

1 **An efficient continuous flow process for the synthesis of a non-conventional**
2 **mixture of fructooligosaccharides**

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18

19 **Abstract**

20 A sustainable and scalable process for the production of a new mixture of
21 fructooligosaccharides (FOS) was developed using a continuous-flow approach based
22 on an immobilized whole cells-packed bed reactor. The technological transfer from a
23 classical batch system to an innovative flow environment allowed a significant
24 improvement of the productivity. Moreover, the stability of this production system
25 was ascertained up to 7 days of continuous working. These results suggest the
26 suitability of the proposed method for a large-scale production of the desired FOS
27 mixture, in view of a foreseeable use as a novel prebiotic preparation.

28

29 **Keywords:**

30 Fructooligosaccharides, Flow chemistry, *Cladosporium cladosporioides*, Dried Alginate
31 Entrapped Enzymes (DALGEE), Prebiotics.

32

33 **1. Introduction**

34 Biocatalysis and flow reactor technology are widely considered some of the key
35 technologies intrinsically compatible with the principle of green chemistry (Bryan, et al. 2013;
36 Ley, 2012). However, even if the effectiveness of their combination has been recently
37 demonstrated (Tamborini, Romano, Pinto, Bertolani, Conti & Molinari, 2012; Tamborini et
38 al., 2013; Itabaiana, de Mariz e Miranda & De Souza, 2013a; Itabaiana, et al., 2013b), the
39 potential of biocatalysis in flow chemistry reactors is far from being fully exploited.

40 In the present paper, we describe an application of this innovative approach in the food
41 field, by proposing a method for preparing fructooligosaccharides (FOS).

42 FOS are alternative sweeteners with a number of nutritional interesting properties: they
43 are calorie free, non-cariogenic and are considered as soluble dietary fibres (Barclay, Ginic-
44 Markovic, Cooper & Petrovsky, 2012). The energy value of FOS is 4.2–9.5 kJ/g. The
45 sweetness of FOS depends on the composition of the mixture and the sweetness of the main
46 components, *i.e.*, 1-kestose, 1-nystose, and 1-fructofuranosyl-nystose, relative to 10% sucrose
47 solution is 31%, 22%, and 16%, respectively (Antošová & Polakovič, 2001). Furthermore,
48 they induce important beneficial physiological effects, such as a prebiotic effect, an improved
49 mineral absorption and decreased levels of serum cholesterol, triacylglycerols and
50 phospholipids (Daubioul, et al., 2002; Giacco, et al., 2004). Currently, FOS are increasingly
51 included in food products and infant formulas due to their prebiotic effect that stimulates the
52 growth of non-pathogenic intestinal microflora (Sabater-Molina, Larqué, Torrella & Zamora,
53 2009).

54 FOS are found in several kinds of plants and vegetables such as banana, onion, asparagus
55 roots and artichokes, however, the supply is rather limited owing to their limited content in
56 natural sources. Therefore, they are industrially produced following two different approaches.
57 The first is based on inulin degradation, whereas the second one employs sucrose

58 transformation catalyzed by fructosyltransferase (FTase) or β -fructofuranosidase (FFase) from
59 microbial sources. Industrial scale production of FOS is commonly performed by either
60 soluble enzymes in batch reactions (Hidaka, Eida, Adachi & Saitoh, 1987) and by
61 immobilized enzymes using continuous fixed-bed reactors (Yun, Kang & Song, 1995; Park,
62 Lim, Kim, Park & Kim, 2005). Immobilized FTase from *Aureobasidium pullulans* and the
63 immobilized whole cells have been used in a packed bed reactors for the continuous
64 production of FOS at a plant scale (Jung, Bang, Oh & Park, 2011; Vaňková, Onderková,
65 Antošová & Polakovič, 2008)

66 Recently, we used the strain of *Cladosporium cladosporioides* MUT 5506 endowed with
67 transfructosylating activity to produce a new mixture of FOS from a 600 g/L solution of
68 sucrose in high yields (Zambelli, et al., 2014; Zambelli et al., 2015). In this mixture, we were
69 able to identify and fully characterize the *non*-conventional disaccharide blastose (6-O- β -D-
70 fructofuranosyl- α , β -D-glucopyranoside), whose prebiotic activity was unknown (Zambelli, et
71 al., 2014).

72 In the present work, we aimed at demonstrating the advantages of performing the
73 previously described biotransformation in a flow-chemistry reactor, with the final aim of
74 predisposing a suitable process for the sustainable and scalable production of the desired FOS
75 mixture, whose potential use as a novel prebiotic preparation can be easily foreseen and is at
76 present under investigation by us. To this purpose, a *C. cladosporioides* MUT 5506 strain was
77 used as an immobilized mycelium into a packed bed reactor to improve the productivity, the
78 efficiency and the scalability of the reported batch biotransformation. The innovative
79 application of whole microbial cells into a flow chemistry reactor combines the advantages of
80 an easy to produce biocatalyst with a process-intensification technology. Moreover, the use of
81 a continuous-flow approach based on a packed bed reactor guarantees improved mass transfer
82 and recyclability of the solid catalyst (Kirschning, Solodenko & Mennecke, 2006). The

83 produced FOS mixture, as well as the isolated *non*-conventional blastose, were submitted to a
84 preliminary *in vitro* study to assess their ability to promote the growth – as sole carbon
85 sources – of selected probiotic strains, thus giving a first indication of their suitability for a
86 potential application as prebiotics.

