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(Article begins on next page)

1 **Gut Microbiota composition after diet and probiotics in overweight breast cancer survivors:**
2 **a randomized open-label **pilot** intervention trial.**

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15 **ABSTRACT**

16 Objective: Breast cancer (BC) is the most diagnosed cancer in women. **Increasing survival rates**
17 **shifts attention to preventive strategies.** Obesity and intestinal microbiota (IM) composition may be
18 associated with BC. Mediterranean Diet (MD) proved to be protective. The aim of this study was to
19 **assess the efficacy of probiotics in addition to MD versus diet alone in influencing gut microbiota**
20 **and metabolic profile** in overweight BC survivors.

21 Methods: 34 BC survivor were randomized to MD for 4 months plus 1 sachet/day of probiotics
22 (*Bifidobacterium longum* BB536, *Lactobacillus rhamnosus* HN001) for the first 2 months
23 (**intervention group**, $n=16$) or MD alone for 4 months (control group, $n=18$). **Anthropometric and**
24 **nutritional assessments, adherence to MD, compliance to physical activity and metabolic**
25 **parameters dosage were performed at baseline (T0), at 2 (T2) and at 4-months (T4).** IM analysis
26 was performed at T0 and T2.

27 Results: After 2-months of probiotic administration **the number of bacterial species ($p=0.01$) and**
28 **the bacterial diversity assessed with the Chao1 index ($p=0.004$) significantly increased, no**
29 **significant variations were detected after diet alone.** The **Bacteroidetes-/Firmicutes ratio**
30 **significantly decreased in the intervention group and increased in controls ($p=0.004$).** Significant
31 reductions of body weight, body mass index (BMI), fasting glucose and **Homeostasis-Model**
32 **Assessment Insulin-Resistance (HOMA-IR)** were observed at T4 in both groups, in the intervention
33 group also waist circumference ($p=0.012$), waist/hip ratio ($p=0.045$) and fasting insulin ($p=0.017$)
34 significantly decreased.

35 Conclusions: Probiotics in addition to MD positively influence the gut microbiota and improve
36 metabolic and anthropometric parameters respect to MD alone.

37
38 **Keywords:** gut microbiota, breast cancer, Mediterranean diet, probiotics

41 **ARTICLE**

42 **Introduction**

43 Breast cancer (BC) is the most common cancer in women worldwide (1). **Disparities in BC death**
44 **rates are evident by state, socioeconomic status, and race/ethnicity, although overall survival rates**
45 **have improved due to advancements in diagnosis and therapies (2).** BC remains a major health
46 **problem, indeed research for primary and secondary prevention strategies represent a biomedical**
47 **priority (3).** Genetic, epigenetic and well-established determinants could explain a limited

48 number of BC cases. Bacterial communities within the host have been considered an additional
49 environmental risk factor related to sporadic BC of unknown aetiology (4).

50 Lifestyle could negatively impact on BC, especially alcohol consumption, fat excess, lack of
51 physical exercise and poor diet (5,6). Overweight and obesity are associated with cancer advanced
52 stage and grade at the diagnosis and resistance to local and systemic therapies (7–9). The largest
53 collection of human-colonizing microorganisms is a complex cellular ecosystem localized at the
54 distal gastrointestinal tract (colon), known as intestinal microbiota (IM) (10–12). The IM influences
55 local and systemic physiological activities such as metabolic and immune functions, which become
56 highly dysregulated during carcinogenesis (13). The composition of the gut microbiota modulates
57 both inflammation and the genomic stability of host cells and thereby is involved in the initiation,
58 progression and dissemination of cancer (14). BC is associated with oestrogen-dependent and
59 oestrogen-independent functions of IM (15–21).

60 Diet contents and quantity have a major role in shaping the gut microbiota composition and
61 function (22). Obesity has been related to a distortion of the microbial homeostasis, with a reduced
62 bacterial biodiversity and an altered expression of bacterial genes, especially those involved in
63 energy extraction from food (23–25). A varied and balanced diet plays an essential role in
64 maintaining the diversity and proper functioning of our gut microbiota. (26). The Mediterranean Diet
65 (MD) is widely regarded as a healthy dietary pattern, due to the high intake of fiber and plant-
66 derived proteins, high levels of polyphenols and other antioxidants and healthy fatty acids (both
67 monounsaturated and polyunsaturated) (27).

68 Dietary supplementation with probiotics, such as bacterial strains exerting beneficial effects on
69 their host, regulates the gut microbiota structure and function through the interaction with the
70 commensal bacteria and the expression of microbial enzymes (28). *Lactobacilli* and *Bifidobacteria*
71 are the most used strains for safety and efficacy. *Lactobacillus rhamnosus* has been reported to
72 improve insulin sensitivity and expression of genes related to glucose and lipid metabolism (29).
73 Furthermore, the combination of the two probiotic strains *Bifidobacterium longum* and *Lactobacillus*
74 *rhamnosus* has shown to be synergistic with positive endosymbiotic functional effects on the IM of
75 the host (30).

76 The aim of this study was to assess the effect of a combination of two well-characterized
77 probiotic strains (*Bifidobacterium longum* BB536, *Lactobacillus rhamnosus* HN001) in addition to
78 MD on body weight, metabolic and inflammatory serum markers and gut microbiota composition
79 compared to MD alone, in a cohort of overweight BC survivors.

81 **Materials and Methods**

82 **Study design**

83 This is a randomized open-label pilot intervention trial.

84 **Recruitment of participants**

85 Participants were recruited from the Breast Unit - San Lazzaro Hospital of the "Città della Salute
86 e della Scienza" of Turin, in the period from January 2017 to January 2018.

87 Inclusion criteria were: female survivors to BC with BMI between 25.0 and 35.0 kg/m², free
88 from cancer disease.

89 Exclusion criteria were: age over 70 years, any other chronic or acute diseases other than the
90 previous BC, use of any supplement, use in the last 8 weeks of drugs for constipation, proton
91 pump inhibitors, probiotics, antibiotics or any other drug potentially impacting on microbiota
92 composition and metabolic parameters.

