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A Clustering Study of Sociodemographic Data, Dietary Patterns, and Gut Microbiota in Healthy and Breast Cancer Women Participating in the MICROMA Study

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Scope: This work is part of the clinical study NCT03885648 registered in ClinicalTrials.gov, aimed at studying the relationship among breast cancer, microbiota, and exposure to environmental pollutants. As a first step, we characterized and evaluated risk factors of the participants.

Methods and results: A case–control study was designed with breast cancer (cases, $n = 122$) and healthy women (controls, $n = 56$) recruited in two hospitals of Andalusia (Southern Spain). Participants answered questionnaires of Mediterranean diet adherence and food frequency. Data were collected from medical histories and microbiota was analyzed on stool samples. Most cases (78.2%) were diagnosed as stages I and II. Cases had higher age, body mass index (BMI), glucose, cholesterol, and potassium values than controls. Cases exhibited higher adherence to the Mediterranean diet and their food consumption was closer to that dietary pattern. A hierarchical cluster analysis revealed that the *Bacillota*/*Bacteroidota* ratio was the most relevant variable in women with breast cancer, which was higher in this group compared with controls.

Conclusion: Although cases exhibited higher adherence to the Mediterranean diet compared with controls, they presented features and microbiota alterations typical of the metabolic syndrome, probably due to their higher BMI and reflecting changes in their lifestyle around the time of diagnosis.

1. Introduction

Female breast cancer was the second leading cause of global cancer incidence in 2022, with an estimated 2.3 million new cases, comprising 11.6% of all cancer cases. The disease is the fourth leading cause of cancer mortality worldwide, with 666 000 deaths (6.9% of all cancer deaths). Among women, breast cancer is the most commonly diagnosed cancer, and it is the leading cause of cancer deaths globally and in 157 countries for incidence and in 112 countries for mortality.[1] By 2040, the burden from breast cancer is predicted to increase to over 3 million new cases and 1 million deaths every year.[2]

Breast cancer is a complex and multifactorial disease whose exact etiology remains unknown. However, a strong contribution of epigenetic, genetic, and environmental factors has been identified.[3,4] Nevertheless, considering the risk factors known so far, only a limited part of the overall burden could be explained, since

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up to 70% of breast cancer cases still constitute sporadic cases, i.e., have an uncertain cause.[4] Among the risk factors identified, there are nonmodifiable factors including advanced age, genetic predisposition (including DNA mutations and family history of breast cancer), early menarche, late menopause, age at first pregnancy, infertility and childlessness, contraceptive use, hormonal treatment after menopause, and no history of breastfeeding. Modifiable factors include type of diet, overweight, and obesity.[5] But the presence of a risk factor does not necessarily imply the development of breast cancer, nor do all risk factors have the same effect, since its influence varies from one person to another, as there may be individual conditions or susceptibilities.[6] On the other hand, several factors decrease the risk of breast cancer, referred to as protective factors, such as breastfeeding and pregnancy.[7] In this regard, one of the best-known risk factors for breast cancer is related to exposure to endogenous hormones (estrogens and progesterone), mainly prolonged exposure to estrogens. As estrogen production is modulated by ovarian function, age of menarche, number of pregnancies, and age at menopause also play a crucial role in the etiology of this disease.[8]

Risk factors can also be classified into nonmodifiable internal factors (e.g., age, inherited genetic characteristics), and alterable external factors. Paradoxically, it is the modifiable factors that contribute most to breast cancer.[9] Among these are some related to unhealthy lifestyles (e.g., smoking, etc.), inadequate diets, or low physical activity (e.g., sedentary lifestyle),[10] in addition to overweight and obesity.[4,10]

On the other hand, although the existing evidence on dietary patterns and their relationship with breast cancer is still scarce, most studies suggest that certain dietary patterns, such as the Mediterranean diet, are a useful strategy for the management of overweight and obesity. It is widely known that adherence to the Mediterranean diet decreases the risk of breast cancer,[11–13] as it constitutes a healthy dietary pattern characterized by a high intake of fruits, vegetables, whole grains, extra virgin olive oil, fish and lean meats, and a low intake of dairy and red meat.[14]

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The Mediterranean diet is also useful for the maintenance of a balanced microbiota. In this perspective, the gut microbiota has been shown to play a crucial role in oncogenesis and tumor progression through the modulation of inflammation and its influence on host cell genomic stability by deregulating different signals/pathways.[15,16] Moreover, some studies suggest that the gut microbiota may favor and even affect the response to cancer treatment, modulating the immune system and toxicity profiles of chemotherapeutic agents, as well as altering the microenvironment, and interfering with systemic levels of endogenous hormones.[15,17,18]

It is known that among the main factors that modify the gastrointestinal microbiota, diet is the most influential.[19] An inadequate diet (e.g., poor in fiber), stressful situations, and/or the use of antibiotics may contribute to an imbalance of the intestinal microbiota (a condition known as dysbiosis).[20,21] In addition, overweight and obesity have been associated with a distortion of microbial homeostasis through a decrease in microbe biodiversity and altered expression of bacterial genes, especially those involved in food metabolism and short-chain fatty acid production.[22] A healthy gut microbiota seems to be mainly composed of *Bacillota* and *Bacteroidota* as dominant bacterial phyla (≈90% of the total bacteria), while the remaining 10% is divided between *Proteobacteria*, *Verrucomicrobia*, and *Actinobacteria* in most people.[21] Likewise, several studies have observed that obese individuals have a higher *Bacillota*/*Bacteroidota* ratio (formerly *Firmicutes*/*Bacteroidetes* ratio), which indicates a higher abundance of *Firmicutes* and a lower abundance of *Bacteroidetes*.[23]

Dietary fiber, short-chain fatty acids, some prebiotics, and bioactive compounds may be relevant dietary elements to promote/maintain the development of the most beneficial intestinal bacterial populations, which would also act as protective factors against cancer development.[24]

Fully identifying the risk factors for breast cancer would help in the design of prevention strategies. Moreover, to develop more precise preventive strategies and therapeutic targets with fewer side effects against this disease, it is not only crucial to understand the molecular mechanisms that give rise to the tumor or its genetic-epigenetic determinants but also a deeper understanding of how the combination of certain breast cancer risk factors can influence the onset and progression of the pathology.

This work is part of a clinical study registered in ClinicalTrials.gov with reference NCT03885648,[15] whose objective was to study the relationship among breast cancer, mammary and intestinal microbiota, and exposure to environmental pollutants (endocrine disruptors). As a first step, we wished to characterize the study population and evaluate the modifiable and nonmodifiable risk factors presented by women at the time of diagnosis.