87

88 **2. Materials and methods**

89 *2.1. Materials*

90 Sucrose, glucose, fructose and *p*-anisaldehyde were purchased from Sigma-Aldrich.
91 Standards of 1-Nystose and 1-kestose were purchased from Fluka (DE). A standard of 1-F-
92 fructofuranosylnystose was purchased from Megazyme. Actilight® was kindly donated by
93 Beghin Meiji. Gluzyme MONO10000 preparation was kindly donated by Novozymes. Yeast
94 extract was purchased from Difco (Difco, MD, USA) and barley malt flour from Diagermal
95 (IT).

96

97 *2.2. Flow chemistry equipment*

98 A R2+/R4 combination flow reactor commercially available from Vapourtec was used.
99 The main R2+ system is driven by two integrated HPLC pumps; the flow rates can be
100 regulated and set at any value between 0.01 to 10 mL/min working with a system pressure of
101 up to 30 bar without any risk. The R4 heater guarantees a precise temperature control over the
102 range room temperature to 150 °C in four independent controlled air-circulating heating
103 zones, with a rapid temperature ramping and cooling (80 °C/min). The four reactor zones can
104 each accept either a packed column or a flow tube arrangement providing reaction volumes of
105 0.1 to 10 mL (or 40 mL in a linked sequential operation). A back-pressure regulator is applied
106 in-line, if necessary. The system is also outfitted with a pair of injection loops that are
107 positioned post the pumps. Finally, at the top, a large drip tray is located for reagents bottles

108 and collection vessels giving the whole system a very small compact foot print which fits
109 comfortably into any fume cupboard.

110

111 2.3. Strains and growth conditions

112 *C. cladosporioides*, previously isolated and deposited at *Mycotheca Universitatis*
113 *Taurinensis* (MUT) as strain MUT 5506, was maintained as previously described (Zambelli,
114 et al., 2014).

115 Five probiotic *Lactobacillus* strains (*Lactobacillus paracasei* DG, *Lactobacillus*
116 *rhamnosus* GG, *Lactobacillus paracasei* SHIROTA, *Lactobacillus johnsonii* LC1,
117 *Lactobacillus reuteri* ATCC55730) used in this study were cultivated in MRS broth (Difco)
118 and incubated at 37 °C for 24 hours. The bacterial cell concentration of an overnight culture
119 was determined microscopically with a Neubauer improved counting chamber (Marienfeld
120 GmbH, Lauda-Königshofen, Germany). For the assessment of *in vitro* blastose fermentation,
121 strains were inoculated in triplicate at a final concentration of 4×10^5 bacteria/mL (from
122 cultures grown overnight to the stationary phase). The medium used to test the growth with
123 different sugars was API 50 CHL medium without glucose, prepared at different dilutions.
124 The medium was added with a 0.5% concentration of different sugars (di- and
125 oligosaccharides): glucose (as positive control), inulin, blastose, FOS-mixture, FOS-mixture
126 without blastose and a commercially available mixture of FOS (Actilight®) (Taveriniti et al.,
127 2012). *In vitro* experiments were carried out in 384 well plates, filled by means of an
128 automated pipetting system epMotion 5070 (Eppendorf, Germany). The microbial growth was
129 monitored with a spectrophotometer (MicroWave RS2, Biotek, USA) programmed for 145-
130 290 readings (OD 600 nm) every 10 min for 24-48 h at 37 °C. At the end of the incubation,
131 the μ Max and the final OD at 600 nm were calculated using the software Gen5 (Biotek, USA)

132 and reported as the mean of three independent measurements \pm standard deviation (Arioli et
133 al., 2014).

134

135 *2.4. Fructofuranosidase activity assays*

136 The enzymatic activity towards sucrose was determined by measuring the initial rate of
137 reducing sugar formation using the dinitrosalicylic acid (DNS) assay adapted to 96-well
138 microplates (Rodriguez, Perez, Ruiz, & Rodriguez, 1995). Dried alginate entrapped (DALGE)
139 mycelium (10 g/L) was incubated with 1 mL of a sucrose solution (100 g/L) in acetate buffer
140 (20 mM pH 6.0) for 20 min at 50 °C and 90 rpm. 50 μ L of the solution, conveniently diluted
141 to fit into the calibration curve, were added to each well. Then, 50 μ L of 10 g/L DNS were
142 added. The plate was incubated for 20 min at 80 °C to develop colour with a seal plate tape
143 (GeneMate). After cooling, 150 μ L of water were added to each well, and the absorbance
144 measured at 540 nm using a microplate reader (model Versamax, Molecular Devices). One
145 unit (U) of activity was defined as that catalysing the formation of 1 μ mol reducing sugar per
146 minute under the above described conditions.

