g. raffinose, galactose, lactose, and maltose) enable the formation of FOS, decreasing levan concentration (Tieking, Ehrmann, Vogel, & Gänzle, 2005; Tieking et al., 2005; Tieking, Kühnl, & Gänzle, 2005).

2.1.3 Microorganism synthesizing EPS

EPS are produced by microorganisms under certain conditions, and their biological role is complex and still unclear. Generally, EPS are thought to play a role in cell recognition (De Vuyst & Degeest, 1999b; Looijesteijn, Trapet, de Vries, Abee, & Hugenholtz, 2001), biofilm formation (De Vuyst, De Vin, Vaningelgem, & Degeest, 2001; Dertli, Mayer, & Narbad, 2015; Leathers & Bischoff, 2011), and cell adhesion (Dertli et al., 2015). Another reported function of EPS is in protecting microbial cells from environmental stresses, e.g. osmotic stress, pH, bacteriophages, metal ions, nisin, and antibiotics (Flemming & Wingender, 2010; Ruas-Madiedo, Hugenholtz, & Zoon, 2002). The microbial producers of homopolysaccharides are bacteria, yeast, and fungi.

2.1.3.1 Dextran producers

Dextran is only produced by lactic acid bacteria (LAB), as the enzyme responsible for dextran synthesis (DSR) is found only in LAB (Galle & Arendt, 2014). Synthesis of dextran is mainly observed in four genera: *Leuconostoc*, *Streptococcus*, *Weissella*, and *Lactobacillus* (Galle et al., 2010; Korakli & Vogel, 2006; Kralj et al., 2004; Ruas-Madiedo et al., 2002; Shukla et al., 2014). *Leuconostoc mesenteroides* NRRL B512F is the strain used for commercial dextran production at the industrial scale (Leathers & Bischoff, 2011), and it produces water soluble dextran composed of 95% α-(1→6) linkages and 5% α-(1→3) branch linkages (Heinze, Liebert, Heublein, & Hornig, 2006; Van Cleve, Schaefer, & Rist, 1956). Some researchers have reported a lower branching degree (4.1%) for dextran produced by this strain (Maina, Tenkanen, Maaheimo, Juvonen, & Virkki, 2008). The average molar mass of dextran produced by *Ln. mesenteroides* NRRL B512F varies from 6.2×10^6 Da to 7.1×10^6 Da (Ruas-Madiedo et al., 2002). Depending on the producer and the

culture conditions, the structure and the molar mass of dextrans can vary considerably. Some representative dextran-producers and the dextrans they produced are summarized in Table 1.

Certain bacteria have shown the ability to produce dextran with various structures, which is attributable to the excretion of different DSRs by the bacteria (Côté & Robyt, 1982; Figures & Edwards, 1981; Zahnley & Smith, 1995). One bacterium can possess more than one DSR; for example, two or more DSRs are found in *Ln. mesenteroides* NRRL B-1299 and *Ln. citreum* FDR241 (Coda et al., 2018; Monchois, Remaud-Simeon, Monsan, & Willemot, 1998; Monchois, Willemot, Remaud-Simeon, Croux, & Monsan, 1996). *Ln. mesenteroides* ATCC 8293 (also known as NRRL B-1118) harbors two DSRs that are separately responsible for the synthesis of soluble and insoluble glucans (Côté & Skory, 2012). Insoluble glucan contains a much higher percentage (50%) of α-(1→3) linked branches compared to soluble glucan (Côté & Skory, 2012). The synthesis of insoluble glucan involves two or more DSRs and uses soluble glucan as the acceptor in the acceptor reaction (Côté & Skory, 2015).

2.1.3.2 Levan producers

Unlike dextran, levan can be produced by both plants and microorganisms (Zannini et al., 2016). Some grasses, e.g. *Agropyron cristatum*, *Dactylis glomerata*, and *Poa secunda*, produce levan as a carbohydrate storehouse, which is usually present in stems and leaf sheaths (Srikanth, Reddy, Siddartha, Ramaiah, & Uppuluri, 2015). In comparison to plant-derived levan, microbial levan is more advantageous, economical, and industrially feasible with various applications (Srikanth et al., 2015). Microbial levan is produced by yeast, fungi, and bacteria (Franken, Brandt, Tai, & Bauer, 2013; Jang et al., 2003; Silbir, Dagbagli, Yegin, Baysal, & Goksungur, 2014). Five different genera of LAB are known to synthesize levan: *Bacillus*, *Leuconostoc*, *Lactobacillus*, *Streptococcus*, and *Weissella* (Ebisu, Kato, Kotani, & Misaki, 1975; Han et al., 2016; Malang et al., 2015; van Hijum, Bonting, van der Maarel, & Dijkhuizen, 2001; Zhang et al., 2014). As with dextran, the molar mass of levan produced by different LAB can vary. For instance, levan produced by *Streptococcus mutans* possesses a molar mass above 2.5×10^7 Da, while levan produced by *Lactobacillus reuteri* shows a molar mass range from 2×10^4 Da to 4×10^6 Da (Carlsson, 1970; van Hijum, Szalowska, van der Maarel, & Dijkhuizen, 2004). Table 1 summarizes some representative levan-producers and the reported structure and molar mass of levans they produce.

Table 1. The structure and molar mass of dextran and levan produced by different LAB.

EPS	Producer	Structure	Molar mass [a] (Da)	Reference
Dextran	*Leuconostoc citreum* E497	85.5% α-(1→6) 11% α-(1→2) 3.5% α-(1→3)	1.85×10^6	Maina et al. (2014); Maina et al. (2008)
	Weissella confusa Cab3	97% α-(1→6) 3% α-(1→3)	1.80×10^7	Shukla et al. (2014)
	Lactobacillus fermentum Kg3	89% α-(1→6) 11% α-(1→3)	–- [b]	Kralj et al. (2004)
	Streptococcus downei Mfe28	90% α-(1→6) 10% α-(1→3)	–-	Gilmore, Russell, and Ferretti (1990)
Levan	*Bacillus methylotrophicus* SK 21.002	100% β-(2→6)	$(4\text{-}5) \times 10^3$	Zhang et al. (2014)
	Leuconostoc citreum BD1707	mainly β-(2→6)	4.3×10^6	Han et al. (2016)
	Lactobacillus reuteri 121	98% β-(2→6) 2% β-(2→1→6)	2×10^4 – $(3\text{-}4) \times 10^6$	van Hijum et al. (2004)
	Streptococcus salivarius HHT	mainly β-(2→6)	–-	Ebisu et al. (1975)
	Weissella confusa F3/2-2	mainly β-(2→6)	2×10^5	Malang et al. (2015)

[a] Molar mass is dependent on strains, culture conditions, and analysis method and valid only under a certain condition. [b] Not reported.

2.1.3.3 LAB synthesizing both dextran and levan

Some LAB strains harbor both DSR and LSR, e.g. *Ln. mesenteroides* ATCC 8293, which has multiple DSR and LSR genes present in its genome (Olvera, Centeno-Leija, & Lopez-Munguia, 2007). Out of all EPS producers, strains belonging to *Streptococcus*, *Leuconostoc*, and *Weissella* genera are reported to produce both dextran and levan (Malang et al., 2015; Mukasa & Slade, 1973; Olvera et al., 2007). Strains from *Streptococcus* spp. have garnered particular attention due to their significance in dental caries, in which both dextran and levan are involved (Gibbons & Banghart, 1967; Hamada & Slade, 1980; Newbrun, 1972). DSRs from *Leuconostoc* spp. have been studied widely due to their potential in commercial dextran production (Morales-Arrieta, Rodríguez, Segovia, López-Munguía, & Olvera-Carranza, 2006). The industrial strain *Ln. mesenteroides* NRRL B512F was found to have LSR activity by Robyt and Walseth (1979). The gene encoding LSR in this strain was isolated, sequenced, and cloned in *Escherichia coli*, and the properties of the

recombinant enzyme were studied (Morales-Arrieta et al., 2006). Strains with both DSR and LSR may produce different products from different substrates. For instance, in the presence of sucrose, both dextran and levan can be produced, while in the presence of raffinose, only levan will be produced.

2.1.4 Rheological properties of EPS solutions

2.1.4.1 Dextran solution

Commercial dextran powder is generally off-white and amorphous in aqueous solution (Heinze et al., 2006). The solubility of dextran in water is dependent on its structure, its molar mass, and the temperature (Côté & Skory, 2012; Heinze et al., 2006). Electron microscopy shows a spherical and compact structure for dextran molecules in aqueous solution (Ingelman & Siegbahn, 1944). Similar to other polysaccharides, the concentration of dextran considerably affects its rheological behavior. According to Arvidson, Rinehart, and Gadala-Maria (2006), dilute solution behavior occurs when the concentration is low enough that the polysaccharide molecules are separated from each other. Concentrated solution behavior occurs when the molecules interact physically with one another, while semi-dilute solution behavior occurs when the molecules are indirectly affected by the presence of other molecules in the solution. The relationship between concentration and specific viscosity has been studied for many polymer–solvent systems in order to determine the concentrations at which the solutions transit between the dilute, semi-dilute, and concentrated regimes (Arvidson et al., 2006). Most disordered or 'random coil' polysaccharides, e.g. dextran, exhibit two distinct linear regions with an abrupt change from one to the other (Morris, Cutler, Ross-Murphy, Rees, & Price, 1981). The concentration at which that abrupt change occurs is called the critical concentration (c*). Three distinct linear regions have been also reported for dextran solutions (McCurdy, Goff, Stanley, & Stone, 1994; Pinder, Swanson, Hebraud, & Hemar, 2006). The rheological properties of dextran solutions are largely dependent on molar mass, and lower c* values are normally found in dextran solutions with higher molar mass (Pinder et al., 2006). McCurdy et al. (1994) reported that for dextran with an average molar mass of 5×10^5 Da, Newtonian behavior is observed at concentrations below 30%. For dextran with high molar mass, slight pseudoplasticity is already observed at concentrations above 1.5%.

The dynamic rheology of dextran solutions has received considerable study, with the common conclusion that dextran cannot form gels in aqueous solutions due to its flexible structure (McCurdy et al., 1994; Pinder et al., 2006). No entanglement process is observed in a dextran-water system due to hindrance caused by the branching characteristic of dextran (McCurdy et al., 1994).

Additionally, frequency affects the viscoelasticity of dextran solutions; at high frequency, a transition from viscous regime to elastic regime is observed (McCurdy et al., 1994; Pinder et al., 2006).

2.1.4.2 Levan solution

Levan is a white crystalline powder (Gupta et al., 2011). Temperature considerably affects the solubility of levan in water solution, with better solubility at higher temperatures (Gupta et al., 2011). The intrinsic viscosities for levan in water range from 0.07 to 0.18 dl/g for the molar mass range of 16-24 million Da (Ehrlich et al., 1975; Newbrun & Baker, 1968; Salvatore S. Stivala & Bahary, 1978), and are much lower than those of typical commercial polysaccharides (cellulose and xanthan). Similar to dextran, aqueous levan solutions also show typical 'random coil' polysaccharide behavior, encompassing dilute, semi-dilute, and concentrated regimes (Arvidson et al., 2006). Levan molecules in aqueous solution do not undergo gelation with increasing concentration and possess a compact, globular structure as confirmed by electron microscopy, light scattering and sedimentation, small-angle X-ray scattering, and viscometry (Arvidson et al., 2006; Bahary, Stivala, Newbrun, & Ehrlich, 1975; Ingelman & Siegbahn, 1944; Khorramian & Stivala, 1982; Stivala, Bahary, Long, Ehrlich, & Newbrun, 1975).

2.1.5 Applications of EPS

2.1.5.1 Applications of dextran

Commercially produced dextran is used in its native or chemically modified form for various purposes. Clinical grade dextran with molar mass of 40000, 60000, or 70000 Da in 6% or 10% aqueous solution is used for the replacement of moderate blood loss (Heinze et al., 2006; Leathers, 2005). Dextran essentially substitutes blood proteins, providing colloid osmotic pressure to pull fluid from the interstitial space into the plasma (Heinze et al., 2006). Due to its low antigenicity and high water solubility, dextran produced by *Ln. mesenteroides* NRRL B512F is a preferred material for clinical use (Heinze et al., 2006). Dextrans with different molar masses are used as standards for molar mass determination in size-exclusion chromatography (Heinze et al., 2006; Kobayashi, Schwartz, & Lineback, 1985). Generally, the most widely used dextran derivatives are obtained under alkaline conditions in the presence of epichlorohydrin to form cross-linked chains (Naessens et al., 2005). Commercial cross-linked dextran is in the form of an insoluble gel, and can be used as a filler in chromatography columns (Leathers, 2005; Naessens et al., 2005). In nanotechnology, dextran sulphate has been used as a stabilizer in the preparation of polyalkyl cyanoacrylate

nanoparticles, which slow down the release rates of drugs (Flexner et al., 1991; Hosoya, Balzarini, Shigeta, & De Clercq, 1991).

Dextran with α-(1→2) linked branches were reported to possess prebiotic properties, and these properties are affected by its molar mass, rather than its structure (Sarbini, Kolida, Deaville, Gibson, & Rastall, 2014; Sarbini et al., 2011). The food use of dextran has been approved in Europe since 2001 (European Commission, 2001). Dextran is incorporated in baked products due to its beneficial emulsifying, moistening, and texture-modifying properties (Heinze et al., 2006; Katina et al., 2009; Korakli et al., 2001; Tieking, Korakli, Ehrmann, Gänzle, & Vogel, 2003). Dextran is also useful in dairy products (Duboc & Mollet, 2001). For instance, McCurdy et al. (1994) reported that frozen dairy products with native dextran added exhibited advanced heat-shock stability and increased melting temperature. The applications of dextran in different industries are summarized in Table 2.

Table 2. Applications of dextran and levan in different industries.

EPS	Application field	Function	Reference
Dextran	Pharmacy	Plasma expander	Heinze et al. (2006); Leathers (2005)
	Chemical industry	Standard for molar mass determination, filler in chromatography columns, and stabilizer in nanoparticles	Flexner et al. (1991); Hosoya et al. (1991); Kobayashi et al. (1985); Leathers (2005); Naessens et al. (2005)
	Food industry	Emulsifier, moistener, stabilizer, texture modifier, and prebiotics	Duboc and Mollet (2001); Heinze et al. (2006); Katina et al. (2009); Korakli et al. (2001); Lynch, Coffey, and Arendt (2018); McCurdy et al. (1994); Tieking et al. (2003)
Levan	Pharmacy	Excipient, emulsifier, encapsulator, and color and flavor enhancer	Han (1990); Srikanth et al. (2015)
	Chemical industry	Adhesive and ingredient in purifying systems and nano film preparation	Hyun Chung, Kyung Kim, Song, Kim, and Rhee (1997); Srikanth et al. (2015)
	Food industry	Stabilizer, emulsifier, flavor enhancer, and prebiotics	Duboc and Mollet (2001); Korakli et al. (2003)

2.1.5.2 Applications of levan

Levan also has various applications in the pharmaceutical, chemistry, and food industry (Table 2). In the chemical industry, levan is used to purify biological materials such as bovine serum albumin,

horse heart myoglobin, and egg albumin through a PEG/Levan two-phase liquid system (Hyun Chung et al., 1997). Levan is involved in the preparation of water-based and cross-linked adhesives, and two forms of levan adhesives are commercially available through Monatana Biotech SE Inc. (Srikanth et al., 2015). Levan derivatives with cationic substituents have been used in home care, fabric applications, hair care products, and oil fields (Srikanth et al., 2015). Furthermore, levan derivatives also find applications in nanofilms. Sima et al. (2011) reported that both pure and oxidized levan can be used in the synthesis of nanostructured thin films, which are widely used as coating materials to reduce the risk of poor adhesion and cracking. Bondarenko et al. (2016) reported the utilization of levan as a coating material for microelement-nanoparticles. In the pharmaceutical industry, levan is an important excipient due to its stability and film-forming properties (Srikanth et al., 2015). It also acts as an emulsifying and encapsulating agent. In the manufacturing of tablets and capsules, levan can be used as a color and flavor enhancer (Han, 1990). It has been reported that levan has positive effects on skin and cell proliferation (Rairakhwada et al., 2007). Levan also possesses several other beneficial biological properties, such as infection prevention and tumor inhibition (Srikanth et al., 2015).

In the food industry, levan is regarded as a potential and functional biopolymer due to its high molar mass and low-viscous nature (Srikanth et al., 2015). Korakli et al. (2003) reported that levan produced by *Lactobacillus sanfranciscensis* LTH 2590 showed prebiotic effects, since it can be converted to small FOS that are considered to be prebiotics (Bello, Walter, Hertel, & Hammes, 2001; Huang, Lee, Ho, Lin, & Pan, 2013). As a prebiotic, levan can significantly modulate the microbiota in the colon by stimulating the growth of endogenous *Bifidobacteria* (Srikanth et al., 2015). Enzymatically or chemically hydrolyzed levan may be used as a sweetener or dietary fiber in beverages (Bello et al., 2001). Levan can also be used in dairy products as a stabilizer, emulsifier, or flavor enhancer (Duboc & Mollet, 2001).

2.2 Fava bean

2.2.1 Composition and nutritional value of fava bean

Fava bean (*Vicia faba* L.), also called broad bean or horse bean, is a legume grown world-wide for food and animal feed in many countries (Duc, 1997). It can tolerate harsh growing conditions e.g. cold climate (Murray et al., 1988) and can produce impressive seed yield in dryland (Loss & Siddique, 1997). Additionally, fava bean is considered to be a sustainable crop due to its ability to contribute nitrogen to the cropping system through biological N_2 fixation, reducing the usage of fertilizer (Jensen, Peoples, & Hauggaard-Nielsen, 2010). The nutritional composition of fava bean

seeds varies depending on origin, cultivar, and growing conditions. In general, fava bean seeds contain about 29% protein and 39% starch, with the remainder comprising vitamins, minerals, and dietary fiber (Jezierny et al., 2010). Some non-nutrient secondary metabolites are also found in the seeds, such as phenols and flavonoids, which have antioxidant activities and show positive effects on human health (Luo et al., 2014). Moreover, fava bean consumption plays a positive role in decreasing LDL-cholesterol levels in plasma and in improving motor symptoms of patients with Parkinson's disease (Frühbeck, Monreal, & Santidrián, 1997; Ramírez-Moreno, Salguero Bodes, Romaskevych, & Duran-Herrera, 2015).

2.2.2 Utilization of fava bean in the food industry

Fava bean is an important source of protein in developing countries, and is mainly used as feed for pigs, horses, and poultry in industrialized countries (Arogundade, Tshay, Shumey, & Manazie, 2006). Recently, the consumption of plant protein has increasingly drawn attention globally due to the various associated health benefits and sustainability, since the production of meat proteins is energy-intensive and unsustainable (Kumar et al., 2017; Orlich et al., 2013). Fava bean is a sustainable source of plant protein and possesses high protein content. In China, fava bean seeds are widely consumed as snacks and dishes. Many attempts have been made to explore the novel usage of fava bean in different foods. For instance, Gularte, Gómez, and Rosell (2012) incorporated fava bean flour in a gluten-free layer cake, with the final product showing increased batter viscosity, higher volume, and lower starch digesting rate. Smith and Hardacre (2011) developed an extruded snack using fava bean flour, which shows high consumer acceptance compared to extruded commercial corn snacks. Partial substitution of cereals with fava bean flour enables the complementation of limiting amino acids in either grain, improving the nutritional quality of cereal-based products (Giménez et al., 2012).

Efforts have been made in the utilization of fermented fava bean flour. Rosa-Sibakov et al. (2016) prepared pasta separately from fava bean flour, the starch-rich fraction of fava bean flour, and fava bean flour fermented with LAB. The pasta made with fava bean flour showed a texture comparable to semolina pasta. Similarly, Rizzello et al. (2017) used fermented fava bean flour to partially replace semolina in pasta, with the final product showing an enhanced nutritional profile with 30% fava bean flour. Fermented fava bean flour has also been reported to be used in wheat bread, which showed high protein content and high protein digestibility (Coda, Varis, Verni, Rizzello, & Katina, 2017). Additionally, there is a potential application for fava bean in meat substitution, where soybean and gluten are commonly used (Kumar et al., 2017; Multari, Stewart, & Russell, 2015).

