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Technological potential of *Bifidobacterium aesculapii* strains for fermented soymilk 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 µg/mL) in soybean fermented milk.

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

1. Introduction

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

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

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

2. Materials and Methods

2.1 Strains

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

2.2 Antibiotic susceptibility

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

2.3 *Quantification of EPS*

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

2.4 *Preparation of soybean fermented milk*

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

2.5 *Reduction of pH*

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

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.

2.7 *Volatile profiles of fermented soy milk*

The analysis of volatile molecules of soybean fermented milks was performed by gas-chromatography-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.

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.

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).

2.10 Determination of lactic and acetic acid

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

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).

3. Results

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.

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

3.3 B. aesculapii strain fermentation kinetics in fermented soymilk

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

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

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 2,3-butanedione 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 and4-ethyl 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.

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

4. Discussion

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

283 Bifidumbacterium 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 ß-galactosidase.

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

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

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 not lactic acid. Theoretically, by the utilisation of carbohydrates through the "Bifidus" metabolic pathway, the bifidobacteria should produce more acetic than lactic acid, which could affect the sensory properties of the final product. However, the final aroma of a product depends from the interaction of different compounds (volatile and not volatile). The GC-MS volatile profiles obtained in this work turned out to be both strain dependent and affected also 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 relation to the desired sensory features.

5. Conclusions

This research is a first challenge to exploit some *Bifidobacterium aesculapii* strain, a novel species recently described, for the production of fermented soymilk enriched in EPS. All the investigated *B. aesculapii* strains grew very well in soymilk, producing considerable amounts of EPS, and resulting in high product viscosity and firmness values. The highest yields in EPS in fermented soymilk were obtained for the strains MRM 4.2, MRM 4.6, MRM 4.7 and MRM 4.8. Moreover, according to the data of the panel test, the fermented milk obtained from MRM 4.6 obtained also the highest scores for general acceptance. Overall, the performances 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 *Bifidumbacterium* 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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Table 1. *Bifidumbacterium* strains used in this study

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 2. Evaluation of minimum inhibitory concentrations (MIC, μg/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

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

Strain	Cell load (log cfu/mL) Glucose 1.5%	EPS (µg/mL) Glucose 1.5%	Cell load (log cfu/mL) Glucose 2%	EPS (µg/mL) Glucose 2%	Cell load (log cfu/mL) Lactose 2%	EPS (µg/mL) Lactose 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±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±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±0.1^{C}	-
MRM_8.7	8.32±1.46^{B}	106.75±8.34^{E}	7.52±0.86^{B,C}	102.10±5.23^{B}	9.40±0.1^{D}	18.08±2.10^{C}
RE06	9.88±1.46^{C}	7.53±2.50^{G}	7.52±0.15 ^{B,C}	15.45±3.10^{E}	9.58±0.1 ^{A,B}	-
DSM 23967	8.92±0.42^{A}	12.88±3.00^{G}	7.90±0.23^{B}	-	8.93±0.20	-

*under the detection limit

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 fermented soymilk (FSM), at the end of fermentation, in relation to the *B. aesculapii* strain employed.

Strain	Cell load (log cfu/mL)	EPS (µg/mL)	acetic/lactic acid molar ratio
FSM MRM_3.1	8.02±0.90^{B}	-*	0.170
FSM MRM_4.2	8.89±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±0.34 ^{A,B}	34.50±1.5^{C}	0.079
FSM MRM_5.13	9.05±0.67 ^{A,B}	5.05±2.2^{D}	0.744
FSM MRM_8.7	8.95±0.37 ^{A,B}	-	0.092
FSM RE06	8.84±0.85 ^{A,B}	-	5.713
FSM DSM 23967	8.69±0.25 ^{A,B}	-	0.465

*under the detection limit

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

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^{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}
***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 *B. saguini* DSM23967	20.03±1.18^{G}	435.05±5.50	8.09±1.11 ^{A}	1.62±0.58^{G}

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

96 **Table 6.** *B. aesculapii* cell loads in fermented soy milk during refrigerated storage