147

148 *2.5. Dried alginate entrapped (DALGE) mycelium*

149 The gel beads were prepared by ionotropic gelation following a slightly modified protocol
150 previously developed by us (Fernandez-Arrojo et al., 2013). A 4% (w/v) sodium alginate
151 solution was prepared in distilled water and stirred until a homogeneous clear solution was
152 formed. The solution was left to settle for 2 h in order to eliminate all the air bubbles. The
153 alginate solution was then gently mixed in a ratio 1:1 (w/w) with 40 g/L of lyophilized
154 mycelia in 20 mM sodium acetate buffer (pH = 6.0) after 5 cycles of sonication at 15 kHz for
155 1 min (Soniprep 150, MSE). The resulting mycelia–alginate mixture was then used as
156 previously described (Fernandez-Arrojo et al., 2013).

157

158 *2.6. Biotransformation with DALGE-mycelium in batch*

159 40 g/L of DALGE-mycelium was added to a 600 g/L sucrose solution in sodium acetate
160 buffer 20 mM pH 6.0 in a final volume of 2 mL, incubated at 50 °C in an orbital shaker at 90
161 rpm. The biotransformations were followed for 144 h and analyzed by HPLC.

162

163 *2.7. Analytical HILIC-HPLC*

164 The analytical data were collected with a HPLC system composed by a Hitachi LaChrom
165 L-7100 pump available from Merck (DE) equipped with a Sedex 75 evaporative light
166 scattering detector (ELSD) available from Alfatech (FR). The chromatographic separation of
167 products was performed with a Luna NH₂ 100 Å column (250 × 4.6 mm, particle size 5 µm,
168 Phenomenex, Aschaffenburg, Germany) equipped with the corresponding guard column (4 ×
169 3.0 mm), and kept at 30 °C with a Merck T-6300 column thermostat. The mobile phase was
170 acetonitrile/water (80:20 v/v) at a flow rate of 1.0 mL/min. The temperature of detection was
171 set to 52 °C. EZ Chrome Elite software by Agilent was used for data management.

172

173 *2.8. Semi-Preparative HILIC-HPLC*

174 The semi-preparative purifications were performed with a commercially HPLC system
175 composed by a Hitachi LaChrom L-7100 pump available from Merck equipped with a Sedex
176 75 evaporative light scattering detector (ELSD) available from Alfatech. The
177 chromatographic separation of products was performed with a Luna NH₂ 100 Å column (250
178 × 10 mm, particle size 5 µm, Phenomenex, Aschaffenburg, Germany) at room temperature.

179 The following gradient of was used:

180 - 0'-30' acetonitrile/water (80:20 v/v)

181 - 30'-35' switch to acetonitrile/water (50:50 v/v)

182 - 35'-40' acetonitrile/water (50:50 v/v)

183 - 40'-45' switch to acetonitrile/water (80:20 v/v)

184 - 45'-60' acetonitrile/water (80:20 v/v).

185 The flow rate was set to 4.7 mL/min. The temperature of detection was set to 52 °C. EZ
186 Chrome Elite software by Agilent was used for data management.

187

188 2.9. Calculation of the T/H index

189 The transfructosylation/hydrolysis ratio (T/H index) was calculated using the following
190 equation (Eq. 1):

$$191 \quad T/H = ([\text{glucose}] - [\text{fructose}]) / [\text{fructose}].$$

192 Eq. 1. Transfructosylation/hydrolysis ratio (T/H)

193

194 2.10. Productivity

195 The productivities for batch and flow biotransformations were calculated at the same
196 degree of conversion using the following equations (Eq. 2 and Eq. 3):

$$197 \quad r_{\text{batch}} = m_p / (t \times m_E)$$

198 Eq. 2. r_{batch} : batch productivity; m_p : amount of the product of interest (mg); t : reaction
199 time (min); m_E : mass of the catalyst used (g).

$$200 \quad R_{\text{flow}} = [P] \times f / m_E$$

201 Eq. 3. r_{flow} : flow productivity; $[P]$: concentration of the product of interest (mg/mL); f :
202 flow rate (mL/min); m_E : mass of the catalyst packed in the reactor (g).

203

204 2.11. Continuous flow biotransformation using alginate mycelia in a packed bed column

205 Alginate beads and celite (1:1 w/w) were packed into an Omnifit® glass Column (15 mm
206 id × 150 mm length). Each gram of alginate beads, mixed with the same volume of celite, can

207 fill about 6 mL of the inner volume of the column. The swelling of the dried beads was
208 achieved with an acetate buffer solution (20 mM, pH 6) before connecting the column. A flow
209 stream of 600 g/L sucrose solution in 20 mM sodium acetate buffer (pH 6) was pumped
210 through the packed bed column, at different flow rates, in order to obtain residence times
211 from 5 hours to 30 hours. The appropriate temperature was set by the R4 block heater. An
212 aliquot (100 µL) of the exiting flow stream was diluted with water (1:3), the sample was
213 filtered on a 0.45 µm nitrocellulose filter and analysed by HLIC-HPLC.

