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In situ synthesis of exopolysaccharides by *Leuconostoc* spp. and *Weissella* spp. and their
rheological impacts in fava bean flour

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Abbreviations:

ANF, anti-nutritional factors; LAB, lactic acid bacteria; EPS, exopolysaccharides; TTA, total
titratable acidity; HPAEC-PAD, high performance anion exchange chromatography with pulse
amperometric detection; WHC, water-holding capacity; RFO, raffinose family oligosaccharides; FQ,
fermentation quotient.

21 ABSTRACT

22 Fava bean flour is regarded as a potential plant-based protein source, but the addition of it at high
23 concentration is restricted by its poor texture-improving ability and by anti-nutritional factors (ANF).
24 Exopolysaccharides (EPS) produced by lactic acid bacteria (LAB) are regarded as good texture
25 modifiers. In this study, fava bean flour was fermented with *Leuconostoc* spp. and *Weissella* spp. with
26 or without sucrose addition, in order to evaluate their potential in EPS production. The contents of
27 free sugars, organic acids, mannitol and EPS in all fermented fava bean doughs were measured.
28 Rheological properties of sucrose-enriched doughs, including viscosity flow curves, hysteresis loop
29 and dynamic oscillatory sweep curves, were measured after fermentation. As one of the ANF, the
30 degradation of raffinose family oligosaccharides (RFO) was also studied by analysing RFO profiles
31 of different doughs. Quantification of EPS revealed the potential of *Leuconostoc*
32 *pseudomesenteroides* DSM 20193 in EPS production, and the rheological analysis showed that the
33 polymers produced by this strain has the highest texturing capability. Furthermore, the viscous fava
34 bean doughs containing plant proteins and synthesized *in situ* EPS may have a potential application
35 in the food industry and fulfill consumers' increasing demands for "clean labels" and plant-originated
36 food materials.

37 Keywords: exopolysaccharides; lactic acid bacteria; fava bean; fermentation; texture modification

38 **1. Introduction**

39 Fava bean (*Vicia faba* L.) is a widely grown crop utilized for food and animal feed in many countries
40 (Duc, 1997). Fava bean seeds are very nutritious due to their high contents of proteins, vitamins,
41 minerals and dietary fibers (Jezierny et al., 2010). Also, fava bean is a rich source of non-nutrient
42 secondary metabolites, such as phenols and flavonoids, which have antioxidant activities and positive
43 effects on human health (Yuwei et al., 2014). Moreover, fava bean consumption shows a positive
44 effect on decreasing LDL-cholesterol levels in plasma and on Parkinson's disease motor symptoms
45 due to the presence of L-3, 4-dihydroxyphenylalanine (L-DOPA) (Frühbeck et al., 1997; Ramírez-
46 Moreno et al., 2015). Although it possesses these beneficial properties, fava bean still remains
47 underutilized, particularly for human consumption in European countries where meat and dairy
48 products are the major protein sources in the diet (Westhoek et al., 2014). One reason for the limited
49 utilization of fava bean is the anti-nutritional factors (ANF) such as raffinose family oligosaccharides
50 (RFO), tannins, protease inhibitors, alkaloids and lectins (Liener, 1990). They are contained in raw
51 fava bean seeds and are known to exert anti-nutritional functions, reducing their digestibility and even
52 leading to some pathological reactions (Gupta, 1987).

53 In some studies, the decrease of ANF in fava bean has been addressed with different processing
54 methods, such as soaking, air classification, extrusion, heat treatment and fermentation (Alonso et al.,
55 2000; Coda et al., 2015; Luo and Xie, 2013). In particular, fermentation with lactic acid bacteria
56 (LAB) has shown to be effective in reducing ANF and at the same time to improve the nutritional
57 values of fava bean (Coda et al., 2015). Another property of LAB is their ability to synthesize
58 exopolysaccharides (EPS), which has been widely exploited for milk and cereal fermentations (Galle
59 et al., 2010; Galle et al., 2012; Gentès et al., 2011; Purwandari et al., 2007) and could be exploited
60 for legume fermentation as well. EPS are long-chain polysaccharides consisting of branched,
61 repeating units of sugars or sugar derivatives (Welman and Maddox, 2003). According to the
62 composition, EPS can be classified as homopolysaccharides, which are composed of only one type

63 of sugar unit (e.g., glucose), and heteropolysaccharides, which are composed of two or more types of
64 sugar units (e.g., galactose and rhamnose). Normally, EPS are secreted into their surroundings during
65 microbial growth and are not attached permanently to the cell surface (Laws et al., 2001), protecting
66 microbial cells from environmental stresses, e.g., nisin, starvation, membrane stress and low pH (Kim
67 et al., 2000; Looijesteijn et al., 2001; Schwab and Gänzle, 2006).

68 Microbial EPS have been regarded as alternatives for plant polysaccharides that are commonly used
69 as thickening, stabilizing, texturizing and gelling agents in the food industry (Galle and Arendt, 2014).
70 In particular, EPS produced by LAB have been applied to improve the rheological, textural and
71 sensory properties of yogurt (Folkenberg et al., 2005; Welman and Maddox, 2003). It was reported
72 that EPS from LAB increased the stay time of milk products in the mouth, imparting an enhanced
73 perception of taste (Duboc and Mollet, 2001). Some health-promoting effects of EPS, e.g., prebiotic,
74 immune-potentiating and cholesterol-lowering activity, were also reported (Chabot et al., 2001; Guo
75 et al., 2013; Hosono et al., 1997; Nakajima et al., 1992). Furthermore, EPS were produced during
76 food fermentation by LAB and hence were not manually added to food products, thus meeting
77 consumers' demands for decreased usage of food additives. To the best of our knowledge, there is
78 still very limited information on the rheological properties of legumes containing EPS synthesized *in*
79 *situ* (Hickisch et al., 2016). The presence of these EPS might contribute to the exploitation of fava
80 bean potential, particularly as a candidate for meat substitution (Multari et al., 2015).