94 **Outcomes**

95 The primary outcome was the changes in the gut microbiota composition after 2 months of MD
96 plus probiotics versus MD alone.

97 Secondary outcomes were changes in body weight, body mass index (BMI), waist
98 circumference and metabolic parameters after 4 months of intervention.

100 **Intervention**

101 Thirty-four female patients were randomized respectively to MD for 4 months plus 1
102 sachet/day of probiotics for the first 2 months of the study (intervention group, n=16) or MD alone

103 for 4 months (control group, $n=18$). AlfaSigma S.p.a. (Bologna, Italy) provided the probiotic
104 product, each sachet containing 4×10^9 colony-forming units (CFU) of *B. longum* BB536 and 10^9
105 colony-forming units (CFU) of *L. rhamnosus* HN001).

106 Data related to health status, use of drugs, supplements or probiotics, usual dietary habits and
107 physical activity were collected from all subjects.

108 All patients were evaluated at enrolment (T0), after 2 (T2) and 4 months (T4) from baseline. At
109 each visit all subjects were assessed with:

110 - nutritional assessment

111 - the Italian Mediterranean Index (IMI) questionnaire

112 - anthropometric measurements, such as height, weight, BMI, waist and hip circumference

113 - metabolic parameters, such as blood count with leukocyte formula, fasting glucose, fasting
114 insulin, glycated haemoglobin (HbA1c), aspartate amino transferase (AST), alanine amino
115 transferase (ALT), γ -glutamyl transferase (GGT), C-reactive protein (CRP), 25OH-vitamin D,
116 triglycerides, total and HDL cholesterol were obtained. LDL cholesterol was calculated with the
117 Friedewald formula.

118 At T0 and T2 faecal samples were collected to analyse the gut microbiota

119 Nutritional assessment, anthropometric measurements and IMI questionnaire distribution were
120 performed by a doctor with a trained dietitian. At the enrolment, after randomization, probiotics
121 were provided to the intervention group. Patients took 1 sachet/day of probiotics 30 minutes
122 before breakfast, for the first 2 months of the study. At T0, for each patient a personalized MD
123 according to WCRF recommendations was elaborated (2) by a trained dietitian. Diet composition
124 ranged from 1200 to 1500 kcal, with 15-18% proteins, 25-35% lipids and 45-55% carbohydrates.
125 Each patient was encouraged to follow a diet rich in whole grains, fish, legumes, vegetables (at
126 least 3 serving/day), fruits (2 serving/day), olive oil and seed oil, with a reduced intake of cheese,
127 butter, meat, potatoes, and a very low content of sugars.

128 Food and beverage consumption were assessed by a validated three-days food record (31,32).

129 All participants were trained by a dietitian to record all food consumed.

130 The compliance with the prescribed diet and physical activity and the adherence to the
131 protocol was performed. A concordance to the prescribed diet ranging from 80 to 100% was
132 arbitrarily defined as a good/very good, from 50 to 80% as mild/moderate and below 50% as
133 none compliance to diet.

134 Physical activity was considered: none <1h/week, moderate 1-2h/week or intense >2h/week.

135 The Italian Mediterranean Index (IMI) questionnaire is a food frequency questionnaire
136 developed and validated by Agnoli et al. (33), to assess the adherence to a MD. The score is
137 calculated from the qualitative and quantitative intake of 11 food items comprehending typical
138 Mediterranean foods (pasta, typical Mediterranean vegetables, fruits, legumes, olive oil and fish)
139 and non-typical Mediterranean foods (soft drinks, butter, red meat, and potatoes). If consumption of
140 typical Mediterranean foods was in the 3rd tertile of the distribution (high intake), the person
141 received 1 point; all other intakes received 0 points. If consumption of non-Mediterranean foods
142 was in the 1st tertile of the distribution (low intake), the person received 1 point. Alcohol receives 1
143 point for intake from 0.71 to 12 g/day; abstainers and persons who consume >12 g/day receive 0
144 (33). Possible scores ranged from 0 to 11, we assumed a good adherence to MD with scores from
145 6 to 11.

146 Each visit was performed at the Department of Clinical Nutrition, San Giovanni Battista
147 Hospital, of the "Città della Salute e della Scienza" of Turin. Blood samples were processed by the
148 main hospital laboratory. Microbiological analysis of the faecal samples was performed at the
149 Department of Agricultural, Forest and Food Sciences, University of Turin.

150

151 Measurements

152 Anthropometric parameters were measured by trained researchers. Body weight was measured
153 to the nearest 0.1 kg, and height was measured to the nearest 0.1 cm with a stadiometer (Seca,
154 Hamburg, Germany) and weight was measured with Tanita Segmental Body Composition
155 Monitor 2012 (Tanita Corporation) with the participants wearing light clothes and no shoes. Waist
156 circumference was measured at the navel level, without clothing by a plastic tape meter to the
157 nearest 0.1 cm. Waist circumference was measured at the navel level, without clothing by a
158 plastic tape meter to the nearest 0.1 cm.

159 **Biochemical analysis**

160 Blood samples were collected after an overnight fast. All laboratory measurements were
161 centralized. Serum glucose, AST, ALT, GGT, triglycerides and cholesterol (total and HDL-
162 cholesterol) and **C-reactive protein (CRP) concentration were tested on COBAS 8000 Roche**
163 **(Roche Diagnostics, Indianapolis). Total 25(OH)vitamin D was measured by Advia Centaur**
164 **Siemens Healthcare Diagnostics analyser.** Insulin was measured by Siemens Immulite analyzer.
165 HbA1c was determined with Tosoff G8 analyzer. The HOMA-IR was calculated according to the
166 published algorithm (34).

167

168 **Microbiological analysis**

169 **DNA extraction**

170 **Stool samples were self-collected** at home by patients and transferred to sterile sampling
171 containers. The samples were immediately refrigerated at 4 ° C and within the next 2 hours stored
172 in a refrigerator at the temperature of -80 ° C.

