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Technological potential of *Bifidobacterium aesculapii* strains for fermented soymilk production

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1 **Technological potential of *Bifidobacterium aesculapii* strains for fermented soymilk**
2 **production**

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Abstract

The present research was aimed to investigate the technological potentialities of seven strains of *Bifidobacterium aesculapii*, a species recently described, in terms of exopolysaccharide (EPS) production and as starter fermentation in soybean milk. The strain production of EPS was firstly evaluated in model system, using different carbon sources. Furthermore, the fermented products obtained by the seven strains of *B. aesculapii* were tested for their EPS content and strain cell loads, the volatile molecule profiles, the texture features and the overall acceptance. The data showed that all the *B. aesculapii* strains were able to produce EPS in *vitro* model in presence of 1.5% and 2% glucose while only four strains were able to produce EPS in presence of lactose 2%. When the strains were employed as fermentation starters in soybean milk, some showed a good growth potential, fermenting the substrate in 14 hours and giving rise to fermented products with good firmness and viscosity indexes. Moreover, five strains out seven showed production of EPS (from 5 to 174 $\mu\text{g/mL}$) in soybean fermented milk.

Keywords: *Bifidobacterium aesculapii*, fermented soymilk, exopolysaccharides, volatile molecule profiles, texture profile

53 1. Introduction

54 Fermented milk obtained from spontaneous microbial fermentation has been traditionally
55 used by nomadic populations from Arabic peninsula, Caucasus and Anatolia, who based their
56 nutrition on milk and milk based products (Oberman & Libudzisz, 1998). International Dairy
57 Federation defined a fermented milk product as “the milk product prepared from skimmed
58 milk or not with specific cultures. The microflora is kept alive until sale to the consumers and
59 may not contain any pathogenic germs” (Panesar, 2011). Depending on the fermenting
60 microflora (lactic acid bacteria and yeasts), fermented milks could be classified as yogurt,
61 acidophilus milk, mayzum, buttermilk, kefir, kumis and leben, with an additional potential
62 functional role when a probiotic bacteria is added (Rivière, Selak, Lantin, Leroy, & De Vuyst,
63 2016). Even though fermented milks containing probiotics can improve the human health,
64 their sensorial features play a crucial role in the product acceptance by consumers. Mainly,
65 strains belonging to *Lactobacillus* spp. and *Bifidobacterium* spp. are used as probiotic bacteria
66 in fermented milk products. However, *Bifidobacterium* strains, due to the presence of oxygen
67 and low pH, are not able to fully explicate their probiotic functionalities when added to milk
68 based products (Kumari, Ranadheera, Prasanna, Senevirathne, & Vidanarachchi, 2015). Also
69 in the human gastrointestinal tract, *Bifidobacterium* strains are more affected by the stomach
70 conditions, such as pH and bile salt concentration compared to *Lactobacillus* ones (Ferdousi
71 et al., 2013). Other factors, such as process parameters, packaging and storage can affect their
72 survival, viability and activity. When probiotics are used in milk as adjuncts or co-starters,
73 *Streptococcus thermophilus* is preferred as starter instead of *Lactobacillus delbrueckii* subsp.
74 *bulgaricus*, to overcome viability losses since *Lactobacillus delbrueckii* subsp. *bulgaricus*
75 increases the acidity of the product during the fermentation (Glušac et al., 2015). When
76 probiotic bacteria, and especially *Bifidobacterium*, are used as unique starter cultures for milk
77 fermentation, the obtained products are often characterized by the lack of desirable sensory
78 features. In particular, structural defects and lack of aroma were reported for milks fermented

79 by *Lactobacillus acidophilus* and *Bifidobacterium* spp. strains (Patrignani et al., 2016).
80 Incorporation of exopolysaccharide (EPS) producing lactic acid bacteria (LAB) in fermented
81 milks can represent a technological challenge when *Bifidobacterium* strains are used. In
82 fact, the EPS-producing LAB strains have increasingly been used as functional starter cultures
83 for manufacturing fermented products due to their ability to improve rheology, texture and
84 mouthfeel, and reducing product syneresis, replacing stabilizers and increasing the mouth
85 thickness. EPS production from *Bifidobacterium* is currently well documented (Hidalgo-
86 Cantabrana et al., 2014; Salazar et al., 2015), and a sugar source modulation on the EPS
87 biosynthesis in *B. longum* subsp. *longum* CRC 002 has been demonstrated by Audy, Labrie,
88 Roy, & LaPointe (2010). However, to date, there is little information on the use of EPS-
89 producing *Bifidobacterium* strains as functional starters in fermented milk products (Prasanna,
90 Bell, Grandison, & Charalampopoulos, 2012). The literature identified *Bifidobacterium*
91 *longum* and *Bifidobacterium pseudolongum* strains as the most resistance to acidity and bile
92 salts. *B. longum* subsp. *longum*, *B. longum* subsp. *infantis* and *B. breve* are the species
93 commonly used in the production of fermented milk (Lankaputhra & Shah, 1995). Recently, a
94 novel species, named *Bifidobacterium aesculapii*, isolated from the faeces of the baby
95 common marmoset (*Callithrix jacchus*), was described by Modesto et al. (2014). These
96 strains were found positive for galactosyl transferase, *cspD*, considered one of the key
97 enzymes involved in the catalyses of the first step of the EPSs-units biosynthesis (Duranti et
98 al., 2017).

99 Thus, principal aim of this research was to investigate the EPS production ability for seven *B.*
100 *aesculapii* strains in model system using two different carbon sources, i.e. glucose or lactose.
101 Furthermore, *B. aesculapii* strains were tested as starters in soybean milk. The fermented milk
102 products obtained were characterized for their EPS content, strain cell loads, the volatile
103 molecule profiles, texture features and overall acceptance.

105 **2. Materials and Methods**

106 *2.1 Strains*

107 The *B. aesculapii* strains used in this study are listed in Error! Reference source not found..
108 *Bifidobacterium longum* subsp. *infantis* ATCC 15697 and *Bifidobacterium saguini* DSM
109 23967^T were also included as controls. All strains were revitalized from freeze-dried state, in
110 TPY medium (BD, Milano, Italy), generally used for the enumeration of *Bifidobacterium*,
111 and incubated anaerobically at 37 °C for 24 h. The anaerobic atmosphere was obtained using
112 the GasPak EZ Anaerobic Pouch system (BD).

