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

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HIGHLIGHTS

- Breast cancer (BC) is the most common cancer in women worldwide and style of life and diet could be impact with their appareance.
- 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.

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

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ABSTRACT

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

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

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

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

Keywords: gut microbiota, breast cancer, Mediterranean diet, probiotics

ARTICLE

Introduction

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

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

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

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

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 (28). *Lactobacilli* and *Bifidobacteria* are the most used strains for safety and efficacy. *Lactobacillus rhamnosus* has been reported to improve insulin sensitivity and expression of genes related to glucose and lipid metabolism (29). Furthermore, the combination of the two probiotic strains *Bifidobacterium longum* and *Lactobacillus rhamnosus* has shown to be synergistic with positive endosymbiotic functional effects on the IM of the host (30).

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

Materials and Methods

Study design

This is a randomized open-label pilot intervention trial.

Recruitment of participants

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

Inclusion criteria were: female survivors to BC with BMI between 25.0 and 35.0 kg/m^2, free from cancer disease.

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

Outcomes

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

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

Intervention

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

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

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

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

- nutritional assessment
- the Italian Mediterranean Index (IMI) questionnaire
- anthropometric measurements, such as height, weight, BMI, waist and hip circumference
- metabolic parameters, such as blood count with leukocyte formula, fasting glucose, fasting insulin, glycated haemoglobin (HbA1c), aspartate amino transferase (AST), alanine amino transferase (ALT), γ-glutamyl transferase (GGT), C-reactive protein (CRP), 25OH-vitamin D, triglycerides, total and HDL cholesterol were obtained. LDL cholesterol was calculated with the Friedewald formula.

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

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

Food and beverage consumption were assessed by a validated three-days food record (31,32). All participants were trained by a dietitian to record all food consumed.

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

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

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

Each visit was performed at the Department of Clinical Nutrition, San Giovanni Battista Hospital, of the “Città della Salute e della Scienza” *o*f Turin. Blood samples were processed by the main hospital laboratory. Microbiological analysis of the faecal samples was performed at the Department of Agricultural, Forest and Food Sciences, University of Turin.

Measurements

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

Biochemical analysis

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

Microbiological analysis

DNA extraction

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

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

Sequencing of the 16S rRNA gene target amplicon

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

Bioinformatic analysis of sequences

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

Statistical analysis

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

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

Randomization

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

Blinding

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

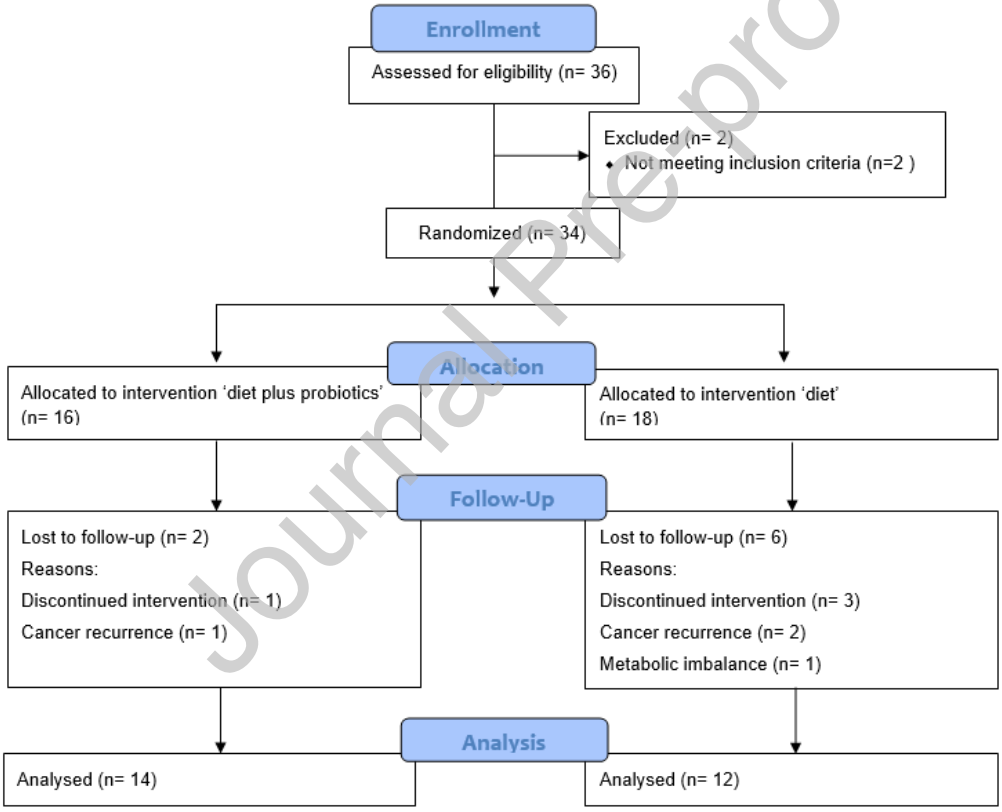
Ethical aspects

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

Results

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

Figure 1. Flow diagram of the trial



Anthropometric, metabolic and lifestyle characteristics

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

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

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

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

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

	Intervention group			**Control group**		
Anthropometric and blood variables	**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/m2)	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

