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Sourdough Bread and Metabolic Risk Factors. A Randomized, Controlled Trial in Subjects with Metabolic Syndrome

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Abstract: Mediterranean diet, featuring sourdough bread, shows promise in managing metabolic syndrome. This study explored the effects of two sourdough breads with differing fermentation times but similar nutritional profiles on inflammation, satiety, and gut microbiota composition in adults with metabolic syndrome. The double-blind clinical trial randomized participants to consume over two-months Elias Boulanger® long fermentation (48h) sourdough bread (EBLong) or Elias Boulanger® short fermentation (2h) sourdough bread (EBShort). We assessed clinical parameters, inflammatory- and satiety-related biomarkers; and richness and abundance of gut microbiota at baseline and follow-up. 31 individuals (mean age: 67, 55.7% female) participated. EBShort was associated with reduced soluble intercellular adhesion molecule (sICAM) levels, and irrespective of the adjudicated intervention, all participants had a decrease in sICAM and diastolic pressure relative to baseline ($p<0.017$). At follow-up, plasminogen activator inhibitor-1 (PAI-1) levels were lower in EBShort (-744 pg/mL; 95%CI: -282 to -1210 pg/mL) compared to EBLong. High-

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quality sourdough bread may offer mild benefits in blood pressure and inflammation for those with metabolic syndrome, however, only EBLong was associated to reduced Enterobacteriaceae abundance. Further research is needed to understand potential benefits and mechanisms of sourdough bread in managing metabolic syndrome and improving cardiovascular health.

Keywords: sourdough bread; metabolic syndrome; sICAM; PAI-1; blood pressure

1. Introduction

Metabolic syndrome, a group of closely interrelated metabolic disorders, is strongly associated with cardiovascular disease incidence and the risk of developing type 2 diabetes mellitus [1,2]. The risk factors for metabolic syndrome include abdominal obesity, high levels of triglycerides, low concentrations of high-density lipoprotein cholesterol (HDLc), elevated blood pressure, insulin resistance and/or hyperglycemia [3]. The adoption of healthy lifestyle habits such as an adequate diet, weight management, regular physical activity, and avoiding smoking can reduce cardiometabolic risk [4]. However, a more holistic strategy is crucial to approach prevention; such strategies could include policies for easier access to healthy eating and physical activity and, innovations in the food industry.

In our context, the Mediterranean diet (MedDiet), which has proved to be effective in cardiovascular disease prevention [5], is characterized by the triad of bread, olives, and wine (but also their derived products) which have shown to improve metabolic syndrome risk factors [6]. Bread has been an important dietary component for Mediterranean populations since ancient times [7]. Sourdough bread, which dates back centuries, is considered a cultural heritage. It contains microorganisms such as yeast and lactic acid bacteria. The dough, made from wheat and/or rye flour and water, is left to naturally ferment at room temperature to increase its content of microorganisms [8]. These microorganisms provide the gas and acidity that give sourdough its aroma, texture, flavor, and keeping qualities [9]. Such characteristics are due to the longer fermentation time, as opposed to more commercial methods with very much shorter periods and higher temperatures [10]. It has yet to be established whether sourdough has a beneficial impact on health and whether a wholegrain sourdough, combining probiotics and prebiotics, could promote bacteria survival and microbiota equilibrium.

The present study aimed to evaluate the effects of two sourdough breads, with long (48 hours) and short (2 hours) fermentation in individuals with metabolic syndrome on clinical parameters, inflammatory biomarkers, and satiety hormones. Additionally, the study aimed to characterize the gut microbiota as a secondary objective. Our aim was to demonstrate a potentially yielding superior results with the long fermentation bread.

2. Materials and Methods

2.1. Study Design and Population

This is a randomized, controlled, double-blind clinical trial with two parallel groups.

Eligible participants were community-dwelling adults who met at least three components of metabolic syndrome according to the updated harmonized criteria of the joint statement from the International Diabetes Federation, the National Heart, Lung and Blood Institute, and the American Heart Association: hypertriglyceridemia [≥150 mg/dL (≥1.7 mmol/L)] or drug treatment for elevated triglycerides; low concentrations of HDLc [<50 mg/dL (<1.3 mmol/L) and <40 mg/dL (<1.03 mmol/L) in women and men, respectively] or drug treatment for low HDLc; elevated blood pressure (systolic ≥130 mmHg and/or diastolic ≥85 mmHg) or being treated for hypertension; high fasting plasma glucose [≥100 mg/dL (≥5.5 mmol/L)] or drug treatment; and elevated waist circumference for European individuals (≥88 cm in women and ≥102 cm in men) [3]. Exclusion criteria included: a) use of antibiotic, prebiotic and/or probiotic supplements in the 3 months prior to the start of the study; b)

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celiac disease; c) inflammatory bowel disease; d) history of bowel resection; e) alcoholism and/or any other drug dependence; f) use of non-steroidal anti-inflammatory drugs; g) use of immunosuppressants, antibiotics, and proton pump inhibitor; h) any disease or condition preventing compliance with the study protocol; and i) inability to provide informed consent.

Participants were assigned to one of the 2 sourdough bread (Elias Boulanger®) intervention arms: a) Elias Boulanger® long fermentation bread (EBLong) and, b) Elias Boulanger® short fermentation bread (EBShort). The volunteers were randomized between the 2 intervention arms using a random sequence generated by a computer program (Cardiovascular Epidemiology and Genetics Group, EGEC, Hospital del Mar Research Institute). The double-blind was maintained until the analysis of the results. No recommendations regarding diet, physical activity, or lifestyle were provided. Participants were instructed to replace their usual bread with the intervention bread while maintaining the same quantity consumed over a two-month period. Participants were instructed to collect the sliced bread from their chosen bakeries once a week or every two weeks (15 distributed throughout the province of Barcelona) and froze it. This trial was carried out at the Hospital del Mar Research Institute in Barcelona, Spain.

2.2. Ethical Aspects

The protocol of the study complied with the Declaration of Helsinki for Medical Research involving Human Subjects. The study protocol was reviewed and approved by the Clinical Research Ethics Committee of the Parc de Salut Mar Barcelona consortium (CEIC-Parc de Salut Mar) registry. Register number: ISRCTN89898870. All participants read and sign an informed consent before enrollment to the study.