97
98

	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).
100

Figure 1

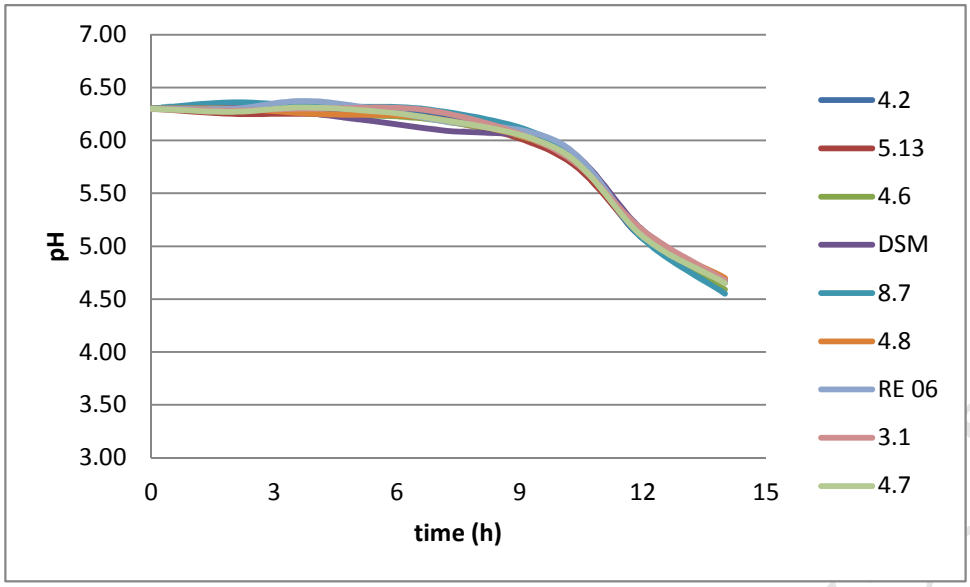
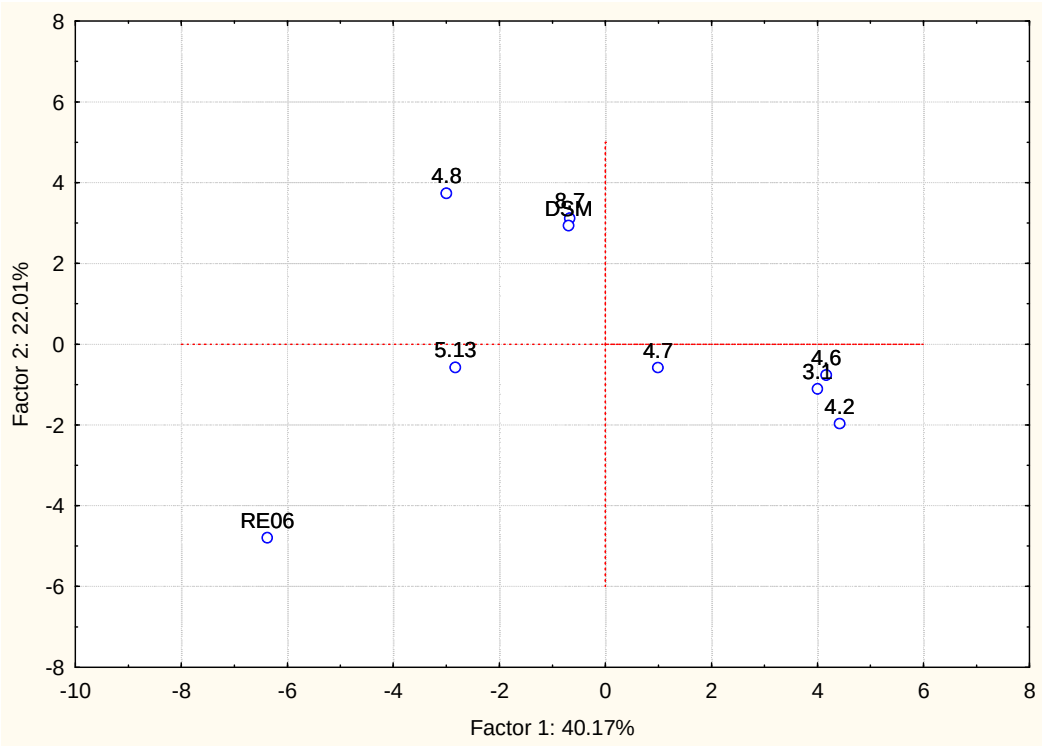


Figure 2

(a)


4.8
8.7
DSM
5.13
4.7
4.6
3.1
4.2
RE06
Factor 2: 22.01%
Factor 1: 40.17%

(b)