However, despite these potential applications of fava bean and the overall global interest in developing plant protein-based foods, fava bean is still underutilized, especially in developed countries where meat is the major source of protein in the diet. One reason for this underutilization is the unsatisfying texture caused by adding fava bean flour or protein concentrate in foods at high concentrations. Another reason is the ANF present in the seeds, which exert anti-nutritional functions, reducing the digestibility of other nutrients or even leading to some pathological reactions (Gupta, 1987).

2.2.3 Elimination of anti-nutritional factors in fava bean

Raw fava bean seeds contain ANF, such as RFO, tannins, protease inhibitors, vicine, and convicine (Liener, 1990; Multari et al., 2015). Different processing methods have been applied to eliminate these ANF, such as soaking, heating, germination, extrusion, and fermentation (Alonso et al., 2000; Coda et al., 2015; Luo & Xie, 2013). Of these, fermentation with LAB has been proven to effectively reduce vicine, convicine, trypsin inhibitor activity, and total condensed tannins (Coda et al., 2015). Due to a long history of safe use and the generally regarded as safe status (Lindgren & Dobrogosz, 1990), LAB are suitable for eliminating ANF in fava bean.

2.3 Combination of EPS production and fava bean fermentation by EPS-producing LAB

2.3.1 Advantages of using LAB for food fermentation

LAB are commonly used in the food industry for the production of fermented food, e.g. sourdoughs and dairy products. They considerably improve the microbial safety of fermented foods by producing lactic acid, which lowers the pH and thus inhibits the growth of spoilage microorganisms (Galle & Arendt, 2014). LAB also contribute to the organoleptic properties of fermented foods through the formation of flavor precursors (Galle & Arendt, 2014). In addition, LAB possess numerous beneficial metabolic activities, such as the synthesis of antimicrobial compounds including bacteriocins, which can inhibit the growth of some food-borne pathogens and spoilage microorganisms (Gänzle, 2009). Furthermore, LAB are able to produce EPS that exhibit a positive effect on the texture, taste perception, mouthfeel, and stability of fermented foods (Katina et al., 2009; Tieking & Gänzle, 2005; Zannini et al., 2016). Since these EPS are produced *in situ* during food fermentation, they are not food additives and do not need to be labelled. This so-called ‘‘clean label’’ technology will meet consumers’ increasing demands for reduced usage of food additives

and should be taken into account during the development of new food products (Asioli et al., 2017). Due to these various benefits of LAB, they are preferable EPS producers in food systems.

2.3.2 Potential applications of LAB EPS in food systems

In the food industry, plant- or seaweed-derived polysaccharides are widely used as thickening, gelling, and stabilizing agents. Microbial EPS are regarded as potential substitutes for plant polysaccharides (Galle & Arendt, 2014). One application for the EPS produced by LAB is in sourdough bread. According to Katina et al. (2009), the addition of dextran-enriched sourdough to wheat bread provided mildly acidic taste and improved bread volume (up to 10%) and crumb softness (25–40%) during storage. EPS produced *in situ* by LAB can also be applied to the preparation of gluten-free food. For instance, Galle et al. (2012) reported that usage of EPS (dextran, fructan, and reuteran) in sorghum sourdough bread improved textural properties and prolonged shelf life. Usage of dextran in rye bread had positive effects on delaying staling of the crumb and improving bread volume and crumb softness (Lacaze, Wick, & Cappelle, 2007). In fermented dairy products, LAB EPS play an important role in improving textural properties (Ruas-Madiedo et al., 2002). For example, Gentès, St-Gelais, and Turgeon (2011) used different LAB strains to ferment milk, and higher values for apparent viscosity, firmness, and whey retention were obtained with EPS-producing strains. Purwandari, Shah, and Vasiljevic (2007) compared the effects of capsular and ropy EPS on the rheological properties of set-type yoghurt, with the highest storage modulus found in samples with capsular EPS.

Compared to the application of EPS in cereal or dairy products, the application of EPS in plant protein-based foods is much less studied but important to explore due to increasing demand for sustainable food sources. Efforts have been made to use LAB-produced EPS in the development of non-dairy milk or yogurt alternatives from soybean, lupin, quinoa, and oat (Table 3). However, no information is available on the utilization of *in situ* EPS produced by LAB in fava bean matrix. The combination of EPS production and fava bean fermentation by EPS-producing LAB could not only improve the resulting food texture, but also eliminate its ANF content. The resulting food would have ‘‘clean label’’ status and could promote fava bean utilization in the food industry.

Table 3. Studies on the applications of *in situ* EPS in plant-protein based foods.

Plant protein source	EPS type	Positive effect	Reference
Soybean	Heteropolysaccharides	Increased viscosity and decreased beany flavor	Li et al. (2014)
Lupin	–- [a]	Good rheological and textural properties	Hickisch, Beer, Vogel, and Toelstede (2016)
Quinoa	Dextran	Improved water holding capacity and viscosity	Zannini, Jeske, Lynch, and Arendt (2018)
Oat	–-	Increased viscosity	Mårtensson, Öste, and Holst (2000)

[a] Not reported.

3 Aims of the study

The overall aim of the thesis was to evaluate the effect of *in situ* produced EPS from LAB on the rheological and textural properties of fava bean matrix. In order to understand the mechanism underlying texture modification of fava bean matrix by EPS, the interactions between isolated dextran and fava bean protein were also evaluated under different conditions.

The specific aims were:

1. To study the possibility of producing *in situ* EPS from EPS-producing LAB in fava bean flour with the addition of sucrose (Studies **I** and **II**)
2. To identify promising EPS producers in fava bean flour (Study **II**)
3. To study the effects of *in situ* produced EPS on the rheological and textural properties of fava bean pastes (Studies **I**-**III**)
4. To study the interactions between dextran and fava bean protein under different conditions (Study **IV**)

4 Materials and methods

This section gives a brief description of the raw materials and analysis methods used. Figure 5 outlines the whole story line of this thesis concerning sample composition and preparation and the analysis methods used in different studies. Fermentation was carried out in Studies **I-III**, with fava bean flour or fava bean protein concentrate (FPC) as the fermentation medium. Rheological and textural properties were evaluated after fermentation. In Study **IV**, the rheological properties were evaluated in a simplified system using only mixtures or conjugates of isolated dextrans and fava bean protein isolate (FPI). Further details are described separately below.

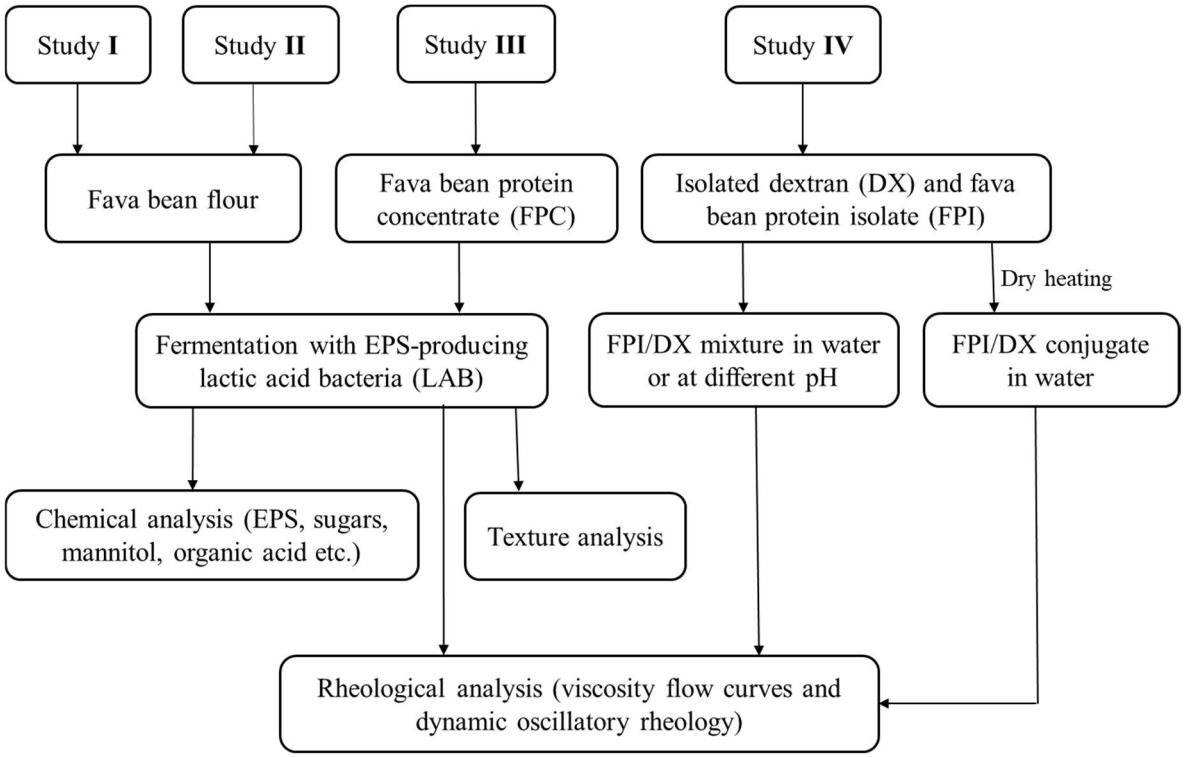

Figure 5. Schematic summary of the sample composition, preparation, and analysis methods used in this thesis.

4.1 Materials

Fava bean flour was purchased from Cerealveneta, (Padova, Italy). FPC was obtained from Vestkorn Milling AS (Norway). *Leuconostoc mesenteroides* subsp. *mesenteroides* DSM 20343, *Leuconostoc pseudomesenteroides* DSM 20193, *Leuconostoc mesenteroides* subsp. *mesenteroides* DSM 20240, *Leuconostoc citreum* DSM 5577 and *Weissella cibaria* DSM 15878 were all purchased from Leibniz Institute DSMZ (Braunschweig, Germany). *Weissella cibaria* Sj 1b and *Weissella confusa* Sj5-4 were obtained from the culture collection of the Division of Food Hygiene

and Environmental Health, University of Helsinki. *Lactobacillus plantarum* DPPMAB24W was obtained from the culture collection of the Department of Soil, Plant and Food Sciences, University of Bari and was proven to be EPS-negative by screening on sucrose- or raffinose-enriched agar plates. *Weissella confusa* VTT E-143403 (E3403) was obtained from the VTT Culture Collection (Espoo, Finland). More detailed information regarding the LAB strains used in this thesis is given in Table 4. All strains were routinely propagated in De Man, Rogosa, and Sharpe (MRS) broth (LAB M Limited, UK) before further use.

Table 4. The name, source, and EPS type of LAB strains used in this thesis.

Strain	Other collection number	Type strain	Source	EPS	Study
Leuconostoc mesenteroides DSM 20343	ATCC 8293, NRRL B-1118	Yes	Fermenting olives	Glucan and fructan	I
Leuconostoc pseudomesenteroides DSM 20193	ATCC 12291	Yes	Cane juice	Glucan	II, III and IV
Leuconostoc mesenteroides DSM 20240	ATCC 10830, NRRL B-512 F	No	–- [a]	Glucan	II
Leuconostoc citreum DSM 5577	ATCC 49370	Yes	Honey dew of rye ear	Glucan	II
Weissella cibaria DSM 15878	LMG 17699	Yes	Chili bo	Glucan	II
Weissella cibaria Sj 1b	No	No	Onion powder	Glucan	II and IV
Weissella confusa Sj5-4	No	No	Onion powder	Glucan	II
Weissella confusa VTT E-143403	No	No	Fava bean	Glucan	III

[a] Not reported.

4.2 Preparation of fava bean pastes (Studies I-III)

Fava bean pastes were prepared slightly different for the three studies, and the paste compositions are presented in Table 5. LAB cells were obtained by centrifugation (10 000 g × 10 min), followed by inoculation at a cell density of approximately 6.0 log cfu/g. Fermentation was conducted in triplicate at 30 ºC for 24 h. In this thesis, fava bean paste refers to all samples made with fava bean flour or FPC, and fava bean dough only refers to samples made with fava bean flour.

Table 5. Compositions of fava bean pastes prepared in this thesis.

Sample code	Matrix type	Matrix (g)	Sucrose (g)	Water (g)	Starter	Study
F0	Fava bean flour	40	0	60	*Ln. mesenteroides* DSM 20343	I
F5	Fava bean flour	35	5	60		
F10	Fava bean flour	30	10	60		
F15	Fava bean flour	25	15	60		
20193_CT	Fava bean flour	40	0	60	*Ln. pseudomesenteroides* DSM 20193	II
20193_S	Fava bean flour	30	10	60		
20240_CT	Fava bean flour	40	0	60	*Ln. mesenteroides* DSM 20240	II
20240_S	Fava bean flour	30	10	60		
5577_CT	Fava bean flour	40	0	60	*Ln. citreum* DSM 5577	II
5577_S	Fava bean flour	30	10	60		
15878_CT	Fava bean flour	40	0	60	*W. cibaria* DSM 15878	II
15878_S	Fava bean flour	30	10	60		
Sj 1b_CT	Fava bean flour	40	0	60	*W. cibaria* Sj 1b	II
Sj 1b_S	Fava bean flour	30	10	60		
Sj5-4_CT	Fava bean flour	40	0	60	*W. confusa* Sj5-4	II
Sj5-4_S	Fava bean flour	30	10	60		
20193C	FPC [a]	20	0	75	*Ln. pseudomesenteroides* DSM 20193	III
20193S	FPC	20	5	75		
3403C	FPC	20	0	75	*W. confusa* VTT E3403	III
3403S	FPC	20	5	75		

[a] Fava bean protein concentrate.

4.3 LAB cell density, pH, and total titratable acidity (Studies I-III)

After inoculation or fermentation, all samples (10 g) were mixed with 90 mL of sterile saline in a Stomacher 400 lab blender (Seward Medical, London, England) followed by serial dilution. The diluted suspensions were plated on MRS agar, and the bacterial colonies were counted after 48 h of incubation at 30 ºC. The pH values of samples were measured by a pH meter (Model HI 99161, Hanna Instruments, Woonsocket, RI, USA) equipped with a food penetration probe. Total titratable

acidity (TTA) was measured by a Mettler Toledo EasyPlus Titrator (Schott, Germany). More details on TTA determination can be found in Study **II**.

4.4 Analysis of sugars, mannitol, and organic acids (Studies I-III)

Sample preparation methods were similar for the analysis of sugars, mannitol, and organic acids. In detail, freeze-dried samples (100 mg) were mixed with 5.0 mL of Milli-Q water and vortexed for 5 min to facilitate the dissolution of free sugars, mannitol, and organic acids. The suspensions were then kept in a boiling water bath for 5 min to inactivate endogenous enzymes and microbes. After cooling and centrifugation (12 000 g × 10 min), 400 µL of the supernatants were filtered using Amicon Ultra-0.5 centrifugal filter units (Millipore, Billerica, MA) in order to remove molecules larger than 10 kDa. Samples were diluted with Milli-Q water and then were analyzed by different chromatographic methods as described below.

4.4.1 Mono-, di-, and oligo-saccharides

High performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) was used for sugar analysis. Mono-, di-, and oligo-saccharides were separated in a CarboPac PA1 column (250 × 4 mm i.d., Dionex, Sunnyvale, CA) at a flow rate of 1 mL/min, and were detected by a Waters 2465 pulsed amperometric detector (Waters, USA). The injection volume was 10 µL. Gradient elution was applied, starting from 2 mM NaOH and gradually progressing to 200 mM NaOH. The sugar standard was composed of galactose (Sigma-Aldrich), glucose (Merck, Germany), sucrose (Merck), fructose (Merck), melibiose (Sigma-Aldrich), raffinose (Sigma-Aldrich), stachyose (Sigma-Aldrich), verbascose (Megazyme, Ireland), and 2-deoxy-D-galactose (Sigma-Aldrich).

4.4.2 Mannitol

Mannitol analysis was performed on a HPAEC-PAD system equipped with a DECADE detector (Antec Leyden, The Netherlands) and a CarboPac MA-1 analytical column (4 × 250 mm i.d., Dionex). The gradient elution started from 40 mM NaOH (25 min) and proceeded first to 620 mM (10 min), then to 40 Mm at a flow rate of 0.4 mL/min. The injection volume was 5 µL. Mannitol (Sigma-Aldrich) was used as the standard for quantification.

4.4.3 Organic acids

Organic acids (lactic acid, acetic acid, and citric acid) were separated by an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, USA) and detected by a Waters 2487 Dual λ Absorbance Detector

(operating at 210 nm). The injection volume was 20 µL. Sulfuric acid (10 mM) was used as the eluent at a flow rate of 0.6 mL/min. The organic acid standard was composed of citric acid (Sigma-Aldrich), lactic acid (Sigma-Aldrich), and acetic acid (Merck) at various concentrations.

4.5 Degradation of RFO in native and autoclaved fava bean flour (Study I)

In order to investigate the effect of endogenous enzymes on the degradation of RFO, fava bean flour was first autoclaved and milled thoroughly for dough preparation. Two sets of doughs were prepared; one the control dough made of native or autoclaved flour (40 g) and water (60 g), the other a raffinose-enriched dough composed of native or autoclaved flour (30 g), raffinose (10 g), and water (60 g). Samples were then fermented by *Ln. mesenteroides* DSM 20343 at an initial cell density of 6.0 log cfu/g at 30 ºC for 24 h. After fermentation, samples were freeze-dried and treated for sugar analysis as described in section 4.4.1. One chemically acidified dough was prepared for comparison using native fava bean flour (40 g) and water (60 g). More details on sample preparation can be found in Study **I**. After freeze-drying, samples were treated for sugar analysis as described above.

4.6 Quantification of EPS in fava bean pastes (Studies I-III)

Glucan was quantified by calculating the content of released glucose after sulfuric acid hydrolysis. Freeze-dried samples (100 mg) were used for glucan quantification. Small sugars were washed out using aqueous ethanol (50%). Starch was degraded with a thermostable α-amylase (Megazyme) in 100 mM (pH 5.0) sodium acetate buffer incubated in a boiling water bath for 16 min. After cooling, amyloglucosidase (Megazyme) was added, and samples were further incubated at 50 °C for 30 min, followed by precipitation with ethanol (99.6%). To allow the complete precipitation of soluble glucan, samples were kept at 4 °C overnight, followed by centrifugation (10 000 *g* × 15 min). The supernatant was removed, and the glucan was hydrolyzed with 1.0 M sulfuric acid at 100 °C for 2 h. The reaction was stopped by adding Na_2CO_3 (1.0 M), and samples were diluted with Milli-Q water for sugar analysis as described in section 4.4.1. Glucose was treated by the same method and used as the standard. More details concerning the quantification of glucan can be found in Study **II**.

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

Fructan was quantified after trifluoroacetic acid (TFA) hydrolysis. Freeze-dried samples (100 mg) were washed twice with aqueous ethanol (80 %) to remove small sugars. Then, 0.5 M TFA (1 mL) was added and the samples were hydrolyzed at 50 °C for 2 h. The reaction was stopped by adding 250 µL of 1.0 M Na_2CO_3. The released fructose was quantified by the HPAEC-PAD system as described in section 4.4.1. Fructan content was calculated as the amount of released fructose minus the fructan in unfermented samples. Fructose was treated by the same method and used as the standard for quantification.

4.7 Preparation of FPI (Study IV)

FPI was obtained by isoelectric precipitation according to Makri, Papalamprou, and Doxastakis (2006), followed by freeze-drying. Details concerning the chemical composition analysis of FPI are provided in Study **IV**.