173 The total DNA was extracted directly from the faecal samples using the RNeasy Power
174 Microbiome kit (Qiagen, Milan, Italy) following the manufacturer's instructions. One microlitre of
175 RNase (Illumina Inc. San Diego, CA) was used for the digestion of RNA in DNA samples, with a 1
176 hour incubation at 37°C. The DNA was quantified using the QUBIT dsDNA Assay kit (Life
177 Technologies, Milan, Italy) and standardized at 5 ng/μL.

178

179 **Sequencing of the 16S rRNA gene target amplicon**

180 DNA extracted directly from the faecal samples was used to evaluate the microbiota by
181 amplification of the V3-V4 region of the 16S rRNA gene using the primers and protocols described
182 by Klindworth et al (35). PCR amplicons were purified with the Agencourt AMPure kit (Beckman
183 Coulter, Milan, Italy) and the resulting products were **tagged** using the Nextera XT Index kit
184 (Illumina Inc. San Diego, CA) according to the **16S metagenomic sequencing library preparation**
185 **instructions.** The paired-end sequencing reaction (2 X 250 bp) was performed using the Illumina
186 MiSeq platform according to the manufacturer's instructions.

187

188 **Bioinformatic analysis of sequences**

189 The paired-end reads were assembled using the FLASH software (36) with the default
190 parameters. The sequences were filtered by quality (Phred <Q20) using the QIIME 1.9.0
191 software (37) and the sequences <250 bp were discarded via Prinseq (38). **After chimeric filtering**
192 **(39), operational taxonomic units (OTUs) were clustered to 97% similarity through UCLUST (40)**
193 **and the representative sequences of each cluster were mapped against the 16S rRNA database**
194 **of Greengenes.**

195

196 **Statistical analysis**

197 The α diversity of the intestinal microbiota was evaluated by the Chao1 index, which
198 estimated the number of different taxa, and the Shannon diversity index, which evaluated the
199 wealth and uniformity of the taxa calculated using the diversity of the **vegan** package (41) in R
200 environment. The OTU table was used to build a principal component analysis (PCA) according
201 to the sampling time using the **made4** package of R. The ADONIS and ANOSIM tests were used
202 to detect significant differences in the general microbial community using the Weighted UniFrac
203 phylogenetic distance matrix and the OTU table. **A principal component analysis (PCA) was**
204 **carried out on the individual datasets (microbiota and anthropometric variable) and the results**
205 **were then integrated using coinertia analysis (CIA), which allows the shared biological trends**
206 **within two datasets.** The statistical package DESeq2 was used to find significant differences in
207 the abundance of microbial taxa.

208 The comparison between groups was performed using the t-Student test or the U-Mann-
209 Whitney test in the case of non-normal distribution variables. The comparison within the same
210 group was evaluated with the t test for paired data **or the Wilcoxon matched pairs test in the case**
211 **of not-normally distributed variables.** A simple correlation analysis between anthropometric and
212 laboratory variables and the individual OTUs (Spearman correlations) was performed. The
213 significant associations were then further evaluated by multiple regression, after adjustment for
214 age, BMI, and probiotic use.

215 **Randomization**

216 A randomization list was drawn up by an operator who did not take part in the study. A number
217 was assigned to each patient. The procedure was completely concealed to researchers.

218
219 **Blinding**

220 The study was not blinded. Indeed, the dieticians who evaluated the questionnaires and the
221 laboratory personnel who analysed the blood and stool samples was blinded to the participants'
222 group assignment.

