

1 **Six week consumption of a wild blueberry powder drink increases bifidobacteria in**
2 **the human gut**

3 Stefano Vendrame^{1,3}, Simone Guglielmetti², Patrizia Riso¹, Stefania Arioli², Dorothy
4 Klimis-Zacas³ and Marisa Porrini¹

5 ¹ Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, sezione
6 Nutrizione. Via Celoria 2, Università degli Studi di Milano, 20133 Milano, Italy.

7 ² Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, sezione Microbiologia
8 Industriale. Via Celoria 2, Università degli Studi di Milano, 20133 Milano, Italy.

9 ³ Department of Food Science and Human Nutrition, University of Maine, Orono, ME
10 04469, USA.

11

12 Corresponding author:

13 Patrizia Riso

14 Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche - Sezione Nutrizione
15 Università degli Studi di Milano

16 Via Celoria 2,

17 20133 Milano, Italy.

18 Tel.: +39-02-50 31 67 26

19 Fax.: +39-02-50 31 67 21

20 E-mail: patrizia.riso@unimi.it

21 **ABSTRACT**

22

23 Wild blueberries are a rich source of polyphenols and other compounds that are highly
24 metabolized by the intestinal microbiota and may at the same time affect the intestinal
25 environment itself.

26 A repeated-measures, crossover dietary intervention on human volunteers was designed
27 to study the effect of six week consumption of a wild blueberry (*Vaccinium*
28 *angustifolium*) drink, versus a placebo drink, in modulating the intestinal microbiota.

29 Relative to total eubacteria, *Bifidobacterium* spp. significantly increased, following
30 blueberry treatment ($P < 0.05$) while *Lactobacillus acidophilus*, increased after both
31 treatments ($P < 0.05$). No significant differences were observed for *Bacteroides* spp.,
32 *Prevotella* spp., *Enterococcus* spp. and *Clostridium coccoides*.

33 Bifidobacteria, which have been largely proposed of benefit for the host, appeared to be
34 selectively favored suggesting an important role of polyphenols and fiber present in wild
35 blueberries. Results obtained suggest that regular consumption of a wild blueberry drink
36 can positively modulate the composition of the intestinal microbiota.

37

38

39 **Keywords:** Wild blueberry, human study, microbiota, prebiotic, *Bifidobacterium*

40

41 INTRODUCTION

42 Compared to other cultivated species of blueberries, wild blueberry (*Vaccinium*
43 *angustifolium*) is characterized by a higher anthocyanin content, as well as significant
44 levels of total fiber, sitosterol, manganese, vitamin B6, vitamin C and vitamin K ⁽¹⁾.
45 The high antioxidant power of wild blueberries may in part explain their protective
46 activity against degenerative processes connected to oxidative stress and the presence of
47 reactive oxygen species, which is also the main reason for the cardiovascular protective
48 and anticarcinogenic activity attributed to phenolic-containing foods in general ⁽²⁾.
49 In addition, an increasing interest in the relationship between intestinal microorganisms
50 and overall health of the human host has been developing in recent years. It is well
51 known that intestinal microbiota is a key factor contributing to digestive processes,
52 producing vitamins, transforming bile acids and generating a multitude of bioactive
53 compounds from food components. For example short-chain fatty acids are derived from
54 the fermentation of fiber, conjugated linoleic acids from linoleic acid, enterodiol and
55 enterolactone from lignans and equol from daidzein, all of which have been linked to
56 anti-cancer, anti-inflammatory and other health-promoting effects ^(3,4). Beneficial
57 intestinal microbiota also play an important role on immunity through the modulation of
58 local and systemic immune response and can prevent the growth of pathogenic bacteria
59 by mechanisms of competition known as ‘barrier effect’ ⁽⁵⁾.
60 Although the composition of intestinal microbiota species and sub-species is extremely
61 variable from person to person, it is relatively constant for every single adult, and it is
62 mostly determined by genetic factors and by intestinal colonization in the early stages of

63 life ⁽⁶⁾. However, its composition can be significantly influenced by several
64 environmental factors, such as antibiotic usage and diet ⁽⁷⁾.

65 Diet can positively modulate intestinal microbiota through consumption of probiotics or
66 prebiotics. Oligosaccharides, such as inulin, lactulose, and dietary fiber in general, have
67 shown a strong prebiotic activity, mostly promoting the selection of lactobacilli and
68 bifidobacteria ⁽⁸⁾. A recent review focused on interactions between phenolic compounds
69 present in foods and intestinal microbiota emphasizing how this two-way interaction
70 affects both the production and bioavailability of bioactive compounds and the selection
71 and/or repression of specific microbial populations ⁽⁹⁾. Only few studies have shown a
72 significant effect of polyphenols (*e.g.* those from tea or soy isoflavones) in modulating
73 intestinal microbiota, although the mechanisms have not been delineated ^(10,11).

74 Since wild blueberries are a good source of polyphenols (in particular anthocyanins, but
75 also other flavonoids and phenolic acids) and dietary fiber, it is likely that they exert a
76 prebiotic activity and modulate the gut microbiota. Such activities have been previously
77 documented *in vitro* and *in vivo* on animal models: in particular, blueberry extracts have
78 been shown to increase the population size of lactobacilli and bifidobacteria in human
79 feces and after administration to rats ⁽¹²⁾.

80 To our knowledge, however, the prebiotic activity of wild blueberries and their ability to
81 influence human intestinal microbiota has never been evaluated in humans. Thus we
82 studied the potential prebiotic activity of wild blueberries and their ability to modulate
83 intestinal microbiota on a subgroup of volunteers enrolled in a larger project that

84 investigated the effects of wild blueberry (*Vaccinium angustifolium*) intake on endothelial
85 function, oxidative stress and inflammation.