81 The objective of this study was to evaluate the potential of different LAB starters in EPS production
82 and texture modification in fava bean flour. The biochemical and rheological properties of fava bean
83 doughs before and after fermentation were assessed and compared. Due to the nutritional importance
84 of RFO, the effect of LAB fermentation on RFO load in fava bean flour was also investigated. Based
85 on the EPS contents and rheological properties of the fermented fava bean doughs, the most promising
86 LAB strain for EPS production could be identified.

87 **2. Materials and methods**

88 **2.1 LAB and raw material**

89 Six LAB strains belonging to *Leuconostoc* spp. and *Weissella* spp., previously selected according to
90 their EPS-producing abilities on sucrose-supplemented MRS agar, were used for fava bean flour
91 fermentation. In detail, *Leuconostoc pseudomesenteroides* DSM 20193, *Leuconostoc mesenteroides*
92 *subsp. mesenteroides* DSM 20240, *Leuconostoc citreum* DSM 5577 and *Weissella cibaria* DSM
93 15878 were purchased from Leibniz Institute DSMZ (Braunschweig, Germany). *Weissella cibaria* Sj
94 1b and *Weissella confusa* Sjs5-4, previously isolated from onion powder, were obtained from the
95 culture collection of the Division of Food Hygiene and Environmental Health, University of Helsinki.
96 *Lactobacillus plantarum* DPPMAB24W, which belongs to the culture collection of the Department
97 of Soil, Plant and Food Sciences, University of Bari, was used as an EPS-negative control. All LAB
98 strains were routinely propagated in MRS broth at 30 °C (Oxoid, Basingstoke, Hampshire, England).

99 Fava bean (*Vicia faba* major, harvest year 2014) flour was purchased from CerealVeneta (San
100 Martino di Lupari, PD, Italy). The composition of the flour was: protein (35.7%), carbohydrates
101 (49.3%), lipids (1.6%), moisture (9.5%) and ash (3.9%), calculating on dry matter base.

102 **2.2 Dough fermentation**

103 Doughs were prepared according to the recipe reported in Table 1. Forty grams of fava bean flour
104 were mixed with 60 mL of distilled water (control doughs). In order to enable EPS formation, 25%
105 (w/w) of fava bean flour was replaced by sucrose and mixed with 60 mL of distilled water (sucrose-
106 enriched doughs). Microbial cells, propagated in MRS broth supplemented with 2% (w/v) sucrose
107 overnight, were sedimented by centrifugation ($10,000 \times g$ for 10 min) and washed with 50 mM
108 sodium phosphate buffer, pH 7.0. Then, cells were re-suspended in distilled water to inoculate doughs
109 at a cell density range of ca. 5.0 – 6.0 log cfu/g. Fermentation was carried out at 30 °C for 24 h.

110 **2.3 LAB cell density, pH and total titratable acidity (TTA)**

LAB cell density was analyzed before and after fermentation. Dough samples (10 g) were homogenized with 90 mL of sterile saline solution in a Stomacher 400 lab blender (Seward Medical, London). Serial dilutions were made and the diluted bacterial suspensions were plated on MRS agar (Oxoid, Basingstoke, Hampshire, England). After 48 h of incubation at 30 °C, bacterial colonies were counted. The pH values were determined by a pH meter (Model HI 99161, Hanna Instruments, Woonsocket, RI, USA) with a food penetration probe. TTA was measured by a Mettler Toledo EasyPlus Titrator (Schott, Germany). Ten grams of sample in 100 mL of distilled water were titrated with 0.1 M NaOH to a final pH of 8.5. TTA was expressed as the volume of NaOH used (mL).

2.4 Analysis of sugars, mannitol and organic acids

One hundred milligrams of freeze-dried samples were mixed with 5.0 mL of Milli-Q water and vortexed for 5 min to allow the complete dissolution of free sugars, mannitol and organic acids. Then, the suspensions were boiled for 5 min to inactivate enzymes and microbes. After cooling, samples (400 µL) were filtered using Amicon Ultra-0.5 centrifugal filter units (Millipore, Billerica, MA) at $12,000 \times g$ for 10 min to remove polymeric molecules (above 10 kDa). Before further analysis, samples were diluted with Milli-Q water.

2.4.1 Sugars

Mono-, di- and oligo-saccharides in fava bean doughs were analyzed by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) system. Sugars were separated in a CarboPac PA1 column (250 × 4 mm i.d., Dionex, Sunnyvale, CA) with a flow rate of 1 mL/min and were detected by a Waters 2465 pulsed amperometric detector (Waters, USA). The gradient elution started from 2 mM NaOH (3 min), then to 60 mM NaOH (32 min), and finally to 200 mM (8 min) followed by washing and regeneration. The injection volume was 10 µL. Glucose (Merck, Germany), fructose (Merck), galactose (Sigma-Aldrich), sucrose (Merck), melibiose (Sigma-Aldrich), raffinose (Sigma-Aldrich), stachyose (Sigma-Aldrich) and verbascose (Megazyme, Ireland) were

135 used as standards and 2-deoxy-D-galactose (Sigma-Aldrich) was used as the internal standard for
136 quantification.

137 In order to confirm the function of microbial enzymes and endogenous α -galactosidase in fava bean
138 flour on RFO degradation, both native and autoclaved fava bean flour were used for control dough
139 preparation and were inoculated with the same starter, as shown in Table 1. After 24 h fermentation,
140 doughs made with both native and autoclaved flour were treated by the same method according to
141 Section 2.4. Then, the sugar profiles of these doughs were analyzed using the same method as stated
142 above.

143 **2.4.2 Mannitol**

144 Mannitol was quantified by the HPAEC-PAD system equipped with a CarboPac MA-1 analytical
145 column (4 × 250 mm i.d., Dionex), a DECADE detector (Antec Leyden, The Netherlands), a Waters
146 717 Autosampler and two Waters 515 pumps. The gradient elution started from 40 mM NaOH (25
147 min), then to 620 mM (10 min), and finally to 40 mM. The flow rate was 0.4 mL/min and the injection
148 volume was 5 μ L. Mannitol (Sigma-Aldrich) was used as the standard for quantification.