2.3. Bread Composition and Fermentation Process

First, a starter was elaborated blending equal weight parts of whole-grain wheat flour (T110 flour, *Triticum dicoccoides*; Moulin de Colagne®, France) and water that rested for 24 hours at room temperature. At day 5 a mixture of infusions, dairy products and fruit (following the baker's own receipt) was added and left at room temperature. The final starter had the following characteristics: a temperature of 34°C, a pH value of 6.68, and a total titratable acidity of 13.73 mL. Sourdough was created by combining the starter (30%), water (50%), and whole-grain wheat flour milled through a stone-mill (70%, T110 flour, *T. dicoccoides*; Moulin de Colagne®). Subsequently, a process known as feeding or back-slopping was carried out, involving the use of the initial mixture to ferment a new blend of water and flour at regular intervals of every 12–24 hours for 3 days and fermented for 4 days at 10-15 °C. This procedure aimed to stimulate the microbial fermentation and promote propagation of sourdough. Details of the high-quality sourdough preparation can be found elsewhere [11].

EBLong was formulated with sourdough (wt/wt; 30% flour basis), whole-grain flour (*Triticum aestivum*), water (wt/wt; 80% flour basis), dry baking yeast (*Saccharomyces cerevisiae*, wt/wt; <0.5% flour basis; Lesaffre (Hirondelle®)), and salt (wt/wt; 1.1% flour basis; Guerande®). Then, in a fermentation chamber (Eurifours®), bread was fermented for 48 hours: 46 hours of maturation at 4-6ºC and 2 hours of dough development at 28ºC.

EBShort was composed by sourdough (wt/wt; 10% flour basis), refined wheat flour (Farinera Corominas®) (being a blend of white bread since the starter contained whole grain flour), water (wt/wt 60% flour basis), yeast (*S. cerevisiae*, 0.010 g/kg of flour; Lesaffre (Hirondelle®)), salt (wt/wt 1.2% flour basis, Sal Costa®), enzymes(α-amylase, endoxylanasse, amyloglucosidanassa; Uniplus®), wheat gluten (0.010 g/kg of flour; Uniplus®), xanthan gum (0.005 g/kg of flour), and additive components: emulsifier E471, antioxidant E-300 (Uniplus®). It was fermented in a chamber (Eurifours®) for 2 hours at 28ºC.

Both breads were baked after the fermentation process in a Eurifours® oven at 200ºC for 90 minutes. Nutritional content of both breads can be found in Supplemental Table S1.

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2.4. General and Life-Style Data

The following variables were recorded at baseline and follow-up: a) a questionnaire for adherence to the MedDiet [12]; b) a three-day food record collected during the three days prior to each visit (one of which at the weekend), which was further translated into nutrients using the Pro-PCN software (Barcelona, Spain) [13]; and c) an abbreviated questionnaire from the Minnesota Leisure Time Physical Activity Questionnaire [14].

2.5. Anthropometric and Exploration Data

The following variables were measured at baseline and follow-up: Participants' weight was recorded in kilograms to one decimal point using a high-quality electronic scale, rounded to the nearest 100 grams, with individuals wearing light clothing and no shoes, jackets, or coats. Height was measured in centimeters with a stadiometer accurate to 1 centimeter. Waist circumference was calculated on expiration at the intermediate level between the last rib and the iliac crest, at the most prominent point of the trochanter. Body mass index (BMI) was derived by dividing weight (in kilograms) by height squared (in meters squared). Blood pressure was assessed while participants were seated with their backs and arms supported to ensure the cuff was at heart level, after refraining from smoking or consuming caffeine for 30 minutes. Measurements were taken on both arms using appropriate cuffs, with the arm displaying the higher mean diastolic blood pressure selected for subsequent measurements throughout the study. If the first two readings differed by more than 5 mmHg, additional readings were taken and averaged.

2.6. Laboratory Analysis

The following parameters were analyzed in fasting ethylenediaminetetraacetic acid (EDTA) plasma at baseline and after two-months follow-up: glucose (Glucose HK CP, Horiba ABX), triglycerides (Triglycerides CP, Horiba ABX), total cholesterol (Cholesterol CP, Horiba ABX) and, HDLc (HDLc Direct CP, Horiba ABX) were measured in an autoanalyzer ABX Pentra (Horiba ABX SAS, Spain). HOMA Index was calculated as (glucose x insulin)/405. We calculated LDLc with the Friedewald formula only when triglycerides were <300 mg/dL, higher values (≥300 mg) implied a missing value for LDLc. The following inflammation markers were analyzed: interleukin 6 and 8 (IL6 and IL8), tumor necrosis factor alpha (TNF-α) (Bio-Plex Cytokine 8-plex, Bio-Rad); soluble intercellular adhesion molecule (sICAM) (Bio-Plex Cytokine 2-plex, Bio-Rad), and Plasminogen activator inhibitor-1 (PAI-1) (Bio-Plex Pro Human Diabetes 10-plex, Bio-Rad). In addition, vascular-related hormones were also analyzed: insulin, C-peptide, ghrelin, leptin, glycoprotein 1 (GLP-1), glucagon, resistin, and vifastin (Bio-Plex Pro Human Diabetes 10-plex, Bio-Rad); a Luminex® xMAP® technology, in a BioPlex system was employed (Bio-Rad, Hercules, California, United States). The lipopolysaccharide binding protein (LBP) (Human LBP, Hycult Biotech) was measured with an Elisa Kit.

2.7. Intestinal Microbiota Analysis

Participants collected fecal samples at home in sterile containers provided for this purpose. They were instructed to keep their samples frozen until delivering them to the study staff the following day. The samples were sent to the Girona Biomedical Research Institute (IdIBGi) with dry ice to maintain the cold chain.