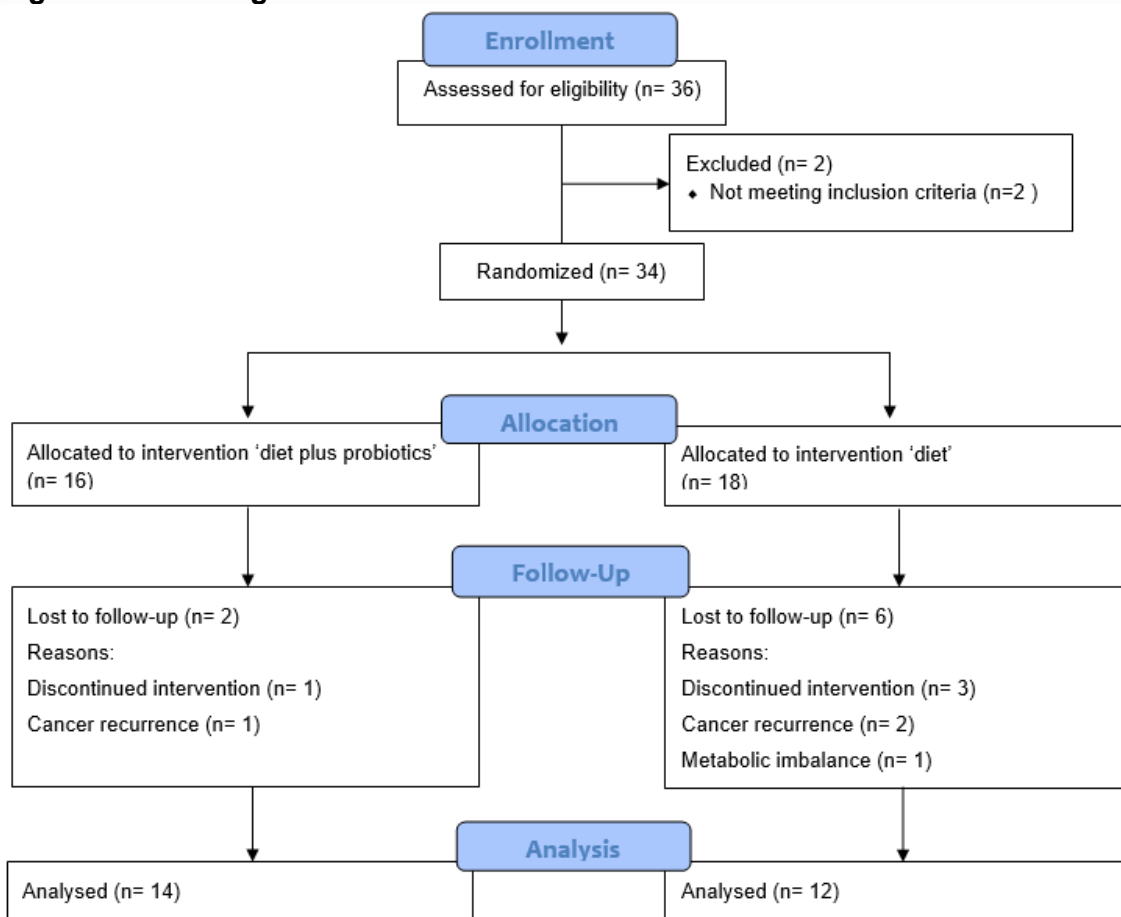
223
224 **Ethical aspects**

225 Each participant gave her written informed consent to participate to the study. The study
226 protocol was approved by the Ethics Committee of the "Città della Salute e della Scienza" Hospital
227 of Turin (approval date: March 30, 2017).

228
229 **Results**

230 Of the 34 participants, respectively 2 and 6 from the **intervention** group and the control group
231 dropped out. The flow diagram of the trial is described in Figure 1.

232
233
234 **Figure 1. Flow diagram of the trial**



235
236
237 **Anthropometric, metabolic and lifestyle characteristics**

238 Anthropometric, metabolic and lifestyle characteristics **were not significantly different between**
239 **the two groups at baseline (p>0.05).**

240 At the end of the study, we observed in **the intervention group** a significant reduction in body
241 weight, BMI, waist circumference, waist/hip ratio, **fasting glucose, insulin and HOMA-IR values**
242 **(Table 1).** The control group showed a significant reduction in body weight, BMI, **fasting glucose**
243 **and HOMA-IR levels** and a **significant increase in vitamin D** (Table 1). **Even if within-group** (Table

244 1) and between-group (data not shown) differences were not significantly different, the CRP values
 245 tended to increase in the controls and to reduce in the intervention group. The delta values (final
 246 value minus baseline value of each variable) were not significantly different between-group, with
 247 the exception of ALT values ($p=0.02$) (data not shown).

248 Participants from the intervention group showed a significant reduction in caloric intake and an
 249 increase of protein intakes (Table 1). All patients at T0 had a medium-low adherence to the MD,
 250 quantified by the Mediterranean Index. During the study, the adherence to the MD was stable in
 251 the intervention group but improved in the control group, though not significantly (Table 1).
 252 Similarly, the adherence to the recommended exercise improved in both groups, with a slightly
 253 higher, though not significantly different, increase in the controls (data not shown).

254
 255 **Table 1. Comparisons of change from baseline for study endpoints in the two study arms.**

Anthropometric and blood variables	Intervention group			Control group		
	T0	T4	p	T0	T4	p
weight (kg)	81.5 ± 10.4	78.8 ± 9.9	0.001	75.5 ± 7.8	72.4 ± 7	0.015
BMI (kg/m ²)	31 ± 3.3	30.1 ± 3.2	0.003	30.1 ± 3.2	28.8 ± 2.5	0.013
waist circumference (cm)	97 ± 10	94.4 ± 9.5	0.012	93.6 ± 10.9	90.4 ± 6.1	0.13
hip circumference (cm)	109.7 ± 8.1	109.5 ± 7	0.87	108.7 ± 8.3	107 ± 6.8	0.16
waist/hip ratio	0.88 ± 0.09	0.86 ± 0.07	0.045	0.86 ± 0.07	0.84 ± 0.04	0.39
fasting glucose (mg/dL)	92.6 ± 10.5	86.7 ± 9.2	0.0025	92.5 ± 7.6	85.7 ± 11	0.017
HbA1c (mmol/mol)	38.6 ± 3.3	38.3 ± 3.5	0.57	37.3 ± 4.3	37.2 ± 4.1	0.079
insulin (μU/mL)	15.1 ± 8.1	12.6 ± 8.3	0.017	11.3 ± 4.6	9.4 ± 5.4	0.11
HOMA-IR (mg/dL*μU/mL/405)	3.5 ± 2.1	2.8 ± 2.1	0.004	2.6 ± 1.1	1.8 ± 1.2	0.024
AST (UI/L)	20.4 ± 9.1	19.6 ± 4.3	0.65	18.1 ± 2.9	17.5 ± 2.7	0.21
ALT (UI/L)	22.9 ± 14.4	21.4 ± 8.4	0.49	18.5 ± 5.1	14.6 ± 3.6	0.001
GGT (UI/L)	23.7 ± 24.3	20.9 ± 17.2	0.67	21.4 ± 13.7	19 ± 11.9	0.16
total cholesterol (mg/dL)	206.6 ± 34.4	202.3 ± 34	0.67	202.1 ± 29.2	193.6 ± 21.7	0.50
HDL-cholesterol (mg/dL)	57.9 ± 14.6	57.1 ± 13.9	0.76	55.7 ± 9.1	61.6 ± 14.5	0.15
LDL - cholesterol (mg/dL)	123.4 ± 31.3	120.5 ± 30.4	0.75	122.3 ± 24.1	115.7 ± 28.9	0.62
triglycerides (mg/dL)	126.5 ± 63.4	123.1 ± 67.5	0.69	104 ± 33.5	94.1 ± 33.1	0.20
C-reactive protein (mg/L)	2.35 (1.30)	2.10 (2.70)	0.45	1.15 (1.95)	1.90 (2.40)	0.12
25OH-vitamin D (ng/mL)	23.7±6.8	25.0±8.7	0.17	22.0±7.8	24.4±9.1	0.02
Food intake						
Proteins (% kcal)	15.8 ± 2.9	18.7 ± 5.15	0.031	15.7 ± 3.5	16.9 ± 2.9	0.29
Lipids (% kcal)	36.5 ± 5.2	38.5 ± 6.7	0.33	38.7 ± 6.4	36.1 ± 6.3	0.41
Carbohydrates (% kcal)	46.5 ± 4.9	44.3 ± 11.0	0.47	42.2 ± 11.1	48 ± 7.3	0.24
Energy (kcal/die)	1431.4 ± 441	1102.5 ± 208.1	0.024	1416.7 ± 503.8	1082.5 ± 191.5	0.058
Mediterranean Index	6 ± 1.2	6 ± 1.1	0.85	5.7 ± 1.3	6.6 ± 0.9	0.075

