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Evaluation of the gut microbiota after metformin intervention in children with obesity: A metagenomic study of a randomized controlled trial

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ABSTRACT

Background: Metformin, a first-line oral antidiabetic agent that has shown promising results in terms of treating childhood and adolescent obesity, might influence the composition of the gut microbiota. We aimed to evaluate whether the gut microbiota of non-diabetic children with obesity changes after a metformin intervention.

Methods: The study was a multicenter and double-blind randomized controlled trial in 160 children with obesity. Children were randomly assigned to receive either metformin (1 g/day) or placebo for 6 months in combination with healthy lifestyle recommendations in both groups. Then, we conducted a metagenomic analysis in a subsample obtained from 33 children (15 metformin, 18 placebo). A linear mixed-effects model (LMM) was used to determine the abundance changes from baseline to six months according to treatment. To analyze the data by clusters, a principal component analysis was performed to understand whether lifestyle habits have a different influence on the microbiota depending on the treatment group.

Results: Actinobacteria abundance was higher after placebo treatment compared with metformin. However, the interaction *time x treatment* just showed a trend to be significant (4.6% to 8.1% after placebo vs. 3.8 % to 2.6 % after metformin treatment, $p = 0.055$). At genus level, only the abundance of *Bacillus* was significantly higher after the placebo intervention compared with metformin (2.5% to 5.7% after placebo vs. 1.5 % to 0.8 % after metformin treatment, $p = 0.044$). Furthermore, different ensembles formed by *Firmicutes*, *Bacteroidetes*, and *Verrucomicrobia* were found according to the interventions under a similar food consumption.

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; FDA, Food and Drug Administration; HDLc, high-density lipoproteins-cholesterol; HFD, high-fat-diet; HOMA-IR, homeostasis model assessment for insulin resistance; KMO, Kaiser-Meyer-Olkin; LB, Hospital Clínico Universitario Lozano-Blesa; LDLc, low-density lipoproteins-cholesterol; MVPA, moderate-to-vigorous physical activity; QUICKI, quantitative insulin sensitivity check index; RCT, randomized controlled trial; RS, Hospital Universitario Reina Sofía; SBP, systolic blood pressure; SENC, Spanish Community Nutrition Society; T2D, type 2 diabetes; US, Hospital Universitario de Santiago de Compostela; VN, Hospital Universitario Virgen de las Nieves: WC Waist circumference.

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Conclusion: Further studies with a large sample size controlled by lifestyle patterns are required in obese children and adolescents to clarify whether metformin might trigger gut microbiota alterations. Trial Registration: Registered on the European Clinical Trials Database (EudraCT, ID: 2010−023061-21) on 14 November 2011.

1. Introduction

Overweight and obesity in children are the most challenging health problems in the 21 st century [1]. In this regard, the composition of gut microbiota during early life has been suggested to influence the development of overweight/obesity in children [2]. Recent evidence suggests that gut microbiota is involved in the control of body weight, energy homeostasis, and inflammation, playing a role in the pathophysiology of obesity [3–6].

Metformin (1, 1- dimethyl biguanide) is an oral anti-hyperglycemic agent approved by the Food and Drug Administration (FDA) to treat type 2 diabetes (T2D) in adults and children older than 10 years of age. Beyond its antidiabetic effects, metformin has been considered a promising compound for the amelioration of childhood and adolescent obesity, especially through the reduction of body mass index (BMI) Z-score and waist circumference [7–9]. Interestingly, its absorption is limited to around 60 % of the administered dose, and the unabsorbed fraction of the drug is excreted unmodified in the feces [10]. Therefore, metformin is accumulated in the intestine at very high concentrations [11,12], which can lead to potential interaction with microbial communities and possibly it plays a role against obesity and its metabolic complications [13]. Hence, recent literature suggests that metformin action may be predominantly mediated via the gut [10,14]. Supporting this idea, studies in obese animal models [15,16] or with high-fat-diet (HFD) [13] show that gut microbiota and their metabolic pathways are altered by metformin treatment. Specifically, metformin enhances the abundance of *Akkermansia muciniphila* in obese animal models [13, 15,17] and decreases amounts of the bacteria which have been linked to the development of obesity, insulin resistance, and diabetes [18]. In humans, while several studies have investigated the effect of metformin on gut microbiota composition in diabetic subjects [19–23], only one has been focused on obese subjects [24]. Global gut microbial diversity indices were unaffected in obese women with a low-calorie diet after the metformin treatment. However, the authors observed an increase in Escherichia/Shigella abundance in the metformin-treated obese women, but *Akkermansia* did not change significantly. In obese children, the composition of gut microbiota after metformin intervention has not been studied. Furthermore, there is a lack of knowledge about how eating patterns in children with obesity are related to intestinal microbiota when they are undergoing an intervention with metformin. Previously, we conducted a randomized control trial (RCT) in 160 children with obesity and demonstrated that a 6-month intervention with 1 g/day of metformin and lifestyle recommendations decreases the BMI Z-score and improves inflammatory and cardiovascular-related obesity parameters [8]. Stomach pain and diarrhea were the most common adverse effects reported in our study, comprising 18.8 % and 13 % of the cases, respectively. Therefore, we pretend 1) to investigate the alteration of the gut microbiota in 33 children with obesity after metformin intervention in comparison with a placebo group, and 2) to assess the relationships between lifestyle characteristics and gut microbiota, highlighting the potential impact of dietary and physical activity patterns on microbiome of children with obesity.