Genomic DNA was extracted from an approximately 0.25 g of faeces using commercial methods (NucleoSpin Soil kit, Macherey-Nagel®). The quality and quantity of the extracted nucleic acids were measured by Nanodrop ND 2000 UV-Vis spectrophotometer (Nanodrop, DE) and Qubit® (ThermoFisher Scientific) according to the manufacturers' instructions. The region corresponding to the variable V3-V4 region of the 16S rRNA gene was determined with specific primers (341F/806R, [15]) and Illumina technology HiSeq 2000 using paired-end reads (generating 300 bp sequences). Briefly, amplification was performed after 25 PCR cycles. In this procedure, positive (CM) and negative (CN) controls were used to ensure quality control. The positive control is a Mock

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Community DNA (Zymobiomics Microbial Community DNA) control and it was processed the same way as the samples.

2.8. Bioinformatic Analysis

Raw demultiplexed forward reads were processed using the following methods and pipelines as implemented in QIIME2 version 2020.11 with default parameters unless stated [16]. DADA2 was used for quality filtering, denoising and amplicon sequence variant calling (ASV, i.e., phylotypes) using qiime dada2 denoise-sinle method [17]. Q16 was used as quality threshold to define read sizes for trimming (parameter: -- p-trunc-len). Reads were truncated at the position when the 75th percentile Phred score felt below Q16: 198 bp. After quality filtering steps, average sample size was 64,907 reads (min: 37,026 reads, max: 153,151 reads) and 1,896 phylotypes were detected. ASVs were aligned using the qiime alignment mafft method [18].The alignment was used to create a tree and to calculate phylogenetic relations between ASVs using qiime phylogeny fasttree method [19]. ASV tables were subsampled without replacement in order to even sample sizes for diversity analysis using qiime diversity core-metrics-phylogenetic pipeline. The smallest sample size was chosen for subsampling (i.e., 37,000 reads). Subsequently, reads were clustered into 1,896 operational taxonomic units (OTUs). The following alpha diversity metrics were calculated: observed ASVs number (i.e., richness) and Pielou's evenness index. Weighted Unifrac distances were calculated to compare community structure [20].

Taxonomic assignment of phylotypes was performed using a Bayesian Classifier [21] trained with Silva database version 138 (99% OTUs full-length sequences) [22], using the qiime feature-classifier classify-sklearn method [23].

2.9. Statistical Analysis

The study described categorical variables by proportions, normally distributed continuous variables by means and SDs, and non-normally distributed continuous variables by medians (1st–3rd quartile). Changes relative to preintervention values were assessed in both groups separately and together by paired t-tests in normally distributed continuous variables and Wilcoxon signed-rank tests in non-normally distributed variables. Multivariable linear regressions were conducted to explore whether there were differences in the follow-up values in the EBLong group relative to the EBShort group. The models were adjusted for baseline levels of each parameter (continuous), age (continuous), sex, BMI (continuous), and MedDiet adherence(continuous).

We applied a Bonferroni correction to account for the multiple comparisons arising from the three sets of variables (clinical parameters, inflammatory-related biomarkers, and satiety-related biomarkers). Results were considered statistically significant if the p-value was less than 0.05/3 (0.017), ensuring a stringent threshold for significance. Analyses were performed using R Software version 4.3.1 [24].

Metagenome statistical analysis were done using Generalized Linear Mixed Models (GLMM). Alpha diversity comparisons were performed using R package NBZIMM version 1.0 [25] for richness and the R package betareg version 3.1-4 [26] for evenness. Beta diversity distance matrices were used to calculate principal coordinates analysis; the significance of groups in community structure was tested using Permanova. Differential abundance of taxa was tested using Negative Binomial Generalized Linear Mixed Models using the R package NBZIMM [25]. P-values were adjusted using false discovery rate (FDR). Significant threshold was set at 0.05.

3. Results

3.1. Study Population

Participants were recruited between July 2019 and February 2020. A total of 292 individuals were contacted and finally 61 were enrolled (55.7% female) (Figure 1). Of these, 31 were randomly assigned to the EBLong intervention and 30 to the EBShort intervention. Finally, due to the COVID pandemic only 31 participants finished the study resulting in 13 participants in EBShort and 18 in EBLong

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group. The participants were older adults, with a mean age of 66.7 years, and 51.6% were female. They exhibited a high prevalence of metabolic risk factors. The only variable in which intergroup differences were observed in baseline values was BMI. No difference in diet according to the Adherence to MedDiet questionnaire, kcal intake, and physical activity performance at baseline were found.

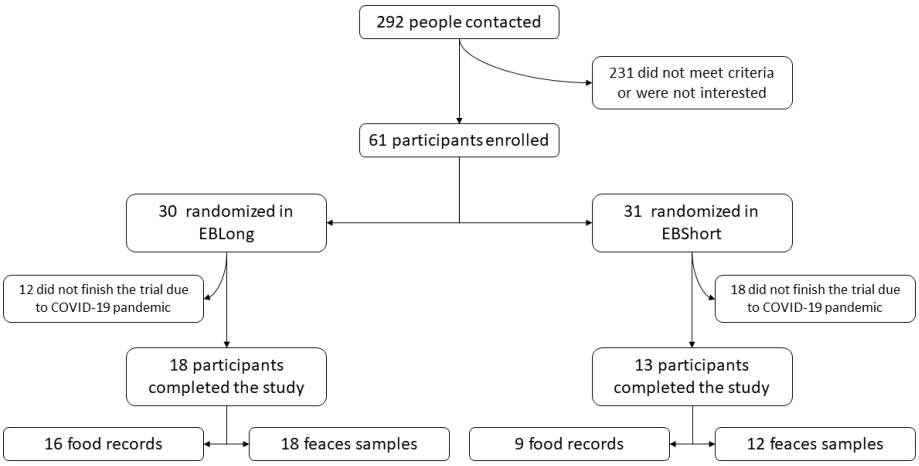

Figure 1. Study flowchart.

Table 1. Basal characteristics of participants.