2. Results

2.1. Characterization of the Study Population

Table 1 summarizes the sociodemographic and lifestyle characteristics of the study population. Of the 178 women recruited (122 cases and 56 controls), 67.1% were from the province of Granada and the rest from the province of Jaen, both in Andalusia, Spain. Cases were older than controls (55.0 ± 0.9 versus 38.0 ± 1.5 years,

Table 1. Sociodemographic and lifestyle characteristics of the participants.

Variables	Cases	Controls	*p* value
Age [years]	55.0 ± 0.9	38.0 ± 1.5	***p* < 0.001**
BMI [kg m^{-2}]	25.3 ± 0.6	22.8 ± 0.7	***p* = 0.003**
Smoking habit [%]			
Smoker	37.6 (35/93)	29.2 (14/48)	*p* = 0.317
No	62.4 (58/93)	71.4 (34/48)	
Alcohol consumption [%]			
Yes	40.0 (36/90)	41.7 (20/48)	*p* = 0.849
No	60.0 (54/90)	50.3 (28/48)	
Educational status [%]			
Elementary school	43.5 (30/69)	26.3 (10/38)	*p* = 0.107
Middle School/High School	23.1 (16/69)	18.4 (7/38)	
Bachelor's degree	33.3 (23/69)	55.3 (21/38)	
Marital status [%)]			
Married	96,1 (50/52)	48.0 (12/25)	***p* < 0.0001**
Never married	3.8 (2/52)	52.0 (13/25)	
History of cancer [%]			
Yes	74.0 (50/54)	50.0 (18/36)	***p* = 0.0194**
No	25.9 (14/54)	50.0 (18/36)	
Other cancer	52.5 (21/40)	38.9 (11/18)	*p* = 0.5418
Breast cancer	47.5 (19/40)	61.1 (7/18)	

Values are presented as mean ± SEM or percentages. For variable age, *n* = 48 controls and 101 cases. For variable BMI, *n* = 48-98. BMI, body mass index; SEM, standard error of mean.

respectively, mean ± standard error of mean [SEM]) (Table 1), with half of cases above 44 years (51.5%) and most controls in the range 25–44 years (67.3%). Cases also showed higher body mass index (BMI) than controls (25.3 ± 0.6 versus 22.8 ± 0.7 kg m^{-2}). Of the cancer patients, 29.5% had overweight, 18.2% had obesity, and 52.3% had a normal weight (**Figure 1**). In contrast, the majority of participants in the control group had a normal weight at the beginning of this study (72.5%), 20.0% had overweight, and 7.5% had obesity (Figure 1). There were also more married women among cases than among controls (96.1% versus 48.0%, Table 1).

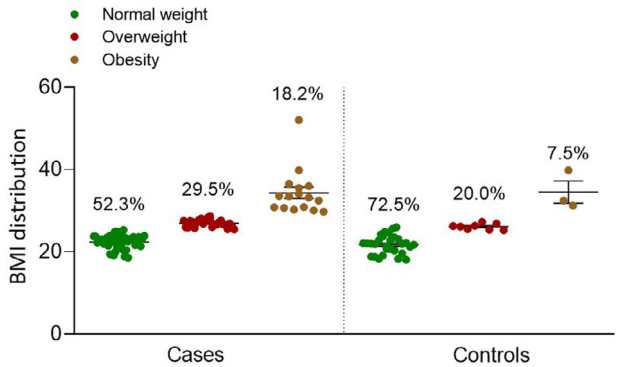

Figure 1. Body mass index (BMI) distribution of cases and controls presented as percentages (%) of the total population. For variable age, *n* = 48 controls and 101 cases. For variable BMI, *n* = 48 controls and 98 cases.

Table 2. Reproductive characteristics of the participants.

Variables	Cases	Controls	*p* value
Menarche [years]	12.5 ± 0.1	13.0 ± 0.2	*p* = 0.5023
Menopause [years]	50.0 ± 0.6	50.0 ± 4.2	*p* = 0.430
Parity [%]	90.8 (89/98)	67.4 (33/49)	***p* = 0.0004**
Age at first pregnancy [years]	27.1 ± 0.6	26.8 ± 10.8	*p* = 0.832
Breast feeding (number of accumulated months)	9.4 ± 1.3	17.0 ± 4.1	*p* = 0.302
Hormonal treatment			
Yes	54.0 (47/87)	46.0 (17/35)	*p* = 0.6893
No	48.6 (40/87)	51.4 (18/35)	

Values are presented as mean ± SEM or percentages. For variables menarche, menopause, age at first pregnancy, and breastfeeding, *n* = 52–101 for cases and *n* = 25–48. Hormonal treatment includes both hormone replacement therapy and contraceptives use. BMI, body mass index; SEM, standard error of mean.

There were no significant differences in the smoking habit, alcohol consumption, and educational level of the participants (Table 1). With respect to family history of cancer it is noteworthy that, although the cases presented a higher percentage of cancer history in general (74.0% versus 50.0%), it was the control group that indicated a higher incidence of breast cancer among their family members (47.5% versus 61.1%) (Table 1).

Reproductive variables were also collected from the participants (**Table 2**). The ages at menarche, menopause, and first pregnancy, as well as the number of accumulated months of breastfeeding, and use of hormone treatment were similar in both groups. Regarding parity, a higher proportion of cases reported having been mothers than controls (90.8% versus 67.4%).

Tumors were classified according to pathological anatomy reports following the criteria established in all clinical guidelines for breast cancer (**Table 3**). Most (87.3%) were classified as non-invasive, and 78.2% as stages I or II. Tumors were also classified according to estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) phenotypes. The majority (>80%) were estrogen and progesterone receptor positive, and 88.4% were HER2 negative. The proliferation rate was estimated by Ki-67 antigen: 46.4% of tumors had an elevated Ki-67 level (>14) and 94.0% were E-cadherin positive (Table 3).

As part of the preoperative protocol, various biochemical parameters were determined in the participants (**Table 4**). Women with breast cancer had significantly higher basal blood glucose, total cholesterol, and potassium values compared to healthy women. However, all parameters analyzed were within reference or normal values in both study groups.