149 **2.4.3 Organic acids**

150 Organic acids were separated by an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, USA) and
151 were detected by a Waters 2487 Dual λ Absorbance Detector (operating at 210 nm). Sulfuric acid (10
152 mM) was used as the mobile phase with a flow rate of 0.6 mL/min. The injection volume was 20 μ L.
153 Citric acid (Sigma-Aldrich), L-lactic acid (Sigma-Aldrich) and acetic acid (Merck) were used as
154 standards for quantification.

155 **2.5 EPS analysis**

156 **2.5.1 Dextran**

157 The amount of low-branched dextran was analyzed by an enzyme-assisted method using a mixture
158 of dextranase (Sigma-Aldrich) and α -glucosidase (Megazyme, Ireland) according to a method
159 reported earlier (Katina et al., 2009).

160 **2.5.2 Glucan**

161 The total glucan content was quantified by calculating the content of released glucose after sulfuric
162 acid hydrolysis. Freeze-dried samples (100 mg) were washed twice with 50% ethanol to remove
163 monosaccharides and oligosaccharides. Then, 3 mL (30 U) of thermostable α -amylase (Megazyme)
164 in 100 mM (pH 5.0) sodium acetate buffer were added. Samples were then incubated in a boiling
165 water bath for 16 min to degrade starch. After cooling, 100 μ L (20 U) of amyloglucosidase
166 (Megazyme) was added, and samples were further incubated at 50 °C for 30 min, followed by
167 precipitation with 10 mL of ethanol (99.6%). Then, samples were kept at 4 °C overnight to allow the
168 complete precipitation of soluble EPS. After centrifugation ($10,000 \times g$, 15 min), the supernatant was
169 removed and 1 mL of 1.0 M sulfuric acid was added to hydrolyze glucan at 100 °C for 2 h. Finally,
170 samples were neutralized with 2 mL of Na_2CO_3 (1.0 M) and diluted for sugar analysis as described
171 in 2.4.1. Glucose was treated under the same conditions and was used as the standard for
172 quantification. The produced glucan contents were calculated by subtracting the glucan contents in
173 unfermented samples.

174 **2.6 Rheological analysis**

175 **2.6.1 Viscosity**

176 Before and after fermentation, samples (60 g) were taken and the viscosity values were measured at
177 20 °C with a RheolabQC rheometer (Anton Paar, Austria) at different shear rates from 2 to 100 1/s
178 (up and down sweeps). To calculate the specific viscosity increase, the viscosity values at the shear
179 rate of 100 1/s were used.

180 **2.6.2 Water-holding capacity (WHC)**

181 Centrifuge tubes containing fermented samples were first weighed and then centrifuged at $8,000 \times g$
182 for 10 min. The liquid that appeared after centrifugation was removed with a needle connected to a
183 syringe. Then, samples were weighed again. WHC was expressed in percentage as the ratio between
184 the weight after liquid removal and the initial weight. Measurements were carried out in triplicate.

185 **2.6.3 Hysteresis loop**

186 The hysteresis loop curve was generated by measuring shear stresses as a function of shear rates from
187 2 to 100 1/s (up and down sweeps) by a HAAKE RheoStress rheometer (RS 50, HAAKE Rheometer,
188 Karlsruhe, Germany). Samples were left for 5 min to equilibrate before measurement. The hysteresis
189 loop area between the upward and downward flow curves was calculated using the RheoWin Pro
190 software.

191 **2.6.4 Dynamic oscillatory sweep**

192 The dynamic moduli (G' , G'') were recorded as a function of frequency from 0.1 to 10 Hz by a
193 HAAKE RheoStress rheometer at 20 °C. Measurements were conducted in triplicate after sample
194 equilibration.

195 **2.7 Statistical analysis**

196 The numerical results of microbial, chemical and rheological analysis in this study are averages of
197 three independent replicates. Data were analyzed by one-way analysis of variance (ANOVA) using
198 Origin 8.6 (OriginLab Inc., USA). The means comparisons were determined by Tukey's test ($P <$
199 0.05).

200 **3. Results and discussion**

201 **3.1 LAB growth and acidification**

202 After 24 h fermentation, the LAB cell density ranged from 8.6 to 9.8 log cfu/g, increasing between
203 2.8 to 4.0 log cycles (Table 2). In detail, the highest cell density increase (Δ log) was observed in

204 5577_S, and the lowest was in 20240_S. Overall, the addition of sucrose did not significantly affect
205 the final cell density and the cell density increase as already reported in a similar study (Kajala et al.,
206 2015).

207 Before fermentation, the pH of the fava bean doughs was ca. 6.6; it dropped 1.6 to 2.3 units after
208 fermentation, reaching values in the range from 4.3 to 5.0 (Table 2). Fermented with the same starter,
209 sucrose-enriched doughs showed slightly lower pH compared with control doughs. According to
210 Table 2, TTA values varied from 10.7 to 19.8 mL, with the highest value in 5577_S and the lowest
211 value in Sj5-4_S. Generally, doughs fermented by *Weissella* spp. were less acidic (higher pH and
212 lower TTA) than doughs fermented by *Leuconostoc* spp., which was also reported earlier (Katina et
213 al., 2009).

214 Citric acid was found in fava bean doughs, before and after fermentation, with some differences
215 among samples (Table 2). In the two doughs fermented by either *Ln. citreum* DSM 5577 (5577_CT
216 and 5577_S) or *W. confusa* Sj5-4 (Sj5-4_CT and Sj5-4_S), citric acid was no longer detectable,
217 indicating its involvement in microbial metabolism. In the other cases, no significant differences were
218 found in citric acid concentration between the control and sucrose-enriched doughs fermented with
219 the same starter. Lactic acid concentration ranged from 9.50 to 21.97 mmol/100 g dough, with the
220 highest concentration in 20193_CT and the lowest in 20240_S. With the addition of sucrose, lactic
221 acid concentration decreased to differing extents. In the case of sucrose-enriched doughs fermented
222 by *Leuconostoc* spp. (20193_S, 20240_S, 5577_S), more acetic acid was formed after sucrose
223 addition, particularly in 5577_S. By contrast, the acetic acid concentration in sucrose-enriched doughs
224 fermented by *Weissella* spp. (15878_S, Sj 1b_S, Sj5-4_S) was less affected by sucrose addition, which
225 was also mirrored by their less varied fermentation quotient (FQ) compared with *Leuconostoc* spp.
226 (Table 2). This difference between the heterofermentative species *Leuconostoc* and *Weissella* might
227 be due to different metabolic pathways of fructose liberated from hydrolysis of the added sucrose by
228 the activity of a glucansucrase. It was previously observed that the mannitol dehydrogenase of

229 *Leuconostoc* spp. could reduce *in vivo* fructose to mannitol, contributing to the formation of acetic
230 acid (Wisselink et al., 2002). On the contrary, *Weissella* spp. ferment fructose but do not reduce it to
231 mannitol, leading to low acetic acid production (Galle et al., 2010; Kajala et al., 2015).