	All	EBLong	EBShort	*p* value
n	31	18	13	
Age, mean (SD)	66.7 (5.94)	66.6 (7.04)	66.8 (4.36)	0.954
Sex, Female, n (%)	16 (51.6%)	8 (44.4%)	8 (61.5%)	0.565
Diabetes, n (%)	26 (83.9%)	17 (94.4%)	9 (69.2%)	0.134
Hypertension, n (%)	30 (96.8%)	18 (100%)	12 (92.3%)	0.419
Triglycerides, mg/dL, median [1st-3rd quartile]	142 [90.5;174]	146 [90.0;168]	139 [92.0;175]	0.889
HDLc, mg/dL, mean (SD)	49.0 (11.7)	50.3 (11.8)	47.2 (11.7)	0.475
BMI, kg/m^2, mean (SD)	32.8 (3.26)	34.1 (2.84)	31.2 (3.13)	0.015
Scholarity				0.895
Elementary School	13 (43.3%)	7 (38.9%)	6 (50.0%)	
Middle school	9 (30.0%)	6 (33.3%)	3 (25.0%)	
Higher education	8 (26.7%)	5 (27.8%)	3 (25.0%)	
Smoking habit				0.634
Non smoker	13 (41.9%)	9 (50.0%)	4 (30.8%)	
Smoker	5 (16.1%)	2 (11.1%)	3 (23.1%)	
Former smoker	13 (41.9%)	7 (38.9%)	6 (46.2%)	
Adherence to MedDiet (14pt), points, mean (SD)	9.71 (2.18)	9.83 (2.07)	9.54 (2.40)	0.724
Basal intake, kcal, mean (SD)	1558 (345)	1552 (387)	1567 (291)	0.900
Physical activity, Mets/day, mean (SD)	2502 (1885)	2320 (1632)	2753 (2234)	0.560

EBLong, Elias Boulanger® long fermentation bread; EBShort, Elias Boulanger® long fermentation bread; BMI, body mass index; SD, standard deviation; HDLc, high density lipoprotein cholesterol; MedDiet, Mediterranean Diet.

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3.1. Dietetic Assesment

Participants of both groups had similar diets before and during the last week of the study. No differences were found in the quantity of energy or macronutrients. The intake of some of the main nutrients recalled by the three-days food records can be found in Supplemental Table S2.

3.2. Sourdough Bread Intervention

Irrespective of the intervention, after two-months of intervention all participants had a decrease in sICAM and diastolic pressure (p<0.025) (Table 2). Whereas we observed a decrease in sICAM and PAI levels in the EBShort group (Table 2), a decrease of diastolic pressure was determined in EBLong group (p<0.025) (Table 2).

Table 2. Intragroup differences between baseline and two-months follow-up.

	EBLong			**EBShort**			**All**		
	Baseline	**Follow up**	***p* value**	**Baseline**	**Follow up**	***p* value**	**Baseline**	**Follow up**	***p* value**
Weight, kg	92.9 (14.7)	93.4 (14.3)	0.710	84 (10.2)	83.5 (9.5)	0.415	89.1 (13.5)	89.1 (13.2)	0.462
Waist, cm	119 (17.3)	114 (12.5)	0.237	111 (10.3)	110 (9.18)	0.796	115 (15.1)	112 (11.2)	0.223
Systolic pressure, mmHg	136 (11.3)	132 (14.5)	0.470	134 (10.1)	135 (9.91)	0.395	135 (10.7)	134 (12.6)	0.830
Diastolic pressure, mmHg	80.2 (12.2)	72.5 (10.2)	0.020	77.6 (12.7)	72.7 (11.3)	0.208	79.1 (12.2)	72.6 (10.5)	0.008
Glucose, mg/dL	125 (30.7)	128 (33.2)	0.162	117 (23.8)	117 (21.9)	0.967	122 (27.8)	124 (29.2)	0.318
Insulin, pg/mL	423 (201)	388 (168)	0.067	484 (282)	490 (287)	0.797	449 (236)	431 (227)	0.241
Glucagon, pg/mL	520 (188)	493 (177)	0.143	541 (117)	539 (180)	0.949	529 (160)	512 (177)	0.376
Homa Index	18.2 (7.97)	17.3 (7.43)	0.286	21.2 (15.6)	21 (13.8)	0.890	19.5 (11.7)	18.8 (10.5)	0.431
Triglycerides, mg/dL	146 [90; 168]	130 [91; 158]	0.862	139 [92; 175]	124 [84; 179]	0.839	139 [92; 175]	124 [84; 179]	0.814
Total cholesterol, mg/dL	199 (39.5)	202 (38.6)	0.378	189 (38.9)	192 (56.3)	0.707	195 (39)	198 (46.3)	0.444
HDLc, mg/dL	50.3 (11.8)	50.6 (11.7)	0.736	47.2 (11.7)	48.7 (15.4)	0.263	49 (11.7)	49.8 (13.1)	0.282
LDLc, mg/dL	120 (28.6)	125 (35.1)	0.123	115 (33.1)	115 (44.2)	0.918	118 (30.2)	121 (38.8)	0.314
C-peptide, pg/mL	1100 (423)	1050 (358)	0.389	1190 (511)	1190 (613)	0.940	1140 (457)	1110 (481)	0.618
Ghrelin, pg/mL	902 (297)	904 (274)	0.952	1180 (777)	1130 (622)	0.444	1020 (558)	998 (458)	0.531
Leptin, pg/mL	8920 (5110)	8540 (5430)	0.434	9170 (4680)	9120 (4910)	0.897	9020 (4850)	8780 (5140)	0.451
GLP1, pg/mL	164 (97.8)	165 (111)	0.960	187 (125)	223 (122)	0.191	174 (109)	189 (118)	0.304
IL6, pg/mL	2.4 (1.73)	3.06 (1.93)	0.106	2.5 (1.5)	2.14 (1.08)	0.350	2.44 (1.62)	2.67 (1.67)	0.426
IL8, pg/mL	4.49 (2.21)	3.86 (2.02)	0.116	4.63 (2.78)	4.4 (2.04)	0.563	4.55 (2.42)	4.09 (2.02)	0.099

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Resistin, pg/mL	4320 (1720)	4360 (1310)	0.883	6260 (3020)	5630 (2240)	0.339	5130 (2510)	4890 (1840)	0.445
TNF a, pg/mL	29.6 (9.59)	29.9 (11.8)	0.898	40.7 (15.4)	35.5 (12.4)	0.032	34.2 (13.3)	32.2 (12.2)	0.246
PAI-1, pg/mL	2740 (1070)	2840 (999)	0.466	2750 (529)	2330 (773)	0.018	2740 (872)	2630 (933)	0.318
Visfatin, pg/mL	1910 (1310)	1730 (1380)	0.133	2030 (1440)	1990 (1400)	0.887	1960 (1340)	1840 (1370)	0.364
sICAM, pg/mL	179000 (67500)	170000 (41800)	0.325	192000 (59300)	160000 (39200)	0.013	184000 (63500)	166000 (40300)	0.014
LBP, ng/mL	15100 (2630)	16500 (4370)	0.095	14200 (3820)	13900 (3690)	0.761	14700 (3160)	15400 (4230)	0.259