4.8 Dextran purification and structure elucidation (Study IV)

Dextran was purified from the slimes produced by LAB on MRS agar plates supplemented with 5% sucrose according to Maina et al. (2008). In brief, the slimes were precipitated with ethonal and dispersed in water, followed by free-drying. The purity of the isolated dextran was evaluated by calculating the percentage ratio between the released glucose content after sulfuric acid hydrolysis and the initial dextran content. Dextran structure was elucidated using nuclear magnetic resonance (NMR) spectroscopy on a 600 MHz Bruker Avance III NMR spectrometer (Bruker BioSpin, Germany) using the Bruker 1D NOESY pulse program (noesygppr1d).

4.9 Size-exclusion chromatography (Study IV)

The molar mass distributions of isolated dextran were determined by size-exclusion chromatography (SEC). Dimethyl sulfoxide (DMSO) was used to dissolve dextran, and samples were analyzed at a concentration of 1 mg/mL using a DMSO-based (DMSO + 0.01 M LiBr) eluent according to Maina et al. (2014).

4.10 Preparation of FPI/dextran (FPI/DX) mixture and conjugate (Study IV)

FPI/DX mixtures and conjugates were prepared with FPC and dextran both at a concentration of 20% (w/v) according to Spotti et al. (2014b). Dextran powder (1.2 g) was first dispersed in 6 mL of Milli-Q water overnight. Next, FPI powder (1.2 g) was added, followed by thorough mixing and

freeze-drying. After this, the obtained powder was incubated at 60 °C for six days at a relative humidity of 63% in order to enable the formation of FPI/DX conjugates. Before rheological analysis, the powders of FPI/DX mixtures and conjugates were dispersed in 6 mL of Milli-Q water overnight. Detailed sample compositions are presented in Table 6.

Table 6. Compositions of fava bean protein isolate (FPI) solution, dextran solutions produced by *Ln. pseudomesenteroides* DSM 20193 (DX_LP) and *W. cibaria* Sj 1b (DX_WC), and FPI/DX mixtures and conjugates (Table 1 in Study **IV**).

Sample code	FPI (g)	Dextran (g)	Dextran producer	Water (mL)	Incubation time (days)
FPI	1.2	0		6	0
DX_LP	0	1.2	*Ln. pseudomesenteroides* DSM 20193	6	0
DX_WC	0	1.2	*W. cibaria* Sj 1b	6	0
FPI/DX mixture					
FPI/LP_M [a]	1.2	1.2	*Ln. pseudomesenteroides* DSM 20193	6	0
FPI/WC_M	1.2	1.2	*W. cibaria* Sj 1b	6	0
FPI/DX conjugate					
FPI/LP_C [b]	1.2	1.2	*Ln. pseudomesenteroides* DSM 20193	6	6
FPI/WC_C	1.2	1.2	*W. cibaria* Sj 1b	6	6

[a] M indicates a mixture. [b] C indicates a conjugate formed by dry-heating (Maillard reaction).

4.11 Protein electrophoresis (Studies III and IV)

Proteolysis of fava bean protein (Study **III**) and the formation of FPI/DX conjugates (Study **IV**) were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), based on an established method (Laemmli, 1970). Fava bean pastes (10 mg) in Study **III** were dissolved in 1 mL of Laemmli sample buffer (Bio-Rad Laboratories, USA). FPI/DX mixtures and conjugates (50 mg) in Study **IV** were dissolved in 1 mL of 0.1 M Tris-HCl buffer (pH 6.8) with 10% glycerol, 2% SDS, 1% β-mercaptoethanol, and 0.02% bromophenol blue. All samples were then heated in a boiling water bath for 5 min, and the insoluble parts were removed by centrifugation. The obtained supernatants were analyzed under a constant voltage of 150 V. After electrophoresis, different

staining techniques were applied. For Study **IV**, protein was stained with Coomassie Brilliant Blue solution (0.1%) and glycoprotein by the Periodic acid-Schiff (PAS) staining technique according to Zacharius, Zell, Morrison, and Woodlock (1969). In Study **III**, only Coomassie Brilliant Blue solution (0.1%) was used for protein staining.

4.12 Heat treatment of FPI/DX mixture and conjugate (Study IV)

Suspensions of FPI, FPI/DX mixtures and conjugates were incubated in a water bath at 90 ºC for 15 min. After cooling to room temperature, the rheological properties were evaluated as described in section 4.14.

4.13 FPI/DX mixture at different pH (Study IV)

FPI/DX mixtures (1.2 g) were dispersed thoroughly in 6 mL of 0.1 M sodium citrate buffer at different pH (6.0, 5.0, 4.0, and 3.0). FPI (1.2 g) was used as the control and dispersed into 6 mL of the same buffer. Subsequently, the rheological properties were evaluated according to section 4.14.

4.14 Rheological analysis

4.14.1 Viscosity flow curves (Studies I-IV)

In Studies **I** and **II**, viscosity flow curves were measured by a RheolabQC rheometer (Anton Paar, Graz, Austria) under different shear rates, from 2 to 100 1/s (up and down sweeps). Samples (60 g) were mixed thoroughly and analyzed at room temperature.

In Studies **III** and **IV**, viscosity flow curves were generated by measuring shear viscosity under different shear rates from 2 to 100 1/s (up and down sweeps) using a HAAKE RheoStress rheometer (RS 50, HAAKE Rheometer, Germany). The hysteresis loop area between the upward and downward flow curves was calculated using RheoWin Pro software.

4.14.2 Dynamic oscillatory rheology (Studies II-IV)

The dynamic moduli (G', G") were recorded as a function of frequency from 0.1 to 10 Hz by a HAAKE RheoStress rheometer after sample equilibration. Measurements were conducted at 20 ºC using a parallel plate system with 2 mm gap in Studies **II** and **III** and 1 mm gap in Study **IV**.

4.15 The role of dextran and levan in viscosity improvement (Study I)

The sucrose-enriched dough fermented by *Ln. mesenteroides* DSM 20343 was separately hydrolyzed by dextranase (Sigma-Aldrich) and levanase (Novozymes, Denmark). Water was added

to the sample as a blank control. Hydrolysis was performed at 30 °C for 2 h. After hydrolysis, the viscosity was measured as described in section 4.14.1. More details are provided in Study **I**.

4.16 Texture analysis (Study III)

Texture analysis was performed by a TA.XT 2i texture analyzer (Stable Micro Systems Ltd., England). The back extrusion method was applied using a 35 mm diameter solid rod (A/BE35) at room temperature. Values for texture evaluation were obtained from the force–time curves using Exponent software (Stable Micro Systems Ltd., England). Four parameters were used for texture evaluation: firmness (peak positive force), consistency (positive area), cohesiveness (peak negative force), and index of viscosity (negative area). More details on the texture analysis can be found in Study **III**.

4.17 Microstructure of fava bean protein (Study III)

Confocal laser scanning microscopy (CLSM) was used to visualize fava bean protein microstructure. The equipment consisted of a Zeiss LSM 710 (Zeiss, Jena, Germany) attached to a Zeiss Axio Imager.Z microscope. The protein was stained using Rhodamine B (Merck, Germany) and examined on a microscope slide with HeNe laser. More details on CLSM are available in Study **III**.

4.18 Statistical analysis

One-way analysis of variance (ANOVA) performed with Origin 8.6 (OriginLab Inc., USA) was used for statistical analyses, and the means comparison was determined by Tukey's test ($p < 0.05$).

5 Results

In the first study, differing amounts of sucrose from 5% to 15% were added to fava bean flour in order to observe the effect of sucrose concentration on EPS production. *Ln. mesenteroides* DSM 20343 was chosen as the starter due to the clear information on what types of EPS it produced. Based on the viscosity increase (Table 3 in Study **I**) and EPS contents (Table 5 in Study **I**) after fermentation, 10% sucrose concentration was determined to be the optimum substrate concentration. Therefore, the second study used only 10% sucrose, and fava bean flour was fermented by different starters to identify promising EPS producers. In particular, *Ln. pseudomesenteroides* DSM 20193 was identified as a promising dextran producer for its high dextran production and was used in Study **III**. *W. confusa* VTT E3403 originated from fava bean was also selected for characterization because of its good performance at dextran production in fava bean flour according to Wang et al. (2018). Samples with 5% sucrose concentration were used in Study **III**, as a pre-experiment demonstrated that 10% sucrose pastes were too thick after fermentation (data not shown). Finally, dextran-protein interactions under different conditions were studied in order to understand the mechanisms behind texture modification of fava bean matrix by EPS (Study **IV**). The detailed results are given below.

5.1 LAB growth in fava bean matrix (Studies I-III)

Fava bean flour was fermented by seven LAB strains with an initial cell density of approximately 6.0 log cfu/g of the final dough. After 24 h of fermentation, the presumptive increase in LAB cell density varied depending on the starter used (Table 7). Higher cell density was found in doughs fermented by *Ln. pseudomesenteroides* DSM 20193 and *Ln. citreum* DSM 5577. The highest cell density increase (Δlog) was observed for the sucrose-enriched dough fermented by *Ln. citreum* DSM 5577 (5577_S). Sucrose addition had only a slight influence on propagation of LAB since doughs fermented by the same starter showed very similar Δlog values with or without sucrose addition as shown in Table 7.

Table 7. The growth of LAB and the acidification in fava bean flour. The table shows presumptive LAB cell density, cell density increase (Δlog), final pH, pH change (ΔpH), TTA, TTA change (ΔTTA), and the starter used.

Sample code [A]	Cell density (log cfu/g)	Δlog	pH	ΔpH	TTA (mL)	ΔTTA (mL)	Starter
F0	8.5 ± 0.1 [a]	2.3	4.4 ± 0.0 [a, d]	1.9	–- [B]	–-	*Ln. mesenteroides* DSM 20343
F10	9.5 ± 0.1 [b, d]	2.5	4.3 ± 0.0 [b]	2.2	–-	–-	
20193_CT	9.7 ± 0.2 [b, d]	3.2	4.4 ± 0.0 [a]	2.1	17.2 ± 0.1 [a]	12.9	*Ln. pseudomesenteroides* DSM 20193
20193_S	9.8 ± 0.1 [b]	3.4	4.3 ± 0.0 [b]	2.2	15.6 ± 1.3 [a, b, e]	12.6	
20240_CT	8.9 ± 0.0 [c, e]	3.1	4.8 ± 0.0 [c]	1.8	14.5 ± 0.0 [b, c, e]	10.5	*Ln. mesenteroides* DSM 20240
20240_S	8.6 ± 0.1 [a, c]	2.8	4.5 ± 0.0 [d]	2.2	15.7 ± 0.1 [a, b]	12.8	
5577_CT	9.5 ± 0.0 [d]	3.9	4.9 ± 0.0 [e, g]	1.7	13.3 ± 0.1 [c, e]	9.4	*Ln. citreum* DSM 5577
5577_S	9.6 ± 0.0 [b, d]	4.0	4.3 ± 0.0 [b]	2.4	19.8 ± 0.2 [d]	17.2	
15878_CT	9.0 ± 0.1 [e]	2.9	4.8 ± 0.0 [c]	1.8	15.0 ± 0.1 [b, e]	11.1	*W. cibaria* DSM 15878
15878_S	9.0 ± 0.0 [e]	2.9	4.6 ± 0.0 [f]	2.1	14.4 ± 0.6 [b, c, e]	11.4	
Sj 1b_CT	9.0 ± 0.0 [e]	3.2	4.8 ± 0.0 [c]	1.8	14.7 ± 0.3 [b, c, e]	10.6	*W. cibaria* Sj 1b
Sj 1b_S	9.0 ± 0.0 [e]	3.3	4.6 ± 0.0 [f]	2.0	14.0 ±0.5 [e]	11.0	
Sj5-4_CT	9.0 ± 0.1 [e]	3.1	5.0 ± 0.0 [g]	1.7	12.6 ± 0.1 [e]	8.7	*W. confusa* Sj5-4
Sj5-4_S	9.1 ± 0.1 [e]	3.2	4.8 ± 0.0 [c]	1.9	10.7 ± 0.1 [f]	7.9	

[A] Sample code details can be found in Table 5. [B] TTA was not measured in Study **I**. [a-g] Values in the same column with different letters are significantly different ($p < 0.05$).

With an initial LAB cell density of ca. 6.0 log cfu/g, the presumptive LAB cell number in FPC pastes increased from 3.0 to 3.5 log cycles after fermentation, reaching a final cell density that varied from 9.5 to 9.7 log cfu/g (Table 8). Similar cell densities and Δlog values were found in pastes fermented by *Ln. pseudomesenteroides* DSM 20193 and *W. confusa* VTT E3403. No big differences were observed in cell density or Δlog values between pastes fermented by the same starter, indicating the minor effect of sucrose addition on LAB growth.

Table 8. The growth of LAB and the acidification in FPC. The table shows presumptive cell density, cell density increase (Δlog), final pH, pH change (ΔpH), TTA, TTA change (ΔTTA) and the starter used.

Sample code [A]	Cell density (log cfu/g)	Δlog	pH	ΔpH	TTA (mL)	ΔTTA (mL)	Starter
20193C	9.6 ± 0.0 [a]	3.0	4.8 ± 0.0 [a]	1.7	12.9 ± 0.2 [a]	8.6	*Ln. pseudomesenteroides* DSM 20193
20193S	9.7 ± 0.0 [a]	3.2	4.4 ± 0.1 [b]	2.1	19.2 ± 0.1 [b]	15.2	
3403C	9.5 ± 0.2 [a]	3.2	5.9 ± 0.0 [c]	0.6	6.8 ± 0.1 [c]	2.5	*W. confusa* VTT E3403
3403S	9.7 ± 0.1 [a]	3.5	5.0 ± 0.0 [a]	1.5	10.9 ± 0.2 [d]	6.9	

[A] Sample code details can be found in Table 5. [a-d] Values in the same column with different letters are significantly different ($p < 0.05$).

5.2 Acidity of fava bean matrix (Studies I-III)

5.2.1 pH and TTA

As an important parameter to evaluate fermentation process, the acidity (pH and TTA) of fava bean matrix was determined. After fermentation, pH values decreased to different extents depending on the starter and whether sucrose was added (Table 7). Lower pH values and higher pH drops (ΔpH) were found in sucrose-added doughs compared to control doughs fermented by the same starter. The added sucrose also had different effects on ΔpH of doughs fermented by different starters. In sucrose-enriched doughs fermented by *Weissella* spp., higher pH and lower ΔpH values were observed compared to those fermented by *Leuconostoc* spp. Similarly, sucrose addition had different effects on TTA increase (ΔTTA). The TTA values of doughs fermented by *Weissella* spp. increased little or even decreased with sucrose addition, whereas doughs fermented by *Leuconostoc* spp. generally showed higher ΔTTA values with sucrose added.

In FPC pastes, sucrose addition decreased the pH and increased the TTA values after fermentation (Table 8). Significantly higher pH values were found in pastes fermented by *W. confusa* VTT E3403 (3403C and 3403S) compared to those fermented by *Ln. pseudomesenteroides* DSM 20193 (20193C and 20193S). A similar phenomenon was also observed for TTA, with 3403C and 3403S showing significantly lower TTA and ΔTTA values.

5.2.2 Organic acids

Citric acid is endogenously present in both fava bean flour and FPC, with various contents detected after fermentation. In Study **I**, citric acid was not found to be involved in microbial metabolism (Table 2 in Study **I**). However, in Studies **II** and **III**, some strains, e.g. *Ln. citreum* DSM 5577 and

W. confusa VTT E3403, metabolised the endogenous citric acid (Table 2 in Studies **II** and **III**). In fava bean flour, lactic acid and acetic acid were both produced after fermentation, with varying contents depending on the starter and sucrose addition (Table 9). The added sucrose increased acetic acid concentration, considerably decreasing the fermentation quotient (FQ, the molar ratio between lactic and acetic acid) in doughs fermented by *Leuconostoc* spp. In contrast, the FQ values in doughs fermented by *Weissella* spp. did not decrease after sucrose addition, except the one fermented by *W. cibaria* Sj 1b (Sj 1b_S), which showed a slight FQ decrease.

Table 9. Concentrations of lactic acid and acetic acid and the fermentation quotient (FQ) in fava bean flour fermented by different starters with or without sucrose addition.

Sample code [A]	Lactic acid [B] (mmol/100g dough)	Acetic acid [B] (mmol/100g dough)	FQ	Starter
F0	12.97 ± 0.21 [a]	3.17 ± 0.30 [a]	4.09	*Ln. mesenteroides* DSM 20343
F10	10.10 ± 0.21 [a]	10.02 ± 0.88 [b]	1.01	
20193_CT	21.97 ± 0.91 [a]	6.46 ± 0.37 [c, f]	3.40	*Ln. pseudomesenteroides* DSM 20193
20193_S	17.63 ± 0.96 [a]	7.46 ± 0.28 [c, d]	2.36	
20240_CT	13.96 ± 0.59 [a]	6.11 ± 0.31 [c]	2.28	*Ln. mesenteroides* DSM 20240
20240_S	9.50 ± 0.32 [a]	9.34 ± 0.45 [b, d]	1.02	
5577_CT	14.08 ± 0.60 [a]	9.44 ± 0.42 [b, d]	1.49	*Ln. citreum* DSM 5577
5577_S	12.45 ± 0.59 [a]	16.24 ± 1.28 [e]	0.77	
15878_CT	17.47 ± 1.69 [a]	7.63 ± 0.57 [b, c, d]	2.29	*W. cibaria* DSM 15878
15878_S	15.98 ± 0.99 [a]	6.56 ± 0.72 [c, f]	2.43	
Sj 1b_CT	14.57 ± 0.73 [a]	7.37 ± 0.30 [c, d]	1.98	*W. cibaria* Sj 1b
Sj 1b_S	9.64 ± 0.26 [a]	7.62 ± 0.58 [b, c, d]	1.26	
Sj5-4_CT	18.41 ± 2.41 [a]	8.65 ± 1.46 [b, d, f]	2.13	*W. confusa* Sj5-4
Sj5-4_S	14.78 ± 0.42 [a]	6.55 ± 0.31 [c, f]	2.26	

[A] Sample code details can be found in Table 5. [B] Concentration was calculated based on wet weight.
[a-f] Values in the same column with different letters are significantly different ($p < 0.05$).

In FPC pastes, the two strains (*Ln. pseudomesenteroides* DSM 20193 and *W. confusa* VTT E3403) also showed different capabilities at producing acetic acid from the added sucrose (Table 10). A significantly higher acetic acid concentration was found for 20193S compared to 3403S. Correspondingly, an obvious FQ drop was observed in 20193S, similar to those in samples fermented by *Leuconostoc* spp. (Table 9). The high acetic acid content found in 3403C and 3403S

may be due to the use of citric acid as an electron acceptor (Corsetti & Settanni, 2007), which is also suggested by the disappearance of citric acid in these two samples (Table 2 in Study **III**).

Table 10. Concentrations of lactic acid and acetic acid and the fermentation quotient (FQ) in FPC fermented by different starters with or without sucrose addition.

Sample code [A]	Lactic acid [B] (mmol/100g)	Acetic acid [B] (mmol/100g)	FQ	Starter
20193C	43.11 ± 2.22 [a]	12.34 ± 0.16 [a]	3.49	*Ln. pseudomesenteroides* DSM 20193
20193S	43.37 ± 0.51 [a]	31.92 ± 0.26 [b]	1.36	
3403C	25.01 ± 0.40 [b]	22.40 ± 0.09 [c]	1.12	*W. confusa* VTT E3403
3403S	38.09 ± 1.10 [c]	18.06 ± 0.65 [d]	2.11	

[A] Sample code details can be found in Table 5. [B] Concentration was calculated based on dry weight.
[a-d] Values in the same column with different letters are significantly different ($p < 0.05$).