2. Methods

2.1. Study design, participants and intervention

The study was a multicenter and double-blind RCT, stratified according to sex and pubertal status in 160 non-diabetic children with obesity. The pubertal stage was determined according to Tanner criteria [25] and obesity defined according to BMI by using the age- and sex-specific cut-off points proposed by Cole et al. (BMI greater than the 95th percentile) [26]. All the children were randomly assigned to receive 1 g/day of metformin or placebo for 6 months after meeting the defined inclusion criteria [8,27]. Both experimental groups included a lifestyle recommendation program. Further details regarding study protocol [27], informed consent and ethics, design, sample size, intervention and participants (participant's data collection and processing, samples codification, randomization method, double-blind condition, and side effects assessment) have been previously described [8,27]. The CONSORT statement (Consolidated Standards of Reporting Trials) has been considered in the study design report and the abstract. The study was registered by the European Clinical Trials Database (EudraCT, ID: 2010−023061-21) on 14 November 2011 (URL: https://www.clinicaltrialsregister.eu/ctrsearch/search?query=2010−023061-21).

2.2. Anthropometric and biochemical measurements

The procedure for the collection of the data concerning anthropometry, blood pressure, and the biochemical parameters measured in the current study have been previously reported [8,27]. The Quantitative Insulin Sensitivity Check Index (QUICKI) and the homeostasis model assessment for insulin resistance (HOMA-IR) were calculated using the fasting plasma glucose and insulin values: fasting insulin (μU/L) x fasting glucose (nmol/L)/22.5 and 1 / (log(fasting insulin) + log(fasting glucose)), respectively.

2.3. Metagenomic analysis

For the metagenomic analysis, a subsample was obtained from 63 children, which both they and their parents/guardians agreed with collecting the stools. Each volunteer provided fecal samples (100−200 g), both at the beginning and the end of the trial. The fecal samples were collected in a hermetically sealed, sterile container provided by the Pediatric Endocrinology Unit of the corresponding study centers. Samples were immediately refrigerated in household freezers and brought in a small fridge with patch of ice to corresponding hospital within 12 h after collection and then transferred to −80 °C until they were used for the analysis. The reception of samples occurred exclusively in the morning (8.30–10.30 a.m.). However, we selected those children with stool samples both at the beginning and at the end of the intervention (n = 33 paired samples: 15 metformin, 18 placebo). Figure S1 describes all the steps for the metagenomics analysis.

2.3.1. DNA extraction

Samples were homogenized in a Stomacher-400 blender. Subsequently, a QIAamp DNA Stool Mini Kit (QIAGEN, Barcelona, Spain) was used for the DNA extraction as indicated by the manufacturer with the exception of the incubation at 70 °C, since the samples were mixed with the lysis buffer and incubated at a temperature of 95 °C to make sure that both Gram-positive and Gram-negative were lysed. The quantification of the DNA was conducted with a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, DE, USA) and the DNA yield was calculated by measuring absorbance ratios spectrophotometrically, including A260/230 nm for salt and phenol contamination and A260/280 nm for protein contamination [28].