256
 257 Body mass index (BMI); glycated hemoglobin (HbA1c), Homeostasis Model Assessment-Insulin Resistance
 258 (HOMA-IR); Alanine aminotranferase (ALT); aspartate aminotransferase (AST); γ-glutamyl transferase (GGT).
 259 Mean ± SD (all such values); median (range)

Composition of intestinal microbiota at baseline (T0) and after 2-months of intervention (T2)

A total of 1,944,328 (2 × 250 bp) were obtained after sequencing. After joining, a total of 1,301,233 reads passed the filters applied by QIIME, with a median value of 24720 (min 5092max 49,644) reads/sample and a sequence length of 440bp. The rarefaction analysis and the estimated sample coverage indicated that there was a satisfactory coverage of all the samples (ESC median value of 96.48%). Moreover, the alpha-diversity showed that there were no differences, in terms of complexity ($P > 0.05$), between the dietary intervention (control vs. probiotic) at baseline as well as across time. Similarly, there was no significant separation by microbiota composition across time, dietary intervention or adherence to the Mediterranean diet (MD) of individuals in PCoA plots based on UniFrac distances (data not shown). However, by taking into the account microbiota composition and nutrients/metabolic variables we performed Coinertia analysis (CIA) (Figure 2) based on PCA of microbiota composition and nutrients/metabolic variables. The results showed a significant relationship between genus-level microbiota composition and probiotic intervention (RV coefficient=0.34; Monte Carlo $p=0.001$).

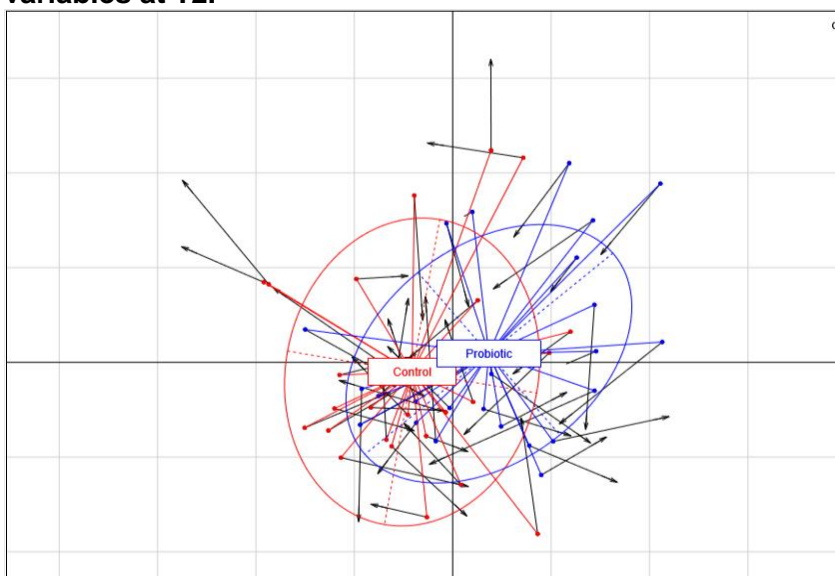
The first component of the CIA (horizontal) accounted for 37.22% of the variance, and the second component (vertical) accounted for another 13.16%. Even if the CIA showed not clear separation of the datasets it is possible to observe a gradient of separation according to probiotic intervention (Figure 2). The statistical package DESeq2 was used to find significant differences in microbial taxa abundance and the boxplot (Figure 3) showed statistically significant differences in several taxa ($P < 0.05$) between T0 and T2.

A significant increase both in number of bacterial species ($p=0.01$) and in bacterial diversity evaluated with the Chao1 index at T2 was observed in the treated subjects but not in controls (Table 2).

At T0 in the intervention group lower levels of Clostridiales and higher levels of *Escherichia* were observed. At T2, in the probiotic treated group a significant increase of *Eubacterium* and *L-Ruminococcus* (*Ruminococcus* assigned to the family Lachnospiraceae) and reduction in *Bacteroides* and *Butyrivibrio* were observed (Figure 3).

The *Bacteroidetes*-/*Firmicutes* ratio was similar in the two groups at T0, but it was significantly reduced in the probiotic treated subjects and increased in controls at T2, due to a reduction in *Bacteroidetes* and a simultaneous increase of *Firmicutes* after probiotic administration.

Figure 2. Coinertia analysis combining PCA of microbiota, nutrient intakes and metabolic variables at T2.