86

87 **EXPERIMENTAL METHODS**

88 *Experimental design*

89 A repeated-measures, crossover dietary intervention was designed. A total of twenty
90 healthy male individuals, ages 45.9 ± 8.6 years and BMI 25.1 ± 2.8 kg/m² were recruited.
91 Volunteers were selected on the basis of a medical history questionnaire and an interview
92 to evaluate their dietary habits and ensure that they were as homogeneous as possible, in
93 particular for fruit and vegetable consumption. This was obtained by means of a food
94 frequency questionnaire previously published and specifically revised to focus on food
95 sources rich in antioxidants ⁽¹³⁾. Exclusion criteria were: high (> 5 portions/day) or low
96 (<2 portions/day) intake of fruits and vegetables; regular use of medications or dietary
97 supplements; habitual alcohol consumption (< 3 drinks per week); adherence to specific
98 vegetarian diets (e.g. vegan or macrobiotic); recent use (less than 1 month) of antibiotics
99 or medications affecting gastrointestinal function; intake of specific prebiotics or
100 probiotics and history of chronic constipation, diarrhea or any other gastrointestinal
101 problem.

102 Participants were randomly divided in two groups. Subjects in the first group received a
103 wild blueberry drink (25 g wild blueberry powder in 250 ml water) every day for 6
104 weeks, in addition to their habitual diet. After a 6 week wash-out period, they received a
105 daily placebo drink (250 ml water, 7.5 g fructose, 7 g glucose, 0.5 g citric acid, 0.03 g

106 blueberry flavour, 280 µl allura red AC 1%, 70 µl brilliant blue FCF 1%) for six weeks.
107 Subjects in the second group followed the opposite sequence: placebo drink – wash-out –
108 wild blueberry drink.

109 The two drinks were freshly prepared each morning and provided to the subjects in
110 appropriate ice boxes. Participants were instructed to keep the drinks under refrigeration
111 and to avoid exposing it to a heat source or light and consume the drink within the
112 morning. Every Friday, subjects received the drinks for the week-end and kept them
113 refrigerated.

114 For the duration of the experiment, volunteers were instructed to maintain their normal
115 dietary and lifestyle habits (as assessed before enrollment) and to abstain from consuming
116 anthocyanin-rich foods (a list of prohibited foods was provided). There is generally good
117 compliance with dietary instructions enrolling in the study subjects with similar dietary
118 habits and asking them to maintain their normal diets. This was also ensured by a 24-hr
119 diet recall (one day before sample collection), a 3-day food record (scheduled randomly
120 during the two experimental periods) and a weekly direct diet interview.

121 Wild blueberries, provided as a composite from Wayman’s (Cherryfield, ME), were
122 freeze-dried and powdered with standard procedures (by FutureCeuticals, Momence, IL).
123 The anthocyanin profile of the wild blueberry powder was determined by LC-DAD
124 MS(MS) and has been previously reported ⁽¹⁴⁾. One serving of wild blueberry drink
125 provided 375 mg of anthocyanins, with peonidin-glucose (49.5 mg), malvidin-galactose
126 (49.5 mg), delphinidin-glucose (33.8 mg) and delphinidin-galactose (29.2 mg) being the
127 most abundant molecules. Chlorogenic acid was the main phenolic compound present

128 (77.5 mg) while only traces of other hydroxycinnamic acids were detected in the freeze
129 dried wild blueberry powder.

130 Content of sugars was determined by ultra-high-performance liquid
131 chromatography/mass spectrometry (UPLC/MS). Glucose (Glc), fructose (Fru),
132 saccharose (Sac) and triethylamine (TEA) were purchased from Sigma-Aldrich (St.
133 Louis, MO, USA). Water was obtained from a MilliQ apparatus (Millipore, Milford,
134 MA). The chromatographic system consisted of an UPLC mod. Acquity (Waters)
135 coupled to a triple quadrupole mass spectrometer mod. Quattromicro (Waters). A 1.7 μm
136 BEH Amide column (150x2.1 mm, Waters) was used for the separation at a flow-rate of
137 0.6 mL/min. The column was maintained at 35°C and the separation was performed in
138 isocratic mode. The eluent was a solution of 0.2% TEA in water:0.2% TEA in CH₃CN
139 (75:25, v/v). The injection volume was 5 μl . The capillary voltage was set to 3 kV and
140 the cone voltage was 15eV. The source temperature was 130°C, the desolvating
141 temperature was 350°C. Data were acquired by Masslinx 4.0 software (Waters) and the
142 analyses were performed in single ion monitoring (SIR) mode monitoring the ions with
143 (m/z)⁻ 179 and 341, with a dwell time of 0.1 s.

144 Each serving provided 17.1 g of sugar, of which 8.8 g fructose and 8.3 g glucose.

145 The total fiber content of each serving was 4.5 g, of which 3.8 g insoluble fiber and 0.7 g
146 soluble fiber, as determined by the AOAC International method 991.43 for the soluble
147 and insoluble fiber and AACC 46.13 for the protein in the residue ⁽¹⁵⁾.

148 Participants were provided with sterile stool containers and they were asked to collect
149 four stool samples, at the beginning and at the end of both experimental periods, which
150 were then stored at -20°C within 12 hours for subsequent analyses.

151 Five volunteers out of the twenty originally involved in the study did not collect fecal
152 samples for the complete duration of the experiment and were excluded from the study.