2.3.2. Sequencing and taxonomic analysis

The amplification of the extracted DNA was done through PCR using the primer pairs, 16S Amplicon PCR forward primer:

5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG, and 16S Amplicon PCR Reverse Primer: 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHV GGGTATCTAATCC targeting the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene [29]. Every single PCR was performed in 25 μL reaction volumes of which 12.5 μL was 2X KAPA HiFi Hotstart ready mix (KAPA Biosystems, Woburn, MA, USA). The rest corresponded to 5 μL of each forward and reverse primers (1 μM) and 2.5 μL of extracted DNA (10 ng). The cycling conditions were also the same for each PCR: initial denaturation at 95 °C for 3 min, followed by cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s, with a final extension at 72 °C for 5 min. AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA) were used for the PCR clean-up to purify the 16S V3 and V4 amplicon away from free primers and primer-dimer species. After this, the index PCR was performed under 95 °C for 3 min; 8 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 138 s; 72 °C for 5 min, and hold at 4 °C, using the Nextera XT Index Kit (Illumina, San Diego, CA, USA) to attach dual indices and Illumina sequencing adapters. Then, before the quantification, the pooled PCR products were purified using AMPure XP beads (Beckman Coulter, Indianapolis, IN, USA). The resultant amplicons were sequenced at MiSeq (Illumina, USA), using paired-end (2 × 300nt) Illumina MiSeq sequencing system (Illumina, San Diego, CA, USA) [28].

Microbial community diversity was analyzed. Hence, diversity (H), Simpson index, inverse Simpson index, Alpha index (Fisher), species richness, Pielou's evenness index, and beta diversity indexes were calculated with Vegan v.2.4−2 in R package with the option ecological diversity indices at the phylum level (Table S1) [29]. The observed relative abundance of each taxon was estimated by counting the number of reads for each taxon and then this was normalized by the total number of reads per sample. The phyla and genera represented in this study were those with a relative abundance higher than ≥ 0.1 %. However, based on the literature in relation to a possible effect of metformin on enhancing the abundance of *A. muciniphila* in obese animal models [13,17,30], its genus, *Verrucomicrobia*, was included although the abundance was < 0.1 %.

Negative controls from laboratory reagents were processed in parallel with the samples to assess for possible microbial contamination.

2.4. Lifestyle monitoring

The dietitians at the centers applied a food frequency questionnaire and a physical activity survey to all subjects at the beginning and at the end of the study, both of them validated and normalized [31]. The food groups were classified as 1) Cereals, pasta and legumes, 2) Vegetables, 3) Fruits and fresh fruit juices, 4) Olive oil, 5) Milk products, 6) Fish, 7) Eggs and white meat, 8) Fat meat and processed cold-meat, 9) Sweets, snacks and soft drinks, 10) Butter, cakes and pastry. Thereupon, the Spanish Community Nutrition Society (SENC) Guidelines for scholar age were used to compare the food habits of the studied children with the standard recommendations [32]. For sedentary habits, we grouped the following data to create the "screen time" variable (measured as 2 h/day in front of the screen): "How much time does your child spend for watching TV, video or DVD", "How much time does your child spend in front of the computer (for internet, video games…)", How much time does your child spend using the game console?", "How much time does your child spend on using the mobile phone?". Furthermore, to obtain the minutes/day of moderate-to-vigorous physical activity (MVPA), we used the data based on the following questions: "How many hours per day do you practice sports with moderate efforts (running, sky, dancing, ball games, swimming…)", How many hours per day do you practice sports with vigorous efforts (sport training)?".

Moreover, the data collected in the lifestyle habits questionnaire were evaluated according to the healthy lifestyle-diet index (HLD-index) described by Manios et al. to ensure a routine quality estimation. The total score on the HLD-index ranges from 0 to 48 [33]. Higher scores on the HLD-index indicate greater adherence to dietary–lifestyle recommendations or to a 'healthy' dietary–lifestyle pattern. Based on this scoring, Manios et al. considered three groups by tertiles of the HLD-index: unhealthy lifestyle-diet pattern = ranging from 1 to 16; moderately healthy lifestyle-diet pattern = ranging from 17 to 32; and healthy lifestyle-diet pattern = ranging from 33 to 48.

2.5. Statistical analysis

Data are given as the mean and standard mean error (SEM). $P < 0.05$ was considered to be statistically significant. Variables that were not normally distributed were log-transformed for analysis, and/or values with ± 3SD of the mean (outliers) were removed (without achieving values loss from samples of up to 15 %). However, the data are presented as untransformed values to ensure a clear understanding. For the clinical characteristics of the participants, differences at baseline and post-treatment per experimental group were assessed by using Student *t*-test or U Mann-Whitney test when variables showed a no normal distribution. For the relative abundances of bacteria (phylum and genus), U Mann-Whitney test was applied for assessing differences at baseline, as well as for the alpha indexes and beta diversity (Table S1). ANCOVA was used at the post-treatment stage when differences at baseline in the clinical characteristics were observed between experimental groups. For categorical variables (sex, pubertal stage, center, screen time, food frequency and gastrointestinal side effects), the $\chi 2$ test was applied.