232 **3.2 Sugar analysis**

233 Before fermentation, sugars in control and sucrose-enriched doughs were analyzed, and the existence
234 of endogenous sucrose was revealed (Table 3). After fermentation, sucrose was utilized by LAB to
235 different extents. In all control doughs without added sucrose, endogenous sucrose was no longer
236 detected, while in two sucrose-enriched doughs, 15878_S and Sj 1b_S, almost half of the total sucrose
237 remained. In other sucrose-enriched doughs, sucrose was depleted or only a small amount (0.30%)
238 was detected after fermentation. Theoretically, sucrose was first utilized for microbial growth during
239 the exponential phase, then for EPS production as slime EPS were produced during the stationary
240 phase (Plante and Shriver, 1998). Through the activity of glucansucrase on sucrose, glucan was
241 formed, liberating fructose. This was further confirmed by the residual fructose in all sucrose-
242 enriched doughs, ranging from 1.76% to 5.37% (Table 3).

243 RFO (raffinose, stachyose and verbascose) were detected in control doughs before fermentation as
244 these oligosaccharides are widely distributed in the leguminous family (Aguilera et al., 2009). After
245 fermentation, the content of RFO decreased in each case, as expected (Table 3). In detail, raffinose
246 was no longer detected while stachyose and verbascose were still detected in control doughs with
247 varying contents, from 0.24% to 0.85% and from 0.31% to 0.99% respectively. Stachyose and
248 verbascose were not detected in sucrose-enriched doughs due to the reduced flour content in those
249 doughs. Galactose, which is a degradation product of RFO by α -galactosidase, was also detected, in
250 agreement with our previous study on fava bean fermentation (Xu et al., unpublished results). In order
251 to assess the role of microbial enzymes and native α -galactosidase of fava bean seeds (Dey and
252 Pridham, 1969) in RFO degradation, the fava bean flour used in this study was autoclaved to
253 inactivate the endogenous enzymes, and used to prepare doughs under the same conditions as native

254 flour. The influence of microbial and endogenous enzymes on RFO degradation was evaluated by
255 comparing the sugar profiles of control doughs made with native or autoclaved flour with the same
256 starter (Fig. 1). According to Fig. 1, the sugar profiles changed after autoclaving. In detail, galactose
257 was not detected after autoclaving, and the contents of RFO in doughs prepared with autoclaved flour
258 were generally higher than those in doughs prepared with native flour, and were comparable to
259 unfermented flour, with the single exception of the dough fermented by *Ln. pseudomesenteroides*
260 DSM 20193. In this dough, raffinose and stachyose disappeared, indicating a presumptive α -
261 galactosidase activity of *Ln. pseudomesenteroides* DSM 20193, as α -galactosidase in fava bean flour
262 was inactivated by autoclaving. The produced galactose might have been consumed during
263 fermentation since *Ln. pseudomesenteroides* DSM 20193 was reported to be able to ferment galactose
264 and raffinose (Farrow et al., 1989). In a departure from our previous work (Xu et al., unpublished
265 results), other degradation products, such as melibiose, manninotriose and manninotetraose, were not
266 detected in this study due to the absence of microbial levansucrase. This was further confirmed by
267 screening all LAB strains on MRS agar containing 2% (w/v) raffinose, on which no EPS formation
268 was observed (data not shown). Notwithstanding the major role of fava bean endogenous α -
269 galactosidase in RFO degradation, the content of residual stachyose and verbascose in doughs
270 fermented without sucrose addition still varied, indicating the effect of different microorganisms on
271 activation of the endogenous enzymatic activities of the flour.

272 **3.3 EPS and mannitol**

273 The addition of sucrose strongly induced dextran and glucan production when comparing the dextran
274 and glucan contents in control and sucrose-enriched doughs fermented with the same starter (Table
275 4). Glucan contents were higher than dextran contents in all cases, as low- and high-branched dextrans
276 were both included in glucans. Dextran content varied from 0.11% to 0.74% in control doughs and
277 from 1.86% to 3.67% in sucrose-enriched doughs. When only endogenous sucrose was available, *W.*
278 *cibaria* Sj 1b produced the highest amount of dextran compared with other starters. However, with

the addition of more sucrose, this strain produced the lowest amount of dextran, indicating a possible repression to glucansucrase of *W. cibaria* Sj 1b due to the high substrate concentration (Shukla and Goyal, 2011). Glucan content ranged from 0.32% to 0.82% in control doughs and from 2.57% to 4.33% in sucrose-enriched doughs, with the highest content in 20240_S and the lowest in Sj5-4_S. In the presence of the same amount of sucrose, *Leuconostoc* spp. produced more glucan than did *Weissella* spp. in sucrose-enriched doughs (Table 4). Additionally, smaller differences were found between dextran and glucan contents in doughs fermented by *Weissella* spp. compared with *Leuconostoc* spp., since *Weissella* spp. preferentially produced only low-branched dextran while *Leuconostoc* spp. tend to produce more than one type of dextran (Cote and Skory, 2015; Maina et al., 2008).