2.2. Dietary Assessment

2.2.1. Consumption of Food Servings in the Study Groups

Table 5 shows the daily serving consumption of the participants. It is noteworthy that control women consumed a greater number of daily servings of processed foods compared to women with breast cancer (0.9 versus 0.5 servings; $p = 0.01$). In relation to the consumption of vegetables, dairy products, fruits, legumes, cereals, protein foods, oils and fats, sugars and alcoholic beverages,

Table 3. Tumor characteristics of women diagnosed with breast cancer.

Variables	
Tumor size	
0.1	0.0 (0/87)
0.1–0.5	9.2 (8/87)
0.5–1	22.0 (19/87)
1–2	50.6 (44/87)
2-5	18.4 (16/87)
Histologic classification	
I	33.7 (34/101)
II	44.5 (45/101)
III	11.9 (12/101)
IV	9.9 (10/101)
Invasion/location	
No	87.3 (55/63)
Perineural	6.3 (4/63)
Lymphovascular	6.3 (4/63)
Progesterone receptors	
Negative	25.6 (22/86)
Positive	86.0 (74/86)
Estrogen receptors	
Negative	10.7 (9/84)
Positive	82.3 (75/84)
HER2 receptor	
Positive	5.8 (4/69)
Negative	88.4 (61/69)
Uncertain	5.8 (4/69)
Antigen Ki-67	
<14	53.6 (45/84)
>14	46.4 (39/84)
E-cadherin	
Positive	94.0 (62/66)
Negative	6.1 (4/66)

Values are presented as percentages. HER2, human epidermal growth factor receptor 2.

both groups had similar daily intakes. However, it should be noted that the daily consumption of sugars in the control group showed a trend of higher consumption (0.8 servings) compared to the cases (0.5 servings).

2.2.2. Dietary Patterns

Using principal component analysis (PCA), three main factors were extracted considering the eight food groups, as mentioned above, which explained between 56.7% for controls and 57.5% for cases, according to the variance explained in the models studied.

For the control women, the first component consisted of a high consumption of processed foods, cereals and grains, protein foods, and sugars which coincide with a Western diet. The second component of the control group was characterized by a high consumption of vegetables and legumes, considered as a Mediterranean diet. The last component was defined as mixed since it included high consumption of dairy products and lower consumption of fruits (**Figure** 2a).

Table 4. Biochemical data of the participants.

Variables	Cases	Controls	*p* value
Glucose [mg dL^{-1}]	90.5 ± 1.6	84.1 ± 1.9	***p* = 0.0191**
Creatinine [mg dL^{-1}]	0.7 ± 0.0	0.7 ± 0.0	$p = 0.8413$
Uric acid [mg dL^{-1}]	4.5 ± 0,3	4.1 ± 0.2	$p = 0.217$
Total cholesterol [mg L^{-1}]	201.5 ± 6.1	179.0 ± 6.9	***p* = 0.026**
HDL cholesterol[mg dL^{-1}]	63.4 ± 2.1	70.8 ± 4.8	$p = 0.248$
LDL cholesterol [mg dL^{-1}]	119.1 ± 5,6	102.4 ± 10.7	$p = 0.206$
AST [U L^{-1}]	17.7 ± 0.9	18.1 ± 0.7	$p = 0.715$
ALT [U L^{-1}]	17.7 ± 1.6	15.3 ± 1.2	$p = 0.279$
γ-GT [U L^{-1}]	22.0 ± 3.9	14.1 ± 1.6	$p = 0.0981$
Triglycerides [mg dL^{-1}]	99.2 ± 14.19	82.2± 6.53	$p = 0.39$
Total bilirubin [mg dL^{-1}]	0.6 ± 0.1	0.6 ± 0,1	$p = 0.240$
Sodium [mEq L^{-1}]	140.3 ± 0.5	139.7 ± 0.6	$p = 0.463$
Potassium [mEq L^{-1}]	4.2 ± 1.4	4.5 ± 2.1	***p* = 0.032**

Values are presented as mean ± SEM. Cases, $n = 10$–103. Controls, $n = 9$–48. ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, γ-glutamyl transferase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SEM, standard error of mean.

For these cases, the first component was defined as Mediterranean diet with a high consumption of vegetables, protein foods, fruits, legumes, and cereals and grains. The second component was defined as mixed/Western-type diet with a consumption of processed foods and dairy products. The last component comprised a high consumption of sugars and fruits and a lower consumption of cereals and grains defined as Western-type diet (Figure 2b).

2.2.3. Adherence to the Mediterranean Diet

The Mediterranean diet adherence questionnaire revealed that most women diagnosed with breast cancer had good adherence, whereas more than half of the controls had low adherence (**Figure** 3a). By age group, patients between 45 and 59 years of age were those with the highest adherence to the Mediterranean dietary

Table 5. Consumption of serving day among the studied groups.

Servings [day]	Cases	Controls	*p* value
Dairy products	1.5 ± 0.1	1.4 ± 0.1	0.258
Fruits	1.4 ± 0.1	1.1 ± 0.1	0.113
Vegetables	1.5 ± 0.1	1.2 ± 0.1	0.184
Legumes	0.3 ± 0.0	0.3 ± 0.0	0.942
Cereals and grains	2.0 ± 0.1	1.7 ± 0.1	0.594
Protein foods	1.7 ± 0.1	1.8 ± 0.1	0.712
Sugars	0.5 ± 0.1	0.8 ± 0.1	0.089
Oils and other fats	1.3 ± 0.0	1.3 ± 0.0	0.715
Processed foods	0.5 ± 0.1	0.9 ± 0.1	**0.010**
Alcoholic beverages	0.4 ± 0.1	0.4 ± 0.1	0.746

Values are means ± SEM. Cases, $n = 100$. Controls, $n = 55$. SEM, standard error of mean.