A linear mixed-effects model (LMM) was used to determine the abundance changes from baseline to six months according to treatment. The specific differences between the treatments were assessed by post hoc Bonferroni tests. The fixed effects included in all the models were sex, pubertal stage, and adherence to the treatment (% based on the following formula: ((Pills ingested – pills returned) / Pills predicted) x 100) and the *time x treatment* interaction, while subject and center were included as random effects. The normality of residuals was checked for all the models and Q—Q plots are available upon request. To analyze the data by clusters, a principal component analysis was performed to understand whether lifestyle habits have a different influence on the microbiota depending on the intervention group, maximizing the information gained for the predominant bacterial variables. We have mixed here bacterial variables and alimentary groups obtained from food frequency questionnaire and some lifestyle habits such as screen time and MVPA. This mathematical model calculates new variables (principal components) that account for the variability in the metagenomic data and enables the study of covariance or correlations between variables (e.g., eggs and white meat (portions/week), fruit and fresh juices (portions/day), among others). The combination of diet and physical activity variables with the greatest amount of variability is the first principal component. The subsequent components (second and third principal components) describe the maximum amount of remaining variability [34]. All of the analyses were performed using SPSS software version 24 (SPSS Inc., Chicago, IL, USA).

3. Results

The general and clinical characteristics of the children at baseline and after 6 months for each intervention group are reported in Table 1. Differences in the prevalence of gastrointestinal side effects were not observed. Differences in the food frequency were not observed per treatment ($p > 0.05$), except the fish intake at baseline ($p = 0.025$) (these data are not presented here, but are available upon request). In bacteria, it did not show any significant differences at baseline ($p > 0.05$) and consequently these are not presented here, but are available upon request.

Tables 2 and 3 show the relative abundances of predominant phyla and genera in the studied children, respectively. Reads were classified into more than 4000 different taxons. All samples were rarefied to prevent bias due to sampling depth. At the phylum level, the relative

Table 1
Clinical characteristics of the study population at baseline and post-treatment stages.

	Baseline			6 months		
	Placebo	Metformin n = 15	*P*-value [a]	Placebo n = 18	Metformin n = 15	*P*-value [a]
Adherence (%)[b]	–	–	–	88.8 (3.9)	93.9 (3.1)	0.078
Sex (Females, %)	33.3	60	0.126	–	–	–
Pubertal stage (Prepubertal, %)	22.2	13.3	0.510	–	–	–
Centre (RS/VN/US/LB)	11.1/22.2/33.3/33.3	0/20/20/60	0.321	–	–	–
Screen time (> 2 h/d) (%)	33.3	33.3	1	33.3	46.7	0.219
MVPA (min/d)[b]	63.1 (17.9)	87.6 (17.6)	0.073	22.5 (7.03)	19.4 (3.09)	0.393
Age (y)	12 (0.6)	12 (0.4)	0.955	12.5 (0.6)	12.8 (0.3)	0.624
Abdominal pain (yes, %)	–	–	–	11.1	26.7	0.239
Diarrhea (yes, %)	–	–	–	5.6	20	0.178
BMI Z-score	3.5 (0.2)	2.8 (0.2)	**0.034**	3.05 (0.19)	2.38 (0.25)	0.433[c]
WC (cm)	97.2 (3)	90.3 (2.9)	0.110	95.2 (2.7)	89.5 (2.2)	0.139
Fat mass (%)	36.8 (1.3)	37.2 (1.2)	0.853	34.7 (1.5)	35.6 (1.3)	0.656
Lean mass (%)	60 (1.9)	52.3 (3)	**0.032**	64.7 (2.3)	67.8 (3.8)	0.272[c]
DBP (mmHg)	69.2 (2.2)	68.2 (1.9)	0.736	68.3 (2.3)	65.7 (2.5)	0.448
SBP (mmHg)	116.7 (3.2)	115.8 (2.8)	0.844	115.9 (2.8)	111.6 (3.5)	0.343
Fasting glucose (mg/dL)	83.7 (1.5)	87.5 (1.5)	0.082	84.1 (1.4)	84.7 (2.2)	0.793
Fasting insulin (μU/mL)	22.5 (2.9)	23.4 (2.6)	0.809	20.4 (1.8)	22.2 (3.2)	0.593
HOMA-IR index	4.8 (0.6)	5.1 (0.6)	0.712	4.2 (0.4)	4.6 (0.6)	0.622
QUICKI index[b]	0.3 (0.03)	0.3 (0.02)	0.516	0.3 (0.02)	0.3 (0.03)	0.729
Total cholesterol (mg/dl)	156.6 (6)	155.1 (6.2)	0.865	154.1 (7.3)	154.1 (5.3)	0.997
TG (mg/dl)	20.4 (1.8)	22.2 (3.2)	0.433	73.4 (7.6)	85.8 (13.9)	0.465
HDLc (mg/dl)	45.8 (2.5)	49.8 (2.4)	0.269	45.7 (2.6)	53.3 (3.2)	0.073
LDLc (mg/dl)	96.4 (5.9)	88.9 (4.6)	0.336	92.3 (6.7)	84.3 (4.8)	0.368