5.3 Sugars in fava bean matrix before and after fermentation (Studies I-III)

Sugars in fava bean flour and FPC were analyzed before and after fermentation in order to evaluate sugar utilization by microbes. Sucrose, raffinose, stachyose, and verbascose are the main endogenous sugars in fava bean flour (Table 11). After fermentation, the endogenous sucrose was completely consumed in control doughs, with no detection of glucose or fructose. In sucrose-enriched doughs, the added sucrose was used to different extents depending on the starter. Generally, *Leuconostoc* spp. showed higher utilization of sucrose compared to *Weissella* spp., as no or little sucrose was detected in *Leuconostoc* fermented doughs while roughly half of the added sucrose was found in 15878_S and Sj 1b_S. Glucose was only found in two doughs fermented by *Ln. mesenteroides* DSM 20343 and *Ln. mesenteroides* DSM 20240. Fructose was also detected after sucrose addition, varying from 0.51% to 5.73% in different samples. Raffinose was totally utilized after fermentation, while the degree of degradation for stachyose and verbascose and the associated release of galactose depended on the starter.

Table 11. Mono-, di-, and oligo-saccharide contents in fava bean flour fermented by different starters with or without sucrose addition.

Sample code [a]	Sugars (%, w/w, wet weight) [b]							Starter
	Glc (%)	Suc (%)	Fru (%)	Gal (%)	Raf (%)	Sta (%)	Ver (%)	
CT_0h [c]	nd [d]	1.33 ± 0.07	nd	nd	0.12 ± 0.01	0.91 ± 0.01	1.94 ± 0.02	–-
S_0h [e]	nd	11.65± 0.05	nd	nd	0.09 ± 0.01	0.68 ± 0.01	1.45 ± 0.01	–-
F0	nd	nd	nd	0.36 ± 0.01	nd	0.11 ± 0.00	0.14 ± 0.00	*Ln. mesenteroides* DSM 20343
F10	0.94 ± 0.02	nd	0.51 ± 0.01	0.41 ± 0.01	nd	nd	0.21 ± 0.00	
20193_CT	nd	nd	nd	0.20 ± 0.01	nd	0.24 ± 0.00	0.31 ± 0.03	*Ln. pseudomesenteroides* DSM 20193
20193_S	nd	nd	5.73 ± 0.10	0.34 ± 0.01	nd	nd	nd	
20240_CT	nd	nd	nd	0.44 ± 0.01	nd	0.58 ± 0.01	0.78 ± 0.04	*Ln. mesenteroides* DSM 20240
20240_S	0.36 ± 0.02	0.30 ± 0.05	3.30 ± 0.02	0.34 ± 0.00	nd	nd	nd	
5577_CT	nd	nd	nd	0.56 ± 0.01	nd	0.85 ± 0.05	0.99 ± 0.02	*Ln. citreum* DSM 5577
5577_S	nd	nd	2.06 ± 0.10	0.45 ± 0.02	nd	nd	nd	
15878_CT	nd	nd	nd	0.48 ± 0.01	nd	0.63 ± 0.01	0.85 ± 0.01	*W. cibaria* DSM 15878
15878_S	nd	5.77 ± 0.37	1.76 ± 0.21	0.34 ± 0.00	nd	nd	nd	
Sj 1b_CT	nd	nd	nd	0.31 ± 0.01	nd	0.85 ± 0.03	0.84 ± 0.03	*W. cibaria* Sj 1b
Sj 1b_S	nd	4.27 ± 0.48	2.19 ± 0.07	0.31 ± 0.00	nd	nd	nd	
Sj5-4_CT	nd	nd	nd	0.30 ± 0.00	nd	0.54 ± 0.00	0.83 ± 0.02	*W. confusa* Sj5-4
Sj5-4_S	nd	nd	5.37 ± 0.03	0.30 ± 0.01	nd	nd	nd	

[a] Sample code details can be found in Table 5. [b] Sugars: Glc, glucose; Suc, sucrose; Fru, fructose; Gal, galactose; Raf, raffinose; Sta, stachyose; Ver, verbascose. [c] Control dough before fermentation. [d] Not detected. [e] Sucrose-enriched dough before fermentation.

Similarly, in FPC, endogenous sucrose was detected, together with stachyose, verbascose, and very small amounts of glucose and galactose (Table 12). After fermentation, sucrose was completely utilized in both control and sucrose-enriched pastes. No glucose was detected in any fermented paste. Fructose was not found in control pastes, but was found in sucrose-enriched pastes, with higher content in the paste fermented by *W. confusa* VTT E3403. No RFO were detected in 20193C, and the added sucrose decreased degradation of RFO and utilization of galactose. In 3403C and 3403S, no substantial difference in RFO content was observed, though more galactose was detected in 3403S.

Table 12. Mono-, di-, and oligo-saccharide contents in FPC pastes fermented by *Ln. pseudomesenteroides* DSM 20193 and *W. confusa* VTT E3403 with or without sucrose addition (adapted from Table 3 in Study **III**).

Sample code [a]	Sugars [b] (%, w/w, dry weight)					
	Glc	Suc	Fru	Gal	Sta	Ver
C_0h [c]	0.20 ± 0.00	1.61 ± 0.06	nd [d]	0.17 ± 0.01	1.13 ± 0.03	3.31 ± 0.22
S_0h [e]	0.20 ± 0.00	22.37 ± 0.21	nd	0.17 ± 0.01	1.13 ± 0.03	3.31 ± 0.22
20193C	nd	nd	nd	nd	nd	nd
20193S	nd	nd	2.66 ± 0.17	0.42 ± 0.02	1.12 ± 0.05	1.45 ± 0.18
3403C	nd	nd	nd	0.21 ± 0.01	1.50 ± 0.13	2.25 ± 0.08
3403S	nd	nd	8.90 ± 0.10	0.42 ± 0.01	1.37 ± 0.12	2.06 ± 0.32

[a] Sample code details can be found in Table 5. [b] Sugars: Glc, glucose; Suc, sucrose; Fru, fructose; Gal, galactose; Sta, stachyose; Ver, verbascose. [c] Control paste before fermentation. [d] Not detected. [e] Sucrose-enriched paste before fermentation.

5.4 Degradation of RFO (Study I)

As RFO are an important ANF in fava bean, their degradation was investigated in both native and autoclaved fava bean flour. In the chemically acidified dough (F0_C), verbascose was degraded, releasing galactose (Figure 6A). This clearly shows the role of endogenous α-galactosidase in RFO degradation. In the control dough made with native flour, galactose was released after fermentation. However, after the inactivation of endogenous α-galactosidase, no galactose was detected, suggesting the absence of microbial α-galactosidase in *Ln. mesenteroides* DSM 20343. The appearance of manninotriose and manninotetraose in F0_A indicated the activity of LSR on RFO. In raffinose-enriched doughs, different sugar profiles were also observed depending on the inactivation of endogenous α-galactosidase (Figure 6B). With α-galactosidase active, galactose, melibiose, and manninotriose were all detected, revealing the joint action of α-galactosidase and

LSR. In contrast, inactivation of α-galactosidase resulted in the detection of only melibiose and manninotriose, confirming the absence of microbial α-galactosidase. Furthermore, raffinose was more efficiently degraded by the joint action of α-galactosidase and LSR, as shown by the minor raffinose peak in FR10_U.

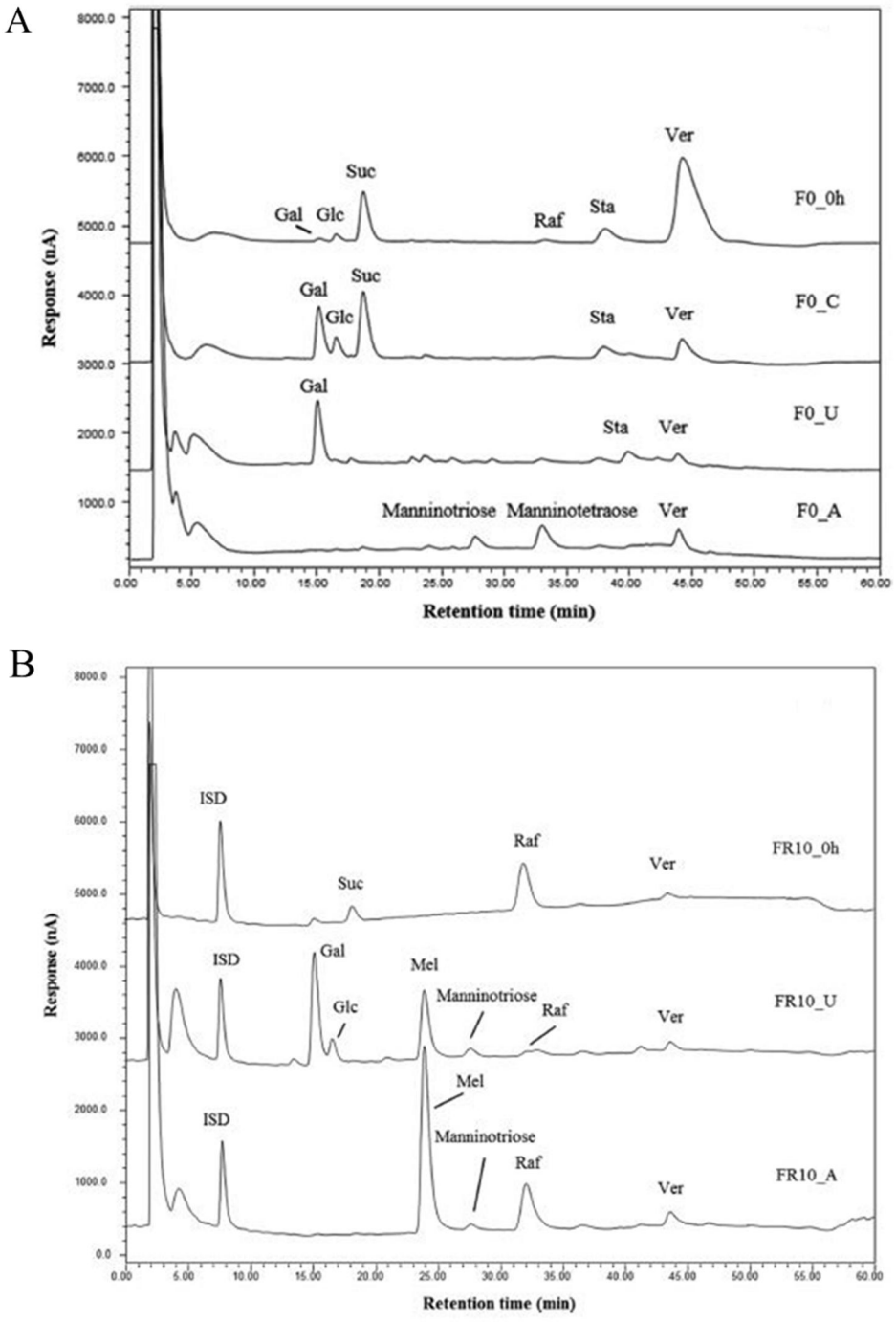

Figure 6. HPAEC-PAD chromatograms of fava bean control (F0) (A) and raffinose-enriched doughs (FR10) (B). 0h, unfermented doughs; C, chemically acidified doughs; U, doughs made with untreated flour; A, doughs made with autoclaved flour; ISD, internal standard; Gal, galactose; Glc, glucose; Suc, sucrose; Mel, melibiose; Raf, raffinose; Sta, stachyose; Ver, verbascose (Adapted from Figure 2 in Study **I**).

Based on the chromatographic analysis of RFO profiles in different fava bean doughs before and after inactivation of endogenous α-galactosidase (Figure 6), two degradation ways for RFO were proposed (Figure 7). In the presence of LSR, RFO could be degraded to melibiose, manninotriose, and manninotetraose, at the same time releasing fructose or forming levan (Figure 7A). With the activity of α-galactosidase, RFO were degraded to galactose and sucrose that was further involved in the formation of glucan through the polymerizing activity of GS (Figure 7B). In Study **I**, both of these degradation ways were observed, while in Study **II**, only the mechanism involing α-galactosidase and GS was observed due to the absence of LSR in the six LAB strains used.

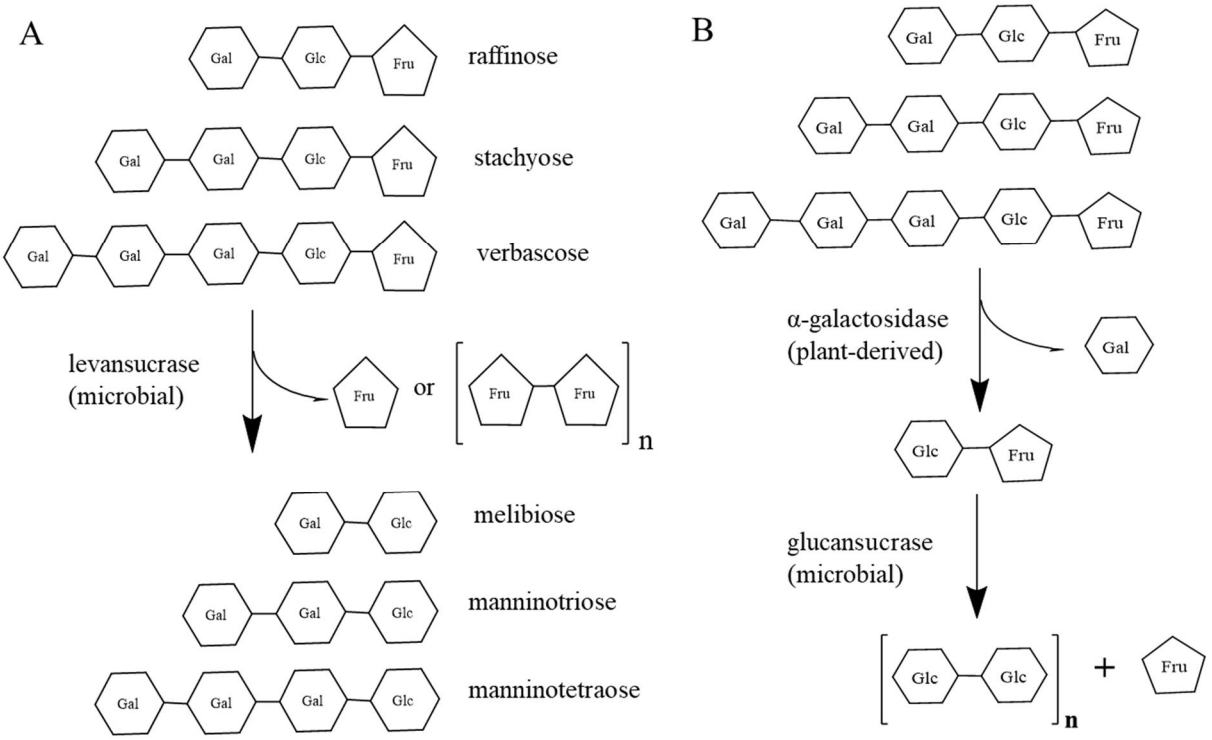

Figure 7. Two degradation ways for RFO: LSR-involved way (A) and α-galactosidase- and GS-involved way (B). LSR and GS are microbial enzymes, while α-galactosidase is an endogenous enzyme in fava bean flour (Figure 5 in Study **I**).

5.5 EPS composition in fava bean matrix (Studies I-III)

Sucrose addition strongly enabled EPS formation, and various EPS constituents (dextran, glucan, and fructan) were detected under different sucrose concentrations (Table 13). Glucan content increased with rising sucrose content, with the highest increase obtained at 10% sucrose. Similarly, the proportion of low-branched dextran increased with more sucrose, with the highest increase observed at 5% sucrose. This finding is consistent with the dextran and glucan ratios, showing that low-branched dextran was preferably produced at low sucrose concentrations. Differences between

glucan and dextran contents suggests the synthesis of high-branched dextran and insoluble glucan produced by *Ln. mesenteroides* DSM 20343. The highest increase of fructan content was found when using 5% sucrose, and the facilitation of fructan formation by low sucrose concentration was also reflected in the ratios of fructan and glucan. The highest EPS yield was achieved when only endogenous sucrose was available; yields decreased gradually with more sucrose.

Table 13. Effect of sucrose concentration on EPS composition in doughs fermented by *Ln. mesenteroides* DSM 20343.

Sample code [A]	Glucan [B] (%)	Dextran [B] (%)	Fructan [B] (%)	EPS yield [C] (%)	Dextran/Glucan	Fructan/Glucan
F0	0.49 ± 0.10 *a*	0.21 ± 0.01 *a*	0.20 ± 0.01 *a*	94.52	0.43	0.41
F5	1.13 ± 0.12 *b*	0.83 ± 0.01 *b*	1.11 ± 0.06 *b*	71.37	0.73	0.98
F10	2.29 ± 0.18 *c*	1.22 ± 0.01 *c*	1.58 ± 0.12 *c*	69.76	0.53	0.69
F15	3.05 ± 0.25 *d*	1.38 ± 0.02 *d*	1.76 ± 0.08 *c*	59.77	0.45	0.58

[A] Sample code details can be found in Table 5. [B] Content was calculated based on wet weight. [C] EPS yield is the percentage ratio between real EPS content and theoretical EPS content. [a-d] Values in the same column with different letters are significantly different ($p < 0.05$).

In samples with the same sucrose proportions, EPS contents varied depending on the starter (Table 14). With only endogenous sucrose, *W. cibaria* Sj 1b produced the highest amount of dextran (glucan). However, with sucrose added, the highest dextran content was found in 20193_S, while the highest glucan content was in 20240_S, indicating that *Ln. mesenteroides* DSM 20240 could produce more high-branched dextran or insoluble glucan. Generally, with added sucrose, higher glucan contents were found in doughs fermented by *Leuconostoc* spp. compared to doughs fermented by *Weissella* spp. No fructan was detected in any doughs except those fermented by *Ln. mesenteroides* DSM 20343.

In FPC pastes, only dextran was quantified since the two strains used mainly produce low-branched dextran. Regardless of sucrose addition, *W. confusa* VTT E3403 was found to possess higher efficiency of dextran production compared to *Ln. pseudomesenteroides* DSM 20193 (Table 15). No fructan was synthesized by these two strains.

Table 14. Glucan, dextran, and mannitol produced in fava bean flour with or without sucrose addition after fermentation.

Sample code [A]	Glucan [B] (%)	Dextran [B] (%)	Mannitol [B] (%)	Starter
F0	0.49 ± 0.10 [a]	0.21 ± 0.01 [a, e]	0.24 ± 0.02 [a, c]	*Ln. mesenteroides* DSM 20343
F10	2.29 ± 0.18 [b]	1.22 ± 0.01 [b]	3.04 ± 0.32 [b, c]	
20193_CT	0.32 ± 0.01 [a]	0.21 ± 0.09 [a, e]	0.16 ± 0.01 [a]	*Ln. pseudomesenteroides* DSM 20193
20193_S	4.15 ± 0.13 [c]	3.67 ± 0.07 [c]	0.27 ± 0.03 [a, c]	
20240_CT	0.49 ± 0.37 [a]	0.42 ± 0.01 [a]	0.46 ± 0.01 [a, c]	*Ln. mesenteroides* DSM 20240
20240_S	4.33 ± 0.30 [c]	2.85 ± 0.11 [d]	2.01 ± 0.98 [c]	
5577_CT	0.53 ± 0.25 [a]	0.11 ± 0.04 [e]	0.28 ± 0.00 [a, c]	*Ln. citreum* DSM 5577
5577_S	3.77 ± 0.11 [c]	2.17 ± 0.03 [f]	4.22 ± 1.31 [b]	
15878_CT	0.50 ± 0.02 [a]	0.46 ± 0.04 [a, g]	0.02 ± 0.00 [a]	*W. cibaria* DSM 15878
15878_S	2.71 ± 0.48 [b, c]	2.11 ± 0.11 [f, h]	0.21 ± 0.10 [a]	
Sj 1b_CT	0.82 ± 0.03 [a]	0.74 ± 0.11 [g]	0.34 ± 0.01 [a, c]	*W. cibaria* Sj 1b
Sj 1b_S	2.66 ± 0.76 [b]	1.86 ± 0.09 [h]	1.39 ± 0.31 [a, c]	
Sj5-4_CT	0.32 ± 0.13 [a]	0.32 ± 0.08 [a, e]	nd [C]	*W. confusa* Sj5-4
Sj5-4_S	2.57 ± 0.28 [b]	2.24 ± 0.13 [f]	nd	

[A] Sample code details can be found in Table 5. [B] Content was calculated based on wet weight. [C] Not detected. [a-h] Values in the same column with different letters are significantly different ($p < 0.05$).