153 This study was conducted according to the guidelines laid down in the Declaration of
154 Helsinki and all procedures involving human subjects were approved by the Ethics
155 Committee of the University of Milan. Written informed consent was obtained from all
156 subjects.

157 *DNA extraction and quantification*

158 DNA was extracted from homogenized feces (200 mg) using the QIAamp DNA stool
159 Mini kit (Quiagen, Hilden, Germany) following the instructions provided by the
160 manufacturer. The final concentration of DNA was determined spectrophotometrically.
161 A260/A280 and A260/A230 ratios were also calculated to exclude major contamination
162 from proteins and salts.

163 DNA samples were subsequently diluted with nuclease-free water to reach a DNA
164 concentration of 5 ng/μl, and stored at -20°C until processing.

165 *Real-time PCR*

166 To evaluate the variation of bacterial levels from faecal samples before and after each
167 treatment, a quantitative real-time polymerase chain reaction (PCR) protocol was
168 performed using specific primers targeting seven different bacterial groups (*Eubacteria*

169 spp, *Bacteroides* spp, *Bifidobacterium* spp, *Prevotella* spp, *Enterococcus* spp,
170 *Lactobacillus acidophilus* and *Clostridium coccooides*), as indicated in **Table 1**.

171 The analysis was performed in duplicates, using a Fast Eva Green Supermix SYBR
172 Green PCR Master Mix (Bio-Rad Laboratories S.r.l., Milano, Italy) in a reaction volume
173 of 20 μ l per well ⁽¹⁶⁾.

174 Quantitative PCR (qPCR) reactions were run on a CFX96 thermocycler (BioRad
175 Laboratories). A gradient PCR was performed initially to standardize the qPCR
176 conditions. qPCR amplification was carried out with initial denaturation at 95°C for 3
177 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at
178 55.9°C for 30 seconds, and extension at 72°C for 20 seconds. Melting curve analysis was
179 always carried out to verify the specificity of the amplification. Data were recorded as
180 threshold cycles (Ct), expressed as means \pm standard deviation, computed using the
181 software BioRad CFX Manager and expressed as normalized quantification ($\Delta\Delta$ Ct) \pm
182 standard error of the mean, calculated on each bacterial group in relation to total
183 eubacteria ^(17,18).

184 The PCR analysis was performed on a subset of eight samples for each set of primers, as
185 a screen. When significant variations or at least a definite trend were observed, the
186 analysis was performed on the remaining samples to confirm the results and a standard
187 curve for calculating the absolute bacterial concentrations was also measured.

188 A standard curve for bifidobacteria was constructed by plotting the Ct values obtained for
189 the standard cultures (different 1:2 dilutions from a mixed culture of *B. longum subsp.*
190 *longum* DSM 20219, *B. longum subsp. infantis* DSM 20088, *B. adolescentis* DSM 20083,

191 *B. bifidum* DSM 20456, *B. animalis* subsp. *lactis* Bb12, *B. breve* DSM 20213;
192 *B.pseudocatenulatum* DSM20438) as a linear function of the base 10 logarithm of the
193 initial number of bifidobacteria in the culture, determined by microscope counts
194 (Neubauer-improved counting chamber, Marienfeld GmbH).

195

196 *Statistical analysis*

197 Statistical analysis was performed on a personal computer with STATISTICA software
198 (Statsoft Inc, Tulsa, OK). Analysis of variance (ANOVA) was used to evaluate the
199 relative variations of the different bacterial groups following wild blueberry
200 consumption. A repeated measures ANOVA with the sequence of treatments (wild
201 blueberry then placebo or placebo then wild blueberry) as the independent factor was
202 performed to evaluate whether a significant carry-over effect was present. When no
203 carry-over effect was observed, data were analyzed with ANOVA considering treatments
204 (wild blueberry and placebo) and time (before and after treatments) as dependent factors.
205 Differences between means were further evaluated by the least significant difference test
206 (LSD). Results were considered significant at $P < 0.05$.

207 Analyses of bacterial levels were performed on stool samples from 8 subjects. The
208 remaining available samples (n=7) were analysed in order to confirm the effect of wild
209 blueberry drink intake on *Bifidobacterium* spp. and *Lactobacillus acidophilus* group.

210

211 **RESULTS AND DISCUSSION**

212 There is paucity of research on the effect of dietary polyphenols and their metabolites on
213 the composition on the gut microbiota while most studies have focused on their
214 antibacterial activity and biotransformation of polyphenols operated by the gut
215 microbiota ^(9, 20).

216 Results from this study presented on Table 2, point to the effect of wild blueberries on
217 different gut bacterial targets. All bacterial groups studied were present in all fecal
218 samples of the subjects tested. Statistically significant variations were observed on the
219 sub-sample of 8 subjects for *Bifidobacterium* spp. (P<0.05) and *Lactobacillus*
220 *acidophilus* group (P<0.05).

221 To increase sample size, the PCR analysis with the same primers was performed on all
222 available samples (n=15) and results obtained from the 8 samples were confirmed. Thus
223 in the 15 samples, *Bifidobacterium* spp. increased from 1.00 ± 0.13 to 2.12 ± 0.44 (P<0.05)
224 following the wild blueberry drink and from 1.25 ± 0.16 to 1.61 ± 0.42 following the placebo
225 drink. *Lactobacillus acidophilus* group (P<0.05) increased from 0.92 ± 0.10 to 6.24 ± 1.20
226 (P<0.05) following the wild blueberry drink and from 1.09 ± 0.14 to 6.03 ± 1.59 following
227 the placebo drink (P<0.05). While *Bifidobacterium* spp. only increased after the wild
228 blueberry drink consumption (2.12 fold compared to eubacteria), the *Lactobacillus*
229 *acidophilus* group increased after both treatments (6.78 and 5.53 fold following wild
230 blueberry and placebo drink respectively).