Data are expressed as mean (standard error of mean) for continuous variables or n for categorical variables.
[a)]Differences between experimental groups at baseline and post-treatment were analyzed using the Student *t*-test or U Mann-Whitney test (marked as [b]) for quantitative variables, or ANCOVA (marked as [c]) when differences at baseline were observed between experimental groups. $\chi 2$ test was used for categorical variables. *P*-value $<$ 0.05 marked in bold. Abbreviations: BMI, body mass index, DBP, diastolic blood pressure, HDLc, high-density lipoproteins-cholesterol, HOMA-IR, homeostasis model assessment for insulin resistance, LB, Hospital Clínico Universitario Lozano-Blesa, LDLc, low-density lipoproteins-cholesterol, MVPA, moderate-to-vigorous physical activity, QUICKI, quantitative insulin sensitivity check index, RS, Hospital Universitario Reina Sofía, SBP, systolic blood pressure, US, Hospital Universitario de Santiago de Compostela, VN, Hospital Universitario Virgen de las Nieves, WC, Waist circumference.

Table 2
Relative abundances of bacteria in fecal microbiota of studied children at the phylum level.

Bacterial variables	Placebo		Metformin		*P*-value [a]
Phylum	Baseline (n = 18)	6 months (n = 18)	Baseline (n = 15)	6 months (n = 15)	
Actinobacteria	4.6 (1.3)	8.1 (1.5)	3.8 (1.5)	2.6 (1.6)	0.055
Bacteroidetes	34.1 (4.1)	28.1 (4.5)	32.4 (5.2)	38.8 (5.6)	0.125
Firmicutes	49.1 (3.6)	50.4 (3.8)	49.3 (4.6)	46.6 (4.8)	0.455
Proteobacteria	0.4 (0.1)	0.6 (0.1)	0.6 (0.2)	0.6 (0.2)	0.660
Verrucomicrobia	3.3 (1.5)	2.9 (1.6)	5.6 (1.8)	4.5 (2)	0.810
Sequences	291,688 (177,933–347,856)	312,575 (211,447–825,954)	294,294 (126,082–656,710)	289,671 (244,278–364,642)	
Alpha diversity	39.5 (18–78)	45.5 (11–68)	37 (5–74)	42 (21–56)	
Unclassified sequences derived from bacteria	4.4 (1.0–16.2)	4.9 (0.7–33.5)	5.2 (1.4–50.9)	7.2 (0.8–18.6)	

Phylum abundance is expressed as mean adjusted (standard error of mean). A linear mixed-effects model (LMM) was used to determine the abundance changes from baseline to six months according to treatment. The specific differences between the treatments were assessed by post hoc Bonferroni tests. The fixed effects included in all the models were sex, pubertal stage, and adherence to the treatment (% based on the following formula: ((Pills ingested – pills returned) / Pills predicted) x 100) and the *time x treatment* interaction, while subject and center were included as random effects. [a] P-value for the *time x treatment* interaction. Table 2 shows only the phylum abundances with a value $\geq$ 0.1 %.

abundance was not different by treatment at the end of the study (Table 2). *Actinobacteria* was higher after placebo treatment compared with metformin. However, the interaction *time x treatment* just showed a trend to be significant (p = 0.055) (Table 2). At the genus level, only the abundance of *Bacillus* was significantly higher after the placebo intervention compared with metformin (p = 0.044) (Table 3).

Principal component analyses were performed with bacteria and lifestyle variables, according to alimentary groups with portions per day or per week, and screen time and MVPA as the variables of sedentary habits and physical activity, respectively. The variables that were finally maintained in the principal component analyses (Fig. 1) were selected according to the Kaiser-Meyer-Olkin (KMO) (p > 0.05), to warrant sampling adequacy for each analysis [34].