After 24 h fermentation, mannitol was detected in all doughs except doughs fermented by *W. confusa* Sj5-4 (Sj5-4_CT and Sj5-4_S) (Table 4). Mannitol content varied from 0.02% to 0.46% in control doughs and from 0.21% to 4.22% in sucrose-enriched doughs, with a strong inducing effect due to sucrose addition. The highest mannitol content was found in 5577_S, corresponding to its highest acetic acid content (Table 2) since the reduction of fructose could induce acetic acid formation (Wisselink et al., 2002). By contrast, the lowest mannitol contents in control and sucrose-enriched doughs were found in 15878_CT and 15878_S. Generally, *Leuconostoc* spp. produce mannitol from fructose by the activity of mannitol dehydrogenase, while *Weissella* spp. ferment fructose but do not reduce it to mannitol, leading to decreased or no mannitol production (Erten, 1998; Galle et al., 2010). However, under the conditions of our study, *W. cibaria* Sj 1b produced a higher amount of mannitol than did *Ln. pseudomesenteroides* DSM 20193 which was previously reported as a good mannitol producer when grown in the presence of fructose or sucrose (Vandamme et al., 1987), indicating that mannitol production was both strain- and substrate-dependent (Galle et al., 2010).

3.4 Sucrose utilization

It was previously shown that during EPS synthesis, sucrose was first utilized for microbial growth during the exponential phase, and then for dextran production during the stationary phase, liberating

fructose (Han et al., 2014; Plante and Shriver, 1998). The liberated fructose was further reduced to mannitol in *Leuconostoc* spp. by mannitol dehydrogenase, while in *Weissella* spp., the liberated fructose was fermented but not reduced to mannitol (Galle et al., 2010). Theoretically, the total content of residual fructose and mannitol should be roughly equal to the content of glucan formed. However, due to the usage of sucrose-liberated glucose for the growth of LAB and other microorganisms (potentially present in fava bean flour), the production of acids and other volatile compounds and the formation of oligosaccharides by glucansucrase through acceptor reaction, the content of glucan was generally lower than the total content of residual fructose and mannitol (Tables 3 and 4). Actually, in a complex system such as any food matrix, the sugar balance is difficult to achieve, as also reported by other researchers who used carrot puree as the medium to produce EPS (Juvonen et al., 2015). Furthermore, significant differences were found in residual fructose and mannitol levels between sucrose-enriched doughs fermented by different LAB strains. The total content of residual fructose and mannitol in sucrose-enriched doughs fermented by *Leuconostoc* spp. (20193_S, 20240_S and 5577_S) was generally in line with the theoretical amount of fructose released from the original and the added sucrose. However, in sucrose-enriched doughs fermented by *Weissella* spp. (15878_S and Sj 1b_S), the residual fructose and mannitol content were lower than the theoretical maximal fructose content. It could be hypothesized that fructose was preferable to glucose for *Weissella* spp. since different strains might have different carbon source preferences (Kajala et al., 2015).

3.5 Rheological properties

3.5.1 Viscosity change and WHC

According to the viscosity flow curves, showing in the supplementary material (Fig. S1 and S2), all doughs showed a typical shear-thinning behavior after fermentation. The initial viscosity values of sucrose-enriched doughs were lower than control doughs due to their lower flour content (Table 5). After fermentation, the viscosity of all doughs increased to different extents, indicating the thickening

ability of EPS. In detail, the viscosity in control doughs ranged from 1.73 to 2.78 Pa·s before fermentation and from 2.59 to 5.87 Pa·s after fermentation, increasing by up to 3.28 Pa·s. The highest viscosity increase was found in Sj 1b_CT, corresponding to its highest dextran content among control doughs (Table 4). In sucrose-enriched doughs, the viscosity ranged from 0.20 to 0.35 Pa·s before fermentation and from 0.82 to 14.77 after fermentation, increasing by up to 14.57 Pa·s. The highest viscosity increase was found in 20193_S, corresponding to its highest dextran content as well (Table 4). Interestingly, doughs fermented by *W. confusa* Sj5-4 (Sj5-4_CT and Sj5-4_S) showed the lowest viscosity after fermentation although their dextran contents were not the lowest. This low viscosity improving effect might be due to the structure and molecular weight of dextran produced by *W. confusa* Sj5-4, which might influence its rheological behavior; however, more evidences are needed to prove this hypothesis.

The WHC of food has been defined as the ability to hold its own and added water during the application of forces, pressing, centrifugation or heating (Zayas, 1997). This is a limiting factor in protein food applications (Zayas, 1997) and is also an important characteristic for evaluating the function of EPS as food hydrocolloids. Among all doughs, 20193_S showed the highest WHC value with significant difference, while the dough fermented by *L. plantarum* DPPMAB24W (B24W_S) showed the lowest value, as it was an EPS-negative control (Table 6). In other sucrose-enriched doughs, WHC values ranged from 83.72% to 89.26%, without much difference.

3.5.2 Hysteresis loop

A hysteresis loop was frequently observed in viscoelastic materials during the shear rate sweep, and was assumed to be the difference between the energy required for structural breakdown and rebuilding (Gambús et al., 2004). Materials with larger hysteresis loop areas tend to have better structural reversibility (Purwandari et al., 2007). In our study, the hysteresis loops of sucrose-enriched doughs fermented by different LAB strains varied considerably (Fig. 2). As expected, 20193_S showed the largest loop due to its highest dextran content, while the EPS negative control B24W_S

showed the smallest loop. In order to get specific loop areas, the loop area of each dough was calculated and is presented in Table 6. Consistent with previous data, the largest loop area (7.98 units) was observed in 20193_S, indicating its best structural reversibility among all doughs. In contrast, B24W_S showed the worst structural reversibility as there was no texture modification from EPS in this dough. No significant difference was found between Sj5-4_S and B24W_S in loop area, indicating a weak texture modification of dextran produced by *W. confusa* Sj5-4. Generally, sucrose-enriched doughs fermented by *Leuconostoc* spp. showed a better structural reversibility than those fermented by *Weissella* spp., except Sj 1b_S, which showed a comparable structural reversibility with 20240_S and 5577_S (Table 6).