Baseline and follow up values are presented as mean (SD) or median [1st-3rd quartile]. EBLong, Elias Boulanger® long fermentation bread; EBShort, Elias Boulanger® short fermentation bread; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; GLP-1, glycoprotein 1; IL6, interleukin 6; Il8, interleukin 8; TNF-a, tumor necrosis factor alpha; PAI-1, plasminogen activator inhibitor-1; sICAM, soluble intercellular adhesion molecule; LBP, lipopolysaccharide binding protein.

When the two groups were compared, no differences were seen in the follow-up values of these variables in non-adjusted model nor in the adjusted model, except for PAI -1 in EBLong (-744 pg/mL; 95%CI: -282 to -1210 pg/mL) (Table 3).

Table 3. Intergroup differences between follow-up values.

	EBLong vs EBShort			
	Non-adjusted (diff. [95% CI])	***p* value**	**Adjusted (diff. [95% CI])**	***p* value**
Weight, kg	9.82 [0.47; 19.2]	0.050	-0.2 [-2.03; 1.62]	0.829
Waist, cm	4.49 [-3.89; 12.9]	0.303	-4.46 [-9.22; 0.3]	0.082
Systolic pressure, mmHg	-3.16 [-12.7; 6.38]	0.522	-11.6 [-21.1; -2.12]	0.026
Diastolic pressure, mmHg	-0.18 [-8.17; 7.81]	0.966	-6.43 [-14.6; 1.76]	0.140
Glucose, mg/dL	11.3 [-9.48; 32]	0.296	5.71 [-4.76; 16.2]	0.296
Insulin, pg/mL	-102 [-262; 58.8]	0.224	-22 [-91.9; 47.9]	0.543
Glucagon, pg/mL	-46.3 [-173; 80.7]	0.480	-2.05 [-96.7; 92.6]	0.966
Homa Index	-3.64 [-11.2; 3.9]	0.352	0.31 [-3.05; 3.67]	0.858
Triglycerides, mg/dL	-9.53 [-53.2; 34.1]	0.672	-33.3 [-66.6; -0.086]	0.062
Total cholesterol, mg/dL	10.2 [-23.2; 43.5]	0.554	-4.44 [-25.3; 16.4]	0.681
HDLc Cholesterol	1.87 [-7.65; 11.4]	0.703	-1.49 [-4.9; 1.91]	0.399
LDL cholesterol, mg/dL	10.2 [-17.7; 38.1]	0.479	2.03 [-16.1; 20.2]	0.829
C-peptide, pg/mL	-149 [-498; 200]	0.411	50.4 [-205; 306]	0.703
Ghrelin, pg/mL	-225 [-548; 97.1]	0.181	-45.4 [-163; 72.1]	0.457
Leptin, pg/mL	-584 [-4310; 3140]	0.761	-276 [-1920; 1370]	0.745
GLP, pg/mL	-58.6 [-141; 24]	0.175	-16.9 [-88.1; 54.2]	0.646
IL6, pg/mL	0.92 [-0.25; 2.08]	0.134	1 [-0.17; 2.16]	0.107
IL8, pg/mL	-0.55 [-1.99; 0.9]	0.466	-0.62 [-1.57; 0.34]	0.219
Resistin, pg/mL	-1270 [-2530; -22.4]	0.056	-5.84 [-1190; 1180]	0.992
TNF a, pg/mL	-5.59 [-14.2; 3]	0.212	3.81 [-3.15; 10.8]	0.295
PAI-1, pg/mL	516 [-135; 1170]	0.131	744 [282; 1210]	0.004
Visfatin, pg/mL	-258 [-1250; 731]	0.613	19.9 [-626; 665]	0.952
sICAM, pg/mL	9530 [-19500; 38600]	0.525	22100 [2250; 42000]	0.040
LBP, ng/mL	2520 [-411; 5450]	0.103	1710 [-1210; 4630]	0.263

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Intergroup comparisons in follow-up values relative to control group were estimated by multivariable linear regression model 1 adjusted for age, sex, BMI, MedDiet adherence (14pt) and baseline values. EBLong, Elias Boulanger® long fermentation bread; EBShort, Elias Boulanger® short fermentation bread; HDLc, high density lipoprotein cholesterol; LDLc, low density lipoprotein cholesterol; GLP-1, glycoprotein 1; IL6, interleukin 6; Il8, interleukin 8; TNF-a, tumor necrosis factor alpha; PAI-1, plasminogen activator inhibitor-1; sICAM, soluble intercellular adhesion molecule; LBP, lipopolysaccharide binding protein.

3.3. Microbiota Characterization

Alpha diversity analysis revealed no discernible differences in richness and evenness either within or between the intervention groups during the follow-up period (Figure 2). Beta diversity, evaluated using weighted UniFrac distance metrics, demonstrated no significant distinctions between the treatment groups throughout the study period (PERMANOVA R2= 0.0072, p = 0.389) (Figure 2).

