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

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28 Abstract

The present research was aimed to investigate the technological potentialities of seven strains 29 of Bifidobacterium aesculapii, a species recently described, in terms of exopolysaccharide 30 (EPS) production and as starter fermentation in soybean milk. The strain production of EPS 31 was firstly evaluated in model system, using different carbon sources. Furthermore, the 32 fermented products obtained by the seven strains of *B. aesculapii* were tested for their EPS 33 content and strain cell loads, the volatile molecule profiles, the texture features and the overall 34 acceptance. The data showed that all the B. aesculapii strains were able to produce EPS in 35 vitro model in presence of 1.5% and 2% glucose while only four strains were able to produce 36 EPS in presence of lactose 2%. When the strains were employed as fermentation starters in 37 soybean milk, some showed a good growth potential, fermenting the substrate in 14 hours and 38 39 giving rise to fermented products with good firmness and viscosity indexes. Moreover, five strains out seven showed production of EPS (from 5 to 174 µg/mL) in soybean fermented 40 milk. 41

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

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53 **1.** Introduction

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

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

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 *Bifodobacterium*, 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

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

127

128 2.3 Quantification of EPS

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

152

153 2.4 Preparation of soybean fermented milk

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

163

164 2.5 *Reduction of pH*

165 The fermented milk pH decrease was monitored by pH meter Hanna Instruments 8519166 (Incofar, Modena, Italy).

167

168 2.6 Cell load viability

The strain cell loads were determined by plating 10-fold serial dilutions in TPY agar (BD,
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

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

177

178 2.8 *Rheological parameters of fermented soymilk*

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

184 2.9 Panel Test

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

189 2.10 Determination of lactic and acetic acid

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

192 2.11 Data Analysis

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

199

200 **3. Results**

201 3.1 Antibiotic susceptibility

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

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

209

3.2 Production of EPS in TPY medium in relation to the Bifidubacterium aesculapii strain
and the carbon source employed

In Table 3, the amounts of EPS produced by the tested strains, after 18 h of anaerobic 212 incubation in TPY at 37 °C, in relation to the carbon source added, are shown. Also the strain 213 cell loads after the incubation are reported. The inoculum levels were about 6 log cfu/mL. In 214 glucose based TPY, regardless the used concentration, all strains were able to grow until 8 log 215 cfu/mL or higher. Also in the 2% lactose based TPY, all strains were able to reach cell loads 216 higher than 8 log cfu/mL, with the exception of *B. aesculapii* MRM 5.13 that reached a level 217 of 6.84 log cfu/mL. All the strains were able to produce EPS in 1.5% glucose. Particularly, 218 the strain MRM 3.1 produced significant high amount of EPS in this condition (P<0.05). In 219 presence of 2% glucose, with the exception of the strain DSM 23967, all the strains produced 220 EPS at levels ranging between 15 and 218 µg/mL. The amount of EPS produced by the strain 221 MRM 4.8 was significant higher (P<0.05) compared to those produced by the other strains. 222 Only the strains MRM 3.1, MRM 4.6, MRM 4.8 and MRM 8.7 were able to produce EPSs in 223 2% lactose. The highest significant concentration was produced by the strain MRM 4.8. 224 3.3 B. aesculapii strain fermentation kinetics in fermented soymilk 225 All the tested strains were able to reach pH 4.6 in soymilk within 14 h of fermentation at 37°C 226

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

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

243

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

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

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

3-hydroxy-2-butanone. The compounds 2,3-butadione, 1-butanol-3-methyl formiate and4ethyl hexadecanol characterized the fermented milks produced with the strains MRM 4.8,
MRM 5.13 and MRM 8.7 while 2-butanone was the main volatile compound of the fermented
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

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

272 4. Discussion

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

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

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.