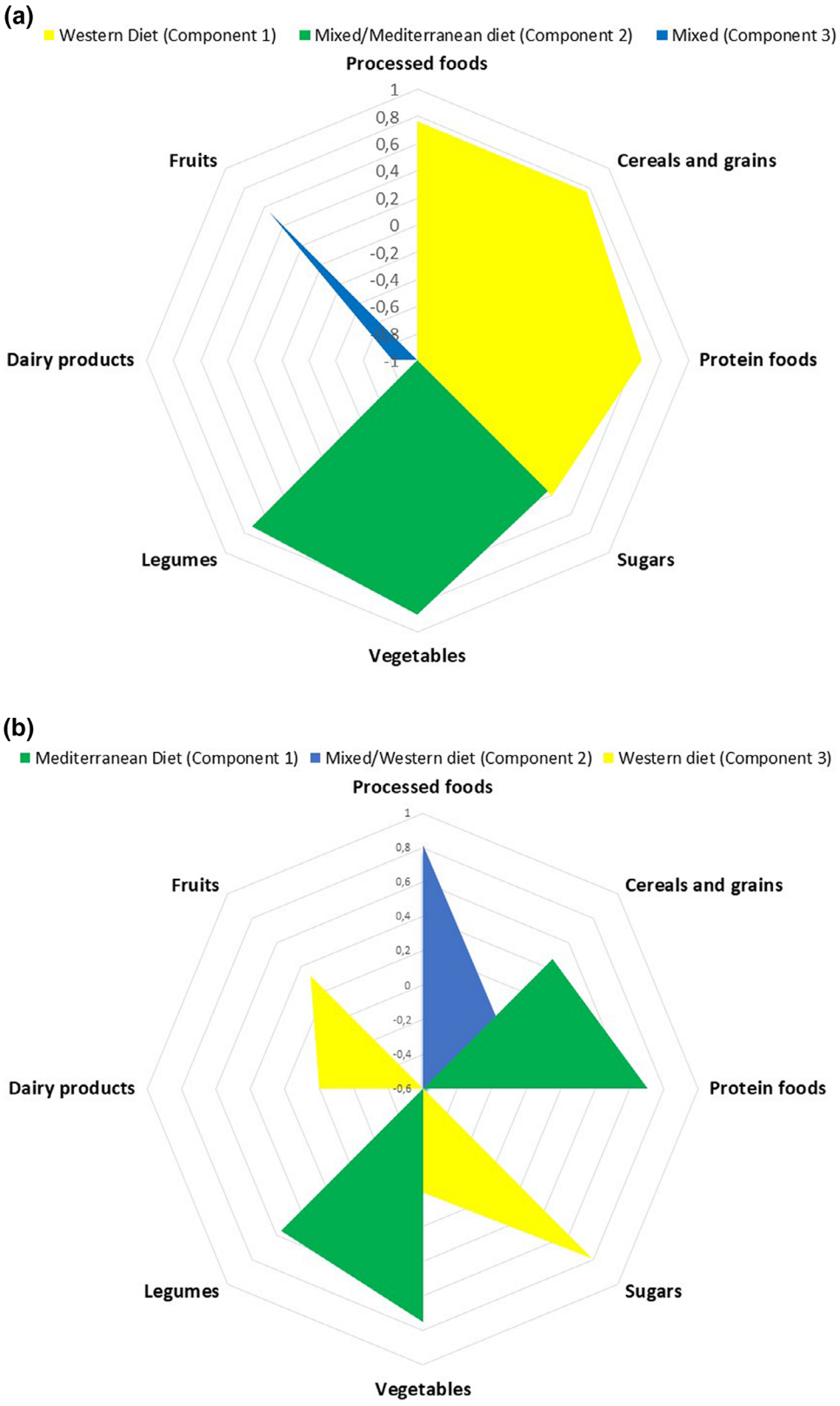

Figure 2. Dietary patterns extracted from the principal component analysis of input variables in the MICROMA study. a) Controls. b) Patients with breast cancer (cases).

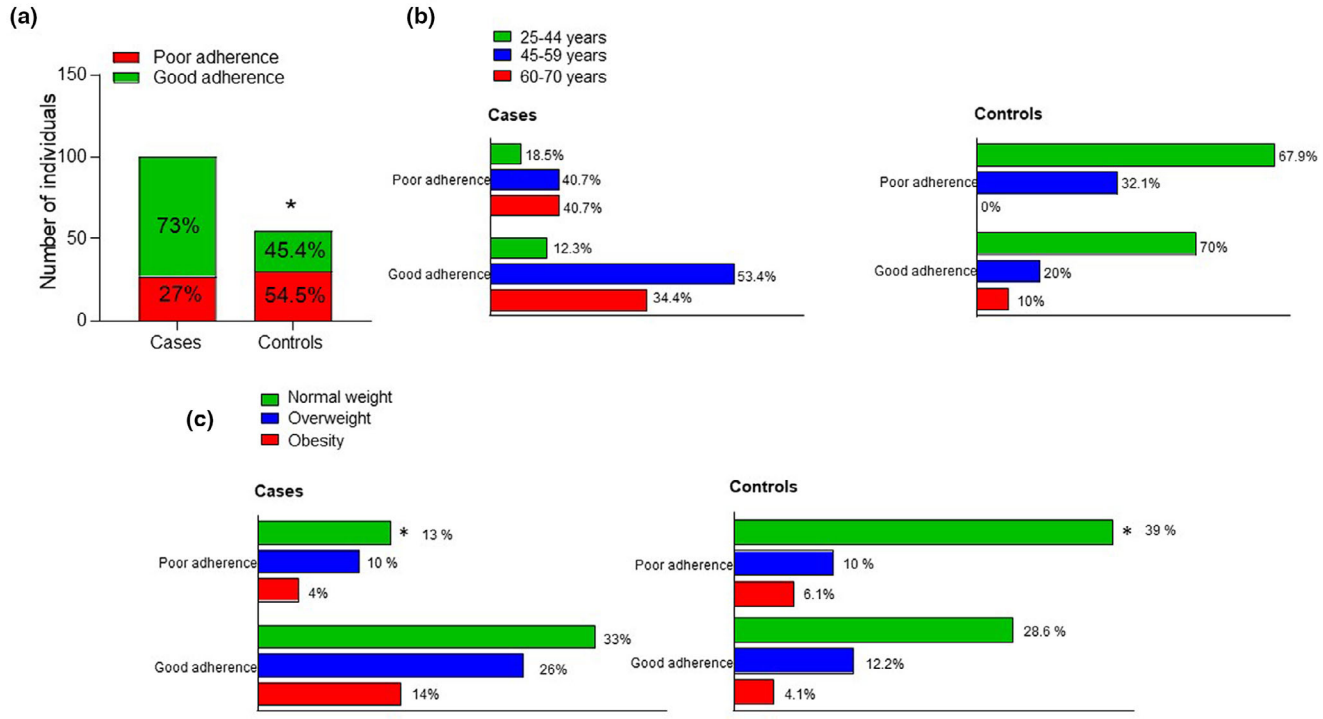

Figure 3. Adherence to the Mediterranean diet of cases and controls. Panel a) shows data based on the total numbers of cases and controls. Panel b) shows data classified by age group. Panel c) shows data classified by body mass index (BMI). Cases, $n = 100$. Controls, $n = 55$. $^*p < 0.05$ versus cases.

pattern. In contrast, in the control group, the age range with the highest adherence was that of the youngest women (25–44 years) (Figure 3b). Adherence to the Mediterranean diet according to BMI of the participants is shown in Figure 3c. Adherence to this diet was greater in cases with normal weight than in controls with normal weight given that 1) only 13% of the cases presented poor adherence to this type of diet compared to 39% of the controls ($p < 0.05$), and 2) 33% of the cases presented good adherence compared to 28.6% of the controls. For the overweight and obese BMI categories, no significant differences were observed.