In placebo group, component one was defined by the consumption of fish, butter, cakes, pastry and *Bacteroidetes* that were correlated inversely with the consumption of milk products, *Firmicutes* and *Verrucomicrobia* (Fig. 1. A). Component two was defined by the consumption of butter, cakes and pastry, eggs and white meat and vegetables that were negatively correlated with MVPA. Finally, component three has shown *Verrucomicrobia* and fruits and fresh juices consumption correlated negatively with butter, cakes and pastry and fatty meat and processed cold-meat. In children that received metformin, component one was defined by the consumption of fatty meat and processed cold-meat, *Firmicutes* and MVPA that were correlated inversely with *Bacteroidetes* and consumption of vegetables (Fig. 1B). Fruits and fresh juices, fish and eggs and white meat consumption established component two and were

Table 3
Relative abundances of bacteria in fecal microbiota of studied children at the genus level.

Bacterial variables	Placebo		Metformin		*P*-value [a]
Genus	Baseline (n = 18)	6 months (n = 18)	Baseline (n = 15)	6 months (n = 15)	
Acetivibrio	1.8 (0.3)	1.6 (0.3)	1.9 (0.4)	2.1 (0.4)	0.543
Acidaminococcus	1.3 (0.6)	1.1 (0.6)	1.6 (0.7)	1.7 (0.8)	0.803
Agreia	3.3 (1.5)	3.2 (1.7)	5.8 (2)	4.7 (2.1)	0.775
Akkermansia	0.011 (0.009)	0.008 (0.010)	0.007 (0.011)	0.021 (0.012)	0.378
Alicycliphilus	4.5 (1.1)	2.6 (1.2)	5.4 (1.4)	5.6 (1.5)	0.319
Avibacterium	0.9 (0.3)	0.6 (0.4)	1.1 (0.4)	1.2 (0.5)	0.603
Bacillus	2.5 (1)	5.7 (1)	1.5 (1.2)	0.8 (1.3)	0.044
Bavariicoccus	0.7 (0.2)	1.3 (0.2)	0.8 (0.2)	0.7 (0.2)	0.061
Bradyrhizobium	1.2 (0.4)	2.1 (0.5)	0.7 (0.5)	1.5 (0.6)	0.937
Chlamydia	2 (0.6)	2.4 (0.7)	2.2 (0.8)	1.5 (0.8)	0.395
Desulfosalina	4.1 (0.7)	4.7 (0.7)	3.8 (0.8)	3.6 (0.9)	0.532
Desulfotomaculum	12.1 (1.8)	11.2 (2)	9.7 (2.2)	7.5 (2.4)	0.678
Flexibacter	0.3 (0.1)	0.4 (0.1)	0.4 (0.1)	0.4 (0.2)	0.51
Marinilabilia	0.4 (0.1)	0.2 (0.1)	0.5 (0.1)	0.4 (0.1)	0.748
Nautilia	6.4 (3.5)	7.4 (3.6)	7.3 (4.3)	9.1 (4.4)	0.825
Oscillochloris	0.5 (0.2)	0.6 (0.2)	0.7 (0.3)	0.5 (0.3)	0.554
Oxalobacter	2.9 (0.7)	4.8 (0.8)	2.1 (0.9)	2.9 (1)	0.435
Paraprevotella	3.9 (0.7)	5.2 (0.7)	4.5 (0.9)	5.4 (0.9)	0.754
Stackebrandtia	5.2 (2.2)	8.4 (2.3)	7.5 (2.8)	7.5 (2.9)	0.275
Streptococcus	0.2 (0.1)	0.2 (0.1)	0.2 (0.1)	0.1 (0.1)	0.137
Veillonella	0.4 (0.1)	0.4 (0.1)	0.2 (0.1)	0.3 (0.1)	0.988

Phylum abundance is expressed as mean (standard error of mean). A linear mixed-effects model (LMM) was used to determine the abundance changes from baseline to six months according to treatment. The specific differences between the treatments were assessed by post hoc Bonferroni tests. The fixed effects included in all the models were sex, pubertal stage, and adherence to the treatment (% based on the following formula: ((Pills ingested – pills returned) / Pills predicted) x 100) and the *time x treatment* interaction, while subject and center were included as random effects. [a] P-value for the *time x treatment* interaction. P-values < 0.05 marked in bold. Table 3 shows only the genus abundances with a value ≥ 0.1 %.

negatively correlated with *Verrucomicrobia* and MVPA. Finally, component three was defined with the positive correlation between the consumption of milk products, butter, cakes and pastry and MVPA correlated inversely with fish consumption and *Verrucomicrobia*.