Table 15. Dextran, fructan, and mannitol produced in FPC with or without sucrose addition after fermentation.

Sample code [A]	Dextran [B] (%)	Fructan [B] (%)	Mannitol [B] (%)	Starter
20193C	0.34 ± 0.01 [a]	nd [C]	0.14 ± 0.01 [a]	*Ln. pseudomesenteroides* DSM 20193
20193S	6.84 ± 0.41 [b]	nd	6.77 ± 0.19 [b]	
3403C	0.72 ± 0.03 [a]	nd	nd	*W. confusa* VTT E3403
3403S	10.00 ± 0.26 [c]	nd	nd	

[A] Sample code details can be found in Table 5. [B] The content was calculated based on dry weight. [C] Not detected. [a-c] Values in the same column with different letters are significantly different ($p < 0.05$).

5.6 Mannitol production in fava bean matrix (Studies I-III)

Mannitol was detected in all fava bean doughs except the one fermented by *W. confusa* Sj5-4 (Table 14). Sucrose addition considerably facilitated mannitol production, especially in doughs fermented by *Ln. mesenteroides* DSM 20343 and *Ln. citreum* DSM 5577. Generally, higher mannitol contents were found in doughs fermented by *Leuconostoc* spp. after sucrose addition, when compared to doughs fermented by *Weissella* spp. Notably, unlike other *Leuconostoc* spp., *Ln. pseudomesenteroides* DSM 20193 produced much less mannitol after sucrose addition in fava bean flour. However, in FPC pastes, added sucrose resulted in this strain producing a high quantity of mannitol (Table 15), indicating that mannitol production was affected by the medium used. *W. confusa* VTT E3403 did not produce mannitol in FPC pastes.

5.7 Rheological properties of fava bean pastes (Studies I-III)

5.7.1 Viscosity flow curves

In fava bean flour, a clear viscosity improvement was observed after fermentation, especially for sucrose-enriched doughs. Figure 8 presents the viscosity flow curves of two representative sucrose-enriched doughs (F0 and 20193_S), showing typical shear-thinning behavior after fermentation. The viscosity values of all samples at 100 1/s were compared. Sucrose content affected both final viscosity and viscosity increase in doughs fermented by *Ln. mesenteroides* DSM 20343 (Figure 9). The highest viscosity value was obtained in F10 and the lowest in F0, demonstrating the thickening effect of EPS. Viscosity increase peaked at 10% sucrose content, therefore, this content was used in Study **II** for the evaluation of fermentation by different LAB strains.

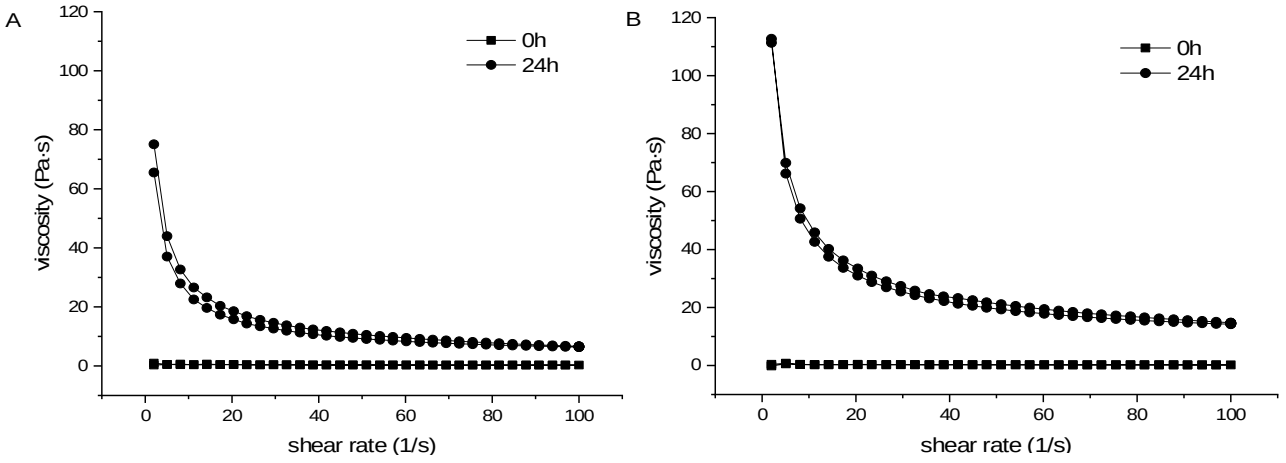

Figure 8. Viscosity flow curves for fava bean doughs fermented by *Ln. mesenteroides* DSM 20343 (A) and *Ln. pseudomesenteroides* DSM 20193 (B). In both samples, 10% of sucrose was added.

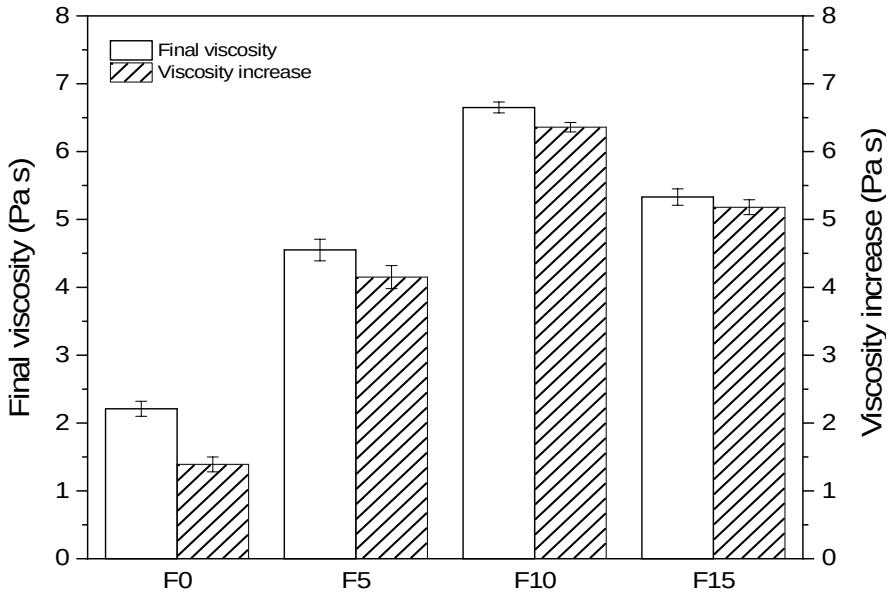

Figure 9. Final viscosity and viscosity increase for fava bean doughs fermented by *Ln. mesenteroides* DSM 20343 at sucrose addition concentrations of 0% (F0), 5% (F5), 10% (F10), and 15% (F15).

The final viscosity and viscosity increase values for fava bean doughs fermented by different starters varied considerably (Table 16). In control doughs, the highest viscosity increase was found in Sj 1b_CT, corresponding to its highest dextran content (Table 14). In sucrose-enriched doughs, 20193_S showed the greatest viscosity increase, which similarly corresponds to its highest dextran content. Doughs fermented by *W. confusa* Sj5-4 showed very little viscosity increase regardless of whether sucrose was added or not.

Similarly, in FPC pastes, significantly higher viscosity values were observed after fermentation in sucrose-enriched pastes than in control pastes, although they had very similar starting viscosities (Figure 10). Pastes fermented by *W. confusa* VTT E3403 showed significantly higher viscosity values over the corresponding pastes fermented by *Ln. pseudomesenteroides* DSM 20193. This is also consistent with the higher dextran contents in pastes fermented by *W. confusa* VTT E3403 (Table 15).

Table 16. Final viscosity and viscosity increase in fava bean flour fermented by different strains with or without sucrose addition.

Sample code [A]	Final viscosity (Pa·s)	Viscosity increase (Pa·s)	Starter
Control doughs			
F0	2.21 ± 0.11 [a]	1.39 ± 0.11 [a]	*Ln. mesenteroides* DSM 20343
20193_CT	3.16 ± 0.20 [b]	1.43 ± 0.38 [a]	*Ln. pseudomesenteroides* DSM 20193
20240_CT	4.57 ± 0.10 [c]	1.79 ± 0.10 [a]	*Ln. mesenteroides* DSM 20240
5577_CT	3.92 ± 0.30 [c]	1.38 ± 0.27 [a]	*Ln. citreum* DSM 5577
15878_CT	4.27 ± 0.20 [c]	1.65 ± 0.22 [a]	*W. cibaria* DSM 15878
Sj 1b_CT	5.87 ± 0.30 [d]	3.28 ± 0.16 [b]	*W. cibaria* Sj 1b
Sj5-4_CT	2.59 ± 0.10 [a, b]	0.00 ± 0.12 [c]	*W. confusa* Sj5-4
Sucrose-enriched doughs			
F10	6.65 ± 0.08 [a]	6.36 ± 0.07 [a]	*Ln. mesenteroides* DSM 20343
20193_S	14.77 ± 0.00 [b]	14.57 ± 0.06 [b]	*Ln. pseudomesenteroides* DSM 20193
20240_S	6.16 ± 0.10 [a]	5.81 ± 0.15 [a]	*Ln. mesenteroides* DSM 20240
5577_S	6.24 ± 0.40 [a]	5.96 ± 0.39 [a]	*Ln. citreum* DSM 5577
15878_S	4.28 ± 0.60 [c]	3.95 ± 0.59 [c]	*W. cibaria* DSM 15878
Sj 1b_S	6.54 ± 0.20 [a]	6.23 ± 0.22 [a]	*W. cibaria* Sj 1b
Sj5-4_S	0.82 ± 0.00 [d]	0.55 ± 0.05 [d]	*W. confusa* Sj5-4

[A] Sample code details can be found in Table 5. [a-d] Values in the same column with different letters are significantly different ($p < 0.05$).

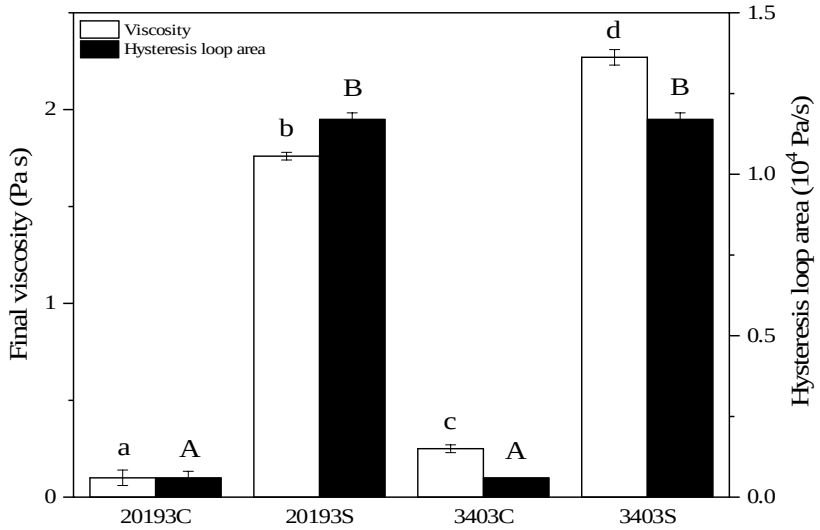

Figure 10. Viscosity and hysteresis loop area of FPC pastes fermented by *Ln. pseudomesenteroides* DSM 20193 and *W. confusa* VTT E3403 with or without sucrose addition. Sample code details can be found in Table 5. [a-d, A-B] Columns with different letters are significantly different ($p < 0.05$).

5.7.2 The role of dextran and levan in viscosity improvement

As *Ln. mesenteroides* DSM 20343 produced both dextran and levan from sucrose, the role of these two polymers in viscosity improvement was evaluated. Dough with 10% added sucrose (F10) was hydrolyzed separately by dextranase and levanase, and various viscosity drops were observed after hydrolysis (Figure 11). In detail, a substantial viscosity drop was observed after dextranase hydrolysis, from 5.51 to 0.79 Pa s. By contrast, a much lower decrease was observed after levanase hydrolysis, from 5.51 to 4.78 Pa s. This difference indicates the major role of dextran in viscosity improvement. As a blank control, the sample with water showed the lowest viscosity drop.

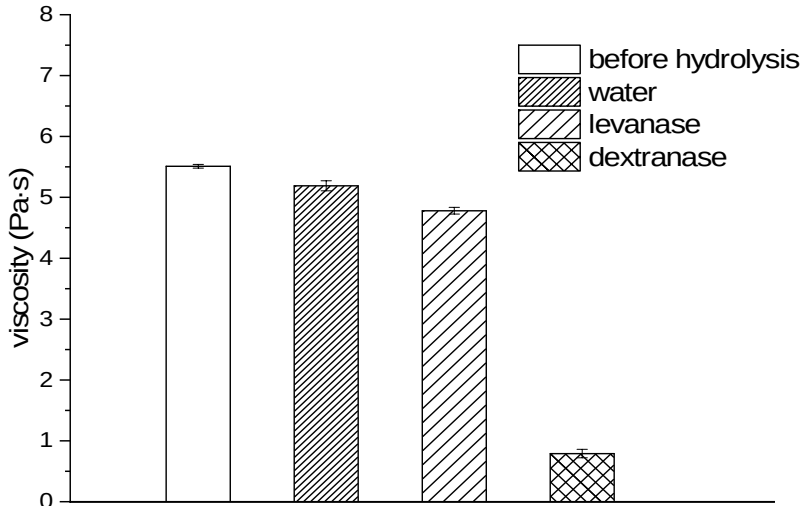

Figure 11. Viscosity changes of the sucrose-enriched fava bean dough fermented by *Ln. mesenteroides* DSM 20343 with 10% sucrose (F10) after hydrolysis by levanase or dextranase. The same volume of water was added to the blank control sample.

5.7.3 Hysteresis loop

Sucrose-enriched doughs fermented by different strains showed diverse differences in their hysteresis loops (Figure 12). The largest loop was found in the dough fermented by *Ln. pseudomesenteroides* DSM 20193, and the smallest in the dough fermented by the EPS-negative strain *L. plantarum* DPPMAB24W. The dough fermented by *W. confusa* Sj5-4 showed a similar loop area to the EPS-negative control dough (B24W_S) even though it contained dextran.

In FPC pastes, an obvious increase in hysteresis loop area was observed after sucrose addition (Figure 10). The two sucrose-enriched pastes fermented by *Ln. pseudomesenteroides* DSM 20193 and *W. confusa* VTT E3403 showed the same loop area. No significant difference was found between the loop areas of control pastes fermented by these two strains.

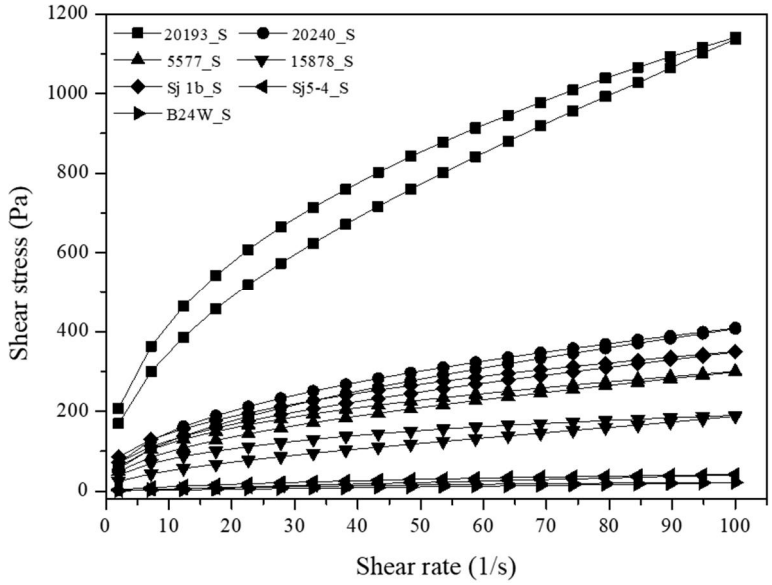

Figure 12. Hysteresis loops of sucrose-enriched fava bean doughs fermented by different strains (Figure 2 in Study **II**). Sample code details can be found in Table 5. B24W_S stands for the sucrose-enriched dough fermented by *L. plantarum* DPPMAB24W.

5.7.4 Dynamic oscillatory rheology

Gel properties were evaluated only in sucrose-enriched doughs since control doughs showed very liquid-like behavior (data not shown). In Study **II**, the elastic modulus (G') increased with rising frequency in all doughs (Figure 13). Two doughs fermented by *Ln. pseudomesenteroides* DSM 20193 (20193_S) and *W. cibaria* Sj 1b (Sj 1b_S), presented considerably higher G' values at any frequency compared to other doughs. The Sj 1b_S dough showed a similar gel elasticity to 20193_S despite containing less dextran, which may indicate superior gel-strengthening capacity for dextran produced by *W. cibaria* Sj 1b. As expected, the EPS-negative control dough (B24W_S) showed the lowest G' values, suggesting a positive effect of dextran on gel strengthening.

In Study **III**, the two sucrose-enriched pastes presented different dynamic rheological profiles (Figure 14). In detail, the paste fermented by *Ln. pseudomesenteroides* DSM 20193 (20193S) showed higher elasticity than that fermented by *W. confusa* VTT E3403, although the latter had a higher dextran content. Furthermore, a lower tan δ (G″/G′) was obtained for 20193S (Table 5 in Study **III**), indicating a more rigid character for this paste.

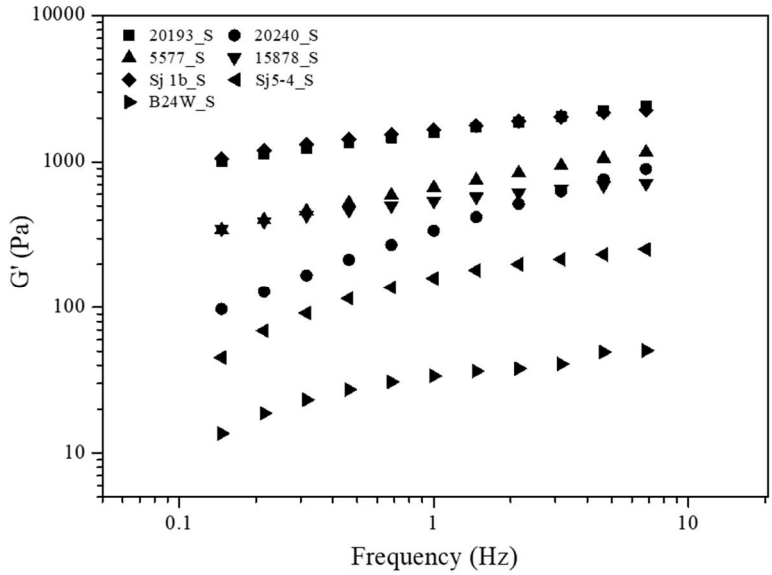

Figure 13. Elastic modulus (G') of sucrose-enriched fava bean doughs with different starters as a function of oscillatory frequency (0.1 – 10 Hz) (Figure 3 in Study **II**). Sample code details can be found in Table 5. B24W_S stands for the sucrose-enriched dough fermented by *L. plantarum* DPPMAB24W.