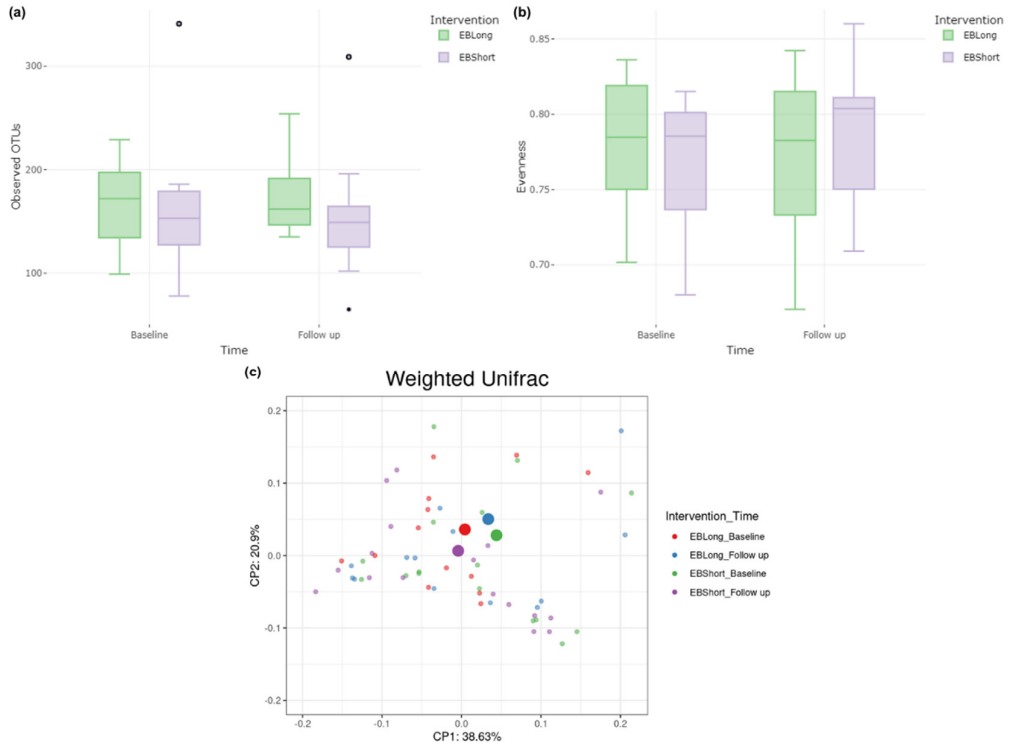

Figure 2. Alpha and beta diversity of EBLong and EBShort intervention. (a) Diversity richness as the number of observed OTUs at baseline and at follow up in each intervention. (b) Pielou's evenness index in each intervention groups at baseline and at follow up. (c) Principal coordinates analysis (PCoA) plot based on weighted UniFrac distances according to interventions and follow up.

Figure 3 illustrates the relative frequencies of the most abundant microbial phyla and family. At the phylum level, the relative abundance observed in both intervention groups was as expected for adult human fecal sample. No differences in baseline relative abundance of phyla were observed between the groups. After the intervention with EBLong bread there was a significant reduction in Synergistota abundance relative to baseline ($p < 0.001$). Additionally, when comparing the two groups at the follow-up assessment, the EBLong group exhibited lower abundance of Synergistota compared to the EBShort group, however its abundance did not exceed 1% (see Supplemental Table 3). At family level, after the two-month intervention, the EBLong group showed a reduction in Enterobacteriaceae and Synergistaceae. In contrast, the EBShort group exhibited a decrease in

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Oscillospiraceae and an increase in Prevotellaceae. At follow-up, the abundance of Oscillospiraceae differed significantly between the groups; however, this difference was also observed at baseline.

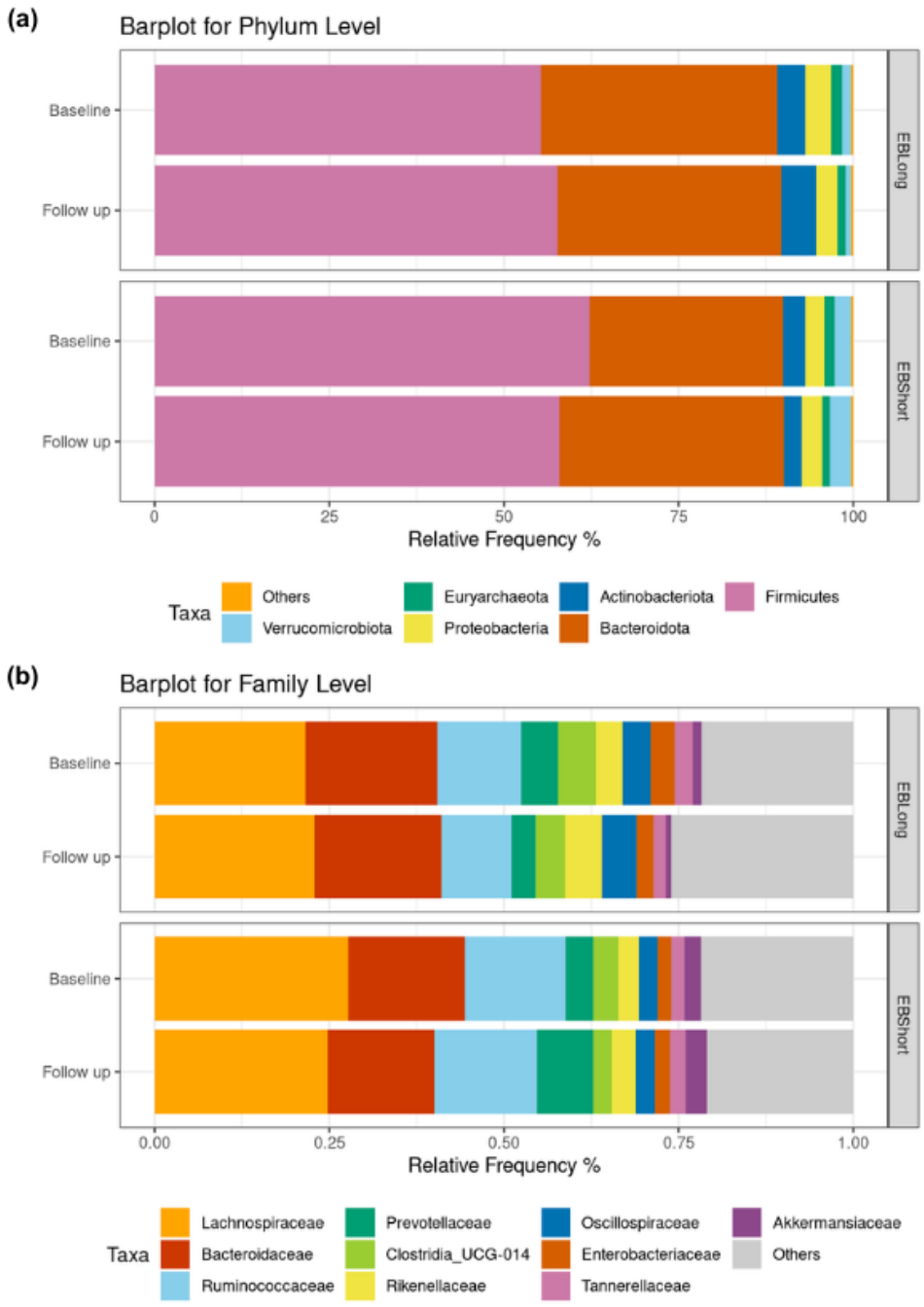

Figure 3. Barplots for relative abundance. (a) Displays abundance at phylum level for each intervention. Phyla with a relative abundance lower than 1% grouped as "Others". (b) Displays abundance at family level of the 10 most abundant taxa.