Body mass index (BMI); glycated hemoglobin (HbA1c), Homeostasis Model Assessment-Insulin Resistance (HOMA-IR); Alanine aminotranferase (ALT); aspartate aminotransferase (AST); γ-glutamil transferase (GGT).
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 1,301,233reads 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).

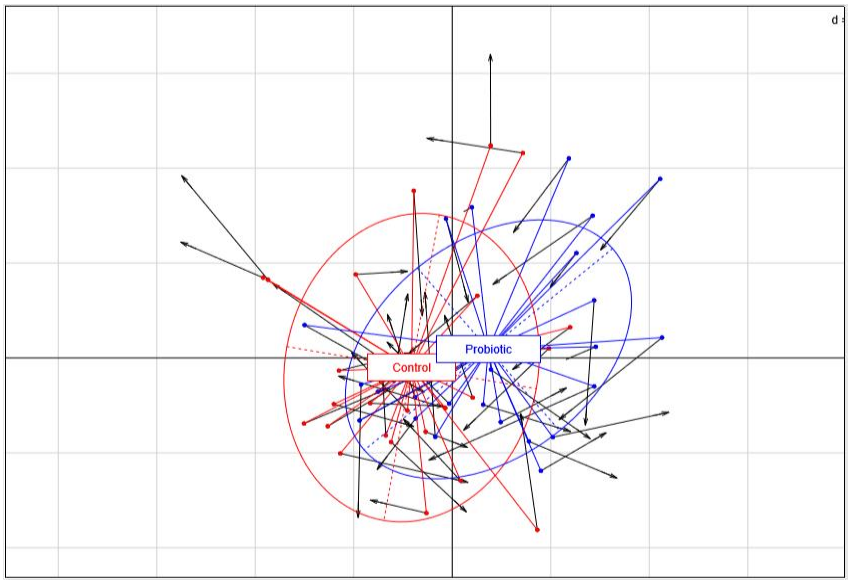
The first component of the CIA (horizontal) accounted for 37.22% of the variance, and the second component (vertical) accounted for another 13.16%. Even if the CIA showed not clear separation of the datasets it is possible to observe a gradient of separation according to probiotic intervention (Figure 2). The statistical package DESeq2was 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 *Butirycicoccus* 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.