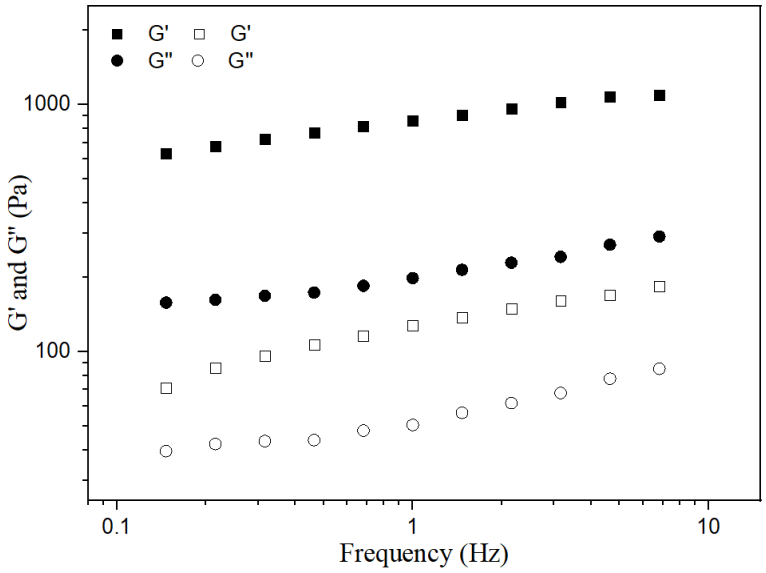

Figure 14. Frequency sweeps of FPC pastes with *in situ* produced EPS. Squares (G') and circles (G") in solid and hollow fill stand for pastes fermented with *Ln. pseudomesenteroides* DSM 20193 and with *W. confusa* VTT E3403, respectively (Figure 2 in Study **III**).

5.8 Textural properties of FPC pastes (Study III)

The textural properties of FPC pastes were analyzed in order to evaluate their macro properties. Four parameters: firmness, consistency, cohesiveness, and index of viscosity for textural evaluation of FPC pastes are shown in Table 17. Compared to control pastes, significantly higher values were observed in sucrose-enriched pastes, especially in the one fermented by *W. confusa* VTT E3403,

corresponding to its highest dextran content (Table 15). This confirms the positive role of dextran in texture modification. No significant difference was observed between control pastes fermented by the two strains for any of the four parameters.

Table 17. Textural properties of FPC pastes fermented by *Ln. pseudomesenteroides* DSM 20193 and *W. confusa* VTT E3403 with or without sucrose addition (Adapted from Table 6 in Study **III**).

Sample code [A]	Firmness (g)	Consistency (g s)	Cohesiveness (g)	Index of viscosity (g s)
20193C	12.18 ± 1.51 [a]	594.09 ± 70.79 [a]	4.77 ± 2.25 [a]	24.27 ± 13.41 [a]
20193S	45.57 ± 0.63 [b]	1131.14 ± 15.16 [b]	27.85 ± 0.19 [b]	766.40 ± 9.10 [b]
3403C	13.16 ± 0.40 [a]	491.57 ± 12.56 [a]	7.08 ± 0.55 [a]	39.32 ± 2.61 [a]
3403S	77.35 ± 5.42 [c]	1734.77 ± 126.93 [c]	34.37 ± 3.79 [c]	1441.36 ± 200.23 [c]

[A] Sample code details can be found in Table 5. [a-c] Values in the same column with different letters are significantly different ($p < 0.05$).

5.9 Proteolysis in FPC pastes (Study III)

The faded protein bands in SDS-PAGE indicated proteolysis in FPC pastes after fermentation (Figure 15). No difference in protein bands was observed between the unfermented sample (lane 1) and the chemically acidified samples (lanes 2 and 5), indicating low protease activity in FPC. Compared to the pastes fermented by *W. confusa* VTT E3403 (lanes 6 and 7), the narrower bands in the pastes fermented by *Ln. pseudomesenteroides* DSM 20193 (lanes 3 and 4) revealed higher extent of proteolysis, which is further confirmed by the lower protein content in these samples (Table 4 in Study **III**).

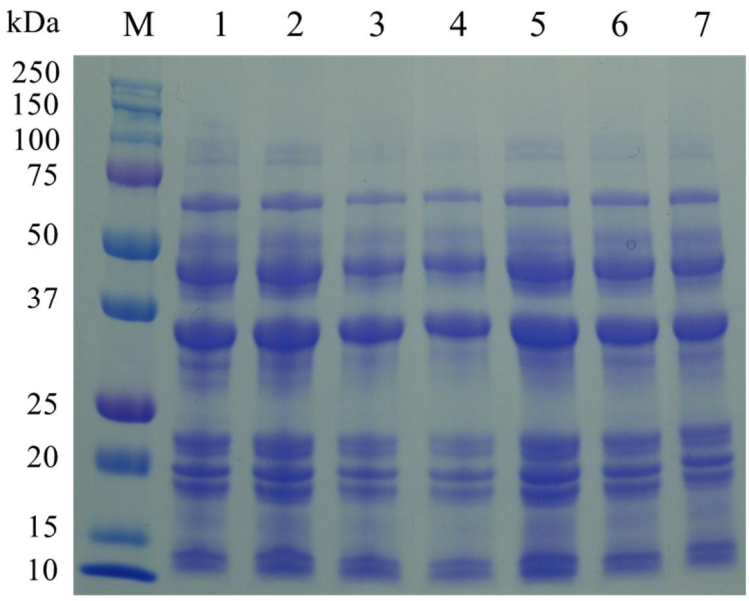

Figure 15. SDS-PAGE of FPC pastes before and after fermentation. M: protein marker; 1: unfermented control (C_0h); 2: chemically acidified control based on the control fermented by *Ln. pseudomesenteroides* DSM 20193 (20193C*); 3: control fermented by *Ln. pseudomesenteroides* DSM 20193 (20193C); 4: sucrose-enriched paste fermented by *Ln. pseudomesenteroides* DSM 20193 (20193S); 5: chemically acidified control based on the control fermented by *W. confusa* VTT E3403 (3403C*); 6: control fermented by *W. confusa* VTT E3403 (3403C); 7: sucrose-enriched paste fermented by *W. confusa* VTT E3403 (3403S) (Figure 1 in Study **III**).

5.10 Microstructure of fava bean protein (Study III)

The microstructure of fava bean protein in FPC pastes before and after fermentation was visualized by CLSM, in which the protein phase showed different structures (Figure 16). In the unfermented paste, proteins were evenly distributed. In fermented pastes, protein aggregates were observed, with larger aggregates present in pastes fermented by *W. confusa* VTT E3403 (Figure 16D and E). In samples with the same starter but differing sucrose content, the aggregates in control pastes (Figure 16B and D) featured a more scattered pattern compared to those in sucrose-enriched pastes (Figure 16C and E), indicating an effect of *in situ* produced dextrans and pH on protein microstructure.

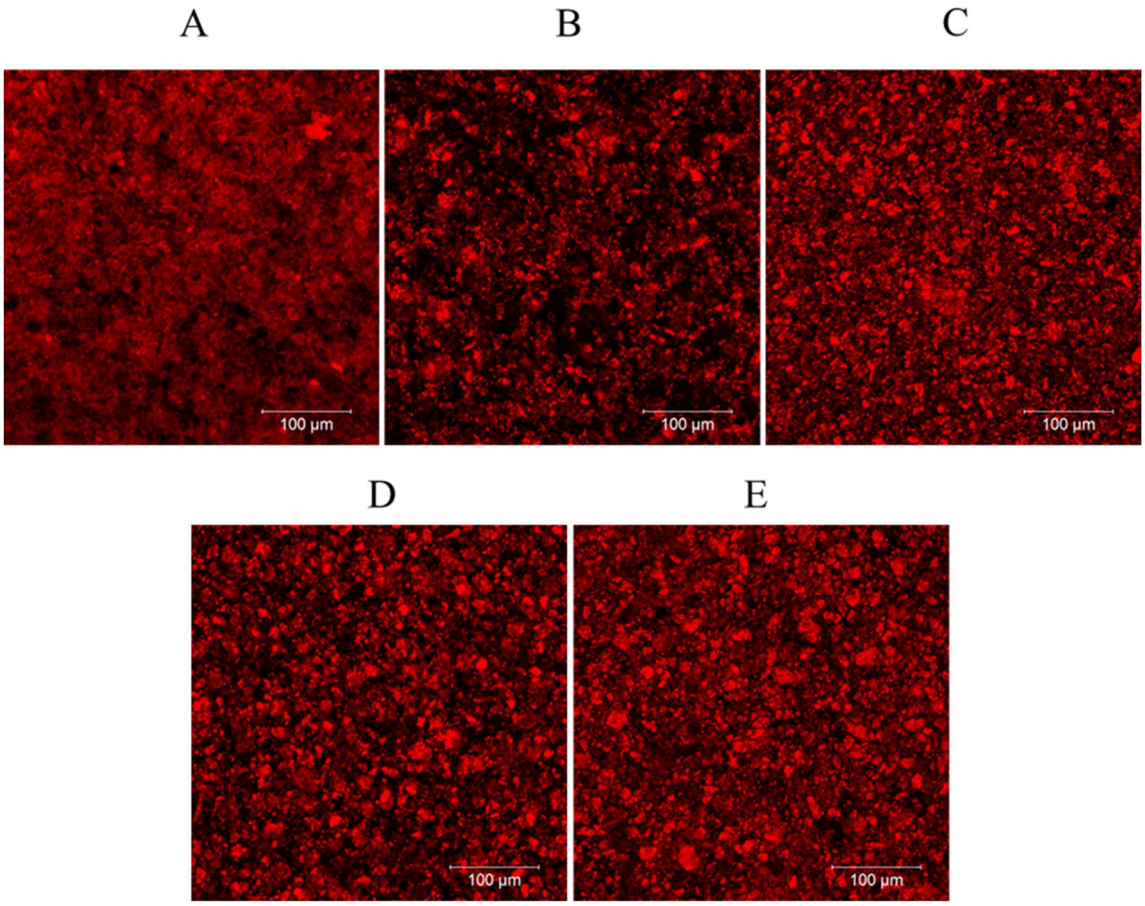

Figure 16. CLSM images of FPC pastes without fermentation (A), fermented by *Ln. pseudomesenteroides* DSM 20193 without (B) or with (C) sucrose addition, and fermented by *W. confusa* VTT E3403 without (D) or with (E) sucrose addition (Figure 3 in Study **III**). Red indicates fava bean protein.

5.11 Structure and macromolecular properties of isolated dextrans (Study IV)

5.11.1 Structure of isolated dextrans

Due to their superior performance in gel strengthening (Figure 13), the water-soluble dextrans from *Ln. pseudomesenteroides* DSM 20193 and *W. cibaria* Sj 1b were isolated from agar plates for structure elucidation. Dextran purity was 92.8 ± 6.1% for that produced by *Ln. pseudomesenteroides* DSM 20193 and was 81.9 ± 3.9% for the dextran produced by *W. cibaria* Sj 1b. According to NMR spectroscopy (Figure 17A), the two isolated dextrans showed typical dextran structure similar to the commercial dextran produced by *Ln. mesenteroides* B512F (Maina et al., 2008). The presence of α-(1→6) linkage was revealed by the peak around 4.98 ppm, and the presence of α-(1→3) branching by the peak around 5.32 ppm (Maina et al., 2008). The branching

degree was 5.8% for DX_LP and 4.1% for DX_WC, as determined by the relative intensities of the ^{1}H anomeric signals.

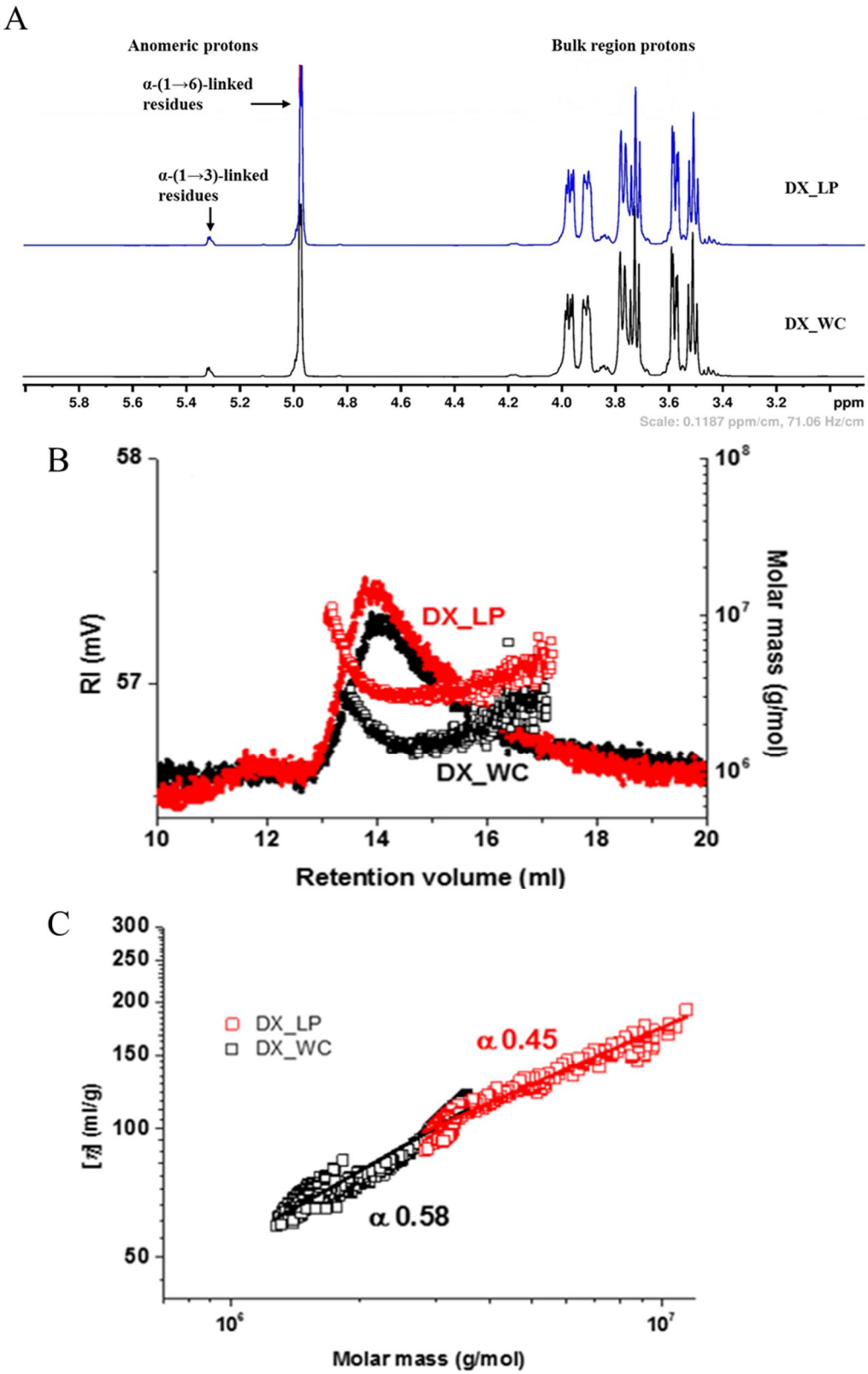

Figure 17. The 1D ^{1}H spectra (A), molar mass distributions (B), and Mark-Houwink plots (C) of dextrans from *Ln. pseudomesenteroides* DSM 20193 (DX_LP) and *W. cibaria* Sj 1b (DX_WC). Squares represent molar mass and lines represent detector signals (B) (Figure 2 in Study **IV**).

5.11.2 Macromolecular properties of isolated dextrans

Molar mass distributions of the two dextrans were analyzed by SEC, presenting similar hydrodynamic sizes based on their overlapped chromatograms (Figure 17B). The initial small peak in DX_LP might indicate the presence of aggregates in the solution. The two dextrans differed in molar masses across the peaks, with DX_LP presenting higher molar mass than DX_WC. As they possessed similar hydrodynamic sizes, the higher molar mass of DX_LP indicated higher molecular density. This density difference was also reflected in the Mark-Houwink plots (Figure 17C), which plotted intrinsic viscosity ([η]) against molar mass. The plot slope (α) was lower for DX_LP than for DX_WC, indicating a difference between the two dextrans in solution conformation (Pitkänen, Virkki, Tenkanen, & Tuomainen, 2009). This might be due to the number of dextran side-chains, since DX_LP contains more branches than DX_WC. The branches in DX_LP might be also longer, as indicated by its higher molecular density.

5.12 Formation of FPI/DX conjugates (Study III)

FPI was extracted from fava bean flour by isoelectronic precipitation method, and its chemical composition was 92.0 ± 4.1% protein, 1.5 ± 0.0% carbohydrate, 6.6 ± 0.3% water, 1.3 ± 0.0% lipid, and 4.8 ± 0.0% ash. After dry-heating with dextran, the presence of FPI/DX conjugate was confirmed by SDS-PAGE with Coomassie brilliant blue staining (Figure 18A) and PAS staining (Figure 18B). Under Coomassie staining, the characteristic bands of fava bean protein faded in lanes 3 and 5 compared to FPI/DX mixtures (lanes 2 and 4). The appearance of the broad bands at the top of lanes 3 and 5 indicate the formation of compounds with high molar mass (Liu, Zhao, Zhao, Ren, & Yang, 2012). The featured pink bands appeared at the top of the stacking and separating gel indicate the presence of glycoproteins only in lanes 3 and 5 (Figure 18B). This is corresponding to the broad bands appeared in Figure 18A, altogether confirming the formation of FPI/DX conjugate.

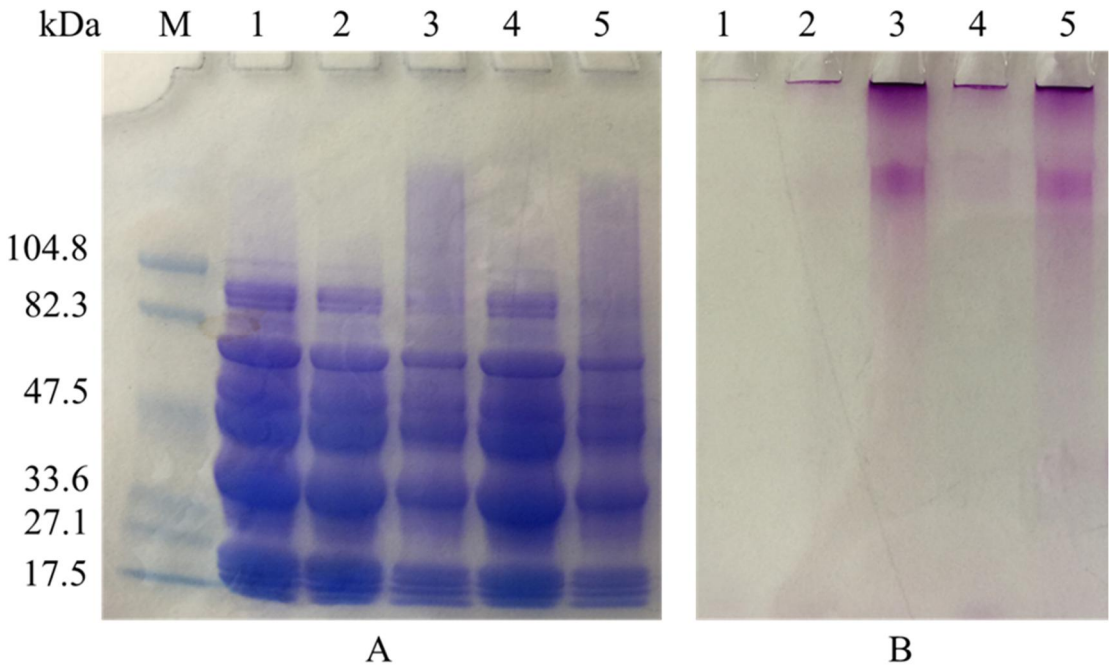

Figure 18. SDS-PAGE of protein marker (M), FPI (1), FPI/DX mixtures: FPI/LP_M (2) and FPI/WC_M (4), and FPI/DX conjugates: FPI/LP_C (3) and FPI/WC_C (5). A: Coomassie Brilliant Blue stain; B: Periodic acid-Schiff (PAS) stain (Figure 3 in Study **IV**).