113 *2.2 Antibiotic susceptibility*

114 The antibiotic susceptibility of the strains was determined using M.I.C.E. evaluator strips
115 (Oxoid Ltd., Basingstoke, UK). The OD600 of 24 h strain cultures were adjusted at 0.6. One
116 hundred microliters of the cell culture (approximately 7 log cfu/mL) were inoculated on MRS
117 agar plates and streaked over the entire surface of the plates. The inoculated plates were dried
118 for about 15 min and finally the M.I.C.E evaluators strips were placed under sterile conditions
119 at the centre of the plates. The plates were then incubated under anaerobic conditions at 37°C
120 for 24 h and the results were read as reported in Thermo ScientificTM OxoidTM
121 M.I.C.EvaluatorTM (M.I.C.E.TM) Strips Interpretation Guide. The tested antibiotics and the
122 relative ranges of concentrations were the followings: Amoxicillin, 256-0.015 µg/mL;
123 Ampicillin, 256-0.015µg/mL; Ciprofloxacin, 32-0.002µg/mL; Clindamycin, 256-0.015µg/mL;
124 Erythromycin, 256-0.015 µg/mL; Gentamicin, 256-0.015µg/mL; Levofloxacin, 32-0.002
125 µg/mL; Penicillin, G 32-0.002µg/mL; Tetracycline, 256-0.015µg/mL and Vancomycin 256-
126 0.015 µg/mL.

127

128 *2.3 Quantification of EPS*

129 To verify the ability of the strains to produce EPS in model medium in relation to the used
130 carbon sources, 10% of an overnight culture of each strain was inoculated and cultivated three
131 times anaerobically at 37 °C for 24h h in TPY prepared by addition of 1.5% (control) or 2%
132 of glucose (Merck, Darmstadt, Germany), or 1.5% or 2% of lactose (Merck). For each trial,
133 three replications were performed. At the end of incubation time, all samples were checked
134 for viable cell loads. The exopolysaccharides (EPS) extraction and quantification, also from
135 fermented milk, were performed according to the method by Goh, Haisman, Archer, & Singh
136 (2005). Briefly, after adjusting the samples to pH 7, 100 µl of Flavourzyme (10%) were added
137 to each sample and vortexed for 15 sec. before incubation at 50 °C for 4 h with gentle stirring.
138 After the incubation, 500 µl aliquots from each sample was transferred into 10 mL plastic
139 tubes containing 2.9 mL of ultra pure water and 7 mL of cold absolute ethanol and finally
140 incubated overnight at 4 °C. After overnight incubation, pellets were recovered by
141 centrifugation at 27000 x g for 40 min at 4 °C, and air dried for 10 min. Further 7 mL of cold
142 absolute ethanol were added to each pellet and incubated overnight at 4 °C. Samples were
143 again centrifuged at 27000 x g for 40 min at 4 °C, and air dried for 10 min, before the pellets
144 were resuspended in 1 mL of ultra pure water. One milliliter of a phenol solution (5%) were
145 added to each sample and vortexed for 15 sec and the tubes were then kept on ice before
146 adding 5 mL of sulphuric acid (97%). After 30 min of incubation, 2 mL of each sample were
147 used for the optical density (OD) measurement at 485 nm by spectrophotometer.
148 Quantifications of EPS concentrations were retrieved by comparison with a standard curve.
149 The standard curve was built based on the OD obtained from water solutions at different
150 concentration of glucose (400 ppm, 200 ppm, 100 ppm, 75 ppm, 50 ppm and 20 ppm) and
151 subjected to the same extraction protocol.

152

153 *2.4 Preparation of soybean fermented milk*

154 All the strains were refreshed and sub-cultivated in 30 mL of TPY broth anaerobically
155 overnight. After that the cells were collected by centrifugation (6000 rpm for 20 min) and
156 washed twice with phosphate buffer saline (PBS). The inoculum was prepared by
157 resuspending the cells in 15 mL of soybean milk (SoyaDrink, Valsoia). For each strain, 100 μ l
158 of inoculum were inoculated in 100 mL of soybean milk, reaching a strain cell load of about 6
159 log cfu/mL. For each strains, ten independent replications were performed. The inoculated
160 soybean milks were incubated at 37 °C until the reaching of pH 4.6, after that they were
161 stored at 4°C for 24 h and characterized for the strain cell loads, EPS concentration, the
162 volatile molecule profiles and the texture features.

163

164 *2.5 Reduction of pH*

165 The fermented milk pH decrease was monitored by pH meter Hanna Instruments 8519
166 (Incofar, Modena, Italy).

167

168 *2.6 Cell load viability*

169 The strain cell loads were determined by plating 10-fold serial dilutions in TPY agar (BD,
170 Milano, Italy). Plates were incubated in anaerobic condition at 37 °C for 24-48 h.

171

172 *2.7 Volatile profiles of fermented soy milk*

173 The analysis of volatile molecules of soybean fermented milks was performed by gas-
174 chromatography-mass spectrometry analysis combined with solid-phase micro extraction
175 (GC/MS-SPME) technique, according to the method proposed by Patrignani et al. (2016). The
176 analyses were performed in triplicate.

177

178 *2.8 Rheological parameters of fermented soymilk*

179 After 24 h from coagulation (reaching of pH 4.6, when possible) and storage at 4 °C, samples
180 were analyzed for their textural features. Firmness, consistency, cohesiveness and viscosity
181 indexes were evaluated using a back extrusion cell (A/AB) on a Texture Analyser TA DHI
182 (Stable Micro System, UK) according to the manufacturer's instructions. A solid rod (35 mm
183 diameter) was thrust into the sterile container holding 100 mL sample using a 5 kg load cell.

184 *2.9 Panel Test*

185 A panel test was performed immediately after 2 days of refrigerated storage. Twenty
186 untrained consumers evaluated colour, flavour, and acceptability of the produced fermented
187 milk. Each parameter was evaluated by consumers on a scale from 0 (very poor) to 5.0 (very
188 good).

