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

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ABSTRACT

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

A repeated-measures, crossover dietary intervention on human volunteers was designed to study the effect of six week consumption of a wild blueberry (*Vaccinium angustifolium*) drink, versus a placebo drink, in modulating the intestinal microbiota. Relative to total eubacteria, *Bifidobacterium* spp. significantly increased, following blueberry treatment ($P<0.05$) while *Lactobacillus acidophilus,* increased after both treatments ($P<0.05$). No significant differences were observed for *Bacteroides* spp., *Prevotella* spp., *Enterococcus* spp. and *Clostridium coccoides*.

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

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

INTRODUCTION

Compared to other cultivated species of blueberries, wild blueberry (*Vaccinum augustifolium*) is characterized by a higher anthocyanin content, as well as significant levels of total fiber, sitosterol, manganese, vitamin B6, vitamin C and vitamin K [(1)]. The high antioxidant power of wild blueberries may in part explain their protective activity against degenerative processes connected to oxidative stress and the presence of reactive oxygen species, which is also the main reason for the cardiovascular protective and anticarcinogenic activity attributed to phenolic-containing foods in general [(2)].

In addition, an increasing interest in the relationship between intestinal microorganisms and overall health of the human host has been developing in recent years. It is well known that intestinal microbiota is a key factor contributing to digestive processes, producing vitamins, transforming bile acids and generating a multitude of bioactive compounds from food components. For example short-chain fatty acids are derived from the fermentation of fiber, conjugated linoleic acids from linoleic acid, enterodiol and enterolactone from lignans and equol from daidzein, all of which have been linked to anti-cancer, anti-inflammatory and other health-promoting effects [(3,4)]. Beneficial intestinal microbiota also play an important role on immunity through the modulation of local and systemic immune response and can prevent the growth of pathogenic bacteria by mechanisms of competition known as 'barrier effect' [(5)].

Although the composition of intestinal microbiota species and sub-species is extremely variable from person to person, it is relatively constant for every single adult, and it is mostly determined by genetic factors and by intestinal colonization in the early stages of

life [6]. However, its composition can be significantly influenced by several environmental factors, such as antibiotic usage and diet [7].

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

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

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

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

EXPERIMENTAL METHODS

Experimental design

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

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

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

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

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

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

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

Content of sugars was determined by ultra-high-performance liquid chromatography/mass spectrometry (UPLC/MS). Glucose (Glc), fructose (Fru), saccharose (Sac) and triethylamine (TEA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was obtained from a MilliQ apparatus (Millipore, Milford, MA). The chromatographic system consisted of an UPLC mod. Acquity (Waters) coupled to a triple quadrupole mass spectrometer mod. Quattromicro (Waters). A 1.7 µm BEH Amide column (150x2.1 mm, Waters) was used for the separation at a flow-rate of 0.6 mL/min. The column was maintained at 35°C and the separation was performed in isocratic mode. The eluent was a solution of 0.2% TEA in water:0.2% TEA in CH_3CN (75:25, v/v). The injection volume was 5 µl. The capillary voltage was set to 3 kV and the cone voltage was 15eV. The source temperature was 130°C, the desolvating temperature was 350°C. Data were acquired by Masslinx 4.0 software (Waters) and the analyses were performed in single ion monitoring (SIR) mode monitoring the ions with $(m/z)^-$ 179 and 341, with a dwell time of 0.1 s.

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

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

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

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

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

DNA extraction and quantification

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

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

Real-time PCR

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

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

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

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

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

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

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

Statistical analysis

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

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

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].