231 Diet can influence intestinal microbiota in both a positive and a negative way. A diet rich
232 in protein promotes the selection of proteolytic metabolic activities whose end products,
233 such as ammonia and N-nitroso compounds, may be toxic for the human host and may

234 increase colon cancer risk ⁽²¹⁾. On the other hand, microbial fermentations of fiber and
235 carbohydrate, with the production of short chain fatty acids, is considered positive for
236 contributing to the digestive process, enhanced absorption of minerals and
237 immunomodulatory and cancer preventive effects ⁽²²⁾. This is specifically associated with
238 the activity of bacterial strains from the genera *Lactobacillus* and *Bifidobacterium*, which
239 exert a range of health promoting effects including inhibition of procarcinogenic
240 enzymatic activities within the microbiota, inhibition of pathogens growth, synthesis of
241 vitamins and other beneficial bioactive compounds from food components ^(3,4,5).

242 For both these bacterial groups a significant variation was observed during this dietary
243 intervention.

244 Our results are in agreement with previous findings in vitro and in the animal model. In a
245 study by Molan et al., addition of a blueberry extract to mixed human fecal bacterial
246 populations resulted in a significant increase in the number of lactobacilli and
247 bifidobacteria ⁽¹²⁾. The same was observed in fecal samples of rats after a 6 days oral
248 administration of the blueberry extract ⁽¹²⁾. Dietary administration of proanthocyanidin-
249 rich extracts also appear to have a similar effect. In rats whose diet was supplemented for
250 16 weeks with a dealcoholized, proanthocyanidin-rich red wine extract, the fecal bacterial
251 composition shifted from a predominance of *Bacteroides*, *Clostridium* and
252 *Propionibacterium* spp. to a predominance of *Bacteroides*, *Lactobacillus* and
253 *Bifidobacterium* spp. ⁽²³⁾. Furthermore, Yamakoshi et al. documented that a
254 proanthocyanidin-rich extract from grape seeds administered for 2 weeks to healthy
255 adults was able to significantly increase the number of bifidobacteria ⁽²⁴⁾.

256 In our study the only bacterial group that increased significantly and exclusively after the
257 wild blueberry treatment, is represented by the genus *Bifidobacterium*. Growth of
258 bifidobacterial population may have benefited from the different components, which
259 were contained in the wild blueberry drink, primarily fiber and anthocyanins ^(25,26).

260 Evolutionarily, bifidobacteria have been subjected to a strong environmental pressure to
261 specialize for the catabolism of a variety of nondigestible plant polymers, glycoproteins
262 and glycoconjugates. While having relatively few proteolytic and lipolytic enzymes, they
263 have many enzymes to hydrolyze oligosaccharides, including those characterized by less
264 common linkages such as hemicelluloses, arabinogalactans, arabinoxylans, gums, inulins,
265 galactomannans and branched starches ⁽²⁷⁾.

266 Since these substrates are poorly metabolized by the most common intestinal bacteria,
267 such adaptation may be at the basis of the ability of bifidobacteria to persist in the colon
268 when an adequate intake of dietary fiber is provided, as it was confirmed by a recent
269 intervention study where a fiber mixture was administered to a group of fifty-nine human
270 volunteers, increasing their bifidobacterial population ⁽²⁸⁾. The β -glucosidase activity of
271 bifidobacteria also suggests their important role in the intestinal metabolism of
272 anthocyanins, which are present in nature mostly as glycosides ⁽²⁹⁾.

273 Additionally, the presence of anthocyanins and other polyphenols in the wild blueberry
274 drink should be considered as a contributor in the creation of a redox environment
275 favourable for the selection of bifidobacteria, which are benefited by low oxidation-
276 reduction potential ⁽³⁰⁾.

277 Moreover, the high content of vitamin K which is characteristic of wild blueberries ⁽¹⁾
278 may have contributed in promoting growth of bifidobacteria. In fact, vitamin K, which
279 cannot generally be produced by bifidobacteria, is a known growth factor for these
280 microorganisms ^(31,32).

281 We also determined the absolute bacterial concentrations for *Bifidobacterium* spp.
282 (Figure 1), calculated by means of a standard curve. Although the trend is similar to the
283 data normalized to total eubacteria, in this case the variations were not statistically
284 significant. However, since the total number of bacteria can vary significantly from one
285 stool sample to another, considering the absolute concentrations of the single bacterial
286 groups can be misleading; while the proportion of each group to the total number of
287 bacteria is more meaningful, especially if variations over time are to be considered. This
288 choice has been already proposed and employed in other studies ⁽³³⁾.

289 The *Lactobacillus acidophilus* group includes the species most commonly employed as
290 probiotics due to their widely recognized health promoting properties (for instance: *L.*
291 *acidophilus*, *L. gasseri*, *L. johnsonii*, *L. crispatus*, *L. helveticus*).

292 Interestingly, the *Lactobacillus acidophilus* group increased after both wild blueberry and
293 placebo drink consumption. The only hypothesis we can formulate to explain such a
294 result involves a determinant role of simple sugars, such as fructose or glucose, which
295 were present in both drinks and whose non-absorbed fraction was likely fermented in the
296 small and large intestine. In fact, lactobacilli, differently from bifidobacteria, colonize
297 also the proximal part of the gastro-intestinal tract ⁽³⁴⁾.