5.13 Rheological properties of FPI/DX mixtures and conjugates (Study IV)

5.13.1 Viscosity and hysteresis loop of FPI/DX mixtures and conjugates in water

Consistent with the thickening properties of EPS observed in Studies **I-III**, the addition of dextran in Study **IV** considerably increased the viscosity of FPI (Table 18). For the same dextran, no significant difference in viscosity was found between the mixture and the conjugate, suggesting little influence of the Maillard reaction on viscosity. No hysteresis loop was observed in either FPI or dextran solution. However, large hysteresis loops were observed in FPI/DX mixtures and conjugates, but with no significant differences between mixtures and conjugates with the same dextran.

Heat treatment increased viscosity of the FPI suspension, mainly due to the thickening effect caused by heat-induced gelation of fava bean protein (Table 18). Viscosity increases were also found in FPI/DX mixtures and conjugates after heat treatment, with higher increases in mixed systems, indicating the importance of FPI-dextran intermolecular interactions in viscosity improvement. The viscosity of FPI/LP_C did not significantly increase after heating, indicating a greater effect from the covalent bonds formed. This differed from FPI/WC_C, which presented a significantly higher

viscosity after heat treatment. A hysteresis loop was observed for heat-treated FPI, which had the lowest loop area compared to other dextran-added samples. Higher loop areas were obtained for FPI/DX mixtures than for FPI/DX conjugates after heating, especially in the system with dextran from *Ln. pseudomesenteroides* DSM 20193, agreeing with the phenomenon observed in viscosity values.

Table 18. The viscosity, hysteresis loop area, G' and tan δ of FPI solution, dextran solutions from *Ln. pseudomesenteroides* DSM 20193 (DX_LP) and *W. cibaria* Sj 1b (DX_WC), and FPI/DX mixtures and conjugates with or without heat treatment (Adapted from Table 3 in Study **IV**).

Sample code [A]	Viscosity [B] (Pa s)	Loop area (10^4 Pa/s)	G′ [C] (Pa)	tan δ
FPI	0.11 ± 0.01 [a]	ns [D]	0.82 ± 0.51 [a]	2.96 ± 1.99 [a]
FPI_H	1.18 ± 0.14 [a]	0.75 ± 0.09 [a]	133.15 ± 31.33 [b, c]	0.44 ± 0.06 [a]
DX_LP	2.34 ± 0.03 [a]	ns	11.09 ± 0.26 [a]	1.74 ± 0.06 [a]
FPI/LP_M	11.10 ± 0.56 [b]	6.79 ± 0.41 [b]	105.61 ± 4.56 [b, d]	1.40 ± 0.03 [a]
FPI/LP_M_H [E]	16.17 ± 1.43 [c]	9.96 ± 0.85 [c]	199.43 ± 2.24 [c, e]	1.14 ± 0.01 [a]
FPI/LP_C	11.07 ± 1.32 [b]	6.78 ± 0.89 [b]	130.88 ± 8.99 [b, c]	1.11 ± 0.02 [a]
FPI/LP_C_H	13.83 ± 0.14 [b, c]	8.27 ± 0.04 [b, c]	205.65 ± 32.90 [c, e]	0.84 ± 0.11 [a]
DX_WC	2.26 ± 0.12 [a]	ns	28.37 ± 1.87 [a, d]	1.48 ± 0.01 [a]
FPI/WC_M	10.34 ± 0.01 [b]	6.62 ± 0.01 [b]	238.10 ± 5.30 [e]	1.03 ± 0.06 [a]
FPI/WC_M_H	16.81 ± 0.23 [c]	10.66 ± 0.20 [c]	1045.74 ± 11.03 [f]	0.59 ± 0.07 [a]
FPI/WC_C	10.94 ± 0.81 [b]	6.97 ± 0.54 [b]	177.24 ± 6.07 [b, c, e]	0.96 ± 0.02 [a]
FPI/WC_C_H	16.57 ± 0.93 [c]	10.46 ± 0.61 [c]	700.51 ± 21.81 [g]	0.46 ± 0.02 [a]

[A] Sample code details can be found in Table 6. [B] Values were taken at the shear rate of 100 1/s. [C] Values were taken at the frequency of 1.0 Hz. [D] Not shown. [E] H means heat treatment. [a-g] Values in the same column with different letters are significantly different ($p < 0.05$).

5.13.2 Dynamic oscillatory rheology of FPI/DX mixtures and conjugates in water

As important properties for proteins with gelling abilities, the dynamic rheological properties of FPI/DX mixtures and conjugates with or without heat treatment were evaluated. Before heat treatment, mixtures and conjugates with the same dextran showed similar G′ values, without significant difference (Table 18). The FPI suspension showed very low gel elasticity before heating. However, heat treatment induced the gelation of FPI, with the G′ value increasing from 0.82 to 133.15 Pa. A significantly higher G′ was obtained with heat treatment in FPI/LP_M, but not in FPI/LP_C. In contrast, the mixture and the conjugate with *W. cibaria* Sj 1b dextran (FPI/WC_M_H

and FPI/WC_C_H) both presented significantly higher G′ values after heating, especially for the mixture. Heat treatment also changed the dependence of G′ on frequency, with lower dependence found in FPI/WC_M_H and FPI/WC_C_H compared to the corresponding samples with *Ln. pseudomesenteroides* DSM 20193 dextran (Figure 19). Formation of covalent bonds decreased the gel elasticity in the system with *W. cibaria* Sj 1b dextran, as demonstrated by the significantly lower G' value in FPI/WC_C_H than in FPI/WC_M_H. However, in the system with *Ln. pseudomesenteroides* DSM 20193 dextran, formation of the covalent bonds had no evident effect on gel elasticity. As an indication of relative viscoelasticity, tan δ also suggested different effects for the two dextrans on fava bean protein gelation (Table 18). Generally, lower tan δ values were found in systems with *W. cibaria* Sj 1b dextran after heat treatment, compared to systems with *Ln. pseudomesenteroides* DSM 20193 dextran.

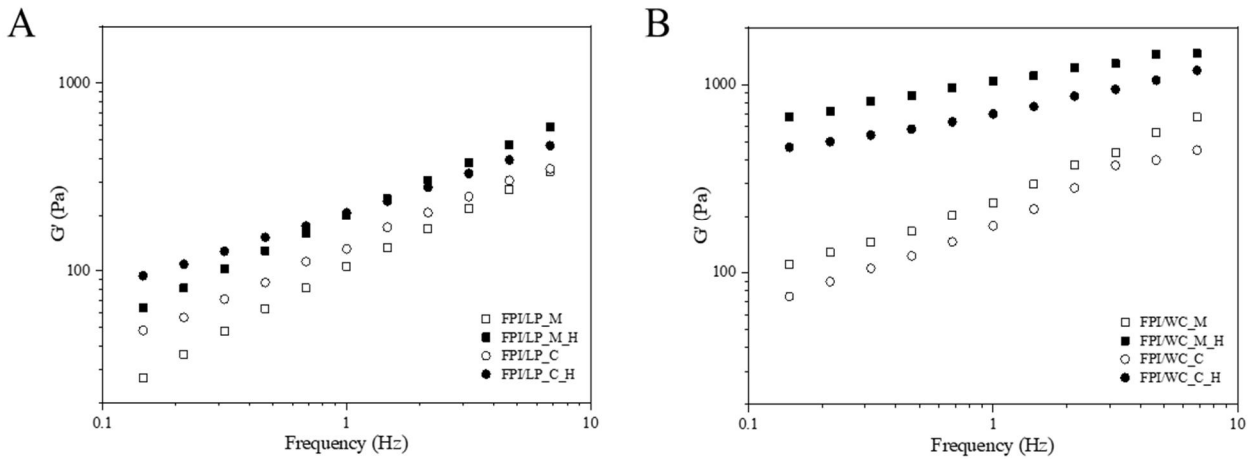

Figure 19. Frequency sweeps of FPI/LP mixtures and conjugates without (FPI/LP_M, FPI/LP_C) or with heat treatment (FPI/LP_M_H, FPI/LP_C_H) (A) and FPI/WC mixtures and conjugates without (FPI/WC_M, FPI/WC_C) or with heat treatment (FPI/WC_M_H, FPI/WC_C_H) (B) (Adapted from Figure 4 in Study **IV**).

5.13.3 Viscosity and hysteresis loop of FPI/DX mixtures at different pH

During LAB fermentation, dextrans are produced, together with pH decrease. Therefore, the effects of pH on the rheological properties of FPI/DX mixtures were studied in order to understand the role of dextran in structure maintenance of fermented food. The pH was found to considerably affect the FPI viscosity. In very acidic buffer, the FPI solution became inhomogeneous; therefore, the viscosity, hysteresis loop areas, G', and tan δ values of FPI_4 and FPI_3 are not shown (Table 19). The thickening properties of dextran was observed in all mixtures at different pH values, with the FPI/WC system showing more stable viscosities, also reflected in its viscosity flow curves (Figure 20). Similarly, pH had less effect on the hysteresis loop areas for the system with *W. cibaria* Sj 1b dextran compared to the system with *Ln. pseudomesenteroides* DSM 20193 dextran.

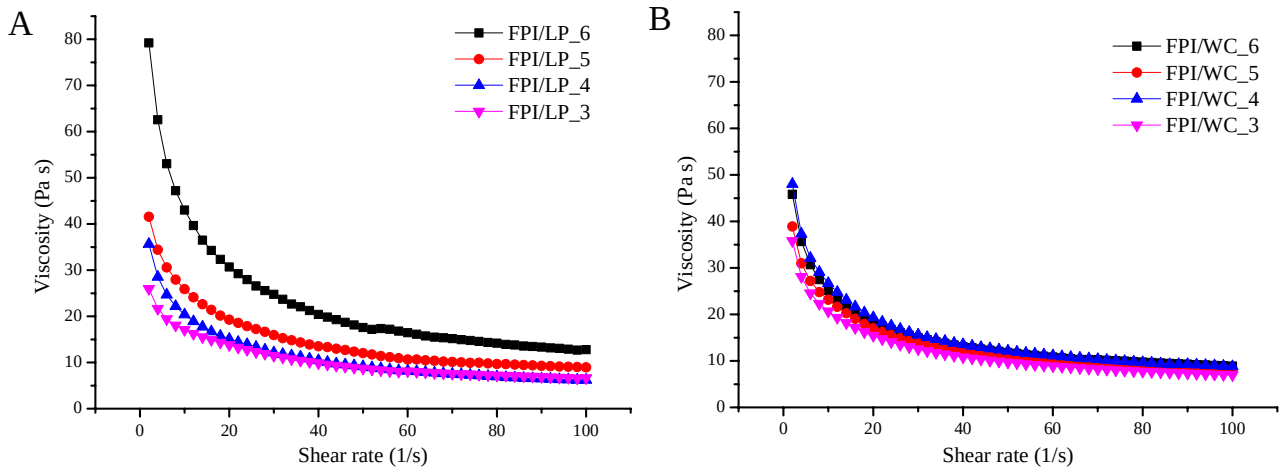

Figure 20. Viscosity flow curves of FPI/DX mixtures at different pH values. FPI/LP and FPI/WC stand for mixtures of FPI and dextran from *Ln. pseudomesenteroides* DSM 20193 (A) and *W. cibaria* Sj 1b (B), respectively; numbers at the end indicate the pH (Figure S6 in Study **IV**).

Table 19. Viscosity, hysteresis loop area, G', and tan δ of FPI and FPI/DX mixtures at different pH values (6.0, 5.0, 4.0, 3.0) (Table 4 in Study **IV**).

Sample name [A]	Viscosity [B] (Pa s)	Loop area (10^4 Pa/s)	G' [C] (Pa)	tan δ
FPI_6	0.12 ± 0.00 [a]	ns [D]	13.65 ± 1.16 [a]	ns
FPI_5	0.11 ± 0.02 [a]	ns	22.35 ± 4.16 [a]	ns
FPI_4	ns	ns	ns	ns
FPI_3	ns	ns	ns	ns
FPI/LP_6	12.78 ± 0.07 [b]	7.92 ± 0.18 [a]	151.04 ± 17.11 [b, d]	1.19 ± 0.01 [a, b, c]
FPI/LP_5	8.94 ± 0.72 [c]	5.45 ± 0.36 [b]	109.47 ± 8.84 [b, c]	1.32 ± 0.05 [a, b]
FPI/LP_4	6.23 ± 0.10 [d]	3.93 ± 0.02 [c]	111.35 ± 8.07 [b, c]	1.19 ± 0.00 [a, c]
FPI/LP_3	6.65 ± 0.97 [d]	3.92 ± 0.57 [c]	90.31 ± 3.62 [c]	1.35 ± 0.05 [b]
FPI/WC_6	8.88 ± 0.08 [c]	5.66 ± 0.06 [b]	163.32 ± 2.36 [d]	1.11 ± 0.02 [c]
FPI/WC_5	8.19 ± 0.25 [c, d]	5.02 ± 0.25 [b, c]	128.59 ± 4.12 [b, c, d]	1.24 ± 0.01 [a, b, c]
FPI/WC_4	8.80 ± 0.14 [c]	5.48 ± 0.12 [b]	148.53 ± 11.33 [b, d]	1.18 ± 0.00 [a, c]
FPI/WC_3	6.97 ± 0.02 [c, d]	4.29 ± 0.03 [b, c]	134.65 ± 7.12 [b, c, d]	1.18 ± 0.02 [a, c]

[A] FPI/LP and FPI/WC stand for the mixtures of FPI and dextran from *Ln. pseudomesenteroides* DSM 20193 and *W. cibaria* Sj 1b, respectively; numbers at the end indicate the pH. [B] Values were taken at the shear rate of 100 1/s. [C] Values were taken at the frequency of 1.0 Hz. [D] Not shown. [a-d] Values in the same column with different letters are significantly different ($p < 0.05$).

5.13.4 Dynamic oscillatory rheology of FPI/DX mixtures at different pH

FPI suspensions had significantly lower gel elasticity compared to other samples with added dextran (Table 19). In the FPI/LP system, gel elasticity varied with pH, and the lowest value was obtained for FPI/LP_3. In contrast, the gel elasticity of the FPI/WC system remained more stable, indicating a stronger stabilizing capacity for *W. cibaria* Sj 1b dextran. Generally, at any given pH, a lower G′ value was found in the FPI/LP system. The tan δ values of the FPI/LP and FPI/WC systems were not significantly different at any given pH, with the only exception at pH 3.0.

6 Discussion

6.1 The growth of LAB and resulting acidification in fava bean matrix

Fava bean flour and FPC are both good media for growing the LAB strains used in this thesis. The final cell density of LAB increased at least 2.3 log cycles after fermentation, reaching values over 8.5 log cfu/g. Sucrose addition showed little influence on the propagation of LAB, but had an obvious effect on acidification of the fermented pastes, especially when fermented by *Leuconostoc* spp. Generally, with sucrose addition, pastes fermented by *Leuconostoc* spp. were more acidic (lower pH and higher TTA values) compared to those fermented by *Weissella* spp. This lesser acidification capacity of *Weissella* spp. has also been observed in other food matrices such as carrot puree and wheat sourdough (Juvonen et al., 2015; Katina et al., 2009).

Compared to *Weissella* spp., *Leuconostoc* spp. had greater ability to synthesize acetic acid from added sucrose, leading to lower FQ values. This difference is probably attributable to different metabolic pathways for the fructose released from sucrose during dextran synthesis. According to a previous report (Wisselink, Weusthuis, Eggink, Hugenholtz, & Grobben, 2002), *Leuconostoc* spp. are able to reduce the released fructose to mannitol by mannitol dehydrogenase, contributing to acetic acid formation. However, *Weissella* spp. normally do not reduce fructose to mannitol, leading to lower acetic acid production (Galle et al., 2010; Kajala et al., 2015). The higher residual fructose in pastes fermented by *Weissella* spp. and the absence of mannitol in pastes with *W. confusa* Sj5-4 and *W. confusa* VTT E3403 in Studies **II** and **III** confirmed this difference (Table 12-15). Unlike the results of previous studies (Galle et al., 2010; Kajala et al., 2015), mannitol production was obsreved in two *Weissella* spp. (*W. cibaria* DSM 15878 and *W. cibaria* Sj 1b), which might suggest different metabolic pathways for fructose or mannitol in these two strains. Limited literature is available on mannitol production in *Weissella* spp. Furthermore, mannitol production is medium-dependent, as *Ln. pseudomesenteroides* DSM 20193 produced little mannitol in fava bean flour even with sucrose addition. However, this strain produced much more mannitol from added sucrose in FPC (Table 15). This might be due to differing availability of carbon sources in different matrices, which affects the conversion efficiency of fructose to mannitol (Wisselink et al., 2002).

6.2 Sucrose addition facilitated EPS production in fava bean matrix

In Study **I**, differing amounts of sucrose were added to study the effect of sucrose concentration on EPS production. For *Ln. mesenteroides* DSM 20343, both glucan and fructan were synthesized with added sucrose due to the presence of GS and FS (Côté & Skory, 2012; Olvera et al., 2007). Glucan and fructan concentrations increased to different extents with rising sucrose concentration. EPS were produced more efficiently at lower sucrose concentrations, indicating a possible inhibition of DSR and LSR by high sucrose concentration (Belghith, Dahech, Belghith, & Mejdoub, 2012; Shukla & Goyal, 2011). According to Côté and Skory (2012), *Ln. mesenteroides* DSM 20343 is able to produce insoluble glucans, which was also confirmed by the observed difference in glucan and dextran contents. With more sucrose added, more insoluble glucans were formed. This might be explained by the synthesis mechanism of insoluble glucans, which were formed from soluble dextrans (Côté & Skory, 2015). The higher glucan contents also suggest that *Ln. mesenteroides* DSM 20343 preferentially produces glucan rather than fructran when sucrose is available for EPS synthesis (Table 13).

In Studies **II** and **III**, only glucan was produced as FS were not active in any of the strains used. This inactivity was confirmed by an initial screening on MRS agar plates supplemented with raffinose, which is a substrate for FS but not for GS. These studies also demonstrated a promoting effect for added sucrose on glucan production. Two strains, *Ln. pseudomesenteroides* DSM 20193 and *W. confusa* VTT E3403, were identified as promising dextran producers in fava bean flour and FPC due to their ability to efficiently use sucrose for dextran synthesis. In particular, the dextran-producing ability of *Ln. pseudomesenteroides* DSM 20193 was not affected by the matrix used, as in fava bean flour this strain produced 3.67% dextran from 10% sucrose, and in FPC it produced 1.71% dextran from 5% sucrose (Table 14 and 15).

6.3 Sucrose utilization in fava bean matrix

The depletion of sucrose and the low content of EPS in all pastes without sucrose addition suggests that the added sucrose was preferentially utilized for microbial growth during the exponential phase (Plante & Shriver, 1998), and then for EPS synthesis during the stationary phase, releasing free fructose (Han et al., 2014). In *Leuconostoc* spp., the released fructose was generally further reduced to mannitol, but not in *Weissella* spp. (Galle et al., 2010), for which fructose was accumulated after fermentation. Therefore, in assuming that the released fructose was not used for microbial growth, the total residual fructose and mannitol should be roughly equal to the amount of glucan formed.

However, in Studies **I-III**, the glucan contents obtained were generally lower than the theoretical values. Possible reasons could be the utilization of sucrose-liberated glucose for the growth of microorganisms, e.g. the starter LAB and other potential microorganisms in fava bean flour, and the formation of GOS by GS through the acceptor reaction. During fructan synthesis, glucose is liberated, and in theory, the amount of fructan should equal the residual amount of glucose. However, in Study **I**, fructan contents were higher than the residual glucose due to possible utilization of the released glucose by microbes. Therefore, based on Studies **I-III**, we can conclude that in a complex system like a food matrix, sugar balance is difficult to achieve due to several overlapping metabolic events during fermentation. Other researchers who used carrot puree as the medium for producing EPS (Juvonen et al., 2015) also reported difficulty in achieving sugar balance.