214

215 *2.12. Glucose oxidation with Gluzyme MONO 10000 BG*

216 The mixture obtained under optimized conditions after 20 hours of residence time has
217 been exposed to the biocatalytic activity of a glucose oxidase preparation from Novozymes
218 (Gluzyme MONO 10.000 BG) in batch (Sheu, Lio, Chen, Lin & Duan, 2001). To a 600 g/L
219 mixture (20 mL) in acetate buffer 20 mM (pH 6) Gluzyme powder (800 mg, 400 U/mL) and
220 CaCO₃ (800 mg) were added. The resulted suspension was stirred at 35 °C insufflating air.
221 The produced gluconic acid precipitated as calcium salt in the reaction flask. At different
222 times, aliquots (100 µL) were withdrawn, diluted with water (1:3) and incubated for 10 min at
223 90 °C to inactivate the enzyme. Each sample was filtered on a 0.45 µm nitrocellulose filter
224 and analysed. After 24 h the whole suspension was centrifuged at 14000 rpm for 30 min,
225 filtered on a cellulose filter paper and used for preparative HPLC purification.

226

227 **3. Results and discussion**

228 In our previous work, we reported a method for the batch production of a new mixture of
229 FOS, starting from a 600 g/L solution of sucrose using lyophilized mycelium of *C.*
230 *cladosporioides* MUT 5506 in 20 mM acetate buffer (Zambelli, et al., 2014). The FOS
231 mixture was characterized by the presence of a high variety of FOS, *i.e.*, kestose (1-, 6- and

232 neo-), nystose (1-, 6-, and neo-), 1-fructofuranosylnystose and, interestingly, an unusual
233 disaccharide named blastose. Given our great interest in investigating the potential prebiotic
234 properties of this new type of FOS mixture, we decided to exploit the flow chemistry facilities
235 to implement its production, with the aim of setting up a suitable process for the sustainable
236 and scalable production of the desired FOS mixture on a large scale. Thus, we decided to
237 perform the biotransformation into a meso-flow reactor, using immobilized mycelia of *C.*
238 *cladosporioides*. Dried alginate entrapped (DALGE)-mycelium was identified as the suitable
239 kind of immobilization for the application in a continuous flow reactor, due to the good
240 stability over the time and during subsequent cycles of biotransformations. Moreover,
241 alginates are economic and easy to prepare (Fernandez-Arrojo, et al., 2013).

242 First, the reaction was performed in batch, following the procedure reported above. The
243 higher conversion (total FOS amount ~ 51% w/w) was achieved after a prolonged reaction
244 time (96 hours) and blastose appeared only after 48 hours with a maximum (4.9% w/w) at 168
245 hours. We then moved to flow. To this aim, a glass column (10 mm id × 100 mm length) was
246 packed with the DALGE-mycelium beads and a 600 g/L sucrose solution in 20 mM acetate
247 buffer (pH 6.0) was flowed through it (Scheme 1). To avoid any unwanted and uncontrolled
248 increase of the pressure due to a clogging, celite was mixed to the alginates before the
249 packaging (alginate beads: celite = 1:1 v/v). In this way, no over-pressure was observed over
250 the reaction time.

251 **Scheme 1 to be inserted here.**

252 We initially optimized the reaction parameters using the transfructosylation/hydrolysis
253 ratio (T/H index) as an index of the transglycosylation rate. First, the temperature effect on
254 the T/H index was evaluated (Figure 1). The residence time was kept constant at 5 h and the
255 temperature was varied between 25 °C and 70 °C. The highest T/H index was obtained at 35
256 °C, with a value of 4.0 ± 0.2 . Then, the effect of the pressure on the biotransformation was

257 evaluated, keeping the temperature constant at 35 °C. Pressurization of the system was easily
258 achieved applying in-line different backpressure regulators (40, 75, 100 and 250 psi). No
259 significant differences in the T/H index were observed (Figure 1) and, for this reason, we
260 decided not to use any back pressure regulator to avoid any negative influence of the
261 prolonged pressurization on the stability of the enzyme and/or alginates over the time.

262 **Figure 1 to be inserted here.**

263 After having set the optimal operating conditions (*i.e.* T = 35 °C, atmospheric pressure),
264 we analyzed the kinetic profile of the packed bed continuous-flow biotransformation and
265 compared it to that obtained in batch with *C. cladosporioides* alginates in buffer. Considering
266 the biotransformation catalyzed by DALGE-mycelium in acetate buffer (pH 6), the main
267 products were 1-kestose, 1-nystose, 1-fructofuranosylnystose and blastose, with a maximum
268 concentration of 139 g/L (23% w/w), 96 g/L (16% w/w), 15 g/L (2.5% w/w), and 30 g/L (5%
269 w/w), respectively. Neo-kestose, neo-nystose and 6-kestose were found in a later stage of the
270 biotransformation (after 48 h of reaction time), with a concentration below 12 g/L (< 2%
271 w/w).