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

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

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

Additionally, the presence of anthocyanins and other polyphenols in the wild blueberry drink should be considered as a contributor in the creation of a redox environment favourable for the selection of bifidobacteria, which are benefited by low oxidation-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 coccoides* 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

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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	GTGSTGCAYGGYTGTCGTCA	50-58.5	147	Maeda et al., 2003 (16)
	EubR1	GAGGAAGGTGKGGAYGACGT			
Bacteroides spp.	g-Bfra-F	AYAGCCTTTCGAAAGRAAGAT	50	495	Matsuki et al., 2002 (17)
	g-Bfra-R	CCAGTATCAACTGCAATTTTA			
Bifidobacterium spp.	g-Bifid-F	CTCCTGGAAACGGGTGG	55	550	Matsuki et al., 2002 (17)
	g-Bifid-R	GGTGTTCTTCCCGATATCTACA			
Prevotella spp.	g-Prevo-F	CACRGTAAACGATGGATGCC	55	513	Matsuki et al., 2002 (17)
	g-Prevo-R	GGTCGGGTTGCAGACC			
Enterococcus spp.	g-Encoc-F	ATCAGAGGGGGATAACACTT	55	337	Matsuda et al., 2009 (18)
	g-Encoc-R	ACTCTCATCCTTGTTCTTCTC			
Lactobacillus acidophilus group of species*	Forward	AGAGGTAGTAACTGGCCTTTA	58.5	391	Malinen et al., 2003 (19)
	Reverse	GCGGAAACCTCCCAACA			
Clostridium coccoides	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
Lactobacillus acidophilus	0.90 ± 0.12[a]	6.18 ± 1.92[b]	1.18 ± 0.20[a]	6.24 ± 1.83[b]
Bifidobacterium spp.	0.75 ± 0.13[a]	2.16 ± 0.70[b]	1.23 ± 0.25[a]	1.05 ± 0.39[a]
Prevotella spp.	0.90 ± 0.08	1.28 ± 0.37	0.68 ± 0.15	0.78 ± 0.46
Enterococcus spp.	0.83 ± 0.12	0.48 ± 0.27	0.54 ± 0.36	0.65 ± 0.17
Bacteroides spp.	0.85 ± 0.11	1.14 ± 0.20	1.07 ± 0.16	1·07 ± 0.32
Clostridium coccoides	1.19 ± 0.18	1.71 ± 0.41	1.51 ± 0.28	1.36 ± 0.20

451

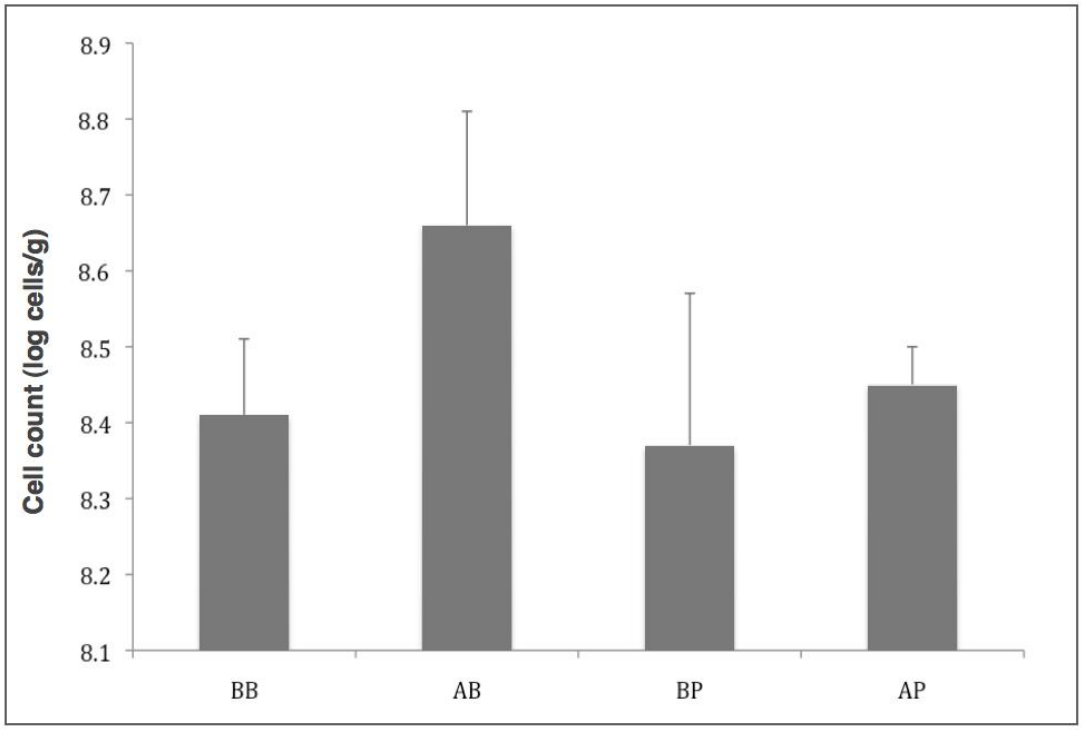
452 Values are expressed as mean ± standard error of mean (SEM) and were normalized to 1
453 at the beginning of the study.