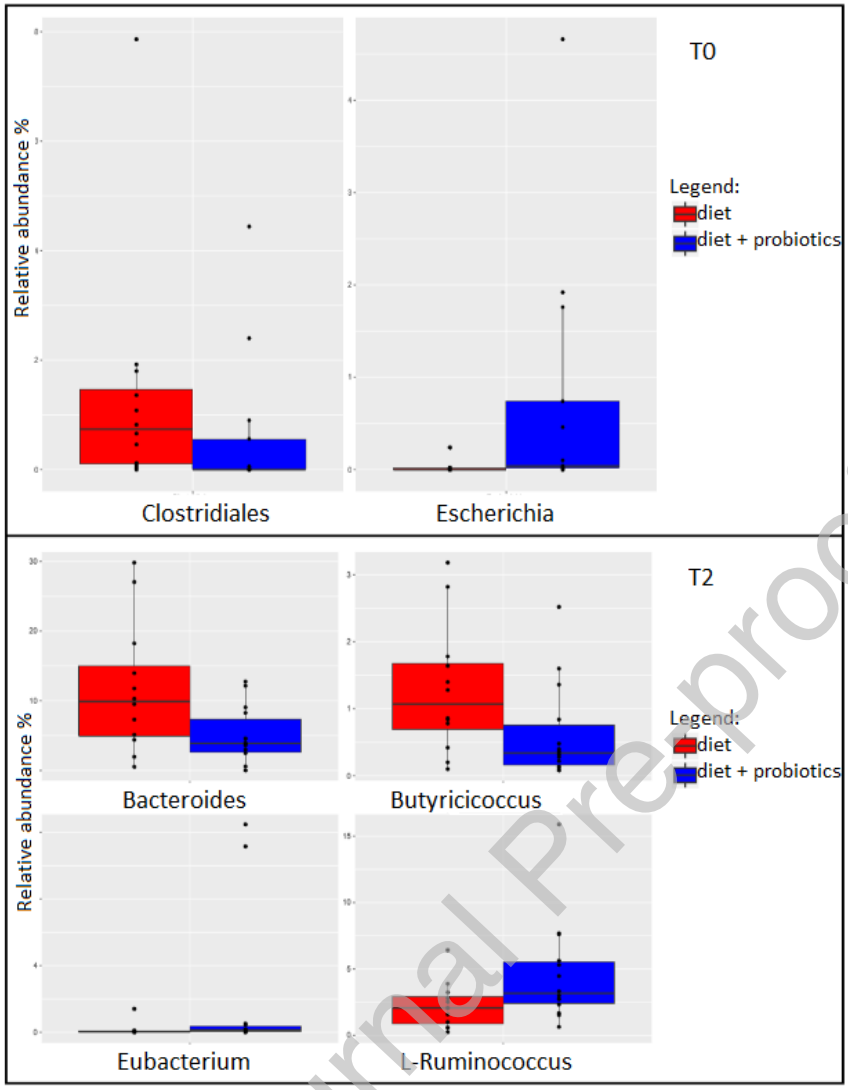


Subjects' clustering and colouring were done according to the intervention (control =red; probiotic=blue). Arrow ends of the line indicate sample position in the microbiota dataset, while black dot end indicates sample position in the nutrient intakes and metabolic dataset. (PCA = Principal component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

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

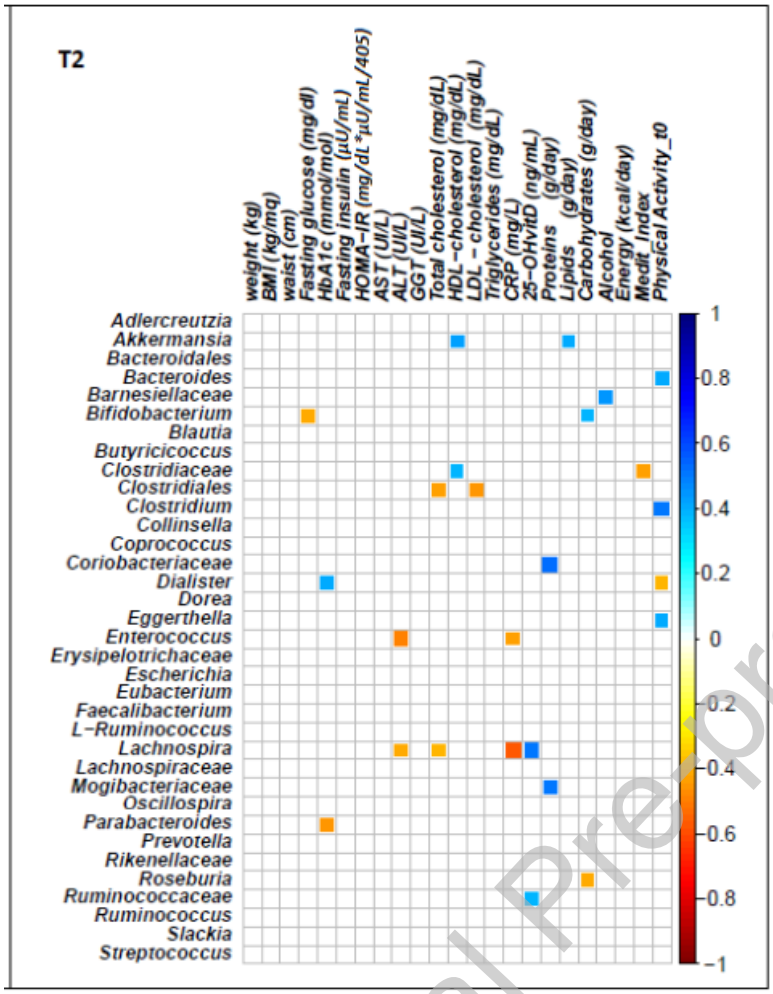
Figure 3. Boxplots showing the relative abundance at genus or family level of the OTUs differentially abundant (P ≤ 0.05) in fecal samples between: control =red; probiotic=blue.