2.3. *Bacillota/Bacteroidota* Ratio

Cases presented a higher *Bacillota/Bacteroidota* ratio than controls: 13.2 ± 2.3 versus 5.5 ± 1.1 (**Figure 4**).

2.4. Cluster Analysis

Data on sociodemographic variables, food consumption, adherence to the Mediterranean diet, and the *Bacillota/Bacteroidota* ratio were used to perform a hierarchical cluster analysis with the aim of forming closed and homogeneous groups from a set of variables of the patients included in the study with different characteristics, but sharing certain similarities (**Figure 5**). Three clusters were extracted in the group of control women. The first is formed by educational level, adherence to the Mediterranean diet, age, BMI, and marital status. The second cluster is formed by the consumption of fruits, legumes, dairy products, and legumes. The third cluster is made up of vaginal delivery, the use of tobacco and the *Bacillota/Bacteroidota* ratio, and the consumption of cereals and grains, sugars, protein, and processed foods standing out (Figure 5a). For women with breast cancer, the first cluster is formed by the *Bacillota/Bacteroidota* ratio, marital status, BMI, age, and educational level. Women with breast cancer share variables with controls, but differ in the *Bacillota/Bacteroidota* ratio and the adherence of the Mediterranean diet. The second cluster is formed by foods that are related to the Mediterranean diet, similar to what was obtained in the PCA. Finally, the third cluster mixes variables related to reproductive health and consumption of processed foods (Figure 5b). These results suggest that women diagnosed with breast cancer have a higher relationship with adherence to the Mediterranean diet than controls, their consumption is closer to such a diet and, in addition, the *Bacillota/Bacteroidota* ratio is the most important variable in this

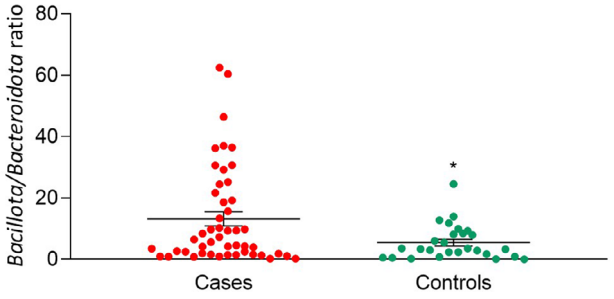

Figure 4. *Bacillota/Bacteroidota* ratio of cases and controls. Cases, $n = 101$. Controls, $n = 56$. $^*p < 0.05$ versus control.

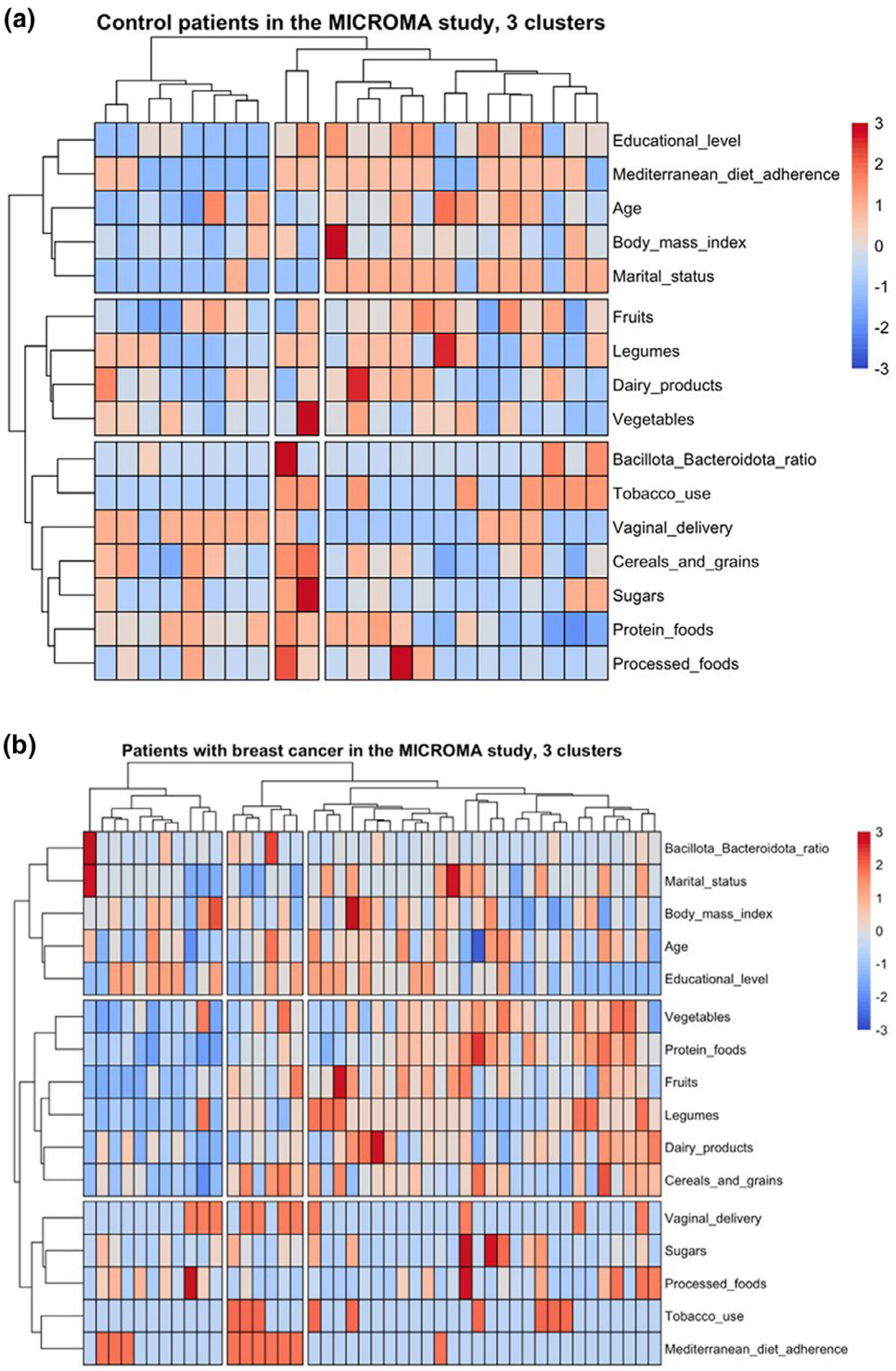

Figure 5. Clusters of subjects and dietary/lifestyle variables were identified via hierarchical clustering in the MICROMA study. The clusters are visually separated by longitudinal marks on vertical and horizontal faces (clusters of subjects or dietary/lifestyle variables, respectively). The vertical and horizontal dendrograms denote the relationship between the clusters, i.e., similar observations. The color bar refers to levels above (red) or below (blue) the mean intake of dietary variables or mean scores of lifestyle variables. Increased color intensities indicate larger differences around the mean. a) Controls. b) Patients with breast cancer (cases).