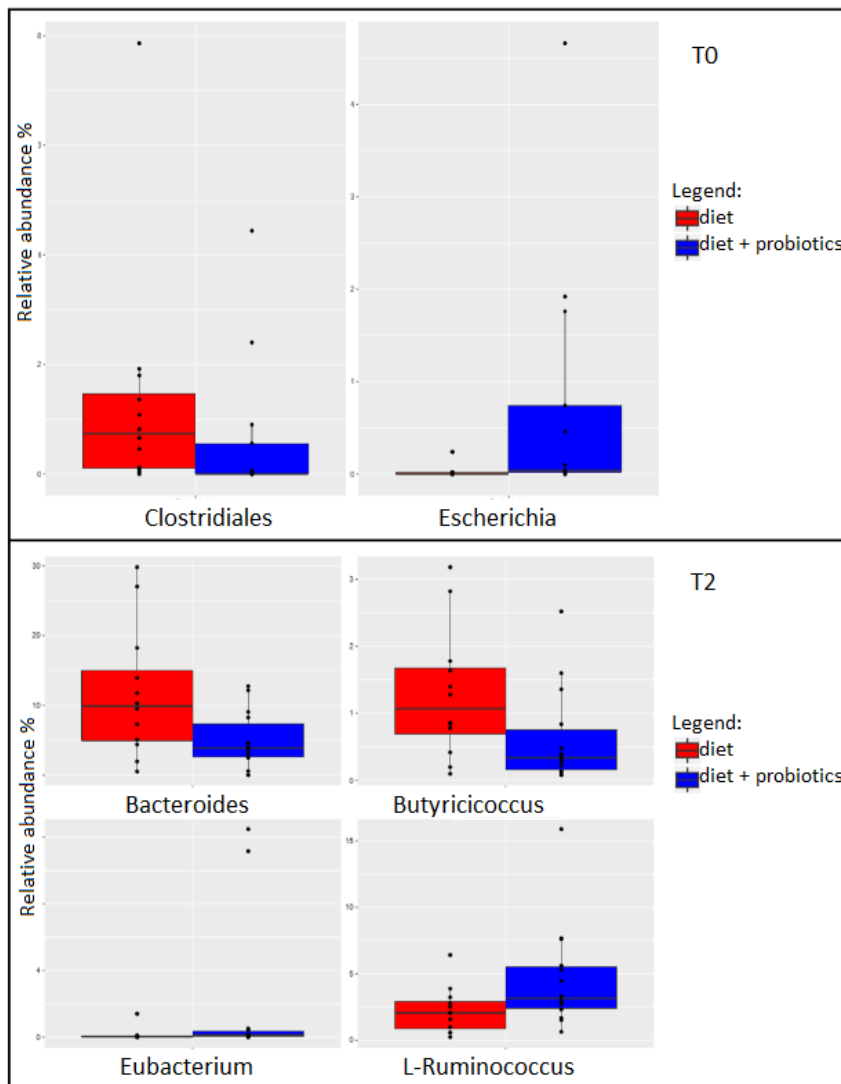
Subjects' clustering and colouring were done according to the intervention (control =red; probiotic=blue). Arrow ends of the line indicate sample position in the microbiota dataset, while black dot end indicates sample position in the nutrient intakes and metabolic dataset. (PCA = Principal component analysis)

300 **Table 2. Number of observed species and bacteria diversity indexes.**

	Intervention group			Control group		
	T0	T2	p	T0	T2	p
Observed species	259.1 ± 50.4	296.4 ± 57.3	0.01	288.5 ± 44.2	288.6 ± 45.4	0.99
Chao1	755.2 ± 171	903.1 ± 232.5	0.004	860.3 ± 193.3	792.8 ± 169.1	0.25
Shannon	4.9 ± 0.5	5 ± 0.5	0.181	5.1 ± 0.4	5.3 ± 0.6	0.12

301
302

303 **Figure 3.** Boxplots showing the relative abundance at genus or family level of the OTUs
304 differentially abundant ($P \leq 0.05$) in fecal samples between: control =red; probiotic=blue.



305

306 Operational taxonomic units (OTU)

307

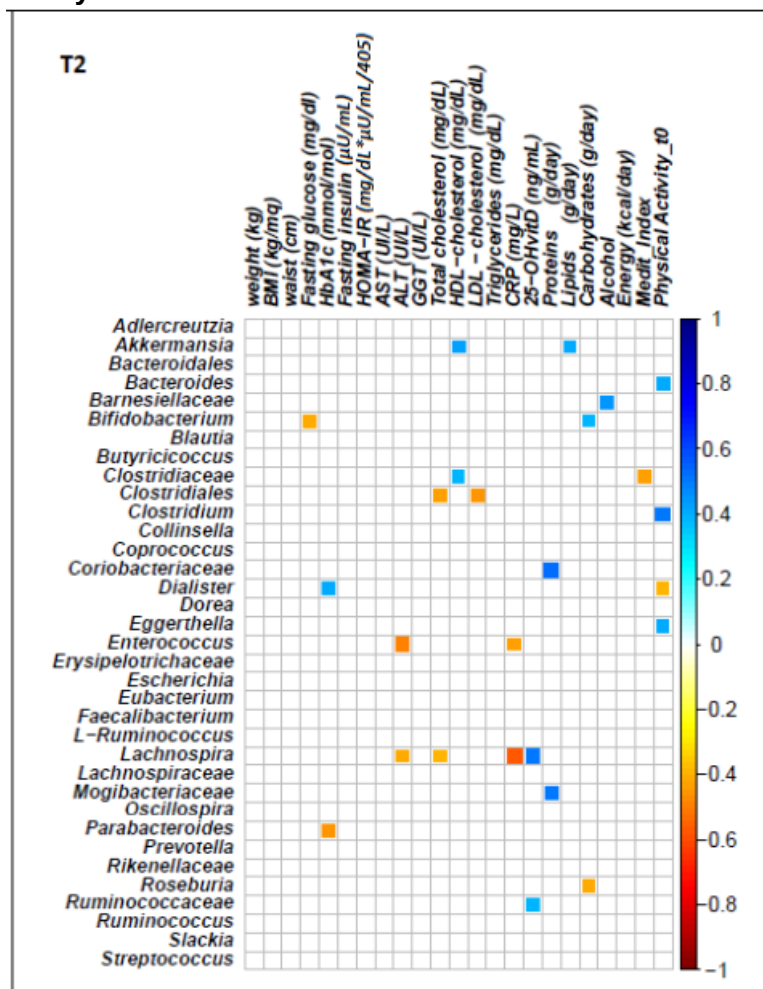
308 **Associations between microbiota, anthropometric, metabolic and lifestyle variables at T2.**