3.5.3 Elastic modulus

The elastic modulus (G') increased with the rising frequency in all sucrose-enriched doughs (Fig. 3). In particular, 20193_S and Sj 1b_S showed the highest G' at any frequency, indicating more elastic and solid characters compared with others. Correspondingly, as an EPS-negative control, B24W_S showed the lowest G' at any frequency. It was previously reported that the dependence of the elastic modulus with frequency gives information about the gel structure (Stading and Hermansson, 1990). According to Fig. 3, 20193_S and Sj 1b_S were less frequency-dependent compared with other doughs, indicating stable and different gel structures formed in these two doughs. The G' values of all sucrose-enriched doughs at the frequency of 1.0 Hz were compared, showing a considerable variation ranging from 33.85 to 1656.03 Pa (Table 6). With EPS synthesized *in situ*, the G' values of the doughs were significantly higher than the EPS-negative control (B24W_S), revealing the function of EPS in gel structure strengthening. Outstandingly, although the dextran content of Sj 1b_S was about half that of 20193_S, they both had G' values over 1500 Pa, indicating a strong network between EPS and fava bean proteins in these two doughs. It was reported that the interaction between EPS and proteins was driven by the chemical structure of EPS (Folkenberg et al., 2006). Therefore, more studies have to be performed on chemical structures, molecular weight distributions and rheological

behaviors of dextrans produced by *Ln. pseudomesenteroides* DSM 20193 and *W. cibaria* Sj 1b. The dynamic oscillatory sweep curves are presented in the supplementary material (Fig. S3), and all sucrose-enriched doughs showed a solid-like behavior, except 20240_S.

Currently, the rheological properties of yogurt containing EPS and the network between EPS and milk proteins have been extensively studied (Amatayakul et al., 2006; Gentès et al., 2013; Purwandari et al., 2007). However, little is known about the influence of EPS on the rheological properties of legume-made doughs and the network between EPS and legume proteins. Therefore, further research is still needed.

To conclude, fava bean doughs fermented by different LAB strains showed different chemical and rheological properties. The addition of sucrose facilitated EPS formation, which further affected the rheological properties of fermented doughs. It has been previously observed that the viscosifying efficiency of EPS also depends on their physical-chemical properties, highlighting the importance of certain structure-function relationships for specific technological applications (Ruas-Madiedo et al., 2002). As a consequence, this also emphasizes the role of selected LAB to produce specific EPS that will behave in a desired way when incorporated into food products. In this work, *Ln. pseudomesenteroides* DSM 20193 showed the highest potential in EPS production and texture modification in fava bean dough. The fermented doughs with synthesized *in situ* EPS may meet consumers' needs for "clean labels" and may have a potential use in the food industry. Further studies should be performed on the application of fermented legumes in different food products.

Supplementary material

The viscosity flow curves of control and sucrose-enriched fava bean doughs fermented by *Ln. pseudomesenteroides* DSM 20193, *Ln. mesenteroides* DSM 20240, *Ln. citreum* DSM 5577, *W. cibaria* DSM 15878, *W. cibaria* Sj 1b and *W. confusa* Sj5-4 before and after fermentation (Fig. S1 and Fig. S2). The dynamic oscillatory sweep curves of sucrose-enriched fava bean doughs fermented

403 by *Ln. pseudomesenteroides* DSM 20193, *Ln. mesenteroides* DSM 20240, *Ln. citreum* DSM 5577,
404 *W. cibaria* DSM 15878, *W. cibaria* Sj 1b, *W. confusa* Sj5-4 and *L. plantarum* DPPMAB24W after
405 fermentation (Fig. S3).

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532

533 Figure captions

534 Fig. 1. HPAEC-PAD chromatograms of control doughs made of native or autoclaved fava bean flour
535 fermented by *Leuconostoc* spp. (A) and *Weissella* spp. (B). The chromatogram of unfermented
536 control dough without starter is also presented (CT_0h). Other chromatograms are: *Ln.*
537 *pseudomesenteroides* DSM 20193 fermented control dough made of native (20193_N) or autoclaved
538 flour (20193_A); *Ln. mesenteroides* DSM 20240 fermented control dough made of native (20240_N)
539 or autoclaved flour (20240_A). The rest of the chromatograms continue like this in the order of *Ln.*
540 *citreum* DSM 5577, *W. cibaria* DSM 15878, *W. cibaria* Sj 1b and *W. confusa* Sj5-4 (Gal, galactose;
541 Suc, sucrose; Raf, raffinose; Sta, stachyose; Ver, verbascose).

542 Fig. 2. Hysteresis loops of sucrose-enriched fava bean doughs fermented by *Ln. pseudomesenteroides*
543 DSM 20193 (20193_S), *Ln. mesenteroides* DSM 20240 (20240_S), *Ln. citreum* DSM 5577 (5577_S),
544 *W. cibaria* DSM 15878 (15878_S), *W. cibaria* Sj 1b (Sj 1b_S), *W. confusa* Sj5-4 (Sj5-4_S) and *L.*
545 *plantarum* DPPMAB24W (B24W_S).

546 Fig. 3. Elastic modulus (G') of sucrose-enriched fava bean doughs fermented by *Ln.*
547 *pseudomesenteroides* DSM 20193 (20193_S), *Ln. mesenteroides* DSM 20240 (20240_S), *Ln. citreum*
548 DSM 5577 (5577_S), *W. cibaria* DSM 15878 (15878_S), *W. cibaria* Sj 1b (Sj 1b_S), *W. confusa* Sj5-
549 4 (Sj5-4_S) and *L. plantarum* DPPMAB24W (B24W_S) as a function of oscillatory frequency (0.1
550 – 10 Hz).

551 Tables

552 Table 1. Sample codes and ingredients of control and sucrose-enriched fava bean doughs fermented
553 by *Leuconostoc* spp., *Weissella* spp. and *Lactobacillus plantarum* DPPMAB24W.

Sample code	Flour (g)	Sucrose (g)	Water (mL)	Starter
20193_CT ^a	40	0	60	<i>Leuconostoc pseudomesenteroides</i> DSM 20193
20193_S ^b	30	10	60	
20240_CT	40	0	60	<i>Leuconostoc mesenteroides subsp.</i> <i>mesenteroides</i> DSM 20240
20240_S	30	10	60	
5577_CT	40	0	60	<i>Leuconostoc citreum</i> DSM 5577
5577_S	30	10	60	
15878_CT	40	0	60	<i>Weissella cibaria</i> DSM 15878
15878_S	30	10	60	
Sj 1b_CT	40	0	60	<i>Weissella cibaria</i> Sj 1b
Sj 1b_S	30	10	60	
Sj5-4_CT	40	0	60	<i>Weissella confusa</i> Sj5-4
Sj5-4_S	30	10	60	
B24W_S	30	10	60	<i>Lactobacillus plantarum</i> DPPMAB24W

554 ^a CT means control dough. ^b S means sucrose-enriched dough.