189 *2.10 Determination of lactic and acetic acid*

190 The determination of lactic and acetic acid was performed by using the enzymatic kit
191 provided by Steroglass (San Martino In Campo PG).

192 *2.11 Data Analysis*

193 All the data are the mean of three repetitions. Microbiological, textural and EPS data were
194 analysed by one-way analysis of variance (ANOVA) using the statistical package Statistica
195 for Windows 6.1 (Statsoft Inc., Tulsa, OK). The ability of each descriptor to discriminate
196 between samples was investigated using the post hoc comparisons of the ANOVA. The
197 volatile molecule data were analyzed by Principal Component Analysis (PCA) using a
198 Statistica software (version 8.0StatSoft., Tulsa, OK).

199

200 **3. Results**

201 *3.1 Antibiotic susceptibility*

202 The antibiotic susceptibility of the *B. aesculapii* with respect to a wide spectrum of antibiotics
203 is reported in Table 2. The results evidenced that among all the considered antibiotics,
204 Amoxicillin, Ampicillin, Oxacillin, Penicillin G and Erythromycin, showed the highest

205 bactericidal effect. One exception was represented by *B. longum* subsp. *infantis* (RE06) which
206 resulted less susceptible to Amoxicillin, with respect to all the other tested strains. In general,
207 all the considered strains showed low susceptibility to Vancomycin (with the exception of
208 MRM_8.7) Gentamycin, and Levofloxacin, with MICs greater than 32µg/mL.

209

210 *3.2 Production of EPS in TPY medium in relation to the Bifidobacterium aesculapii strain* 211 *and the carbon source employed*

212 In Table 3, the amounts of EPS produced by the tested strains, after 18 h of anaerobic
213 incubation in TPY at 37 °C, in relation to the carbon source added, are shown. Also the strain
214 cell loads after the incubation are reported. The inoculum levels were about 6 log cfu/mL. In
215 glucose based TPY, regardless the used concentration, all strains were able to grow until 8 log
216 cfu/mL or higher. Also in the 2% lactose based TPY, all strains were able to reach cell loads
217 higher than 8 log cfu/mL, with the exception of *B. aesculapii* MRM 5.13 that reached a level
218 of 6.84 log cfu/mL. All the strains were able to produce EPS in 1.5% glucose. Particularly,
219 the strain MRM 3.1 produced significant high amount of EPS in this condition (P<0.05). In
220 presence of 2% glucose, with the exception of the strain DSM 23967, all the strains produced
221 EPS at levels ranging between 15 and 218 µg/mL. The amount of EPS produced by the strain
222 MRM 4.8 was significant higher (P<0.05) compared to those produced by the other strains.
223 Only the strains MRM 3.1, MRM 4.6, MRM 4.8 and MRM 8.7 were able to produce EPSs in
224 2% lactose. The highest significant concentration was produced by the strain MRM 4.8.

225 *3.3 B. aesculapii strain fermentation kinetics in fermented soymilk*

226 All the tested strains were able to reach pH 4.6 in soymilk within 14 h of fermentation at 37°C
227 without significant differences among the strains (Figure 1).

228 *3.4 EPS quantification strain cell loads and acetic/lactic acid ratio in fermented soymilk*

229 In Table 4, the production of EPS in fermented soymilk in relation to the *B. aesculapii*
230 employed strain is reported. Strains MRM 5.13, MRM 4.2, MRM 4.6, MRM 4.7 and MRM

231 4.8 were able to produce EPS at level of 5.05, 131.35, 174.50, 33.41, 34.50 $\mu\text{g/mL}$,
232 respectively, while the remaining strains were unable to produce EPS at the adopted
233 conditions. In particular, the highest significant concentration ($P<0.05$) was produced by the
234 strain MRM 4.6 when compared to the others. In Table 4, also the cell loads of the employed
235 strains are reported after the fermentation. The strains were inoculated in milk at level of
236 about 6 log cfu/mL and all the strains increased their cell load of almost 2 log cycles. During
237 the refrigerated storage (Table 6), the strains lost their viability. After 30 days, only the strains
238 MRM 5.13, MRM 4.7, MRM 4.8 and 8.7 maintained cell loads significant higher than 7 log
239 cfu/mL with respect to the other strains. In table also the molar ratio between acetic and lactic
240 acid was reported. The data evidenced that the highest quantity of acetic acid was produced
241 by the strain RE06. On the other hand, the fermented milk obtained with this stain received
242 the lowest scores for colour, flavour and acceptance.

243

244 *3. 5 Volatile profiles of fermented milks in relation to the employed strain*

245 The volatile profiles of the soybean fermented milks were characterized by GC/MS-SPME
246 analysis, which permitted to identify 40 molecules belonging to different classes of
247 compounds such as alcohols, ketones, acids, esters and aldehydes. In all the samples, in a
248 strain dependent way, ethanol and acetic acid deriving from *Bifidobacterium* metabolic
249 pathway were found. In addition, also 2-butanone, 3-hydroxy-2-butanone and 2,3-
250 butanedione were found.

251 Further, to better understand the relations between the strains employed and the volatile
252 profiles obtained, the volatile results were analysed using a principal component analysis
253 (PCA). In Figure 2a and 2b, the projection of samples and volatile molecules are reported and
254 the PCA analysis was able to explain more than 70% of the total variance among the samples.
255 In particular, the fermented milk from strains MRM 4.6, MRM 3.1, MRM 4.2 and MRM 4.7
256 clustered together and they were characterized by the presence of acetic acid, ethyl decanol,

257 3-hydroxy-2-butanone. The compounds 2,3-butanone, 1-butanol-3-methyl formiate and 4-
258 ethyl hexadecanol characterized the fermented milks produced with the strains MRM 4.8,
259 MRM 5.13 and MRM 8.7 while 2-butanone was the main volatile compound of the fermented
260 milks obtained with the use of strain RE06.