298 In addition to bifidobacteria and lactobacilli, this exploratory study also assessed other
299 microbial groups chosen on the basis of their well- known impact on host physiology.
300 *Bacteroides* spp. are the largest portion of the mammalian gastrointestinal microbiota
301 (10¹⁰-10¹¹ cells per gram), where they play a fundamental role in the processing of
302 complex carbohydrates ⁽³⁵⁾. *Prevotella* spp. and *Enterococcus* spp. are two of the
303 predominant genera in human feces. Changes in their number and enzymatic activities
304 have been connected to specific host physiologic conditions ^(36,37). Finally, the
305 *Clostridium coccooides* group, also predominant in the human gut, includes species that
306 are known butyrate-producing bacteria, thereby contributing to processes important to
307 colonic health ⁽³⁸⁾.

308 However, no statistically significant variation or trend could be observed for any of the
309 above microbial groups following the wild blueberry drink consumption.

310 In conclusion, the results of this study suggest that regular consumption of a wild
311 blueberry drink is able to favorably modulate the composition of the intestinal
312 microbiota, increasing in particular, bacterial strains from the genus *Bifidobacterium*,
313 which is considered among the most health promoting ⁽⁵⁾.

314 Considering the discovery-based nature of this investigation, further experiments on a
315 larger population group investigating specific species of bifidobacteria is strongly
316 recommended.

317 In addition, the wild blueberry drink may also be interesting as a basis for the formulation
318 of a “functional” drink that could combine the positive prebiotic activity observed of the

319 wild blueberry components with the probiotic activity of live *Bifidobacterium* and/or
320 *Lactobacillus* strains ⁽³⁹⁾.

321

322 **ACKNOWLEDGMENTS**

323 The authors would like to thank the Wild Blueberry Association of North America for
324 contributing the wild blueberries, and FutureCeuticals (Monmence, IL) for processing the
325 wild blueberry powder. This work was supported by grants (2007.5810 and 2010.2303)
326 from Cariplo Foundation (Italy).

327

328 **LITERATURE CITED**

- 329 1. Beattie, J.; Crozier, A.; Duthie, G.G. Potential health benefits of berries. *Curr Nutr*
330 *Food Sci.* **1995**, *1*, 71-86.
- 331 2. Bomser, J.; Madhavi, D.L.; Singletary, K.; Smith, M.A. In vitro anticancer activity of
332 fruit extracts from *Vaccinum* species. *Planta Med*, **1996**, *62*, 212-216.
- 333 3. Oozeer, R.; Rescigno, M.; Ross, R.P., et al. Gut health: predictive biomarkers for
334 preventive medicine and development of functional foods. *Br J Nutr*, **2010**, *103*, 1539-
335 1544.
- 336 4. Beards, E.; Tuohy, K.; Gibson, G. A human volunteer study to assess the impact of
337 confectionery sweeteners on the gut microbiota composition. *Br J Nutr*, **2010**, *104*, 701-
338 708.
- 339 5. Bruzzese, E.; Volpicelli, M.; Squaglia, M., et al. Impact of prebiotics on human health.
340 *Dig Liver Dis*, **2006**, *38*, S283–S287.
- 341 6. Eckburg, P.B.; Bik, E.M.; Bernstein, C.N., et al. Diversity of the human intestinal
342 microbial flora. *Science*, **2006**, *308*, 1635-1638.
- 343 7. Gibson, G.R.; Probert, H.M.; Loo, J.V.; et al. Dietary modulation of the human colonic
344 microbiota: updating the concept of prebiotics. *Nutr Res Rev*, **2004**, *17*, 259-275.
- 345 8. Younes, H. ; Coudray, C. ; Bellanger, J.; et al. Effects of two fermentable
346 carbohydrates (inulin and resistant starch) and their combination on calcium and
347 magnesium balance in rats. *Br J Nutr*, **2001**, *86*, 479-485.

- 348 9. Selma, M.V.; Espín, J.C.; Tomás-Barberán, F.A. Interaction between phenolics and gut
349 microbiota: role in human health. *J Agric Food Chem*, **2009**, *57*, 6485-6501.
- 350 10. Lee, H.C.; Jenner, A.M.; Low C.S.; Lee Y.K. Effect of tea phenolics and their
351 aromatic fecal bacterial metabolites on intestinal microbiota. *Res Microbiol*, **2006**, *157*,
352 876-884.
- 353 11. Parkar, S.G.; Stevenson, D.E.; Skinner, M.A. The potential influence of fruit
354 polyphenols on colonic microflora and human gut health. *Int J Food Microbiol*, **2008**,
355 *124*, 295-298.
- 356 12. Molan, A.L.; Lila, A.A.; Mawson, J.; et al. In vitro and in vivo evaluation of the
357 prebiotic activity of water-soluble blueberry extracts. *World J Microbiol Biotechnol*,
358 **2009**, *25*, 1243-1249.
- 359 13. Porrini, M.; Gentile, M.G.; Fidanza, F. Biochemical validation of a self-administered
360 semi-quantitative food-frequency questionnaire. *Br J Nutr*. **1995**, *74*, 323-333.
- 361 14. Del Bo', C.; Ciappellano, S.; Klimis-Zacas, D.; et al. Anthocyanin absorption,
362 metabolism, and distribution from a wild blueberry-enriched diet (*Vaccinium*
363 *angustifolium*) is affected by diet duration in the Sprague-Dawley rat. *J Agric Food*
364 *Chem*, **2010**, *58*, 2491-2497.
- 365 15. AOAC Method 991.43, Total, Insoluble and Soluble Dietary Fiber in Food-
366 Enzymatic-Gravimetric Method, MES-TRIS Buffer. Official Methods of Analysis, 16th
367 ed. AOAC International, Gaithersburg, MD, **1995**.