group of women compared to controls. The Mediterranean diet, a key variable associated with health maintenance, as well as the microbial component of some noncommunicable diseases such as obesity, were both associated with the group of women diagnosed with breast cancer.

3. Discussion

In the present study, we characterized a Spanish population that included both healthy women and women with breast cancer. We evaluated and ranked the modifiable and nonmodifiable risk factors they presented at diagnosis[15] and found that women with breast cancer had higher BMI, basal blood glucose, total cholesterol, and potassium values than healthy women. Also, our PCA showed that women with breast cancer had a first component defined as a Mediterranean diet whereas healthy women had a first component that coincides with a Western diet. Our hierarchical cluster analysis showed that for women with breast cancer, the first cluster was formed by the *Bacillota*/*Bacteroidota* ratio, marital status, BMI, age, and educational level. In contrast, women diagnosed with breast cancer had a higher relationship with adherence to the Mediterranean diet than controls, their consumption was closer to such a diet and, in addition, the *Bacillota*/*Bacteroidota* ratio was the most important variable in this group of women compared to healthy women.

Nowadays, the incidence of breast cancer is increasing worldwide, being one of the leading causes of mortality globally.[25] In addition, an increase in more aggressive neoplasms has been observed in young women in recent years.

Many nutrients (e.g., fatty acids, some vitamins, carotenoids, phytoestrogens, and fiber) have been found to have a potential impact on tumor development, in both animal experiments and epidemiological studies.[26] In general, the dietary patterns refers to the combination of foods recommended for healthy living or the foods and beverages consumed over a period of time.[27] A healthy diet should take into account personal preferences, cultural traditions, and budgetary restrictions in order to benefit everyone, regardless of their age, race, ethnicity, or current health status.[28] It is important to make several decisions regarding how input variables are to be formatted and transformed, how many input variables are to be analyzed and how food grouping schemes are to be used, how the estimation method are to be used, and what criteria should be used to select the model, including how many dietary patterns to include.[29] In spite of significant differences in approaches and interpretations, it is possible to identify underlying eating patterns through either PCA or cluster analysis[30] and most identified dietary patterns yield valid dietary constructs indicating the dietary characteristics of the populations studied.[31] The PCA indicated that women diagnosed with breast cancer had a first component defined as a Mediterranean diet (containing a high intake of vegetables, protein foods, fruits, legumes, cereals, and grains). The vast majority of studies and medical guidelines point out that dietary patterns that include vegetables and limit saturated fats and red and processed meats can reduce breast cancer risk.[32]

A recent meta-analysis notes that high consumption of fruits, vegetables, soy protein, and soy isoflavone significantly reduced breast cancer risk, whereas high alcohol consumption significantly increased the risk.[33] Consumption of meat, soy, and green tea was not significantly associated with breast cancer risk.[33] Likewise, high adherence to a healthy dietary pattern may reduce breast cancer risk.[34] Conversely, adherence to unhealthy dietary patterns may increase the risk of breast cancer.[33] Although no single food alone can cure or prevent disease, following a dietary pattern that emphasizes these foods, such as the Mediterranean diet, is recommended for an optimal health.[34] Numerous studies have evaluated the impact of adherence to the Mediterranean diet and breast cancer risk. Turati et al. analyzed this association in a multicenter study of 3034 breast cancer cases and 3392 controls, finding that women with higher adherence to the Mediterranean diet had a 20% risk reduction compared to women with worse adherence.[11] Similarly, other studies have shown that even small changes toward greater adherence to the Mediterranean diet are associated with reductions in both premenopausal[12] and postmenopausal breast cancer risk.[13]

The Mediterranean diet is also inversely associated with overweight and obesity.[35] In our study, it is paradoxical that women with breast cancer presented a higher adherence to the Mediterranean diet but also a higher BMI than healthy women. This discrepancy could be due to: i) the subjective component of the dietary questionnaires; ii) women switching their lifestyle habits to healthier ones after the diagnosis of the breast cancer disease; and iii) the age difference between cases and controls. This difference, in turn, is due to the fact that the control group consisted mostly of women undergoing breast augmentation, i.e., young women, while the majority of cases were postmenopausal women; a factor that, together with age, contributes to weight gain.[36]

Cluster analysis and factor analysis use different statistical approaches to approximate dietary patterns. Experts have recommended comparing these methods in relation to disease outcome to better understand different dietary patterns.[37–39] Cluster analysis finds people who share similar frequency patterns in food consumption, whereas factor analysis finds foods that are correlated and scores people based on the degree to which their diets show the same pattern of variation.[40] Based on cluster analysis, we show that women diagnosed with breast cancer have a crucial association with *Bacillota*/*Bacteroidota* ratio, in contrast to the healthy control group, who adhered to the Mediterranean diet to a lesser degree.

The successful development of breast cancer prevention strategies requires the identification of biomarkers of dietary exposure, as well as the search for new strategies to address this disease. In this sense, it has been described that the human microbiota could play a role in the development of breast cancer, but to date its potential for intervention or its usefulness as a biomarker of diagnosis and/or progression of the disease has not been sufficiently exploited.

A study by Yang et al.[41] has demonstrated a direct correlation between that specific gut microbiota and various clinicopathological characteristics, such as Ki67, HER2 levels, and tumor grade. In our study, similar approximations were made with these variables, but no correlation was observed. This was perhaps due to the early stages of breast cancer in our study population.