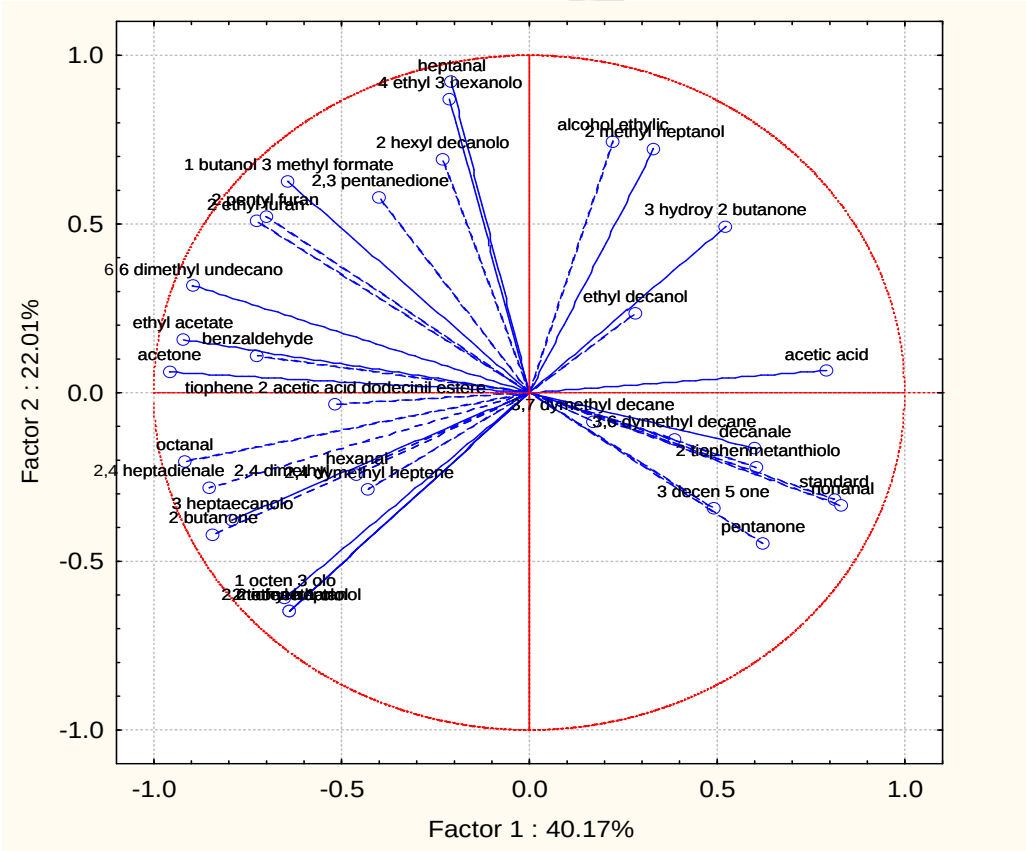
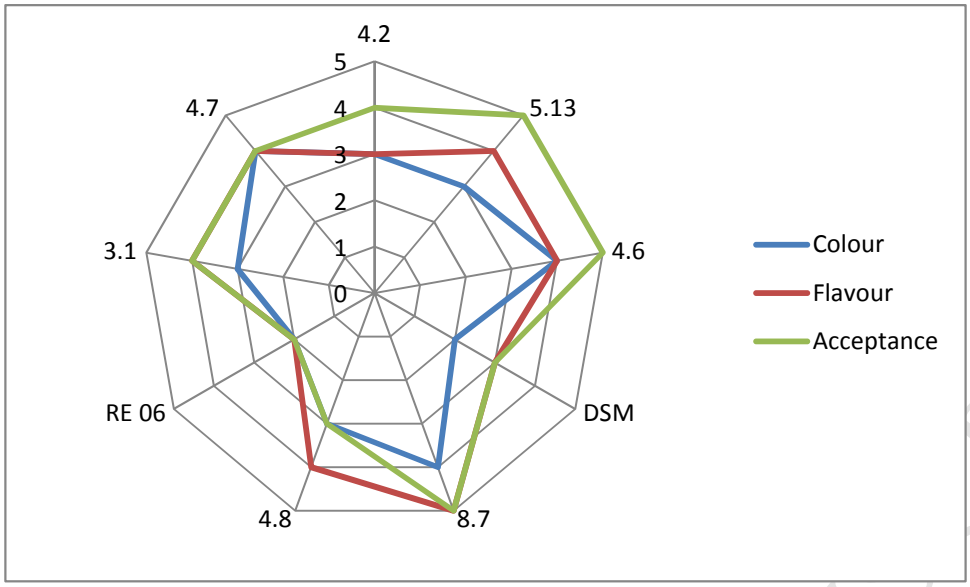

heptanal
4 ethyl 3 hexanolo
2 hexyl decanolo
alcohol ethylic
2 methyl heptanol
1 butanol 3 methyl formate
2,3 pentanedione
2 pentyl furan
2 ethyl furan
3 hydroy 2 butanone
6 6 dimethyl undecano
ethyl decanol
ethyl acetate
benzaldehyde
acetone
acetic acid
tiophene 2 acetic acid dodecinil estere
3,7 dymethyl decane
3,6 dymethyl decane
decanale
octanal
2 tiophenmetanthiolo
2,4 heptadienale
hexanal
standard
nonanal
3 decen 5 one
3 heptaecanolo
2 butanone
pentanone
1 octen 3 olo
Factor 2 : 22.01%
Factor 1 : 40.17%

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.