272 In flow, working at atmospheric pressure and at a temperature of 35 °C, we considered
273 the residence time and we evaluated the conversion in terms of total amount of FOS formed
274 (Figure 2). The maximum amount of FOS was reached at 20 h of residence time and remained
275 constant up to 30 h. At 20 h of residence time, the concentration of FOS was 313 g/L (52.2 %
276 w/w), as represented in figure 2, with a concentration of 1-kestose, 1-nystose, 1-
277 fructofuranosylnystose and blastose of 139 g/L (23.2 % w/w), 86 g/L (14.3 % w/w), 39 g/L
278 (6.5% w/w) and 28 g/L (4.7% w/w) respectively, and a concentration of neo-kestose, 6-
279 kestose and neo-nystose of 9 g/L (1.5% w/w), 6 g/L (1% w/w) and 6 g/L (1% w/w),
280 respectively. It is worth noting that, after only 10 h of residence time, a 47.4 % (w/w) of
281 conversion was already reached and that the total amount of FOS only slightly increased in

282 the following 10 hours. A similar conversion (48% w/w) was obtained in batch after 72 hours
283 of reaction. A significant reduction of the reaction time is a typical benefit achieved on
284 passing from batch to flow conditions, both for heterogeneous chemo- or bio-catalyzed
285 reactions (Kirschning, Solodenko & Mennecke 2006; Puglisi, Benaglia & Chiroli, 2013).
286 Focusing on blastose formation, as shown in Figure 2, blastose could be identified in the
287 reaction flow stream after only 5 hours (residence time), and it reached a concentration of 28
288 g/L (4.7% w/w) after 20 hours, whereas a similar concentration (30 g/L) could be obtained in
289 batch only after 168 hours. Moreover, running the reaction in flow for additional ten hours
290 (30 hours of total residence time), the amount of blastose increased up to 31 g/L (5.2% w/w)
291 of the total solution.

292 **Figure 2 to be inserted here.**

293 To compare the batch and flow efficiencies for the FOS production, we determined the
294 productivity values for both total FOS and blastose, at different reaction times, in these two
295 different environments. In batch conditions (72 hours, total FOS ~ 48.0% w/w), we obtained a
296 complex mixture of different FOS, with different degree of polymerization and structures, and
297 a productivity of 1.66 mg/g*min. In flow, a similar FOS concentration (47.4%) was reached
298 with a residence time of 10 hours. In these conditions, the productivity value was equal to
299 2.84 mg/g*min, about 1.7 times the productivity obtained in batch. Considering blastose, the
300 maximum value (30 g/L) in batch was reached after 168 hours. This corresponds to a
301 productivity 0.07 mg/g*min. In flow, a similar concentration of blastose was obtained after 20
302 hours of residence time: the productivity is 0.14 mg/g*min, two times the productivity
303 obtained in batch.

304 After the optimization of reaction parameters, we exploited the packed bed flow system
305 for the continuous production of the new mixture of FOS. First, we evaluated the stability of
306 the packed bed alginate beads over the time; to this aim, the conversion at 5 h of residence

307 time was evaluated during 7 days of continuous work. The reaction outcome was checked the
308 first time, after 5 hours of residence time and, then, every 24 hours, for one week. A similar
309 concentration of residual sucrose was observed in the monitored reaction time (Figure 3). The
310 same packed column was then washed with the acetate buffer 20 mM and conserved at 4 °C
311 for two months. The column was used again under the same reaction conditions and a similar
312 conversion was obtained. This outcome highlights the possibility of using this set up for a
313 continuous production over the time, without the need of replacing or recycling the
314 biocatalyst.

315 **Figure 3 to be inserted here.**

316 Subsequently, we selected a residence time of 20 h, because the highest amount of total
317 FOS is formed, including a 4.6% (w/w) of blastose, and we followed the production over 7
318 days of continuous work, as represented in figure 4. It is important to note that, using the flow
319 system, once fixed the residence time, a constant mixture of FOS can be produced. Indeed,
320 the total FOS amount remains in a range of 51.7-53.0% (w/w) during the monitored reaction
321 time.