261

262 *3.6 Textural analysis and panel test of fermented milks in relation to the strains and matrix*

263 Rheological parameters, such as firmness, cohesion, adhesion and viscous index, were
264 analysed for each fermented milk and results are summarized in Table 5. In particular, the
265 strain MRM 4.7 showed the highest significant firmness value and viscosity index (1071.2 g
266 and 1071.2 g*s, respectively), but the lowest consistency value, 23.96 g*s; while MRM 4.6
267 gave rise products with the significant highest consistency ($P < 0.05$) and cohesiveness
268 (1399.55 g*s and 36.93 g) and it also showed good results for both the firmness and the
269 viscosity indexes. Also the data of the panel test confirmed the good quality of the obtained
270 fermented soy milk, showing the highest scores of acceptance for the fermented milk
271 produced by MRM 5.13, MRM 4.6, MRM 8.7 (figure 3).

272 **4. Discussion**

273 The *B. aesculapii* strains used in this research were isolated from faecal samples of baby
274 common marmosets (*Callithrix jacchus*) and they were described for the first time by
275 Modesto et al. (2014). The strains were found non-haemolytic and able to grow in whole milk
276 under aerobic, microaerophilic and anaerobic conditions at temperatures between 25-42 °C
277 and pH ranging between 4.5–7.0. Due to these features, they were tested as potential starters
278 for the production of fermented milks using soymilk as substrate. Since the determination of
279 the antibiogram is considered a prerequisite in protocols for the selection of starter, co-starter
280 or functional microorganisms by EFSA (Wedajo, 2015), the strains were investigated using a
281 wide gamma of antibiotics- The antibiogram results are in agreement with literature data
282 (Ammor et al., 2007; Nueno-Palop and Narbad, 2011; Fguiri et al., 2015). In fact, the

283 *Bifidobacterium* showed a variable spectrum of susceptibility in relation to the strain
284 considered. The majority of the tested strains resulted very sensitive to Amoxicillin,
285 Ampicillin, Oxacillin, Penicillin G and Erythromycin. Some strains were found to be less
286 susceptible to different antibiotics. In these case, further studies are needed to better
287 characterize the resistance mechanism, before including these strains in food products. In
288 addition, these strains were previously found to be able to codify for the galactosyl
289 transferase, *cspD*, glycosyltransferases, considered to be a key enzyme involved in EPS
290 production (Duranti et al., 2017). In fact, with a complex pathway, several *Bifidobacterium*
291 strains can synthetize heteroexopolysaccharides (hEPS). Hypothesis about the biosynthesis
292 have been proposed based on the functional analysis of few genes and on sequences
293 homology studies. Briefly, the EPS biosynthesis process includes three steps such as the
294 assimilation of simple sugars and conversion into nucleotide derivatives, the assembly of
295 pentasaccharide subunits attached to a lipid transporter (*p-gtf*) and the polymerisation of
296 repeating units of pentasaccharide and extracellular secretion (Hidalgo-Cantabrana et al.,
297 2014). For this reason, the first step of this research was comparing the *B. aesculapii* strains
298 and control strains (*B. longum* subsp. *infantis* and *B. saguini*) for their ability to produce EPS
299 in a TPY medium with different carbon source and concentrations. All the strains were able to
300 produce EPS in presence of glucose, independently on the concentration employed. Also *B.*
301 *saguini*, which according a previous work (Michelini et al., 2015) should not produce EPS
302 due to the absence of the priming glycosyl transferase (pGTF), produced little amount of EPS.
303 Probably this pGTF, even if it is not present in the cluster of EPS genes, can be present in
304 different positions in the genome and contribute to the EPS production.

305 On the other hand, only the strains MRM 3.1, MRM 4.6, MRM 4.8 and MRM 8.7 were able,
306 although in strain-dependent way, to produce EPS in presence of lactose 2% when used as
307 unique carbon source. According to the hypothesis previously reported, these strains seems to
308 be able to hydrolyze lactose into glucose and galactose due to the presence of β -galactosidase.

309 This is a fundamental enzyme which also permits the assimilation of human milk
310 oligosaccharides by bifidobacteria and the bifidobacteria colonization in intestine of newborn
311 infants (Miwa et al., 2010). Although generally during the milk fermentation process,
312 bifidobacteria like other lactic acid bacteria, utilise lactose after the hydrolysis by β -
313 galactosidase to produce monosaccharides, the activity rate of this enzyme is strain dependent
314 and some authors have reported the treatment of some bifidobacteria strain with high intensity
315 ultrasounds to increase the carbohydrate metabolisms in the strains (Nguyen et al., 2012). Li
316 et al. (2012) found a relatively strong α and β -galactosidase activity in *Bifidobacterium*
317 *animalis* subsp. *lactis* V9 and BB12. Osman, Tzortzis, Rastall, & Charalampopoulos (2010),
318 with the development of a mathematical model, demonstrated that the hydrolysis of lactose in
319 *B. bifidum* NCIMB 41171 was dependent on lactose concentration, temperature, cell biomass
320 and cultivation time. In this research, the strains MRM 3.1, MRM 4.6, and MRM 4.8 can be
321 regarded as high producers of EPS *in vitro* model in all the condition tested, reaching also cell
322 loads higher than 8.0 log cfu/mL. Due to their effect on rheological properties such as
323 stabilizing and improving the viscosity, the use of EPS producing microbial strains has been
324 proposed as strategy to improve textural properties of fermented milks also produced from
325 low fat milk, generally characterized by scarce textural properties (Becker, 2015). Also
326 Mende, Rohm, & Jaros (2016) have underlined the impact of microbial EPS from lactic acid
327 bacteria (LAB) on dairy products such as yoghurt, cheese, or milk based desserts. Moreover,
328 exopolysaccharides from microbial source have attracted recent attention, mainly due to their
329 potential health promoting functions (Badel, Bernardi, & Michaud, 2011; Hidalgo-Cantabrana
330 et al., 2014). EPSs has been reported to have beneficial effects on the cholesterol-lowering
331 and antitumor activities (Pigeon, Cuesta, & Gililliand, 2002), as well as immunomodulating
332 and prebiotic effects (Salazar, Gueimonde, Hernández-Barranco, Ruas-Madiedo, & de los
333 Reyes-Gavilán, 2008; Vinderola, Perdigón, Duarte, Farnworth, & Matar, 2006). Li et al.
334 (2014) found a significant beneficial effect on gut microbiota for EPS from *Bifidobacterium*

335 *bifidum* WBIN03, and they also found that the EPSs produced had antimicrobial and
336 antioxidant activity (Li, Huang, et al., 2014). Moreover, López et al. (2012) found that the
337 EPS-producing bifidobacteria showed good adherence properties to the human colon cell
338 lines CaCo2 and HT29, which could be of interest for a transitory colonisation of the gut.
339 Most purified EPS were able to slightly stimulate the proliferation of peripheral blood
340 mononuclear cells and their cytokine production pattern, depending on the polymer type
341 tested.