- 368 16. Maeda, H.; Fujimoto, C.; Haruki, Y.; et al. Quantitative real-time PCR using TaqMan
369 and SYBR Green for *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*,
370 *Prevotella intermedia*, tetQ gene and total bacteria. *FEMS Immunol Med Microbiol*, **2003**,
371 39, 81-86.
- 372 17. Matsuki, T.; Watanabe, J.; Fujimoto, Y.; et al. Development of 16S rRNA-gene-
373 targeted group-specific primers for the detection and identification of predominant
374 bacteria in human feces. *Appl Environ Microbiol*, **2002**, 68, 5445-5451.
- 375 18. Matsuda, K.; Tsuji, H.; Asahara, T.; et al. Establishment of an analytical system for
376 the human fecal microbiota, based on reverse transcription-quantitative PCR targeting of
377 multicopy rRNA molecules. *Appl Environ Microbiol*, **2009**, 75, 1961-1969.
- 378 19. Malinen, E.; Kassinen, A.; Rinttilä, T.; Palva, A. Comparison of real-time PCR with
379 SYBR Green I or 5'-nuclease assays and dot-blot hybridization with rDNA-targeted
380 oligonucleotide probes in quantification of selected faecal bacteria. *Microbiology*, **2003**,
381 149, 269-277.
- 382 20. Nohynek, L. J.; Alakomi, H. L.; Kahkonen, M. P.; Heinonen, M.; Helander, I. M.;
383 Oksman-Caldentey, K. M.; Puupponen-Pimia, R. H. Berry phenolics: antimicrobial
384 properties and mechanisms of action against severe human pathogens. *Nutr. Cancer*
385 **2006**, 54, 18-32
- 386 21. Manning, T.S.; Gibson, G. Microbial gut-interaction in health and disease. *Best Pract*
387 *Res Clin Gastroenterol*, **2004**, 18, 287-298.

- 388 22. Mortensen, P.B.; Clausen, M.R. Short-Chain Fatty Acids in the Human Colon:
389 Relation to Gastrointestinal Health and Disease. *Scand J Gastroenterol*, **1996**, *31*, 132-
390 148. (1)
- 391 23. Dolara, P.; Luceri, C.; Filippo, C. D.; et al. Red wine polyphenols influence
392 carcinogenesis, intestinal microflora, oxidative damage and gene expression profiles of
393 colonic mucosa in F344 rats. *Mutat Res*, **2005**, *591*, 237–246.
- 394 24. Yamakoshi, J.; Tokutake, S.; Kikuchi, M. Effect of proanthocyanidin-rich extract
395 from grape seeds on human fecal flora and fecal odor. *Microb Ecol Health Dis*, **2001**, *13*,
396 25-31.
- 397 25. Iwata, E.; Hotta, H.; Goto, M. The screening method of a bifidogenic dietary fiber
398 extracted from inedible parts of vegetables. *J Nutr Sci Vitaminol*, **2009**; *55*:385-388.
- 399 26. Avila, M.; Hidalgo, M.; Sanchez-Moreno, C.; Pelaez, C.; Requena, T.; de Pascual-
400 Teresa, S. Bioconversion of anthocyanin glycosides by Bifidobacteria and Lactobacillus.
401 *Food Res Int*, **2009**; *42*:1453–1461.
402
- 403 Schell, M.A.; Karmirantzou, M.; Snel, B.; et al. The genome sequence of
404 Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract. *Proc*
405 *Natl Acad Sci*, **2002**, *99*, 14422-27.
- 406 26. Depeint, F.; Tzortzis, G.; Vulevic, J.; et al. Prebiotic evaluation of a novel
407 galactooligosaccharide mixture produced by the enzymatic activity of Bifidobacterium

408 bifidum NCIMB 41171, in healthy humans: a randomized, double-blind, crossover,
409 placebo-controlled intervention study. *Am J Clin Nutr*, **2008**, *87*, 785-791.

410 27. Keppler, K.; Humpf, H.U. Metabolism of anthocyanins and their phenolic
411 degradation products by the intestinal microflora. *Bioorg Med Chem*, **2005**, *13*, 5195-
412 5205.

413 28. Jayamanne, V. S.; Adams, M. R. Modelling the effects of pH, storage temperature
414 and redox potential (Eh) on the survival of bifidobacteria in fermented milk. *Int J Food*
415 *Sci Technol*, **2009**, *44*, 1131-1138.

416 29. Collins, M.D.; Jones, D. Distribution of isoprenoid quinone structural types in
417 bacteria and their taxonomic implications. *Microbiol Rev*, **1981**, *45*, 316-54.

418 30. Hojo, K.; Nagaoka, S.; Murata, S.; et al. Reduction of vitamin K concentration by
419 salivary Bifidobacterium strains and their possible nutritional competition with
420 Porphyromonas gingivalis. *J Appl Microbiol*, **2007**, *103*, 1969-1974.

421 31. Gong, J.; Yu, H.; Liu, T.; et al. Characterization of ileal bacterial microbiota in
422 newly-weaned pigs in response to feeding lincomycin, organic acids or herbal extract.
423 *Livestock Science*, **2008**, *116*, 318-322.