4. Discussion

The current study evaluates the fecal microbiota in children with obesity after a metformin intervention vs. another based-on placebo, both with lifestyle recommendations.

Although the *time x treatment* interaction was not significant, we observed an interesting change in the *Actinobacteria* abundance from 4.6 to 8.1% after placebo treatment, in comparison with metformin which decreased from 3.8 to 2.6 %. Furthermore, the abundance of *Bacillus* species increased significantly from 2.5 to 5.7 % in placebo compared with metformin group, that showed a slight reduction from 1.5 to 0.8 %. It is known that obesity contributes to disturb microbial diversity [35]. Concretely, both the abundance of *Actinobacteria* and *Bacillus* are increased in obesity status [36–39]. In this context, metformin might slightly attenuate the increment of them in children with obesity, although further evidence is needed to elucidate the effect of metformin on *Actinobacteria* abundance on this population. Recently, a study showed that the increased abundance of *Actinobacteria* observed in T2D mice decreased in the group treated with metformin [40]. Dietary and physical activity patterns have important effects on the microbiota [18, 41]. Moreover, in rodents, metformin has been shown to modify gut microbiota composition and diversity, but in a diet-dependent manner [2526 genes]. Actually, Shin et al. [25] showed that there are significant differences in the abundance of *Firmicutes* and *Bacteroidetes*, between metformin-treated and non-treated mice, but only under a HFD. Similarly, Lee and Ko [26] observed that metformin induces a decrease in bacterial diversity in mice under a HFD. Thus, we considered to take a further step and to analyze jointly the lifestyle and the gut microbiota in each intervention group. When data about lifestyle were introduced as

a)

Variables for Placebo group	PRINCIPAL COMPONENTS		
	Component 1	Component 2	Component 3
Bacteroidetes	-0.904		
Firmicutes	0.711		
Verrucomicrobia	0.757		-0.302
Fruits and fresh juices (portions/day)			-0.680
Milk products (portions/day)	0.776		
Fish (portions/week)	-0.369		
Eggs and white meat (portions/week)		0.827	
Vegetables (portions/day)		0.549	
Butter, cakes and pastry (portions/week)	-0.507	0.304	0.533
Fatty meat and processed cold-meat (portions/week)			0.881
MPVA (minutes/day)		-0.875	
Variance (%)	30.9	19.3	12.5
Cumulative proportion of variance (%)	30.9	50.2	62.7
KMO (*P*-value)	0.227		

b)

Variables for Metformin group	PRINCIPAL COMPONENTS		
	Component 1	Component 2	Component 3
Bacteroidetes	-0.839		
Firmicutes	0.631		
Verrucomicrobia		-0.790	-0.381
Fruits and fresh juices (portions/day)		0.887	
Milk products (portions/day)			0.703
Fish (portions/week)		0.660	-0.546
Eggs and white meat (portions/week)		0.699	
Vegetables (portions/day)	-0.693		
Butter, cakes and pastry (portions/week)			0.815
Fatty meat and processed cold-meat (portions/week)	0.889		
MPVA (minutes/day)	0.393	-0.341	0.494
Variance (%)	27.6	19.7	17.5
Cumulative proportion of variance (%)	27.6	47.4	64.9
KMO (*P*-value)	0.194		

Fig. 1. Principal component analysis between intestinal microbiota and lifestyle variables in children treated with metformin and placebo after 6 months of the intervention. Strong loading was defined as a value < 0.6, moderate as 0.3-0.59 and low as <0.3. KMO, Kaiser-Mayer-Olkin. a) Placebo group, b) Metformin group.

variables to build principal components with the fecal microbiota, we detected interesting predominant variables grouped differently according to the interventions despite both treatment groups had a similar food intake. Here, although *Bacteroidetes*, *Firmicutes* and *Verrucomicrobia* were the phyla that finally constituted the principal components in both experimental groups, the correlations with lifestyle variables within the components and the importance of them differed by groups. For instance, *Verrucomicrobia* composed the components one and three in placebo at the end of the intervention, whereas in the metformin group was presented in components two and three with similar loadings, but its correlations showed opposed directions with the lifestyle variables (Fig. 1A and B); fish, butter, cakes and pastry, and fatty meat and processed cold-meat consumption in the placebo group, and fruits and fresh juices, fish, eggs and white meat, milk products and butter, cakes and pastry in the metformin group. *Bacteroidetes* and *Firmicutes* were located in the same component at the end of the intervention in both groups (Fig. 1A and B), whereas the lifestyle patterns in component one were differently expressed by treatment. According to our data in the placebo group, several genera belonging to phylum *Bacteroidetes* were negatively associated with milk products, concretely yoghurt intake, in a study with younger children [42] and with young adults [43]. Furthermore, *Verrucomicrobia* constituted component one in the placebo group compared with the metformin. Our results have shown that *Verrucomicrobia* is more correlated with the placebo group in opposite with data previously published [13] in which this phylum was significantly increased in the obese mice treated with metformin.