6.4 Two degradation ways for RFO in fava bean flour during fermentation

Although many LAB have shown the capability to produce α-galactosidase (Duszkiewicz-Reinhard, Gujska, & Khan, 1994; Songré-Ouattara et al., 2008; Yoon & Hwang, 2008), microbial α-galactosidase activity was not detected in the strains used in Studies **I** and **II.** According to Dey and Pridham (1969), fava bean seeds contain two forms of α-galactosidase, with the optimum pH from 4.0 to 5.0. This further supports the endogenous α-galactosidase activity observed in this thesis, which was involved in the degradation of RFO, affecting the activity of LSR from *Ln. mesenteroides* DSM 20343. In the presence of α-galactosidase and a low concentration of the inducer raffinose (Teixeira, McNeill, & Gänzle, 2012), LSR was not expressed or activated. In the absence of endogenous α-galactosidase, LSR was expressed even under low raffinose concentration, as shown by the appearance of manninotriose and manninotetraose in the sample made with autoclaved flour (Figure 6). When raffinose concentration was high enough, α-galactosidase and LSR acted together on RFO, releasing galactose, melibiose, manninotriose, and manninotetraose. In Study **II,** RFO were mainly degraded by the endogenous α-galactosidase in fava bean flour due to the absence of LSR in the six strains used. *Ln. pseudomesenteroides* DSM 20193 was found to be able to use galactose and stachyose as carbon sources in both fava bean flour (Table 3 in Study **II**) and FPC (Table 3 in Study **III**), especially when only endogenous sucrose was available. With externally added sucrose, this strain preferentially used sucrose, and the RFO were less degraded (Table 12). This is probably because sucrose-derived glucose or fructose is a preferable carbon source for this strain, compared to galactose and RFO. Different strains showed various capabilities

in hydrolyzing RFO; this is attributable to many factors such as the expression of LSR, carbon source preference, and pH of the system during fermentation. Teixeira et al. (2012) reported that in *Lactobacillus* spp., LSR, sucrose phosphorylase, and α-galactosidase are the main enzymes contributing to the degradation of RFO. A similar function for LSR was identified in this thesis, and for the first time, the role of endogenous α-galactosidase in RFO degradation and its effects on LSR expression were elucidated.

6.5 The effects of EPS on rheological properties of fava bean pastes

The produced EPS changed the rheological properties of fava bean pastes to different extents, depending on the sucrose concentration, the starter, and the fermentation medium. In this thesis, a clear viscosity increase was observed after fermentation in most sucrose-enriched pastes due to the formation of EPS acting as thickening agents (Galle & Arendt, 2014). The *in situ* synthesized dextran, rather than levan, played a major role in viscosity improvement, mainly due to the low molar mass of levan produced by *Ln. mesenteroides* DSM 20343 (Olvera et al., 2007). For fava bean flour, the sucrose-enriched dough fermented by *Ln. pseudomesenteroides* DSM 20193 showed the highest viscosity increase (Table 16). For FPC, the sucrose-enriched paste fermented by this strain also showed high viscosity (Figure 10), suggesting minor influence of fermentation medium on the thickening ability of dextran produced by this strain. Greater influence of the fermentation medium on thickening ability was observed for dextran produced by *W. cibaria* Sj 1b, with higher viscosity found in fava bean flour (Table 16) and lower viscosity in FPC (data not shown). This highlights the importance of EPS-producer selection for a desired rheological property in a certain medium, further customizing the fermentation of fava bean matrix. The hysteresis loop reveals the structural reversibility of the viscoelastic materials (Gambuś, Gumul, & Juszczak, 2004), and was observed in EPS-positive pastes. With sucrose addition, larger loops were found after fermentation, suggesting better structural reversibility (Purwandari et al., 2007). Generally, the viscosity increase showed a positive correlation with EPS content, with high values obtained at high EPS contents (Studies **I**-**III**). The only exception was the sucrose-enriched dough fermented by *W. confusa* Sj5-4, which showed little viscosity increase (Table 16), but contained a large amount of dextran (Table 14). This might be due to the low molar mass of dextran produced by this strain, since low molar mass results in low viscosity (Kasaai, 2012). Another possibility is that the dextran produced by this strain is highly branched, contributing little to viscosity increase.

The dynamic oscillatory rheology was evaluated for all pastes in order to observe the influence of EPS on viscoelastic properties. After fermentation, most samples with *in situ* produced EPS showed

solid-like behavior, demonstrating the gel-strengthening ability of EPS in fava bean matrix. It is known that dextran itself cannot form a gel structure in water solution (McCurdy et al., 1994). However, in the presence of protein, e.g. fava bean protein, these two polymers interact with each other, resulting in changed viscoelastic behavior for polysaccharide/protein systems (Doublier, Garnier, Renard, & Sanchez, 2000; Mounsey & O'Riordan, 2008). Notably, the dough fermented by *W. confusa* Sj5-4 showed typical liquid-like behavior even with high dextran production, revealing the effect of dextran molar mass or structure on its gel-strengthening ability. This effect was further corroborated by the superior gel elasticity of the sucrose-enriched dough fermented by *W. cibaria* Sj 1b, which did not contain the highest amount of dextran. In addition, the FPC paste fermented by *Ln. pseudomesenteroides* DSM 20193 showed higher gel stiffness (higher G′ and lower tan δ) at a lower dextran concentration compared to the corresponding paste fermented by *W. confusa* VTT E3403. These three phenomena altogether suggest the dependence of gel stiffness on dextran molecular properties (structure, molar mass, and spatial conformation), which is also confirmed later in Study **IV**.

6.6 The *in situ* produced EPS modified the texture of FPC pastes

In order to evaluate the role of EPS in texture modification in a protein-rich system, the textural properties of FPC pastes with or without sucrose addition were analyzed after fermentation. Sucrose-enriched pastes demonstrated significantly increased firmness, consistency, cohesiveness, and index of viscosity, especially the one fermented by *W. confusa* VTT E3403, confirming a positive role for EPS in texture modification. Generally, an improved texture, e.g. high viscosity, high firmness, and low syneresis, is often observed after the synthesis of EPS in protein-rich foods (Guzel-Seydim, Sezgin, & Seydim, 2005; Han et al., 2016). The reason behind this is, as a hydrocolloid, EPS are able to absorb water and interact with proteins in food systems, leading to increased water-holding capacity and improved textural properties (Ruas-Madiedo et al., 2002). The presence of EPS can also affect the integrity of the protein matrix, with EPS randomly filling holes in the protein network (Hassan, Frank, & Qvist, 2002; Hassan, Ipsen, Janzen, & Qvist, 2003; Li et al., 2014). This may further cause phase separation, affecting the structure of the polysaccharide/protein systems and leading to a difference in textural properties (Döring, Nuber, Stukenborg, Jekle, & Becker, 2015; Hassan et al., 2003; Solowiej et al., 2015).

In this thesis, *W. confusa* VTT E3403 showed high capability for dextran production and texture modification, agreeing with the results reported by Wang et al. (2018). They also reported improved bread volume and reduced crumb hardness in wheat bread after the incorporation of fava bean

sourdough containing dextran produced by *W. confusa* VTT E3403. This indicates a potential application for this strain in sourdough or gluten-free bread, which can improve bread texture with *in situ* produced dextran. Furthermore, as shown in Study **III** and in Wang et al. (2018), this strain has low acidifying capacity, producing fermented foods with mild flavor that are more acceptable to consumers.

6.7 Proteolysis is weak in FPC pastes

Besides the produced EPS during fermentation, fava bean protein, as a main component of FPC, may also contribute to the texture of FPC pastes. Since proteolysis is a common phenomenon during LAB fermentation (Kunji, Mierau, Hagting, Poolman, & Konings, 1996) and was crucial to the texture of the fermented food (Gänzle, Loponen, & Gobbetti, 2008), the proteolysis of fava bean protein was evaluated in Study **III**. As measured by the Bradford method, a decrease in protein content of fermented pastes was noticed (Table 4 in Study **III**). In particular, lower protein content was found in pastes fermented by *Ln. pseudomesenteroides* DSM 20193, indicating a greater degree of proteolysis than with *W. confusa* VTT E3403 fermentation. The amine content and the free amino nitrogen (FAN) were additionally analyzed as extra parameters for proteolysis evaluation. The amine contents revealed that *Ln. pseudomesenteroides* DSM 20193 may use the released peptides for growth, and the FAN values suggested utilization of free amino acids by both strains. Consistent with the decreased protein content previously mentioned, SDS-PAGE showed faded protein bands in fermented samples. The slightly narrower protein bands in samples fermented by *Ln. pseudomesenteroides* DSM 20193, together with their lower protein contents, suggest higher proteolytic activity in this strain. According to a previous experiment, proteinase activity in FPC is weak; the highest activity was found at a pH of 4.5 (data not shown). Taken together, it can be concluded that proteolysis is weak in FPC pastes after fermentation. Therefore, fava bean protein is not a primary determiner of the changes in rheological or textural properties of FPC pastes after fermentation, emphasizing a positive effect of EPS on texture modification.

6.8 LAB fermentation and EPS production changed the microstructure of fava bean protein

Due to the modified texture of sucrose-enriched FPC pastes after fermentation, changes in the protein network after EPS formation were hypothesized. Then, observation of the microstructure of fava bean protein in unfermented and fermented pastes confirmed clear differences in protein networks. The presence of protein aggregates in fermented pastes (Figure 16) was mainly due to

low pH, which could affect the protein network, forming aggregates (Arogundade et al., 2006). The more concentrated protein phase in sucrose-added pastes clearly indicated an effect for EPS in protein network arrangement, likely through intermolecular interactions. The difference in protein structure between fermented samples with or without sucrose addition was mainly due to the produced EPS filling pores in the protein network (Hassan et al., 2002; Hassan et al., 2003). With more EPS in the network, the protein phase became more concentrated. Due to exclusion effects, the combination of EPS and fava bean protein normally resulted in a less homogenous protein network, which could also be seen in the protein microstructure of fermented pastes (Figure 16). The larger protein aggregates found in pastes fermented by *W. confusa* VTT E3403 indicated a possible effect of dextran structure or molar mass on the protein network. In addition, the pH values of the two samples fermented by *Ln. pseudomesenteroides* DSM 20193 were closer to the isoelectric point of fava bean protein (Arogundade et al., 2006), but greater protein aggregation was not observed. This further supports an effect of dextran molecular properties on the protein network. In order to confirm this speculation, further work is needed to elucidate the structure of *in situ* produced EPS and the effects of their molecular properties on EPS-protein interactions. In this thesis, the difference in rheological and textural properties between EPS-abundant and EPS-insufficient pastes was illustrated by their protein microstructure, which was not characterized for fava bean protein.

6.9 Interactions between dextran and fava bean protein

In Studies **I-III**, the positive effects of EPS on texture modification were observed, attributable to the commonly-known EPS-protein interactions (Doublier et al., 2000). Previous studies have documented interactions between dextran and whey protein (Spotti et al., 2014a, 2014b; Sun et al., 2011) and peanut protein (Liu et al., 2012), but no study has explored dextran-legume protein interactions. Furthermore, due to the good performance of the dextran produced by *Ln. pseudomesenteroides* DSM 20193 in viscosity improvement, and the surprisingly high ability of *W. cibaria* Sj 1b dextran in gel strengthening, these two strains were selected as dextran producers. Then, interactions between FPI and purified dextrans were studied in order to understand the mechanism underlying the improvement of rheological properties after dextran formation in the fava bean protein system. The interactions between dextran and FPI are either intermolecular, resulting from mixing the polymers together, or intramolecular, resulting from the Maillard reaction between the polymers. Formation of the conjugate of dextran and FPI accounted for 6.6% to 7.2% of the total protein (Table 3 in Study **IV**). This low glycosylation level was due to the high molar mass (10^6 Da) of dextrans used (Table 2 in Study **IV**), as polysaccharides with lower molar mass

have easier access to amine groups, leading to a higher propensity to the Maillard reaction (Spotti et al., 2014a).

6.9.1 Dextran-FPI interactions in water system

The thickening ability of dextran was also observed in a simplified system containing only dextran, FPI, and water. The viscosities of FPI/DX mixtures were much higher than the sum viscosity of dextran and FPI solutions, indicating a contribution of FPI/DX interactions to viscosity improvement. In systems incorporating the same dextran, changes in interaction (Maillard reaction) between FPI and dextran had no obvious influence on viscosity or hysteresis loop area (Table 18). This might be due to the low incidence of the Maillard reaction, which was not sufficient to make a visible difference in the rheological properties of the system. Neither FPI solution nor dextran solution formed the hysteresis loop that is a typical characteristic of viscoelastic materials and an indication of structural reversibility (Purwandari et al., 2007). Hysteresis loops were only formed after the addition of dextran to the FPI solution, suggesting a structural change in the protein system due to FPI/DX interactions; this was also supported by the microstructure of fava bean protein in Study **III** (Figure 16).

Since heat treatment is an important processing method in the food industry, the FPI/DX mixtures and conjugates in water system were all heated in order to observe the effect of dextran on protein gelation. Viscosity increases were observed in all heated samples, mainly due to the thickening effect caused by protein gelation. The system with dextran from *Ln. pseudomesenteroides* DSM 20193 was more sensitive to interaction changes compared to the system with dextran from *W. cibaria* Sj 1b, which might be due to its higher molar mass and molecular density (Figure 17). The importance of intermolecular interactions between dextran and FPI in determining the rheological properties of the system was indicated by the higher viscosities and hysteresis loop areas obtained for mixed systems after heat treatment.

As in Studies **I-III**, the capacity of dextran to strengthen gels was also evaluated in Study **IV**, with the dextran produced by *W. cibaria* Sj 1b showing higher ability in gel strengthening. This effect was more obvious after heat treatment of the system, in which fava bean protein was gelated. The significantly improved gel elasticity (Table 18) and gel stability (Figure 19) in the mixture and conjugate with *W. cibaria* Sj 1b dextran are consistent with the high gel elasticity observed in the dough fermented by this strain (Figure 13). This difference in gel strengthening between the two dextrans partially explains the various properties of fava bean pastes fermented by different starters in Studies **I-III**. According to previous studies, dextran itself is not able to form a gel structure in

water solution (McCurdy et al., 1994); its gel-strengthening ability in a protein system is driven by microphase separation between protein and dextran molecules and the low entropy of the mixing process (Spotti et al., 2014b; Turgeon, Beaulieu, Schmitt, & Sanchez, 2003). After heat treatment, the reduced increase in G' values for conjugated systems with dextrans from *Ln. pseudomesenteroides* DSM 20193 and *W. cibaria* Sj 1b suggested the importance of intermolecular interactions between dextran and FPI in gel structure formation and maintenance.

6.9.2 Dextran-FPI interactions at different pH

In all fermented pastes in Studies **I-III**, EPS were produced alongside a reduction in pH. Therefore, the effects of pH on the rheological behaviors of FPI/DX systems were studied in order to understand the role of dextran in maintaining the structure of fermented protein-rich food. These were determined only in FPI/DX mixtures becasue in fermented pastes, only intermolecular interactions exist. Decreasing pH was found to affect the thickening ability of dextran from *Ln. pseudomesenteroides* DSM 20193, mainly due to the aggregation of fava bean proteins near or at its isoelectronic point (Arogundade et al., 2006). In contrast, the relatively stable viscosity observed for the mixture with *W. cibaria* Sj 1b dextran suggested the stabilizing ability of this dextran against protein aggregation. The different behaviors of the two dextrans in protein system under different pH also illustrate the effect of dextran molecular properties on maintenance of the protein network.

In contrast to previous studies on rheological properties of fermented milk (Gentès et al., 2011; Hassan et al., 2003), the addition of dextran significantly strengthened the gel network of fava bean protein (Table 19). The gel elasticity of FPI/DX mixtures was not substantially affected by pH decrease, especially in the system with *W. cibaria* Sj 1b dextran, further confirming the capability of this dextran to maintain the protein network. The greater stabilizing ability of *W. cibaria* Sj 1b dextran might be due to its lower molar mass, which makes it a better filler in the protein network. In fava bean protein solution, the initial protein network was destabilized by decreasing pH, as shown by the formation of inhomogeneous FPI solutions at pH values of 3.0 and 4.0. However, after dextran addition, various interactions between dextran and fava bean protein occurred, interfering the aggregation of protein molecules, and further leading to a relatively stable structure across the pH range of 3.0–6.0. This also explains the high viscosity and the relatively homogenous structure observed after fermentation for fava bean pastes with *in situ* produced EPS in Studies **I-III**.

6.10 Connection of EPS production to the fermentation of plant protein food

In this thesis, EPS were produced *in situ* during the fermentation of fava bean flour and FPC by LAB. The increased viscosity and gel elasticity and the decreased RFO content in fava bean matrix emphasized the advantage of connecting EPS production to the fermentation of legume protein-rich foods. By selecting EPS producers with different properties, the texture of the fava bean matrix may be tailored, thus meeting various consumer needs. Since EPS were produced from sucrose by LAB during fermentation, the resulted pastes possess the ‘‘clean-label’’ status that is commonly pursued nowadays. Furthermore, fermentation with EPS-synthesizing LAB is cost-effective due to the considerably lower price of sucrose compared to commercial dextran. Simultaneous EPS production and fermentation of fava bean matrix may broaden applications for fava bean protein in novel plant-protein food development and meat substitution, which has gained increasing attention globally. Finally, the method developed in this thesis may be applied to other food matrices rich in plant protein, increasing the utilization of plant protein in the food industry.

7 Conclusions

In this thesis, EPS were produced *in situ* by LAB in fava bean flour or in FPC. Focus was given to the evaluation of rheological properties of the fermented pastes in order to investigate the function of EPS in texture modification. The interactions between dextran and FPI were studied for a better understanding of the mechanisms underlying the texture modification by EPS in the fava bean protein system.

Obvious increases in viscosity and gel elasticity were observed with EPS production. In doughs fermented by *Ln. mesenteroides* DSM 20343, dextran played a major role in viscosity improvement. *Ln. pseudomesenteroides* DSM 20193 and *W. confusa* VTT E3403 are promising dextran producers for use in fava bean matrix, having the potential to achieve a range of textures comparable to products based on animal proteins, e.g. yogurt and pudding. Due to different utilization of glucose and fructose deriving from sucrose and several overlapping metabolic events in fava bean matrix, sugar balance cannot be achieved.

RFO in fava bean flour were degraded by two ways depending on endogenous α-galactosidase activity. In the presence of endogenous α-galactosidase, RFO were preferentially degraded by this enzyme, producing galactose and sucrose that could be further used for EPS synthesis. With no α-galactosidase activity, RFO were degraded by LSR, producing melibiose, manninotriose, and manninotetraose. These two ways co-exist in the presence of a high amount of raffinose. No microbial α-galactosidase was detected in the strains used.

Texture modification of the FPC pastes was mainly due to the positive effect of EPS, as proteolysis of fava bean protein was very weak after fermentation. Fermentation of FPC induced the appearance of protein aggregates, and *in situ* produced EPS changed the arrangement of those aggregates, resulting in a more concentrated protein phase. In this thesis, the rheological and textural differences between EPS-abundant and EPS-insufficient pastes were further revealed by their differences in protein microstructure.

Intermolecular interactions between dextran and fava bean protein played a major role in determining the rheological properties of FPI/DX mixtures. During fava bean protein gelation, covalent bonds formed through the Maillard reaction decreased viscosity and gel elasticity. During acidification, dextran stabilized the network of fava bean protein through intermolecular interactions. The molar mass and conformation of dextran affected the rheological properties of FPI/DX mixtures, especially during protein gelation.

In conclusion, this thesis for the first time evaluated the effects of EPS produced *in situ* by different LAB strains on texture modification of fava bean matrix. The feasibility of connecting EPS production to the fermentation of legume protein-rich food by LAB was demonstrated, which may contribute to the development of novel plant protein-rich foods. Two strains with high EPS-producing capability in fava bean matrix were selected, and have the potential to be utilized for more applications in the food industry. Visualization of the fava bean protein microstructure and investigation of fava bean protein-dextran interactions enhanced the understanding of the role of EPS in protein network arrangement. Finally, this study demonstrated the potential to tailor the texture of fava bean matrix by selecting certain EPS producers, a new method that can be used in other plant protein-based food matrices as well.

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