309 Several simple associations between microbiota and anthropometric, metabolic and lifestyle
310 variables at T2 were detected (Figure 4). In summary, there were positive (direct) associations
311 between: *Akkermansia* and lipid intake and HDL cholesterol levels; *Barnesiellaceae* and alcohol
312 intake; *Bifidobacterium* and carbohydrate intake; *Clostridiaceae* and HDL levels; *Clostridium*,
313 *Bacteroides* and *Eggerthella* and physical activity; *Coriobacteriaceae* and *Mogibacteriaceae* and
314 protein intake; *Dialister* and HbA1c levels and *Lachnospira* and vitamin D. On the contrary,

315 negative (inverse) associations were found between: *Roseburia* and carbohydrate intake;
 316 *Enterococcus* and *Lachnospira* and **CRP** and ALT levels; *Lachnospira* and *Clostridiales* and total
 317 cholesterol levels; *Clostridiales* and LDL cholesterol; *Dialister* and physical activity; *Bifidobacterium*
 318 and blood sugar; *Clostridiaceae* and Mediterranean Index; *Parabacteroides* and HbA1c (Figure 4).
 319 In the multivariate model, after adjustment for age, BMI at T2 **with** probiotic use, a significant and
 320 inverse association between HbA1c values at T2 and *Parabacteroides* levels (Table 3) and
 321 between *Roseburia* and carbohydrate intake (Table 4), and a significant and direct association
 322 between *Coriobacteriaceae* and protein intake (Table 4) were detected.

323

324 **Figure 4. Simple associations between microbiota and anthropometric, metabolic and**
 325 **lifestyle variables at T2.**



326

327 Spearman's rank correlation matrix of OTUs with > 0.2% abundance in at least 10 fecal samples, dietary
 328 information and blood variables. Strong correlations are indicated by large squares, whereas weak
 329 correlations are indicated by small squares. The colors of the scale bar denote the nature of the correlation,
 330 with 1 indicating a perfectly positive correlation (dark blue) and -1 indicating a perfectly negative correlation
 331 (dark red) between the two datasets. Only significant correlations (P < 0.01) are shown.

332

333 **Table 3. Multivariate analysis of the associations between metabolic and inflammatory**
 334 **variables and bacteria at T2.**

335

Variables	Bacteria	beta	SE	p
Fasting Glucose (mg/dL)	<i>Bifidobacterium</i>	-0.44	0.32	0.18
HbA1c (mmol/mol)	<i>Dialister</i>	0.11	1.64	0.94
	<i>Parabacteroides</i>	-3.31	1.21	0.012
ALT (UI/L)	<i>Enterococcus</i>	1.27	2.50	0.62

	<i>Lachnospira</i>	-1.51	2.09	0.48
Total Cholesterol (mg/dL)	<i>Clostridiales</i>	-16.8	9.00	0.08
	<i>Lachnospira</i>	-7.54	6.99	0.29
HDL-Cholesterol (mg/dL)	<i>Akkermansia</i>	0.24	0.60	0.70
	<i>Clostridiaceae</i>	23.8	19.1	0.23
LDL-Cholesterol (mg/dL)	<i>Clostridiales</i>	-13.8	8.51	0.12
CRP (mg/L)	<i>Lachnospira</i>	-0.36	0.50	0.48
	<i>Enterococcus</i>	-0.13	0.61	0.83
Vitamin D (ng/mL)	<i>Lachnospira</i>	2.46	1.88	0.20

336
337 Multivariate regression, after adjustment for age, BMI at T2 and use of probiotics.
338 Glycated hemoglobin (HbA1c); Alanine aminotranferase (ALT).
339 SE= standard error

340

341 **Table 4. Multivariate analysis of the associations between food intakes, lifestyle and bacteria at**
342 **T2.**

Variables	Bacteria	beta	SE	p
Proteins (g/die)	<i>Coriobacteriaceae</i>	0.070	0.029	0.024
	<i>Mogibacteriaceae</i>	0.007	0.008	0.40
Lipids (g/die)	<i>Akkermansia</i>	0.14	0.08	0.08
Carbohydrates (g/die)	<i>Bifidobacterium</i>	0.015	0.014	0.33
	<i>Roseburia</i>	-0.05	0.02	0.04
Alcohol intake	<i>Barnesiellaceae</i>	3.15	1.82	0.10
Mediterranean Index	<i>Clostridiaceae</i>	-0.18	0.36	0.63
Physical Activity	<i>Bacteroides</i>	0.29	0.69	0.67
	<i>Clostridium</i>	-0.24	0.27	0.38
	<i>Dialister</i>	0.99	0.88	0.27
	<i>Eggerthella</i>	-0.12	0.14	0.37

343 Multivariate regression, after adjustment for age, BMI at T2 and use of probiotics.
344 SE= standard error

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346 Discussion

347 The ability of *Lactobacillus rhamnosus* HN001 and *Bifidobacterium longum* BB536 to colonize
348 the intestinal environment and positively modulate the gut microbiota composition was previously
349 reported in healthy subjects (42). BC survivors were assessed in our study and regression
350 analyses have been adjusted for BMI, indeed data analysed separately in patients with overweight
351 (n=12) and obesity (n=14) did not change significantly. In the **intervention** group a better glycidic
352 homeostasis could be explained by an additional effect of probiotics, according to the literature
353 (43–45).

354 The close dietary follow-up and repeated nutritional counselling have probably led to a better
355 compliance in dietary habits and food choices. **Agrarian diet** lead to an increase in *Prevotella*, while
356 diets rich in proteins and fats to an increase in *Bacteroides* and *Clostridiales* (46–48). **Here we**
357 **observed a reduction in *Bacteroides* in probiotic treated subjects** probably **due to** a reduction in
358 protein and lipid intakes from T0 to T2. At T2 the Bacteroidetes/Firmicutes ratio decreased in the
359 intervention group and increased significantly in controls, probably due to a progressive
360 improvement in the adherence to the Mediterranean diet in the control group.