424 32. Sartor R.B. Microbial influences in inflammatory bowel diseases. *Gastroenterology*.
425 **2008**, *134*, 577-594.

426 33. Wexler, H.M. Bacteroides: the good, the bad, and the nitty-gritty. *Clin Microbiol Rev*,
427 **2007**, *4*, 593-621.

428

- 429 34. Heimesaat, M.M.; Nogai, A.; Bereswill, S.; et al. MyD88/TLR9 mediated
430 immunopathology and gut microbiota dynamics in a novel murine model of intestinal
431 graft-versus-host disease. *Gut*, **2010**, *59*, 1079-1087.
- 432
- 433 35. Šefčíková, Z.; Bujňáková, D.; Raček, P.; Kmet, V.; Mozeš, Š. Developmental
434 changes in gut microbiota and enzyme activity predict obesity risk in rats arising from
435 reduced nests. *Physiol Res*, **2011**, *60*, 337-346.
- 436
- 437 36. Hayashi, H.; Sakamoto, M.; Kitahara, M.; Benno, Y. Diversity of the Clostridium
438 coccoides group in human fecal microbiota as determined by 16S rRNA gene library.
439 *FEMS Microbiol Lett*, **2006**, *257*, 202-207.
- 440 37. Worthley, D.L.; Le Leu, R.K.; Whitehall, V.L.; et al. A human, double-blind,
441 placebo-controlled, crossover trial of prebiotic, probiotic, and synbiotic supplementation:
442 effects on luminal, inflammatory, epigenetic, and epithelial biomarkers of colorectal
443 cancer. *Am J Clin Nutr*, **2009**, *90*, 578-586.
- 444

445 **Table 1** - List of primers and their sequences used for the PCR analysis

Target	Primer	Sequence (5'-3')	Annealing temp (°C)	Product size (bp)	Reference
Eubacteria	EubF1	GTGSTGCAYGGYTGTCTGCA	50-58.5	147	Maeda et al., 2003 (16)
	EubR1	GAGGAAGGTGKGGAYGACGT			
<i>Bacteroides</i> spp.	g-Bfra-F	AYAGCCTTTCGAAAGRAAGAT	50	495	Matsuki et al., 2002 (17)
	g-Bfra-R	CCAGTATCAACTGCAATTTTA			
<i>Bifidobacterium</i> spp.	g-Bifid-F	CTCCTGGAAACGGGTGG	55	550	Matsuki et al., 2002 (17)
	g-Bifid-R	GGTGTCTTCCCGATATCTACA			
<i>Prevotella</i> spp.	g-Prevo-F	CACRGTAAACGATGGATGCC	55	513	Matsuki et al., 2002 (17)
	g-Prevo-R	GGTCGGGTTGCAGACC			
<i>Enterococcus</i> spp.	g-Encoc-F	ATCAGAGGGGGATAAACTT	55	337	Matsuda et al., 2009 (18)
	g-Encoc-R	ACTCTCATCCTTGTTCTTCTC			
<i>Lactobacillus acidophilus</i> group of species*	Forward	AGAGGTAGTAACTGGCCTTTA	58.5	391	Malinen et al., 2003 (19)
	Reverse	GCGGAAACCTCCCAACA			
<i>Clostridium coccoides</i>	g-Ccoc-F	AAATGACGGTACCTGACTAA	50	440	Matsuki et al., 2002 (17)
	g-Ccoc-R	CTTTGAGTTTCATTCTTGCGAA			

446 * *L. acidophilus* probe detects *L. acidophilus*, *L. amylovorus*, *L. amylolyticus*, *L.*

447 *crispatus*, *L. gasseri* and *L. johnsonii*.

448

449 **Table 2** - Relative proportion of bacterial groups compared to total eubacteria in human
 450 fecal samples (n=8).

	Wild blueberry drink		Placebo drink	
	Before	After	Before	After
<i>Lactobacillus acidophilus</i>	0.90 ± 0.12 ^a	6.18 ± 1.92 ^b	1.18 ± 0.20 ^a	6.24 ± 1.83 ^b
<i>Bifidobacterium</i> spp.	0.75 ± 0.13 ^a	2.16 ± 0.70 ^b	1.23 ± 0.25 ^a	1.05 ± 0.39 ^a
<i>Prevotella</i> spp.	0.90 ± 0.08	1.28 ± 0.37	0.68 ± 0.15	0.78 ± 0.46
<i>Enterococcus</i> spp.	0.83 ± 0.12	0.48 ± 0.27	0.54 ± 0.36	0.65 ± 0.17
<i>Bacteroides</i> spp.	0.85 ± 0.11	1.14 ± 0.20	1.07 ± 0.16	1.07 ± 0.32
<i>Clostridium coccooides</i>	1.19 ± 0.18	1.71 ± 0.41	1.51 ± 0.28	1.36 ± 0.20