342 Due to their capability to produce EPS *in vitro* models, and to their ability to grow in whole
343 milk both in aerobic and microaerophilic conditions, the *B. aesculapii* strains were
344 investigated as potential starters in soy milk fermentations. In fact, even if *Bifidobacterium*
345 strains are already used in dairy products, they usually have less technological features, such
346 as texture and aroma influence, compared with traditional lactic acid bacteria, which hinder
347 their possible applications as single starter cultures. Furthermore, the bifidobacteria generally
348 exhibit weaker growth in cows' milk and require long fermentation times, anaerobic
349 conditions and low redox potential for optimal growth (Gomes, Malcata, Klaver, & Roy,
350 1998). In this research, encouraging results were obtained since all the *B. aesculapii* strains
351 were able to ferment the substrate, in overnight cultivation at 37 °C, reaching pH values
352 between 4.2 and 4.6, and cell load increased with respect to the initial inoculums. It may be
353 hypothesized that the strains were able to secrete α -galactosidase enzyme, which is necessary
354 for growth in soybean milk rich of galactose based-(oligo)saccharides (mainly α -galactosides)
355 (Havas, Kun, Perger-Mészáros, Rezessy-Szabó, & Nguyen, 2015). The acidification kinetics
356 were very similar among the strain, which reached pH 4.6 in 14 hours. Moreover, the results
357 obtained are in accordance with the data reported by Horáčková, Mühlhansová, Sluková,
358 Schulzová, & Plocková (2015) who described a good growth of *Bifidobacterium animalis*
359 subsp. *lactis* BB 12 and *Bifidobacterium bifidum* CCDM 94 in soybean milk at 37 °C in 16 h,
360 although the bifidobacteria, compared to the yoghurt culture, were only able to acidify the

361 media to the half of the pH values. Havas et al. (2015) also showed that bifidobacteria strains
362 were able to grow well on a native soymilk medium without any additional nutrients. The
363 fermentation processes with initial cell concentrations of 10^5 – 10^7 cfu/mL reached the
364 maximum cell load of 10^8 cfu/mL already after 8–12 h of incubation in soymilk, and those
365 levels were maintained to the end of fermentation. Li, Chen, et al. (2014) demonstrated that
366 fermented soymilk produced with single culture of *Bifidobacterium animalis* subsp. *lactis* V9
367 and Bb12 was characterized by high cell load levels, especially for *B. animalis* subsp. *lactis*
368 Bb12, which was all over 9.0 log cfu/mL. They also found increasing contents of bioactive
369 substances in soymilk, including γ -aminobutyric acid, vitamin B6, and total isoflavone
370 aglycone. The strain employed in this research, particularly MRM 5.13, MRM 4.6, MRM 4.7,
371 and MRM 4.8. maintained also a viability higher than 7 log cfu/mL during the product
372 refrigerated storage satisfying also the criteria for probiotic bacteria (Patrignani et al., 2017).
373 The highest production of EPSs from the *B. aesculapii* strains was found for strains MRM
374 4.2, MRM 4.6, MRM 4.7, MRM 5.13 and MRM 4.8. The production of EPS during
375 fermentation probably affected the textural parameters. In fact, the rheological properties of
376 milk products may depend on several factors related to EPS, such as the EPS location
377 (capsular or free), EPS structure (the molecular mass, possible side chains, stiffness, charge),
378 EPS concentration, and from the EPS interaction with other compounds in the product such as
379 proteins, minerals, or even the bacteria themselves.

380 The technological aspects to be considered in probiotic strain selection for fermented milk
381 include the phage resistance, viability throughout processing and storage, ability to give rise
382 to fast fermentation in a proper substrate such as milk, and to improve good sensory
383 properties (Mattila-Sandholm, Mättö, & Saarela, 1999). The latter aspect plays an important
384 role in consumer acceptance (Gardini, Lanciotti, Elisabetta Guerzoni, & Torriani, 1999).
385 Generally, fermented milks obtained from the direct and sole use of probiotic strains are often
386 characterised by the lack of desirable sensory features. In particular, structural defects and

387 absence of aroma were reported for milk fermented solely by *Bifidobacterium* spp., due to the
388 lack of alcohol dehydrogenase able to convert ethanol in acetaldehyde (Marshall & Cole,
389 1983). However, other molecules considered as key compounds for positive aroma profile of
390 fermented milks were found, such as for example 2,3-butanedione and 2-butanone. In
391 particular, 2,3-butadione, 1-butanol-3-methyl formiate and 4-ethyl hexadecanol characterized
392 the fermented milks produced with the strains MRM 4.8, MRM 5.13 and MRM 8.7.

393 The volatile profiles analysis by GC/MS-SPME technique permits to detect acetic acid, but
394 not lactic acid. Theoretically, by the utilisation of carbohydrates through the “Bifidus”
395 metabolic pathway, the bifidobacteria should produce more acetic than lactic acid, which
396 could affect the sensory properties of the final product. However, the final aroma of a product
397 depends from the interaction of different compounds (volatile and not volatile). The GC-MS
398 volatile profiles obtained in this work turned out to be both strain dependent and affected also
399 by the initial substrate. The profiles obtained can be considered as product fingerprints,
400 allowing to discriminate among the tested strains, in order to select the best candidate in
401 relation to the desired sensory features.