To the best of our knowledge, this is the first study that analyses the gut microbiota in children with obesity after metformin treatment. As the effect of metformin on gut microbiota composition seems to differ under healthy and diabetes status [44,45], this could also occur in obesity conditions. To date, only one study in obese subjects has been performed to evaluate the effect of metformin (in addition to low-calorie diet) on the gut microbiota [24]. The authors reported no significant changes in the overall fecal microbial composition and diversity. However, we have to consider that the population studied was adult women and it is known that sex and age are important factors that influence gut microbiota [46,47]. Furthermore, as a decreased abundance of *A. muciniphila* has been associated with the presence of T2D [17], this bacteria is proposed to be a biomarker for glucose intolerance [48]. Moreover, it has also been inversely associated with several metabolic complications in humans [49–51]. *Akkermansia* is the main genus reported as increased after metformin treatment in diabetic mice and humans [17,19], and it is suggested as a possible mechanism of action to exert the antidiabetic effects [17]. However, to date, any study in non-diabetic obese subjects treated with metformin has proposed that *A. muciniphila* could be increased using metformin in obese or healthy populations [24,52]. Indeed, de la Cuesta-Zuluaga et al. observed an increment of the relative abundance of *A. muciniphila* after taking metformin, in participants with T2D, but not in those without T2D [19]. The no-effect of metformin on this specie in our subjects could be explained due to the no alteration of *Akkermansia* in the gut microbiota. Unfortunately, we have not a control group based on healthy children to compare the abundance of *Akkermansia*.

Evidence that abnormalities in the microbiota composition can have a major role in the development of obesity and diabetes is constantly growing, and also that metformin may mediate its action by gut bacteria [53]. Nevertheless, the evidence is still fairly scarce in the young population and nothing has been reported in terms of obesity in children and adolescents. One of the major concerns is that obese children are highly prone to becoming obese adults and developing severe co-morbidities such as metabolic syndrome, T2D and cardiovascular disease [54]. For these reasons, the prevention and management of obesity must begin before the adult stage. Unfortunately, single-strategy lifestyle intervention is not always effective [55]. Indeed, we previously observed a reduction of BMI Z-score in the current study, but the results were much more promising in the metformin arm of prepubertal children after the 6 months of the intervention [8].

A limitation of the current study was the reduced sample size for the metagenomic analysis due to the low level of involvement from the subjects or their parents/guardians to bring the fecal samples. The limited sample size also hampered that we could achieve a greater number of variables included, such as the bacterial genera, for the component analyses. Furthermore, the single nutrient intake collected by 24 -h recalls might have provided more information in relation to the specific nutrients supplied by the participant's diet. However, this study provides the first data regarding metformin and gut microbiota in obese children that might encourage to increase the evidence on this relevant issue by further metagenomic studies with greater sample size. We highlight the importance to perform future longer term RCTs with a large sample size that enable an appropriate stratification by pubertal stage to investigate possible and interesting links between the benefits demonstrated by metformin along with a healthy lifestyle in terms of weight loss and metabolic complications and the alteration of gut microbiota. Indeed, the role of metformin on gut microbiota is rising as relevant research focus to elucidate novel mechanisms for its therapeutic effect.

5. Conclusions

Actinobacteria showed to be higher, but not significantly, after placebo treatment compared with metformin. At the genus level, only the abundance of Bacillus was significantly higher after the placebo intervention compared with metformin. These findings could contribute to explain a possible attenuation in the increment of these bacteria by metformin in children with obesity. Moreover, different ensembles of predominant variables were found according to the interventions, under a similar food consumption. Here, *Firmicutes*, *Bacteroidetes*, and *Verrucomicrobia* characterized different patterns along with specific food groups. Further studies with a large sample size controlled by lifestyle patterns are needed in obese children and adolescents to clarify the possible alterations that metformin might trigger in gut microbiota.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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