454 n: number of subjects.

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

456

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

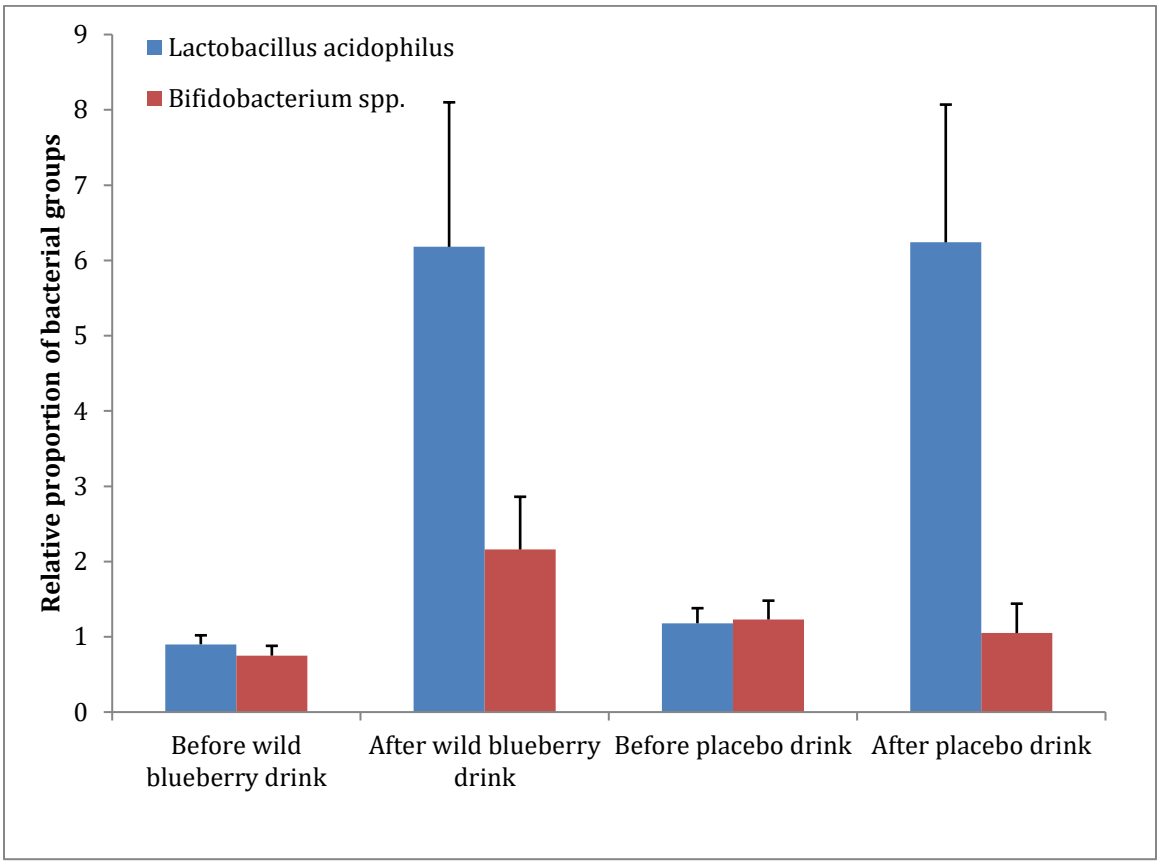


Bifidobacterial cell counts versus wet fecal weight, expressed as log (cells/g) mean + SEM.

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

471

TOC Graphic



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