402

403 **5. Conclusions**

404 This research is a first challenge to exploit some *Bifidobacterium aesculapii* strain, a novel
405 species recently described, for the production of fermented soymilk enriched in EPS. All the
406 investigated *B. aesculapii* strains grew very well in soymilk, producing considerable amounts
407 of EPS, and resulting in high product viscosity and firmness values. The highest yields in EPS
408 in fermented soymilk were obtained for the strains MRM 4.2, MRM 4.6, MRM 4.7 and MRM
409 4.8. Moreover, according to the data of the panel test, the fermented milk obtained from
410 MRM 4.6 obtained also the highest scores for general acceptance. Overall, the performances
411 of these newly isolated were comparable with those reported by the literature for the industrial

412 *Bifidobacterium* strains (Havas et al., 2015). So these results are very promising and useful
413 for the further scaling-up of the process to obtain function fermented soymilk.

414 **Figure Legend**

415 **Figure 1.** Fermentation kinetics of *B. aesculapii* strains in soy milk

416 **Figure 2.** Principal component analysis loading plot of fermented milks (1a) and volatile
417 molecules (1b) in relation to the *Bifidobacterium* strain used

418 **Figure 3.** Sensory data of soy milk fermented milk, in relation to the strains used after 2days
419 of storage at 4 °C.

420

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ACCEPTED MANUSCRIPT

1 **Table 1.** *Bifidumbacterium* strains used in this study

Species	Strain	Collection Number
<i>B. aesculapii</i>	MRM 3.1	DSM 26737 ^T
<i>B. aesculapii</i>	MRM 4.2	DSM 26738
<i>B. aesculapii</i>	MRM 4.6	-
<i>B. aesculapii</i>	MRM 4.7	-
<i>B. aesculapii</i>	MRM 4.8	-
<i>B. aesculapii</i>	MRM 5.13	-
<i>B. aesculapii</i>	MRM 8.7	-
<i>B. longum</i> subsp. <i>infantis</i>	RE 06	ATCC 15697
<i>B. saguini</i>	-	DSM 23967 ^T

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25 **Table 2.** Evaluation of minimum inhibitory concentrations (MIC, $\mu\text{g/mL}$) of various antibiotics against *Bifidumbacterium* strains

Sample	Amoxicillin	Vancomycin	Oxacillin	Linezolid	Ciprofloxacin	Ampicillin	Penicillin G	Gentamycin	Erythromycin	Tetracycline	Levofloxacin	Clyndamicin
MRM_4.2	0.03	> 256	1	2	>32	0.015	0.03	> 256	0.25	4	>32	4
MRM_4.6	0.03	> 256	0.5	1	>32	0.03	0.03	> 256	2	8	>32	4
MRM_5.13	0.06	> 256	0.5	1	>32	0.015	0.03	> 256	4	4	>32	1
MRM_4.8	0.03	> 256	0.5	1	>32	0.06	0.03	> 256	16	4	>32	8
MRM_8.7	0.015	0.25	0.5	1	>32	0.06	0.06	> 256	16	4	>32	8
Re06	> 256	> 256	0.25	0.06	>32	0.015	0.03	> 256	1	0.12	>32	> 256
DSM 23967	0.6	> 256	1	2	>32	0.015	0.06	> 256	4	8	>32	8
MRM_4.7	0.6	> 256	1	2	>32	0.015	0.03	> 256	8	8	>32	4
MRM_3.1	0.6	> 256	0.25	1	>32	0.015	>32	> 256	4	8	>32	4

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32 **Table 3.** Cell loads and Exopolysaccharides (EPS) detected in TPY medium in relation to the
 33 carbon source added and the employed strain.

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Strain	Cell load	EPS	Cell load	EPS	Cell load	EPS
	(log cfu/mL)	(μ g/mL)	(log cfu/ mL)	(μ g/mL)	(log cfu/mL)	(μ g/mL)
	Glucose 1.5%	Glucose 1.5%	Glucose 2%	Glucose 2%	Lactose 2%	Lactose 2%
MRM_3.1	8.94 \pm 0.27 ^A	231.61 \pm 5.25 ^A	8.67 \pm 0.05 ^A	123.92 \pm 10.20 ^A	8.14 \pm 1.0 ^A	101.18 \pm 4.90 ^A
MRM_4.2	8.82 \pm 0.30 ^A	83.46 \pm 2.20 ^B	7.92 \pm 0.10 ^B	92.51 \pm 8.45 ^B	8.77 \pm 0.2 ^B	-*
MRM_4.6	8.15 \pm 0.14 ^B	196.48 \pm 8.50 ^C	7.85 \pm 0.09 ^B	127.67 \pm 6.34 ^A	8.28 \pm 0.72 ^{A,B}	103.47 \pm 5.20 ^A
MRM_4.7	8.55 \pm 0.42	162.88 \pm 7.35 ^D	7.68 \pm 0.13 ^B	135.89 \pm 3.45 ^A	8.74 \pm 0.1 ^{A,B}	-
MRM_4.8	8.34 \pm 0.15 ^B	114.01 \pm 7.28 ^E	8.47 \pm 0.16 ^A	218.99 \pm 8.12 ^C	7.74 \pm 0.7 ^{A,B}	143.65 \pm 7.34 ^B
MRM_5.13	8.72 \pm 0.21 ^A	44.52 \pm 6.32 ^F	8.18 \pm 0.86 ^A	41.82 \pm 2.10 ^D	6.84 \pm 0.1 ^C	-
MRM_8.7	8.32 \pm 1.46 ^B	106.75 \pm 8.34 ^E	7.52 \pm 0.86 ^{B,C}	102.10 \pm 5.23 ^B	9.40 \pm 0.1 ^D	18.08 \pm 2.10 ^C
RE06	9.88 \pm 1.46 ^C	7.53 \pm 2.50 ^G	7.52 \pm 0.15 ^{B,C}	15.45 \pm 3.10 ^E	9.58 \pm 0.1 ^{A,B}	-
DSM 23967	8.92 \pm 0.42 ^A	12.88 \pm 3.00 ^G	7.90 \pm 0.23 ^B	-	8.93 \pm 0.20	-

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36 *under the detection limit

37 For each column considered, values with the same superscript letter are not statistically different (P > 0.05).

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54 **Table 4.** Cell loads, Exopolysaccharides (EPS), and acetic/lactic acid molar ratio detected in
 55 fermented soymilk (FSM), at the end of fermentation, in relation to the *B. aesculapii* strain
 56 employed.