322 **Figure 4 to be inserted here.**

323 Finally, we set up a suitable method for the purification of the FOS mixture from the
324 reaction medium and for the isolation of the *non*-conventional sugar blastose, in order to
325 submit them to biological investigation. Glucose is the main undesirable by-product in the
326 synthesis of FOS catalyzed by *trans*-fructofuranosydases (Duan, Chen & Sheu, 1994). In the
327 exiting flow stream, glucose represents the 26% (w/w) of the mixture and its presence
328 complicates the purification of the FOS mixture, and, in particular, the isolation of blastose.
329 Therefore, we exploited a method reported in the literature for glucose removal based on its
330 enzymatic oxidation to gluconic acid catalyzed by a commercially available glucose oxidase
331 (Gluzyme® mono 10000 BG, from Novozymes); the formed gluconate can be precipitated as

332 calcium salt and easily removed (Sheu, Lio, Chen, Lin & Duan, 2001; Biyela, Du Toit, Divol,
333 Malherhe & Van Renshurg, 2009). A final semi-preparative HPLC step was necessary to
334 obtain either the pure FOS mixture or the blastose (97% purity).

335 In the last decade the concept of “prebiotic”, substrate that selectively stimulates the
336 growth and activity of health-promoting *Lactobacilli* and *Bifidobacteria*, has assumed much
337 interest in terms of improving human host health (Gimeno-Pérez, Linde, Fernández-Arrojo,
338 Plou & Fernández-Lobato, 2014). In order to investigate the ability of probiotic strains to use
339 blastose as the sole carbon source and thus its suitability for a potential application in
340 symbiotic-prebiotic mixtures, an *in vitro* fermentation study was carried out on 5 well
341 characterized probiotic *Lactobacillus* strains, easier to cultivate than *Bifidobacteria*: *L.*
342 *paracasei* DG (Ferrario et al, 2014), *L. rhamnosus* GG (Segers and Leber, 2014); *L.*
343 *paracasei* SHIROTA (Aoki et al., 2014); *L. johnsonii* LC1 (Isobe et al., 2012); *L. reuteri*
344 ATCC55730 (Di Nardo et al, 2014; Valeur, Engel, Carbajal, Connolly, & Ladefoged, 2004).
345 In detail, *Lactobacillus* strains were grown in presence of glucose (considered as positive
346 control) and two well known prebiotic substrates, *i.e.* inulin and FOS Actilight®. Then, the
347 growth kinetic parameter (μ_{\max} value) and the final OD after 48 h of incubation were
348 compared to that obtained in presence of our new FOS-mixture, our FOS-mixture without
349 blastose and only blastose. The results are summarized in Table 1.

350 **Table 1 to be inserted here.**

351 All the *Lactobacillus* probiotics were able to growth in the presence of blastose as carbon
352 source, except for *L. reuteri* strain, able to growth only in presence of glucose. Among the
353 probiotics tested, *L. paracasei* DG and *L. johnsonii* LC1 showed the most efficient growths in
354 presence of the *non-conventional* disaccharide, with an increase of the final OD of 50% and
355 109% and higher μ_{\max} s (>62% and >77%) in respect to glucose. These data indicate a growth
356 stimulation effect of blastose. No increases of biomass production or μ_{\max} values were

357 observed for *L. rhamnosus* GG and *L. casei* Shirota, probably due to a less efficient
358 disaccharide transport system in these species. More in general, the microorganisms, in the
359 presence of blastose as sole carbon source, are able to reach higher OD and μ_{\max} than in the
360 presence of other prebiotic substrates such as inulin and the FOS mixture Actilight®.
361 Surprisingly, our new FOS mixture seems to be better metabolized than FOS Actilight® by *L.*
362 *rhamnosus* and *L. casei* cells, reaching a major final OD value and an increased μ_{\max} . This
363 effect is probably due to a different 1F-FOS amount compared to Actilight®, as well as the
364 presence of about 5% of blastose (Zambelli et al., 2014). These positive results provide a first
365 preliminary evidence on the potential *in vitro* prebiotic effect of the newly isolated blastose
366 and of our new FOS mixture. Nevertheless, further *in vitro* and *in vivo* studies are needed in
367 order to assess the effect of the new compound on other probiotic microorganisms and its
368 possible use in prebiotic formulations.

369

370 **4. Conclusions**

371 A continuous production of a new mixture of fructooligosaccharides was performed
372 exploiting the innovative combination of immobilized whole cells with a continuous flow
373 chemistry reactor, leading to a significant improvement of the total FOS productivity (1.7
374 times) and blastose productivity (2 times), compared to classical batch methods. Moreover,
375 we ascertained the stability of our production system over the time and, indeed, we performed
376 a 7 days continuous production cycle, being able to produce a mixture of FOS characterized
377 by a constant composition. Hence, the use of a continuous flow reactor allowed to overcome
378 some of the typical limitations of batch biotransformations, such as long reaction times,
379 product inhibition, and scalability.

380 These results suggest the suitability of the proposed method for a sustainable and scalable
381 production of the desired FOS mixture, whose potential use as a novel prebiotic preparation

382 can be easily foreseen and is at present under investigation by us. The results presented here,
383 even if only preliminary, indicate that blastose has prebiotic properties similar or higher than
384 the reference prebiotics, *i.e.*, inulin and a commercialized FOS mixture (Actilight®),
385 depending on the probiotic species analyzed. This result represents the first indication that
386 blastose has a positive influence on the growth of probiotic microorganisms and this can
387 justify further *in vivo* assays conducted on animal models or human volunteers to ensure and
388 better assess its prebiotic properties.