Table 2. LAB growth and acidification of control and sucrose-enriched fava bean doughs fermented by *Leuconostoc* spp. and *Weissella* spp.. The table shows cell density, cell density increase, pH, TTA, citric acid, lactic acid and acetic acid concentrations together with their ratios (FQ).

Sample code ^A	Cell density ^D (log cfu/g)	$\Delta \log$ ^E	pH	TTA (mL)	Citric acid (mmol/100 g dough)	Lactic acid	Acetic acid	FQ ^F
CT_0h ^B	-	-	6.6 ± 0.00	4.0 ± 0.05	2.29 ± 0.05	nd	nd	-
S_0h ^C	-	-	6.7 ± 0.01	2.9 ± 0.03	1.80 ± 0.02	nd	nd	-
20193_CT	9.7 ± 0.2 ^{a, c}	3.2 ± 0.2 ^{a, d}	4.4 ± 0.00 ^a	17.2 ± 0.11 ^a	2.69 ± 0.10 ^a	21.97 ± 0.91 ^a	6.46 ± 0.37 ^{a, d}	3.40
20193_S	9.8 ± 0.1 ^a	3.4 ± 0.1 ^a	4.3 ± 0.00 ^b	15.6 ± 1.27 ^{a, b, e}	2.00 ± 0.07 ^{a, b}	17.63 ± 0.96 ^{b, e}	7.46 ± 0.28 ^{a, b, d}	2.36
20240_CT	8.9 ± 0.0 ^{b, d}	3.1 ± 0.0 ^{a, b, d}	4.8 ± 0.00 ^c	14.5 ± 0.04 ^{b, c, e}	1.39 ± 0.26 ^b	13.96 ± 0.59 ^{b, d}	6.11 ± 0.31 ^a	2.28
20240_S	8.6 ± 0.1 ^b	2.8 ± 0.1 ^{b, d}	4.5 ± 0.01 ^d	15.7 ± 0.05 ^{a, b}	1.21 ± 0.12 ^b	9.50 ± 0.32 ^c	9.34 ± 0.45 ^{b, d}	1.02
5577_CT	9.5 ± 0.0 ^c	3.9 ± 0.0 ^c	4.9 ± 0.02 ^e	13.3 ± 0.14 ^{c, e, f}	nd	14.08 ± 0.60 ^{b, d}	9.44 ± 0.42 ^{b, d}	1.49
5577_S	9.6 ± 0.0 ^{a, c}	4.0 ± 0.1 ^c	4.3 ± 0.00 ^b	19.8 ± 0.24 ^d	nd	12.45 ± 0.59 ^{c, d, f}	16.24 ± 1.28 ^c	0.77
15878_CT	9.0 ± 0.1 ^d	2.9 ± 0.1 ^d	4.8 ± 0.01 ^c	15.0 ± 0.07 ^{b, e}	1.73 ± 0.38 ^{a, b}	17.47 ± 1.69 ^{b, e}	7.63 ± 0.57 ^{a, b, d}	2.29
15878_S	9.0 ± 0.0 ^d	2.9 ± 0.1 ^d	4.6 ± 0.04 ^f	14.4 ± 0.58 ^{b, c, e}	1.26 ± 0.35 ^b	15.98 ± 0.99 ^{b, d, e}	6.56 ± 0.72 ^{a, d}	2.43
Sj 1b_CT	9.0 ± 0.0 ^d	3.2 ± 0.1 ^{a, d}	4.8 ± 0.02 ^c	14.7 ± 0.33 ^{b, c, e}	0.94 ± 0.59 ^b	14.57 ± 0.73 ^b	7.37 ± 0.30 ^{a, b, d}	1.98
Sj 1b_S	9.0 ± 0.0 ^d	3.3 ± 0.1 ^a	4.6 ± 0.04 ^f	14.0 ± 0.52 ^{e, f}	1.22 ± 0.31 ^b	9.64 ± 0.26 ^c	7.62 ± 0.58 ^{a, b, d}	1.26
Sj5-4_CT	9.0 ± 0.1 ^d	3.1 ± 0.1 ^{a, b, d}	5.0 ± 0.01 ^e	12.6 ± 0.13 ^f	nd	18.41 ± 2.41 ^{a, e}	8.65 ± 1.46 ^d	2.13
Sj5-4_S	9.1 ± 0.1 ^d	3.2 ± 0.1 ^{a, b, d}	4.8 ± 0.01 ^c	10.7 ± 0.11 ^g	nd	14.78 ± 0.42 ^{b, f}	6.55 ± 0.31 ^{a, d}	2.26

^A Details about the sample code can be found in Table 1. ^B CT_0h, control dough without starters before fermentation. ^C S_0h, sucrose-enriched dough without starters before fermentation. ^D LAB cell density after fermentation. ^E The increase of LAB cell density after fermentation. ^F FQ, fermentation quotient. ^{a-g} Values in the same column with different letters are significantly different (p < 0.05).

560 Table 3. Mono-, di- and oligo-saccharides in control and sucrose-enriched fava bean doughs fermented by *Leuconostoc* spp. and *Weissella* spp.
561 before and after fermentation.

Sample code ^a	Sugars ^d (% , w/w, wet matter)						
	Glc (%)	Suc (%)	Fru (%)	Gal (%)	Raf (%)	Sta (%)	Ver (%)
CT_0h ^b	nd ^e	1.33 ± 0.07	nd	nd	0.12 ± 0.01	0.91 ± 0.01	1.94 ± 0.02
S_0h ^c	nd	11.65 ± 0.05	nd	nd	0.09 ± 0.01	0.68 ± 0.01	1.45 ± 0.01
20193_CT	nd	nd	nd	0.20 ± 0.01	nd	0.24 ± 0.00	0.31 ± 0.03
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
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
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
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
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
Sj5-4_S	nd	nd	5.37 ± 0.03	0.30 ± 0.01	nd	nd	nd

562 ^a Details about the sample code can be found in Table 1. ^b CT_0h, control dough without starters before fermentation. ^c S_0h, sucrose-enriched
563 dough without starters before fermentation. ^d Sugars: Glc, glucose; Suc, sucrose; Fru, fructose; Gal, galactose; Raf, raffinose; Sta, stachyose; Ver,
564 verbascose. ^e nd = not detected.