Breast cancer patients have an altered gut microbiota compared to healthy individuals. This indicates that certain microbes may be related with breast cancer development and treatment.[42] In our study, we have analyzed the impact of the microbiota,

one of the modifiable risk factors, in breast cancer patients through the *Bacillota/Bacteroidota* ratio considering, in addition, the great impact of diet on microbiota composition. In fact, BMI represents an indicative parameter to predict gut microbiota dysbiosis.[43] Furthermore, overweight and obesity have been associated with a distortion of microbial homeostasis, since it decreases bacterial biodiversity and alters the expression of bacterial genes, especially those involved in the metabolism of nutrients and hormones (estrogens) and in the production of short-chain fatty acids.[22] Likewise, several studies have observed that individuals with obesity have a higher *Bacillota/Bacteroidota* ratio.[23] These results are in agreement with our findings and with those of He et al.,[44] who observed a significantly higher *Bacillota/Bacteroidota* ratio in premenopausal women with breast cancer compared to healthy women. Precisely, in our study we have identified that the *Bacillota/Bacteroidota* ratio is the most important variable of breast cancer cases in cluster analyses, suggesting that this microbiological trait may have an important impact on the occurrence and development of breast cancer.

The diagnosis of cancer can motivate patients to modify their lifestyle habits. A minority of breast cancer survivors follow a healthy lifestyle that includes both recommended intakes of vegetables–fruits and moderate levels of physical activity.[45] In a UK cohort of 1560 breast cancer patients, diet was assessed ≈1 year after diagnosis of the disease. Intake of fruit and vegetables, whole grains, and lean sources of protein increased significantly after diagnosis.[46] Another study showed that greater adherence to the Mediterranean diet was associated with better physical and health states, as well as less pain and insomnia symptoms, suggesting a possible role of this diet in the quality of life of women newly diagnosed with breast cancer.[47]

The present study has some limitations: i) some of the variables analyzed were difficult to collect and obtain high levels of participants' compliance; ii) although most of the cases were classified as stages I and II, 12 patients were stage III and 10 were stage IV; and iii) the control group was made of women who underwent breast augmentation and, accordingly, they were younger than the cases.

In conclusion, although cases exhibited higher adherence to the Mediterranean diet compared with controls, they presented features and microbiota alterations typical of the metabolic syndrome, probably due to their higher BMI and reflecting changes in their lifestyle after diagnosis.

4. Experimental Section

Study Design, Subjects, Inclusion, and Exclusion Criteria for Participation: A cross sectional, case–control study was conducted in women with newly diagnosed breast cancer (cases) and matched healthy women (controls). The study was registered in ClinicalTrials.gov under reference NCT03885648 (*Breast Cancer and Its Relationship with the Microbiota*, *MICROMA study*). The Andalusian Biomedical Research Ethics Coordinating Committee granted approval for the study (reference 9/2017).

A total of 178 women were recruited: 122 newly diagnosed with breast cancer (cases) and 56 healthy women (controls). Cases were defined as women diagnosed and surgically intervened of incident breast cancer, preferably stages I and II. Women who had received antibiotic treatment during the 3 months prior to recruitment, or any neoadjuvant therapy (chemical, radiological, or hormonal) were excluded from the study. Controls were selected among women undergoing surgery for breast reduction or augmentation, with no history of oncology, gynecology or endocrine disease, and those who had not received antibiotic treatment during the 3 months prior to recruitment were excluded from the study.[15] Women between 25 and 70 years of age were included in the study.

Recruitment and Sampling: Women were recruited at the Surgery and Breast Pathology Units of the University Hospital Clínico San Cecilio (Granada, Spain) and the University Hospital of Jaén (Jaén, Spain). All were informed of the objectives of the study during the hospital stay and signed an informed consent form. Collaboration in the study involved answering questions about their anthropometric, sociodemographic, and reproductive characteristics, obtaining information from their medical records, and agreeing to donate a stool sample during their hospital stay.

Ethical Aspects: Participation in the project was voluntary and the request for participation was made as a health research proposal, independent of the conventional health care procedure followed by the health system to treat the patients. Participants were informed in writing of the nature of the research and the use to be made of the information obtained. In addition to verbal information, participants were presented with a written informed consent form that they had to sign in order to be included in the study. They were also informed that they could withdraw from the study whenever they wished without having to give any explanation.

The rules described in Organic Law 3/2018, of December 5, on Personal Data Protection and Guarantee of Digital Rights (Spain) were followed, which guaranteed confidentiality, treatment in a strictly anonymous manner, and availability of health data for the participants.

The study was carried out in accordance with the standards recognized by the Declaration of Helsinki (52nd General Assembly, Edinburgh, Scotland, October 2000), the Standards of Good Clinical Practice, and in compliance with current Spanish legal regulations governing clinical research in humans (Law on Biomedical Research, Royal Decree 561/1993 and 033/2004).

In order to protect the confidentiality of participants' personal information, the following measures were taken: i) all data that could identify the participants was kept separate from the rest of the information collected in the different questionnaires of the study; ii) each case in the study had an identification number that was the one in the databases and the sample was double coded with a standardized procedure; iii) the analysis of the information was always done on an aggregate basis and never individually; and iv) all the researchers involved in the project undertook to comply with the necessary rules to preserve the confidentiality of the information provided by the participants.

Anthropometric and Sociodemographic Data: An ad hoc questionnaire was used to obtain information on age, race, weight (according to WHO classification[48]), height, area of residence (rural/urban), education, current work activity, marital status, smoking habit, and alcohol consumption.[49]

Reproductive Characteristics: Data were also collected on age at menarche and menopause, number of pregnancies, number of children, age at first and last full-term pregnancy, lactation, total duration of lactation (sum of all months of lactation), use of hormonal contraceptives, and/or hormone replacement therapy.

Tumor Characteristics: Tumor characterization protocols were similar in the participating hospitals and were performed following the criteria established in the breast cancer clinical guidelines: histological type and grade (International Classification of Oncological Diseases, ICD-O-3.1); tumor stage: TNM classification of malignant tumors[50]; hormone receptors (estrogen and progesterone); and HER2 and tumor markers (E-cadherin, Ki-67 antigen).