4. Discussion

A two-months intervention with two sourdough bread, with similar nutrient composition but different fermentation time, decreased sICAM and diastolic pressure levels in all the participants. In the assessment of each bread, we observed a decrease in sICAM levels in the consumers of EBShort

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bread, relative to baseline values. When comparing the effects of each bread, PAI-1 levels were lower after the intervention with EBShort bread compared to EBLong bread. No changes were observed in any other clinical parameter or satiety-related biomarker. In the microbiota analysis, we found comparable composition between groups after the intervention, with no changes in the diversity and abundance, suggesting that gut microbial communities remain stable after both interventions. Nevertheless, at the phylum level, the abundance of Synergistota exhibited a reduction following the intervention with EBLong. At the family level, the intervention groups differed in the abundance of Oscillospiraceae at follow-up; however, these differences were already present at baseline.

Regarding inflammatory biomarkers, we have not found any difference in cytokines, similar to what has been reported in a previous article [27]. Nevertheless, after two-months of swapping their habitual bread for sourdough bread (either with short or long fermentation) participants had lower values of sICAM, an adhesion molecule that plays an important role in the development of atherosclerosis [28]. Comparable outcomes were found in a study with a 5-weeks intervention with a prebiotic antioxidant bread composed by wheat–rye bread, tomato paste, green tea powder and herbs [29]. In the previous study by Seidel et al., ICAM-1 significantly decreased after the intervention in non-smoker adults. Despite this bread not containing sourdough, it was enriched similarly to our starter, which included infusions, dairy products, and fruit resulting in a bread rich in flavonoids, pre and probiotics that could provide this effect. An in vitro study suggests that some flavonoids could inhibit ICAM-1 expression [30] and a clinical trial showed a decrease in ICAM-1 expression after the consumption of a probiotic sausage containing *Lactobacillus paracasei* [31]. Because of the limited number of participants involved in this study, besides the variability of flavonoids and probiotics in diet, more evidence is required to draw any conclusions regarding any mechanism associated with this observation. PAI-1 is a primary inhibitor of fibrinolysis, playing a critical role in the development of thrombosis, atherosclerosis, and cardiovascular risk [32]. Contrary to our hypothesis that bread with a higher sourdough proportion would reduce inflammation, PAI-1 levels were lower in the EBShort group compared to EBLong intervention at follow-up. Since our objective was to compare different sourdough breads, distinguishing between interventions is challenging without a poor-quality control bread for reference. Other authors, that compared the consumption of whole grain wheat sourdough bread to refined white bread in normoglycemic-normoinsulinemic and hyperglycemic-hyperinsulinemic patients observed no differences in PAI-1 levels after 6 weeks of consumption [33]. Different sample size of our intervention groups, the greater proportion of diabetics, differences in BMI (being greater in EBLong) and different breads may explain discrepancies in the findings presented here.

Regarding clinical parameters, after consuming for two-months sourdough bread there was a decrease in diastolic pressure. In contrast, another clinical trial comparing the effect of whole grain wheat sourdough to non-fermented white bread did not find differences in blood pressure after 6 weeks of treatment [34]. In vitro studies, have described an anti-hypertensive effect in spelt flours with 96 hours of fermentation [35], and quinoa and wheat flours with 46 hours of fermentation [36]. The authors reported that mechanism underlaying may be the release during fermentation of bioactive peptides involved in the inhibition of the Angiotensin-Converting Enzyme (ACE) activity, a molecule that plays a crucial role in the control of blood pressure with a vasoconstrictor effect [35,36]. Different conditions such as the types of flour, time, and microorganisms engaged in the fermentation process could have different impacts on the results. Future studies could assess polymorphisms in the ACE gene associated with hypertension to tailor recommendations for sourdough bread consumption. Despite minor variations in blood pressure, we did not observe any additional differences in lipid or glucose metabolism following the 2-month intervention. Similar outcomes were found in a previous clinical trial, which compared consumption of whole grain wheat sourdough bread and white bread over 6-weeks. Notably, no differences in serum lipids or glycemic parameters were observed in adults with normoglycemia nor hyperglycemia [34]. On the other hand, another clinical trial with healthy young adults found that after an intervention of 4 weeks with an ancient grain “Verna” bread (leavened with sourdough or yeast), LDL cholesterol decreased. Fasting glucose was higher in the group eating yeast-fermented bread, but when comparing both types of

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bread, there were no differences in lipids or glucose levels, and sourdough bread didn't offer any extra benefits [27]. Although there's no evidence that sourdough affects long-term glucose control, a meta-analysis suggested it might help lower post-prandial blood sugar compared to other breads[37].

As far as we know, no other study has examined long-term interventions of sourdough bread on satiety, although a few studies have looked for its acute effects. When appetite was assessed using subjective scales, the evidence was inconclusive [38–40]. However, when satiety was tested with an ad libitum meal after the consumption of different types of bread, sourdough breads did not seem to influence energy intake [38,39]. Some studies have specifically measured incretins. One study found that the postprandial concentrations of gastric inhibitory peptide (GIP) and GLP-1 were lower after consuming sourdough bread compared to whole wheat and whole wheat barley breads [41]. Another study described a lower ghrelin AUC after the consuming einkorn sourdough bread compared to commercial breads. However, there were no differences among the consumption of different sourdough breads[42]. The differences between these studies may be attributed to various factors, such as the use of different measurement scales, variations in control breads, and differences in the duration of the studies.