Table 4. The contents of dextran, glucan and mannitol in control and sucrose-enriched fava bean doughs fermented by *Leuconostoc* spp. and *Weissella* spp.^A

Sample code ^B	Dextran (%)	Glucan (%)	Mannitol (%)
20193_CT	0.21 ± 0.09 ^{a, e}	0.32 ± 0.01 ^a	0.16 ± 0.01 ^{a, c}
20193_S	3.67 ± 0.07 ^b	4.15 ± 0.13 ^b	0.27 ± 0.03 ^{a, c}
20240_CT	0.42 ± 0.01 ^{a, e}	0.49 ± 0.37 ^a	0.46 ± 0.01 ^{a, c}
20240_S	2.85 ± 0.11 ^c	4.33 ± 0.30 ^b	2.01 ± 0.98 ^a
5577_CT	0.11 ± 0.04 ^a	0.53 ± 0.25 ^a	0.28 ± 0.00 ^{a, c}
5577_S	2.17 ± 0.03 ^d	3.77 ± 0.11 ^{b, c}	4.22 ± 1.31 ^b
15878_CT	0.46 ± 0.04 ^e	0.50 ± 0.02 ^a	0.02 ± 0.00 ^c
15878_S	2.11 ± 0.11 ^{d, f}	2.71 ± 0.48 ^c	0.21 ± 0.10 ^{a, c}
Sj 1b_CT	0.74 ± 0.11 ^e	0.82 ± 0.03 ^a	0.34 ± 0.01 ^{a, c}
Sj 1b_S	1.86 ± 0.09 ^d	2.66 ± 0.76 ^c	1.39 ± 0.31 ^{a, c}
Sj5-4_CT	0.32 ± 0.08 ^{a, e}	0.32 ± 0.13 ^a	nd ^C
Sj5-4_S	2.24 ± 0.13 ^f	2.57 ± 0.28 ^c	nd

^A The contents were calculated on wet weight base. ^B Details about the sample code can be found in Table 1. ^C nd = not detected. ^{a-f} Values in the same column with different letters are significantly different (p < 0.05).

Table 5. Initial, final viscosity and viscosity increase of control and sucrose-enriched fava bean doughs fermented by *Leuconostoc* spp. and *Weissella* spp..^A

Sample code ^B	Initial viscosity (Pa·s)	Final viscosity (Pa·s)	Viscosity increase (Pa·s)
Control dough			
20193_CT	1.73 ± 0.20 ^a	3.16 ± 0.20 ^a	1.43
20240_CT	2.78 ± 0.20 ^b	4.57 ± 0.10 ^b	1.79
5577_CT	2.54 ± 0.20 ^b	3.92 ± 0.30 ^b	1.38
15878_CT	2.62 ± 0.00 ^b	4.27 ± 0.20 ^b	1.65
Sj 1b_CT	2.59 ± 0.10 ^b	5.87 ± 0.30 ^c	3.28
Sj5-4_CT	2.59 ± 0.10 ^b	2.59 ± 0.10 ^a	0.00
Sucrose-enriched dough			
20193_S	0.20 ± 0.00 ^a	14.77 ± 0.00 ^a	14.57
20240_S	0.35 ± 0.00 ^b	6.16 ± 0.10 ^b	5.81
5577_S	0.28 ± 0.00 ^{c, e}	6.24 ± 0.40 ^b	5.96
15878_S	0.33 ± 0.00 ^{b, d}	4.28 ± 0.60 ^c	3.95
Sj 1b_S	0.31 ± 0.00 ^{c, d}	6.54 ± 0.20 ^b	6.23
Sj5-4_S	0.27 ± 0.00 ^e	0.82 ± 0.00 ^d	0.55

^A The viscosity values of all samples were taken at the shear rate of 100 1/s. ^B Details about the sample code can be found in Table 1. ^{a-e} Values in the same column with different letters are significantly different (p < 0.05).

575 Table 6. Water holding capacity (WHC), hysteresis loop area and elastic modulus (G') of sucrose-
576 enriched fava bean doughs fermented by *Leuconostoc* spp., *Weissella* spp. and *Lactobacillus*
577 *plantarum* B24W.

Sample code ^A	WHC (%, w/w)	Hysteresis loop area (Unit) ^B	G' ^C (Pa)
20193_S	99.16 ± 0.33 ^a	7.98 ± 0.63 ^a	1588.28 ± 58.16 ^a
20240_S	88.33 ± 1.65 ^b	2.82 ± 0.01 ^b	339.42 ± 29.17 ^{b, d}
5577_S	86.41 ± 1.43 ^{b, c}	2.14 ± 0.02 ^{b, c}	667.77 ± 65.49 ^c
15878_S	87.77 ± 1.26 ^{b, c}	1.40 ± 0.04 ^c	539.92 ± 76.84 ^{b, c}
Sj 1b_S	89.26 ± 1.51 ^b	2.52 ± 0.22 ^b	1656.03 ± 93.94 ^a
Sj5-4_S	83.72 ± 1.37 ^{c, d}	0.28 ± 0.01 ^d	159.70 ± 105.35 ^{d, e}
B24W_S	81.00 ± 0.59 ^d	0.14 ± 0.01 ^d	33.85 ± 13.87 ^e

578 ^A Details about the sample code can be found in Table 1. ^B Unit = 10⁶ Pa/s. ^C G' values were taken at
579 the frequency of 1.0 Hz. ^{a-e} Values in the same column with different letters are significantly different
580 (p < 0.05).

581 Fig. 1

A

582

B

583

584 Fig. 2

585

586 Fig. 3

587