389

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394

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510

511 **Figure and scheme captions**

512

513 **Scheme 1.** Schematic representation of the continuous system configuration used for
514 pressure and temperature optimization. BPR: back pressure regulator.

515

516 **Figure 1.** Graph of the T/H index in function of temperature (A) and pressure (B). A)
517 Conditions: [sucrose]: 600 g/L in acetate buffer 20 mM (pH 6); alginate beads: 1 g; celite: 1
518 g; residence time: 5 h; atmospheric pressure. B) Conditions: [sucrose]: 600 g/L in acetate
519 buffer 20 mM (pH 6); alginate beads: 1 g; celite: 1 g; residence time: 5 h; T = 35 °C. Results
520 are the average of three trials with standard deviations were lower than 5%.

521

522 **Figure 2.** Total (A) and single (B) FOS amount at different residence times using a
523 packed bed continuous flow reactor. Conditions: [sucrose]: 600 g/L in acetate buffer 20 mM
524 (pH 6); alginate beads: 2 g; celite: 2 g; T = 35 °C; atmospheric pressure. Results are the
525 average of three trials with standard deviations were lower than 5%.

526

527 **Figure 3.** Sucrose residual concentration during 7 days of continuous work. Conditions:
528 [sucrose]: 600 g/L in acetate buffer 20 mM (pH 6); alginate beads: 2 g; celite: 2 g; T = 35 °C;
529 atmospheric pressure. Results are the average of three trials with standard deviations were
530 lower than 5%.

531

532 **Figure 4.** Continuous production of FOS under optimized conditions. Conditions:
533 [sucrose]: 600 g/L in acetate buffer 20 mM (pH 6); alginate beads: 2 g; celite: 2 g; T = 35 °C;
534 atmospheric pressure. Results are the average of three trials with standard deviations were
535 lower than 5%.

536

537 **Table 1.** Growth parameters (OD 600 nm and μ_{\max}) of probiotics during the *in vitro*

538 prebiotic assay.

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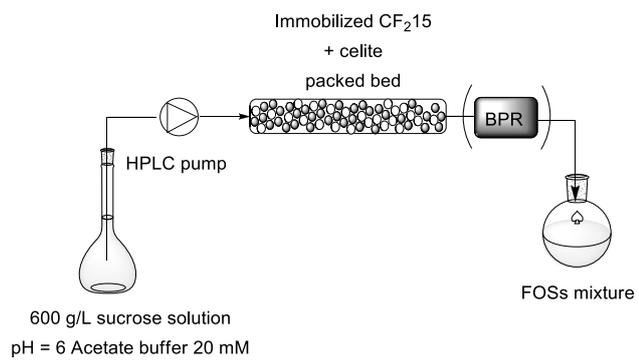
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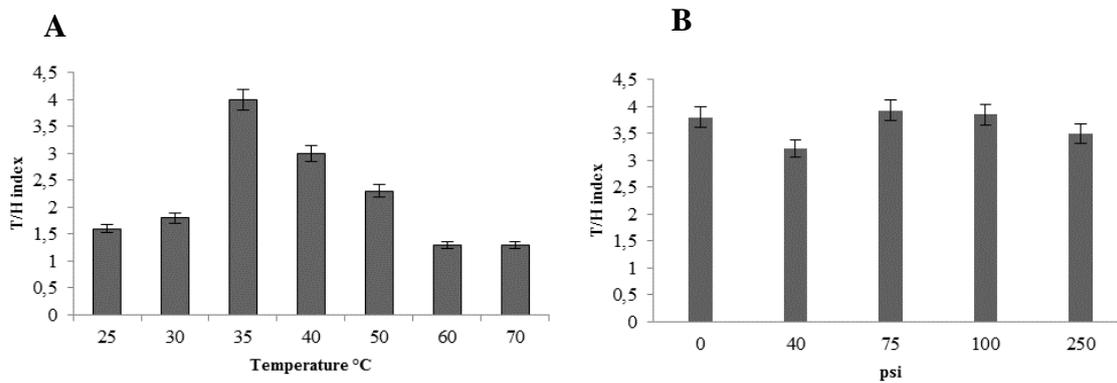