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Strain	Cell load	EPS	acetic/lactic acid
	(log cfu/mL)	($\mu\text{g/mL}$)	molar ratio
FSM MRM_3.1	8.02 \pm 0.90 ^B	-*	0.170
FSM MRM_4.2	8.89 \pm 0.35 ^{A,B}	131.35 \pm 2.0 ^A	0.844
FSM MRM_4.6	8.90 \pm 0.26 ^{A,B}	174.50 \pm 2.4 ^B	0.014
FSM MRM_4.7	9.08 \pm 0.15 ^A	33.41 \pm 1.8 ^C	0.833
FSM MRM_4.8	8.81 \pm 0.34 ^{A,B}	34.50 \pm 1.5 ^C	0.079
FSM MRM_5.13	9.05 \pm 0.67 ^{A,B}	5.05 \pm 2.2 ^D	0.744
FSM MRM_8.7	8.95 \pm 0.37 ^{A,B}	-	0.092
FSM RE06	8.84 \pm 0.85 ^{A,B}	-	5.713
FSM DSM 23967	8.69 \pm 0.25 ^{A,B}	-	0.465

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*under the detection limit

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For each column considered, values with the same superscript letter are not statistically different (P > 0.05).

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79 **Table 5.** Texture parameters detected for fermented soymilk (FSM) in relation to the used
 80 *Bifidumbacterium* strain

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Strain	Firmness (g)	Consistency (g*s)	Cohesiveness (g)	Viscosity index (g*s)
FSM MRM 3.1	27.71±2.70 ^A	550.43±34.45 ^A	8.24±1.04 ^A	4.50±0.94 ^A
FSM MRM 4.2	29.60±1.82 ^{A,B}	600.97±12.34 ^B	11.97±0.98 ^B	15.17±1.30 ^B
FSM MRM 4.6	63.27±2.56 ^C	1399.55±32.39 ^C	36.93±2.13 ^C	28.53±3.29 ^C
FSM MRM 4.7	1071.42±29.10 ^D	23.96±1.26 ^D	10.04±1.02 ^{A,B}	1071.42±18.67 ^D
FSM MRM 4.8	25.21±1.78 ^A	512.51±23.76 ^A	9.80±1.10 ^A	0.82±0.10 ^E
FSM MRM 5.13	28.23±2.62 ^{A,B}	515.42±17.45 ^A	9.77±1.67 ^{A,B}	3.07±0.60 ^A
FSM MRM 8.7	40.14±2.45 ^E	768.22±11.10 ^E	19.48±1.08 ^D	9.49±1.10 ^F
<i>FSM B. infantis</i> RE06	14.14±1.21 ^F	329.14±24.22 ^F	6.72±0.23 ^E	1.14±0.34 ^{E,G}
<i>FSM B. saguini</i> DSM23967	20.03±1.18 ^G	435.05±5.50	8.09±1.11 ^A	1.62±0.58 ^G

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 83 For each column considered, values with the same superscript letter are not statistically different (P > 0.05).

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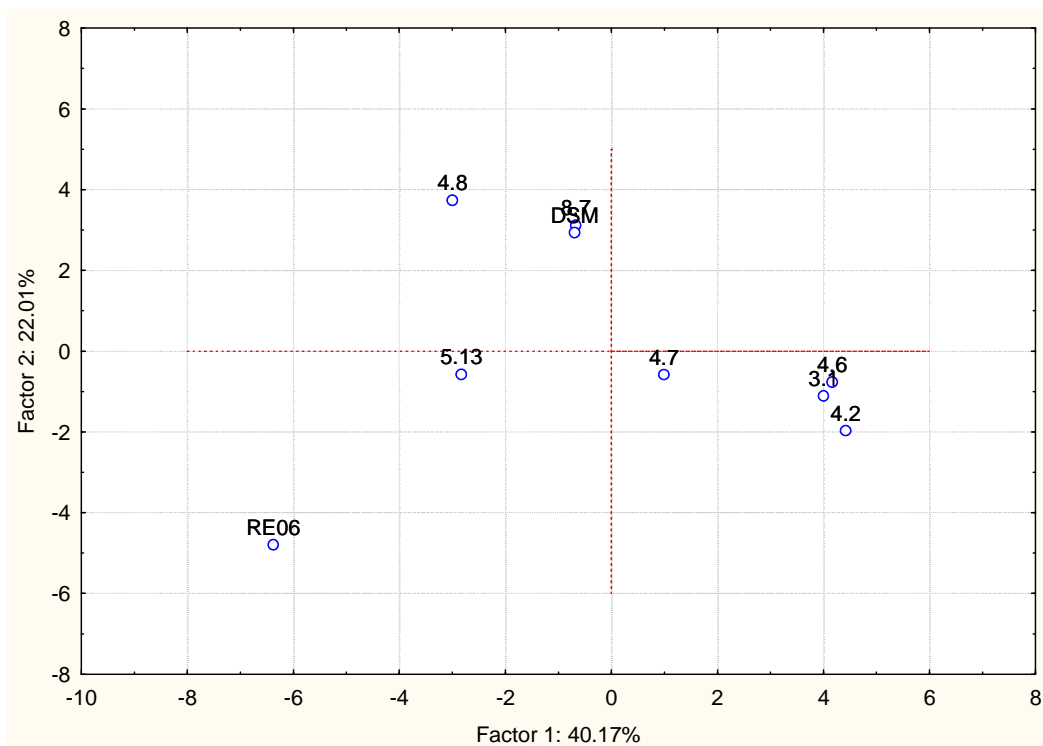
96 **Table 6.** *B. aesculapii* cell loads in fermented soy milk during refrigerated storage97
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	0 d	14 d	30 d
Strain	Cell load (log cfu/mL)	Cell load (log cfu/mL)	Cell load (log cfu/mL)
FSM MRM_3.1	8.02±0.90 ^B	7.50±0.10 ^A	6.80±0.15 ^A
FSM MRM_4.2	8.89±0.35 ^{A,B}	7.70±0.15 ^A	6.70±0.30 ^A
FSM MRM_4.6	8.90±0.26 ^{A,B}	7.50±0.25 ^A	6.90±0.10 ^A
FSM MRM_4.7	9.08±0.15 ^A	8.20±0.20 ^B	7.25±0.13 ^B
FSM MRM_4.8	8.81±0.34 ^{A,B}	8.35±0.16 ^B	7.15±0.25 ^B
FSM MRM_5.13	9.05±0.67 ^{A,B}	8.10±0.25 ^B	7.10±0.10 ^B
FSM MRM_8.7	8.95±0.37 ^{A,B}	8.25±0.25 ^B	7.30±0.10 ^B
FSM RE06	8.84±0.85 ^{A,B}	6.90±0.15 ^C	6.10±0.30 ^C
FSM DSM 23967	8.69±0.25 ^{A,B}	7.00±0.10 ^C	6.14±0.15 ^C