Regarding microbiota, the lack of changes in alpha diversity, which indicates species richness and diversity within individual samples, suggested consistent levels of microbial diversity regardless of sourdough bread consumption and its fermentation duration. Likewise, the consistency in beta diversity, which measures the differences in microbial community composition between different samples, suggests that there were no changes in microbial composition across the interventions. These results are consistent with a similar trial conducted in subjects diagnosed with ulcerative colitis, which examined the effects of a two-month intervention with two different sourdough breads (differing in percentage) [43]. Consistent with our results, the above-mentioned study with 23 subjects reported no differences microbiota diversity. Furthermore, although the bread treatment suggestively reduced the Firmicutes/Bacteroidetes ratio, both groups experienced some relief from symptoms resembling intestinal bowel disease [43]. Likewise, a one-week clinical trial involving 20 healthy subjects that compared sourdough bread consumption to white bread found similar results, with no noticeable differences in alpha and beta diversity or relative abundances at the phylum level [44]. Despite not finding differences in diversity, we have identified some differences in the abundances of the most prevalent phylum and families. We observed a reduction in the abundance of the Synergistota phylum following sourdough bread consumption, although its abundance did not surpass 1%. Although this phylum is poorly characterized, it is known to inhabit human soft tissues and the gut, and its main function is their role in amino acid degradation [45]. Interestingly, at family level, after the consumption of EBLong, the abundance of Enterobacteriaceae decrease. This family thrive in inflamed environments, commonly associated with conditions such as inflammatory bowel disease, obesity, colorectal cancer, celiac disease, and after antibiotic use [46]. EBLong may contribute to reducing the conditions for Enterobacteriaceae proliferation, consequently lowering their abundance. Further investigations could explore these alterations and their potential effects on gastrointestinal symptoms following sourdough consumption, as previously reported. For EBShort, we observed a decrease in the abundance of the Oscillospiraceae family. Within this family, the genus Oscillospira is the most studied and is associated with leanness, metabolic health, and reduced inflammation [47]. Additionally, we found an increase in the Prevotellaceae family, which has been described as more abundant in individuals with obesity [48]. It is important to note that our analysis did not extend to the genus level, making it difficult to draw specific conclusions about the implications of these changes. Furthermore, the weight-related effects described for these families are not reflected in the clinical data from our study. These collective findings suggest that the intestinal microbiota exhibits resilience to changes induced by bread consumption, particularly in the case of sourdough bread.

Our study has some limitations. First, due to the health contingency caused by the Covid-19 pandemic, the clinical trial was interrupted, affecting the disparity between the intervention groups in number and characteristics, especially in BMI. Nevertheless, we tried to minimize it by adjusting covariates (for age, sex, BMI, MedDiet adherence (14pt) and baseline values). Second, breads

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composition was very similar between them, both of them contained sourdough which could explain the findings, and the nutritional composition in terms of energy, carbohydrate, total fat, protein, fiber, and sodium was very similar. Third, intake of bread and nutritional assessment were auto-reported, which could imply some self-report bias. Fourth, due to the high number of determinations of the study, and even though we corrected our results for multiple testing, our results should be interpreted cautiously. Finally, we conducted the study in a very specific population, which could complicate the generalization of the results.

5. Conclusions

Sourdough bread may offer some mild benefits in blood pressure and inflammation markers in individuals with metabolic syndrome. Gut microbiota, however, did not exhibit differences when comparing both interventions, suggesting that it remains stable to changes in sourdough consumption. Apart from a higher abundance of Enterobacteriaceae in the group that consumed EBL, no further modifications were observed with a bread with longer fermentation time. More studies with larger samples comparing different fermented sourdough breads and control breads, are needed in order to verify our results and to fully understand the potential benefits and mechanisms of action of sourdough bread.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Supplemental Table S1: Nutritional composition of EBLong and EBshort breads; Supplemental Table S2: Difference in dietetic assessment of food records in EBShort and EBLong groups; Supplemental Table S3: Differential abundance at phylum taxonomic level.

Author Contributions: Conceptualization, María-Dolores Zomeño, Montserrat Fitó and Olga Castañer; Data curation, Karla Alejandra Pérez-Vega and Álvaro Hernáez; Formal analysis, Karla Alejandra Pérez-Vega, Nerea Carrón and Pedro González-Torres; Funding acquisition, Marc Llirós, Isidre Elias, Xavier Aldeguer and Montserrat Fitó; Investigation, Karla Alejandra Pérez-Vega, Albert Sanllorente, María-Dolores Zomeño, Ana Quindós, Júlia Muñoz-Martínez, Daniel Muñoz, Sònia Gaixas and Gemma Blanchart; Methodology, María-Dolores Zomeño, Daniel Muñoz, Montserrat Fitó and Olga Castañer; Project administration, Montserrat Fitó; Resources, Ana Aldea-Perona, Isidre Elias, Núria Elias-Masiques and Montserrat Fitó; Supervision, Álvaro Hernáez and Montserrat Fitó; Validation, Montserrat Fitó; Visualization, Montserrat Fitó and Olga Castañer; Writing—original draft, Karla Alejandra Pérez-Vega and Montserrat Fitó; Writing—review & editing, Albert Sanllorente, María-Dolores Zomeño, Ana Quindós, Júlia Muñoz-Martínez, Mireia Malcampo, Ana Aldea-Perona, Álvaro Hernáez, Aleix Lluansí, Marc Llirós, Isidre Elias, Núria Elias-Masiques, Xavier Aldeguer, Helmut Schröder, Javier Hernando-Redondo, Nerea Carrón, Pedro González-Torres, Valentini Konstantinidou and Olga Castañer. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The generation and analysis of the data sets within this study are not projected to be open to access beyond the core research group. This is because the participants' consent forms and ethical approval did not include provisions for public accessibility. However, we follow a controlled data-sharing collaboration model, as the informed consent documents signed by the participants allowed for regulated collaboration with other researchers for study-related research. The data described in the manuscript, alongside the codebook and analytic code, will be available upon request. Researchers interested in this study can reach out to corresponding author Montse Fito (mfito@researchmar.net).

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