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Scheme 1

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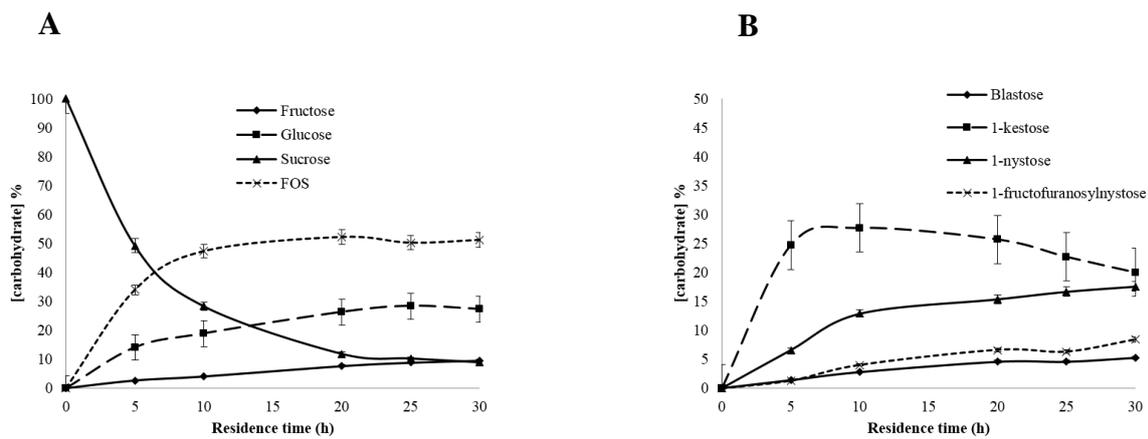
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Figure 1.

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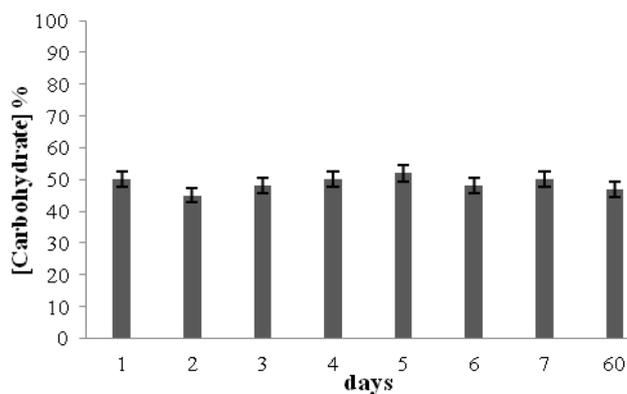
555 **Figure 2.**

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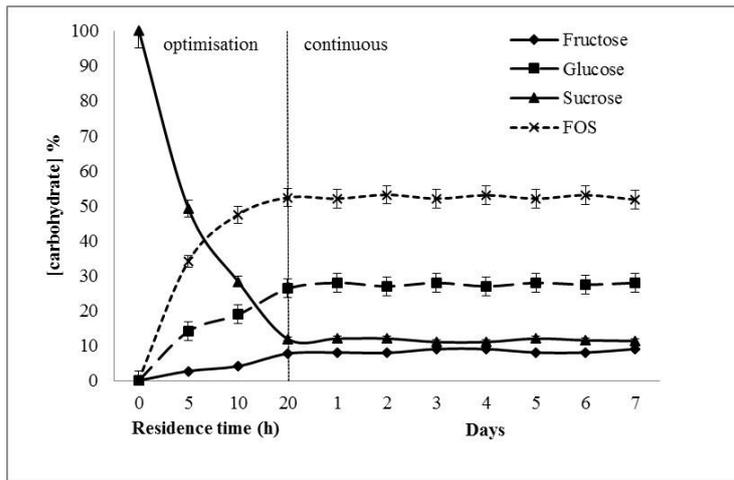
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560

561 **Figure 3.**

562



563

564 **Figure 4.**

565

566 **Table 1.** Growth parameters (OD_{600nm} and μ_{max}) of probiotics during the *in vitro* prebiotic
 567 assay.

Carbohydrate	<i>L. paracasei</i>		<i>L. rhamnosus</i>		<i>L. casei</i>		<i>L. johnsonii</i>		<i>L. reuteri</i>	
	OD _{600nm}	μ_{max} mOD/min								
Glucose	1.2±0.07	0.29±0.07	1.9±0.03	0.71±0.002	1.6±0.08	0.43±0.09	1.1±0.01	0.49±0.05	1.5±0.01	0.1±0.01
Inulin	0.9±0.03	0.26±0.03	0.9±0.01	0.32±0.03	1.1±0.03	0.28±0.00	0.8±0.01	0.21±0.02	n.d	n.d
FOS Actilight®	1.5±0.02	0.34±0.03	0.74±0.05	0.13±0.03	n.d.	n.d.	1.8±0.05	0.86±0.02	n.d	n.d
FOS mixture	1.4±0.05	0.39±0.03	1.2±0.07	0.40±0.03	1.6±0.03	0.36±0.01	1.9±0.05	0.82±0.02	n.d	n.d
FOS mixture (-blastose)	1.4±0.04	0.38±0.09	1.1±0.07	0.35±0.02	1.1±0.04	0.35±0.03	2.0±0.01	0.21±0.02	n.d	n.d
Blastose	1.8±0.05	0.47±0.06	1.8±0.07	0.57±0.02	1.6±0.04	0.53±0.02	2.3±0.05	0.87±0.02	n.d	n.d

568 n.d: not detected growth after 48h of incubation at 37 °C

569