Dietary Assessment: The adherence of the participants to the Mediterranean diet pattern was assessed through a short and specific known questionnaire, called Mediterranean Diet Adherence Screener (MEDAS) (see Supplementary Material), which initially included nine questions[51,52] but was later modified and validated to 14 items for its application in the PREDIMED study.[53,54] The test score is obtained by assigning a value of 1 or 0 to each of the items, depending on whether or not the response is in accordance with the characteristics of the Mediterranean pattern, and then adding the 14 values obtained. To determine the degree of adherence to the Mediterranean diet, two groups of women were established, those

whose total score was ≥9, indicating good adherence, and those who obtained a total sum <9, with a low level of adherence.[55]

A food frequency questionnaire (FFQ) validated for the Spanish population was also applied[56] (see Supplementary Material). To analyze FFQ, foods were grouped according to the United States Department of Agriculture and Human Services,[57] as well as according to the Spanish Database of Food Composition (BEDCA, 206AD),[58] calculating each of the servings (grams) per day for each participant. Percentages of macronutrients (carbohydrates, lipids, and proteins) as well as food groups consumed were obtained.

The foods were grouped into ten different categories: dairy products (e.g., whole milk, yogurt, and cheese), fruits (e.g., orange, banana, apple, and natural juice), vegetables (e.g., chard, spinach, lettuce, and vegetable soups), legumes (e.g., lentils, chickpeas, and beans), protein foods (e.g., egg, chicken, pork, sausage, white fish, and blue fish), cereals and grain foods (e.g., puffed cereals, cookies, potatoes, rice, pasta, bread, and nuts), oils and other fats (e.g., olive oil, butter, and sunflower oil), sugars (e.g., chocolate, commercial juice, sweetened beverages, and candy), processed foods (e.g., chocolate cookies, donuts, sponge cake, croissant dairy desserts, ice cream, and snack bags), and alcoholic beverages (e.g., beer, wine, and sangria). In order to calculate the intake of each food, a coding was made according to each response where: never or almost never = 0; 1 time per week = 0.14; 2–4 times per week = 0.42; 5–7 times per week = 0.85; 8–10 times per week = 1.28; 11–13 times per week = 1.7; 14–16 times per week = 2.14; 17–19 times per week = 2.57; 20–22 times per week = 3; and 23–25 times per week = 3.42. These values were obtained by dividing the average number of times consumed per week by 7 (e.g., 2–4 times per week = 3/7 = 0.42) to obtain the servings consumed per day.

Dietary Patterns: A PCA was performed to identify underlying dietary patterns using the average weight consumed (g day^{-1}) by each individual from eigjt food groups as input variables. Multicollinearity was evaluated by looking at the determinant of the R-matrix Bartlett's test of sphericity, and the Kaiser–Meyer–Olkin (KMO) measure of sampling adequacy was used to verify the appropriateness of factor analysis. To assess the degree of intercorrelations between variables, a value >0.60 for the KMO was adopted.[59] Factors were orthogonally rotated (the Varimax option) to enhance the difference between loadings, which facilitated interpretability. Those were retained based on the following criteria: factor eigenvalue >1.2, identification of a breakpoint in the screen plot, the proportion of variance explained, and factor interpretability.[59]

The strength and direction of the associations between patterns and food groups were described through a rotated factor loading matrix. Food groups with factor loadings >0.30 and communality >0.30 were retained in the patterns identified. The factor score for each pattern was constructed by summing observed intakes of the component food items weighted by the factor loading. A high factor score for a given pattern indicated a high consumption of the foods constituting that food factor, and a low score indicated a low intake of those foods. Radar charts were used to display multivariate data in the form of a two-dimensional chart of eight food groups (input variables) represented on axes starting from the same point.[59]

A two-step cluster analysis procedure was used as an exploratory tool to reveal natural clusters within the dataset that would otherwise not be apparent and automatically determine the "best" number of clusters using SPSS v.25 (IBM, Chicago, IL, USA). Thereafter, using R v.3.6.3 package, unsupervised hierarchical clustering analysis was applied on the FFQ and variables (anthropometric and sociodemographic data and *Bacillota*/*Bacteroidota* ratio) to build clusters of subjects with similar characteristics (R package pheatmap). The distance matrix was defined by Euclidean distances, and Ward's method was used as linkage criteria to group the clusters.

Metagenomics: DNA was isolated from feces samples with the QIAamp cador Pathogen Mini kit (QIAGEN, Barcelona, Spain), following the manufacturer's directions. DNA concentration and purity were evaluated in a NanoDrop2000c (Thermo Fisher Scientific, Waltham, MA, USA). The Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) was used for metagenomics library construction. The amplicon tagment mix (ATM) in Nextera XT, which includes the enzyme used for tagmentation, was diluted 1:10 in nuclease-free water for library construction using 1–100 pg input DNA. Each 20 µL of the tagmentation reaction mixture consisted of 10 µL TD buffer, 5 µL of input DNA, and 5 µL of diluted ATM. PCR cycles for library construction were 12, 14, 17, and 20 cycles for 1000, 100, 10, and 1 pg DNA, respectively, following the manufacturer's protocol. The manufacturer recommends 12 cycles of the PCR reaction for no less than 1 ng input DNA. Amplified libraries were purified using AMPure XP (Agencourt, Brea, CA, USA). The quality of the purified libraries was assessed using an Agilent High Sensitivity DNA Kit on an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA). The sequencing libraries were further quantified using the KAPA Library Quantification Kit. Metagenomic libraries were mixed with PhiX Control v3 (Illumina) at a ratio of 9:1 and sequenced with an Illumina MiSeq Reagent Kit v3 (600 cycles). The raw data samples were analyzed using MetaPhlAn version 3.0.[60]

Statistical Analysis: For each of the variables, a descriptive analysis was performed and frequencies and contingency tables obtained. Results were expressed as the mean ± SEM for continuous variables, and as a percentage for discrete variables. To determine differences in means between cases and controls, continuous variables were first analyzed with the Kolmogorov–Smirnov normality test and, depending on their behavior, by the Student's *t*-test or the nonparametric Mann–Whitney *U* test, respectively. For categorical variables, the *p* value was calculated through the χ^2 test (chi-square). A statistically significant difference was considered when $p < 0.05$. Two statistical programs were used, SPSS v.26 and GraphPad Prism version 8.0.1.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

C.M.R.-M. and A.I.Á.-M. share first authorship. M.F.F. and L.F. share senior authorship. M.F.F. and L.F. contributed to the study concept and design and obtained funding. C.M.R.-M., T.G.-A., M.T.S.-B., and S.R.L. recruited patients, collected samples and questionnaires, and participated in the acquisition of the biochemical data. C.M.R.-M., A.I.A.-M., and J.P.D. participated in the acquisition of the rest of the data for this study and did the statistical analysis. All authors took part in the analysis and interpretation of data. A.I.A.-M., J.P.D., A.R.-L., M.F.F., and L.F. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Data are available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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