99 For each column considered, values with the same superscript letter are not statistically different (P > 0.05).
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Figure 2

(a)



(b)

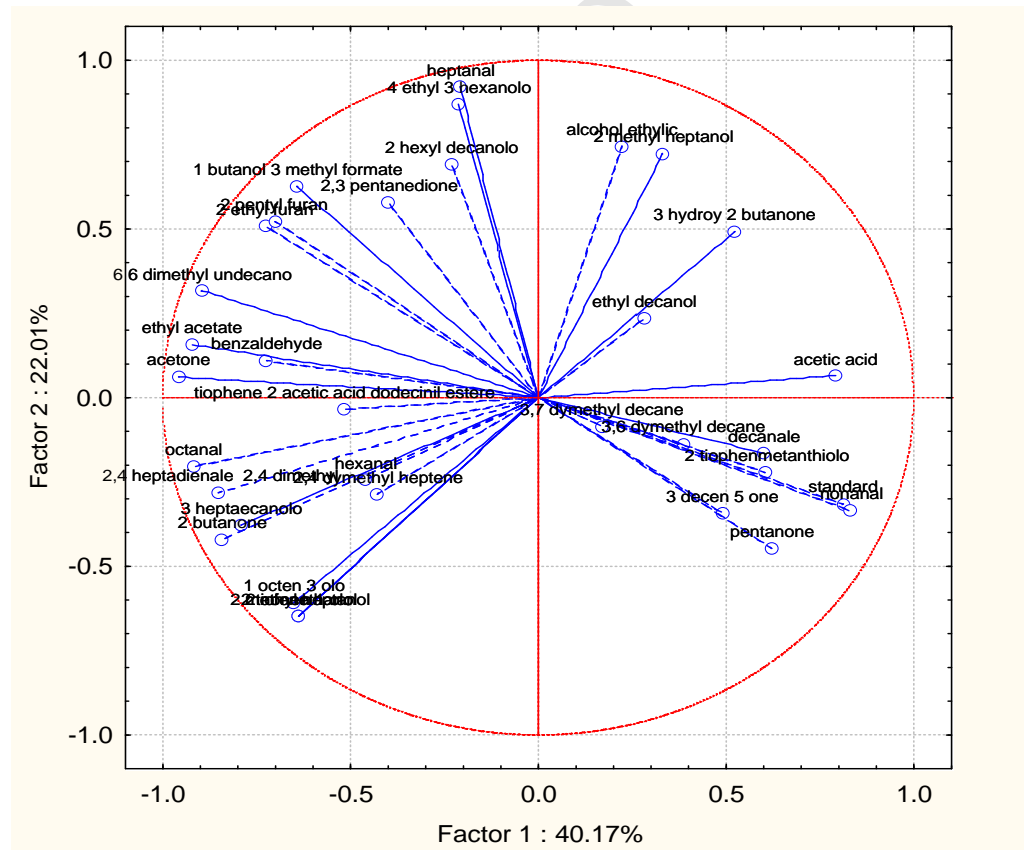
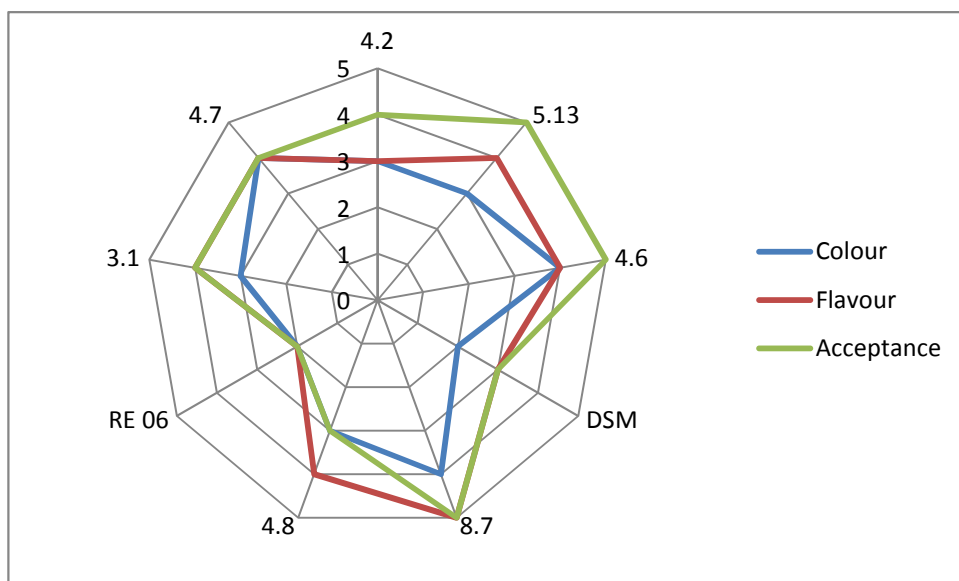


Figure 3

The *B. aesculapii* strains showed good potential to be used as starter in soymilk

The *B. aesculapii* strains gave rise to fermented products with good firmness and viscosity indexes

Five strains out seven showed production of EPS in soybean fermented milk.

ACCEPTED MANUSCRIPT