361 *L-Ruminococcus* has been positively associated with omnivorous diets and particularly with **animal**
362 **based food** (49). The decrease of *L-Ruminococcus* in controls could reflect a change in the dietary

363 habits on this group towards more vegetarian diets. Conversely, in the intervention group the
364 increase of L-*Ruminococcus* could also be explained by probiotic administration (50).
365 Even if within-group (Table 1) and between-group (data not shown) differences were not
366 significantly different, the CRP values tended to increase in the controls and to reduce in the
367 intervention group. We then observed in probiotic treated patient an increased in *Butyricoccus*
368 often associated with the low-fat diets (51), that could be beneficial because its ability of reducing
369 the incidence and severity of inflammation or insulin sensitivity (52). It should be pointed out that
370 by the correlation analysis we observed an inverse relationship between *Lachnospira* and CRP
371 value may have a protective role in inflammatory conditions (53). In addition, a positive association
372 between these taxa with Vitamin D level was also observed. In a healthy gut microbial environment
373 there is a link between microbes and vitamin D adsorption (54) and a positive effect of *Lachnospira*
374 could be suggested. Obesity, diet and microbiota composition impact on Vitamin D blood levels,
375 which is reduced in concomitant metabolic syndrome and gut dysbiosis related to a low-grade
376 persistent inflammatory status (54–56). Interestingly Vitamin D increased significantly in the
377 controls only, even if the between-group difference were not statistically different. This might be
378 due a slight better compliance to physical activity and to MD with better food choice in the control
379 group, although both assessed parameters did not reach statistical significance.
380 Since patients increased the consumption of plant food stuff, an increase in dietary fiber intake
381 could be related to the significant decrease of *Eubacterium* at T2 in both groups, as previously
382 reported (57).

383 The direct association between *Coriobacteriaceae* and protein intake could be explained by the
384 substitution of animal with plant-derived proteins, mainly deriving from legumes, including soy.
385 *Coriobacteriaceae* perform important intestinal functions such as the conversion of bile and steroid
386 salts and the activation of food polyphenols (58,59).

387 388 **Limitations**

389 The small sample size and the limited follow-up represent both limitations of the present study,
390 not allowing for a more detailed interpretation of the results. However, these are preliminary data of
391 an explorative pilot trial in order to design a larger trial with a longer follow-up. Further limitations
392 are the lack of gut microbiota analysis at T4 to assess later microbial shifts, as microbial
393 communities are resilient and resistant to change (60), the lack of evaluation of psychological and
394 cognitive aspects of participants, owing to the known interaction between those characteristics and
395 the gut microbiota (61), and the lack of quality of life assessment, that could be modified by the
396 microbiota modulation (62).

397 398 399 **Conclusions**

400 The present study contributes to interpreting the correlations between diet, lifestyle and gut
401 microbiota in a selected group of breast cancer survivors. We found that the combination of
402 probiotics *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001, administered daily
403 for two months, positively influenced the microbiota composition. Importantly, a close follow up
404 improved dietary habits, metabolic and anthropometric parameters; these findings were more
405 evident in the group that took probiotics. Therefore, further studies are needed to demonstrate an
406 effective correlation between the administration of probiotics, the lifestyle of the study subjects and
407 the detectable changes of microbiota.

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414 415 **Statement of Authorship**

416 FC, PM, IM and AD designed research. FC, PM, IM, VR and MT conducted research. FI, BS
417 and PM analysed data and performed statistical analyses. PM, IM, BS and FC wrote the paper. FC
418 and PM had primary responsibility for final content. All Authors read and approved the final
419 manuscript.

420

421 **Conflict of Interest**

422 The Authors declare that they have no conflict of interest.

423

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431 **Appendix**

432 **Table 5. Tertiles of intake of the Italian Mediterranean Index components (gr/day).**

433

Tertiles of intake of the Italian Mediterranean Index components (gr/day). Adapted from (33).			
ITEMS	1° TERZILE	2° TERZILE	3° TERZILE
Pasta	0 – 37,9	38 – 71,8	71,9 – 431,5
Olive oil	0 – 19,3	19,4 – 29,8	>29,9
Mediterranean vegetables	0 – 96,6	96,6 – 160	>160
Fruits	0 – 249	249 – 391,8	>391,9
Fish	0 – 20,1	20,2 – 38,5	>38,6
Legumes	0 – 11,8	11,9 – 23,5	>23,5
Red meat	0 – 69	69,1 – 111,9	112- 666,5
Butter	0 – 0,2	0,3 – 1,3	1,4 – 101,1
Potatoes	0 – 16,6	16,7 – 34,6	34,7 - 420,9
Soft drinks	0 – 0,5	0,6 – 14,3	14,4 – 3000
Alcohol	0 – 0,71	0,71 – 12,3	12,3 – 198,6

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435

436 IMI scores are calculated from qualitative and quantitative intake of 11 food items. 1 point is
437 assigned for consumption of typical Mediterranean foods (pasta, typical Mediterranean vegetables,
438 fruits, legumes, olive oil and fish) in the 3rd tertile and for non-Mediterranean foods (soft drinks,
439 butter, red meat, and potatoes) in the 1st tertile of the distribution. Alcohol receives 1 point for
440 intake from 0.71 to 12 g/day; abstainers and persons who consume >12 g/day receive 0.

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- 620

HIGHLIGHTS

- Breast cancer (BC) is the most common cancer in women worldwide and style of life and diet could be impact with their apparence.
- Obesity and intestinal microbiota composition may be associated with breast cancer and with a distortion of the microbial homeostasis, and reduced bacterial biodiversity.
- Overweight and obesity are associated with cancer advanced stage and grade at the diagnosis and resistance to local and systemic therapies.
- Dietary supplementation with probiotics, such as bacterial strains exerting beneficial effects on their host, regulates the gut microbiota structure and function through the interaction with the commensal bacteria and the expression of microbial enzymes.

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PC Supervision SB, Data curation IF,LC