451

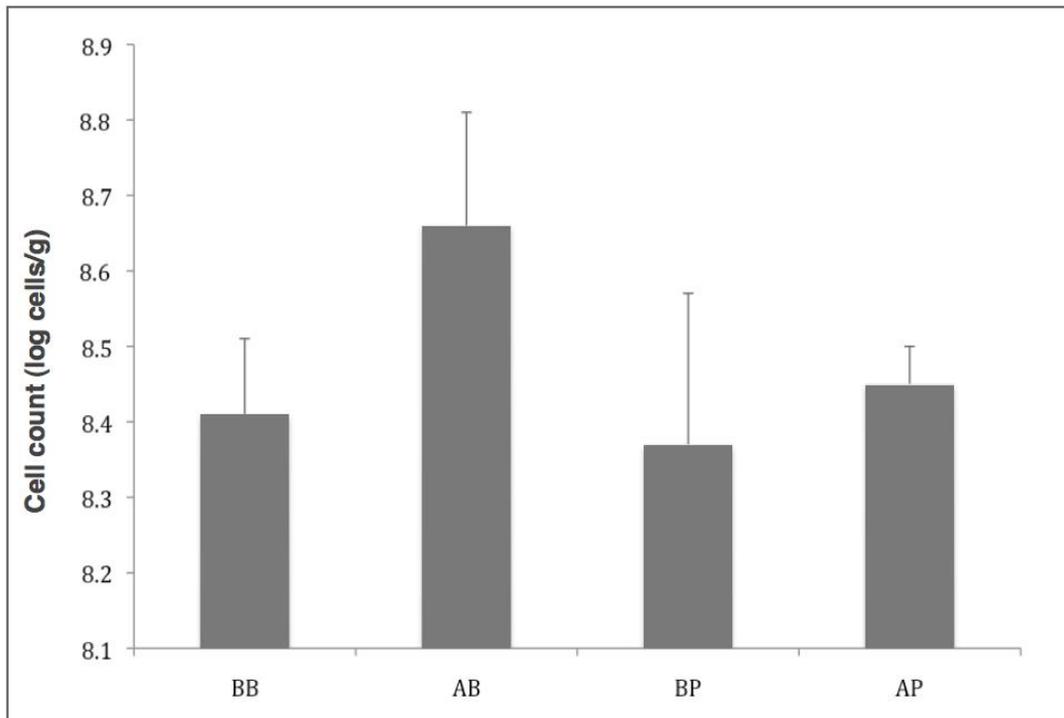
452 Values are expressed as mean ± standard error of mean (SEM) and were normalized to 1
 453 at the beginning of the study.

454 n: number of subjects.

455 a,b: mean values within a row with different letters were significantly different (P<0.05).

456

457 **Figure 1** - Absolute concentration of bifidobacteria in human fecal samples (n=15).



458

459 Bifidobacterial cell counts versus wet fecal weight, expressed as log (cells/g) mean +
460 SEM.

461 BB, before blueberry; AB, after blueberry; BP, before placebo; AP, after placebo.

462

463

464

465

466

467

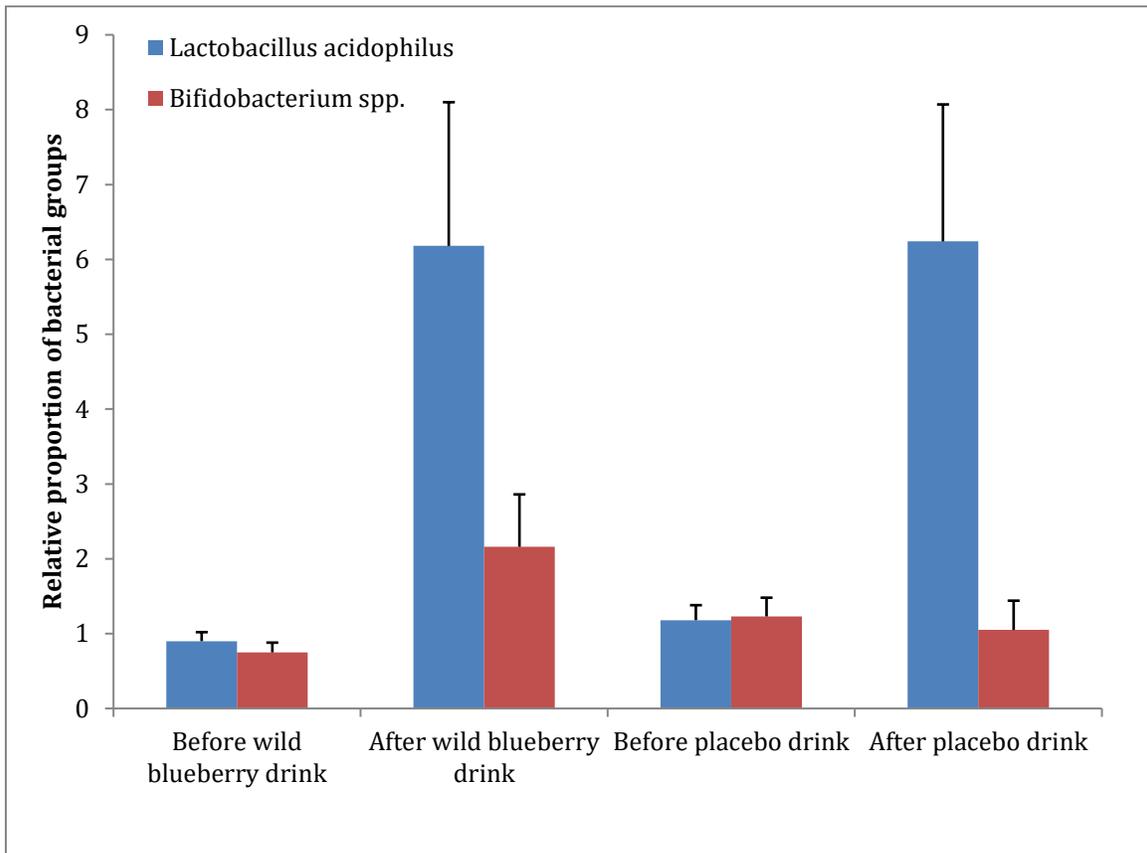
468

469

470

471

TOC Graphic



472