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

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

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

media to the half of the pH values. Havas et al. (2015) also showed that bifidobacteria strains 361 were able to grow well on a native soymilk medium without any additional nutrients. The 362 fermentation processes with initial cell concentrations of 10^5-10^7 cfu/mL reached the 363 maximum cell load of 10^8 cfu/mL already after 8–12 h of incubation in soymilk, and those 364 levels were maintained to the end of fermentation. Li, Chen, et al. (2014) demonstrated that 365 fermented soymilk produced with single culture of *Bifidobacterium animalis* subsp. *lactis* V9 366 and Bb12 was characterized by high cell load levels, especially for *B. animalis* subsp. *lactis* 367 Bb12, which was all over 9.0 log cfu/mL. They also found increasing contents of bioactive 368 substances in soymilk, including γ -aminobutyric acid, vitamin B6, and total isoflavone 369 aglycone. The strain employed in this research, particularly MRM 5.13, MRM 4.6, MRM 4.7, 370 and MRM 4.8. maintained also a viability higher than 7 log cfu/mL during the product 371 refrigerated storage satisfying also the criteria for probiotic bacteria (Patrignani et al., 2017). 372

The highest production of EPSs from the *B. aesculapii* strains was found for strains MRM 4.2, MRM 4.6, MRM 4.7, MRM 5.13 and MRM 4.8. The production of EPS during fermentation probably affected the textural parameters. In fact, the rheological properties of milk products may depend on several factors related to EPS, such as the EPS location (capsular or free), EPS structure (the molecular mass, possible side chains, stiffness, charge), EPS concentration, and from the EPS interaction with other compounds in the product such as proteins, minerals, or even the bacteria themselves.

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

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

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

402

403 **5. Conclusions**

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

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

420

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

Table 1. *Bifidumbacterium* strains used in this study



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

Table 2. Evaluation of minimum inhibitory concentrations (MIC, μg/mL) of various antibiotics against *Bifidumbacterium* strains

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

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

36 *under the detection limit

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

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.

<u> </u>	Cell load	EPS	acetic/lactic acid
Strain	(log cfu/mL)	(µg/mL)	molar ratio
FSM MRM_3.1	8.02±0.90 ^B	_*	0.170
FSM MRM_4.2	$8.89 \pm 0.35^{A,B}$	131.35±2.0 ^A	0.844
FSM MRM_4.6	8.90±0.26 ^{A,B}	174.50±2.4 ^B	0.014
FSM MRM_4.7	9.08 ± 0.15^{A}	33.41±1.8 ^C	0.833
FSM MRM_4.8	$8.81 \pm 0.34^{A,B}$	$34.50 \pm 1.5^{\circ}$	0.079
FSM MRM_5.13	$9.05 \pm 0.67^{A,B}$	5.05±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

- ⁵⁸ *under the detection limit
- 59 For each column considered, values with the same superscript letter are not statistically different (P > 0.05).

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Table 5. Texture parameters detected for fermented soymilk (FSM) in relation to the used

- *Bifidumbacterium* strain

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 [°]	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	$0.82{\pm}0.10^{E}$
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	$19.48{\pm}1.08^{D}$	9.49±1.10 ^F
FSM B. infantis RE06	14.14±1.21 ^F	329.14±24.22 ^F	6.72 ± 0.23^{E}	1.14±0.34 ^{E,G}
FSM <i>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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Table 6. *B. aesculapii* cell loads in fermented soy milk during refrigerated storage

	0 d	14 d	30 d
	Cell load	Cell load	Cell load
Strain	(log cfu/mL)	(log cfu/mL)	(log cfu/mL)
FSM MRM_3.1	8.02 ± 0.90^{B}	$7.50 \pm 0.10^{\text{A}}$	6.80±0.15 ^A
FSM MRM_4.2	$8.89 \pm 0.35^{A,B}$	$7.70\pm0.15^{\rm A}$	6.70 ± 0.30^{A}
FSM MRM_4.6	$8.90\pm0.26^{A,B}$	$7.50\pm0.25^{\rm A}$	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	$8.81 \pm 0.34^{A,B}$	8.35±0.16 ^B	7.15 ± 0.25^{B}
FSM MRM_5.13	$9.05 \pm 0.67^{A,B}$	8.10±0.25 ^B	$7.10{\pm}0.10^{B}$
FSM MRM_8.7	$8.95 \pm 0.37^{A,B}$	8.25 ± 0.25 ^B	7.30 ± 0.10^{B}
FSM RE06	$8.84{\pm}0.85^{A,B}$	$6.90 \pm 0.15^{\rm C}$	$6.10 \pm 0.30^{\circ}$
FSM DSM 23967	$8.69 \pm 0.25^{A,B}$	$7.00 \pm 0.10^{\circ}$	6.14 ± 0.15^{C}

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



Figure 1

Figure 2

(a)



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.