Operational taxonomic units (OTU)

Associations between microbiota, anthropometric, metabolic and lifestyle variables at T2. Several simple associations between microbiota and anthropometric, metabolic and lifestyle variables at T2 were detected (Figure 4). In summary, there were positive (direct) associations between: *Akkermansia* and lipid intake and HDL cholesterol levels; *Barnesiellaceae* and alcohol intake; *Bifidobacterium* and carbohydrate intake; *Clostridiaceae* and HDL levels; *Clostridium, Bacteroides* and *Eggerthella* and physical activity; *Coriobacteriaceae* and *Mogibacteriaceae* and protein intake; *Dialister* and HbA1c levels and *Lachnospira* and vitamin D. On the contrary, negative (inverse) associations were found between: *Roseburia* and carbohydrate intake; *Enterococcus* and *Lachnospira* and CRP and ALT levels; *Lachnospira* and *Clostridiales* and total cholesterol levels; *Clostridiales* and LDL cholesterol; *Dialister* and physical activity; *Bifidobacterium* and blood sugar; *Clostridiaceae* and Mediterranean Index; *Parabacteroides* and HbA1c (Figure 4).
In the multivariate model, after adjustment for age, BMI at T2 with probiotic use, a significant and inverse association between HbA1c values at T2 and *Parabacteroides* levels (Table 3) and between *Roseburia* and carbohydrate intake (Table 4), and a significant and direct association between *Coriobacteriaceae* and protein intake (Table 4) were detected.

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



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

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

Variables	Bacteria	beta	SE	p
Fasting Glucose (mg(dL)	*Bifidobacterium*	-0.44	0.32	0.18
HbA1c (mmol/mol)	*Dialister*	0.11	1.64	0.94
	Parabacteroides	-3.31	1.21	**0.012**
ALT (UI/L)	*Enterococcus*	1.27	2.50	0.62
	Lachnospira	-1.51	2.09	0.48
Total Cholesterol (mg/dL)	*Clostridiales*	-16.8	9.00	0.08
	Lachnospira	-7.54	6.99	0.29
HDL-Cholesterol (mg/dL)	*Akkermansia*	0.24	0.60	0.70
	Clostridiaceae	23.8	19.1	0.23
LDL-Cholesterol (mg/dL)	*Clostridiales*	-13.8	8.51	0.12
CRP (mg/L)	*Lachnospira*	-0.36	0.50	0.48
	Enterococcus	-0.13	0.61	0.83

Vitamin D (ng/mL)	*Lachnospira*	2.46	1.88	0.20

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

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

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

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

Discussion

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

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

L-Ruminococcus has been positively associated with omnivorous diets and particularly with animal based food (49). The decrease of L-*Ruminococcus* in controls could reflect a change in the dietary habits on this group towards more vegetarian diets. Conversely, in the intervention group the increase of L-*Ruminococcus* could also be explained by probiotic administration (50).

Even if within-group (Table 1) and between-group (data not shown) differences were not significantly different, the CRP values tended to increase in the controls and to reduce in the intervention group. We then observed in probiotic treated patient an increased in *Butyricicoccus* often associated with the low-fat diets (51), that could be beneficial because its ability of reducing

the incidence and severity of inflammation or insulin sensitivity (52). It should be pointed out that by the correlation analysis we observed an inverse relationship between *Lachnospira* and CRP value may have a protective role in inflammatory conditions (53). In addition, a positive association between these taxa with Vitamin D level was also observed. In a healthy gut microbial environment there is a link between microbes and vitamin D adsorption (54) and a positive effect of *Lachnospira* could be suggested. Obesity, diet and microbiota composition impact on Vitamin D blood levels, which is reduced in concomitant metabolic syndrome and gut dysbiosis related to a low-grade persistent inflammatory status (54–56). Interestingly Vitamin D increased significantly in the controls only, even if the between-group difference were not statistically different. This might be due a slight better compliance to physical activity and to MD with better food choice in the control group, although both assessed parameters did not reach statistically significance.
Since patients increased the consumption of plant food stuff, an increase in dietary fiber intake could be related to the significative decrease of *Eubacterium* at T2 in both groups, as previously reported (57).

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

Limitations

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

Conclusions

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

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Statement of Authorship

FC, PM, IM and AD designed research. FC, PM, IM, VR and MT conducted research. FI, BS and PM analysed data and performed statistical analyses. PM, IM, BS and FC wrote the paper. FC

and PM had primary responsibility for final content. All Authors read and approved the final manuscript.

Conflict of Interest
The Authors declare that they have no conflict of interest.

Author Contribution: Conceptualization Methodology Investigation: MP, CF, MI, TM, RV PC Supervision SB, Data curation IF,LC

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Appendix

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

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

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

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