Open Access Library Journal
2024, Volume 11, e12342
ISSN Online: 2333-9721
ISSN Print: 2333-9705

Two-Sample Mendelian Randomization Analysis of the Causal Relationship between Intestinal Flora and Childhood Obesity

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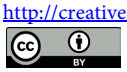
How to cite this paper: Xu, D.W. and Wang, H. (2024) Two-Sample Mendelian Randomization Analysis of the Causal Relationship between Intestinal Flora and Childhood Obesity. *Open Access Library Journal*, **11**: e12342.
https://doi.org/10.4236/oalib.1112342

Received: September 19, 2024
Accepted: October 15, 2024
Published: October 18, 2024



Abstract

Objective: To evaluate the potential causal relationship between 182 gut microbiota and childhood obesity by two-sample Mendelian randomization. Methods: The data source was the summary data of genome-wide association study (GWAS), and 182 intestinal microbiota contained 874 single nucleotide diversity (SNPs). The childhood obesity data contained 2,442,739 SNPs. Mendelian randomization analysis was performed using three methods: inverse variance weighting (IVW), weighted median, and MR Egger regression. Subsequently, heterogeneity test, horizontal pleiotropy test, MR PRESSO method and leave one out analysis were used to detect outliers. Outcome: IVW analysis showed that class Erysipelotrichia (OR = 0.530, 95% CI: 0.293 - 0.958, P = 0.035), family Verrucomicrobiaceae (OR = 0.475, 95% CI: 0.311 - 0.726, P = 0.001), and genus Akkermansia (OR = 0.476, 95% CI: 0.311 - 0.726, P = 0.001) and order Verrucomicrobiales (OR = 0.475, 95% CI: 0.311 - 0.726, P = 0.001) were protective factors for the development of childhood obesity, while genus Ruminiclostridium 9 (OR = 2.051, 95% CI: 1.069 - 3.932, P = 0.031) was potential risk factor for childhood obesity. Conclusion: Genus Ruminiclostridium 9 is positively correlated with childhood obesity, while class Erysipelotrichia, family Verrucomicrobiaceae, genus Akkermansia, and order Verrucomicrobiales are negatively correlated.

Subject Areas

Bioinformatics, Microbiology, Pediatrics, Public Health

Keywords

Childhood Obesity, Intestinal Flora, Mendelian Randomization, Causality

1. Introduction

Obesity is a metabolic disease in which an imbalance between long-term energy intake and expenditure leads to excessive energy storage in the form of fat. It is considered a "common ground" for many chronic noncommunicable diseases [1]. Overweight and obesity are usually measured by the Body Mass Index (BMI), which = weight (kg)/height squared (m^2) [2]. Studies predict that by 2035, more than 4 billion people may be affected, and the prevalence of obesity alone (BMI ≥ 30 kg/m^2) is expected to rise from 14% to 24%, affecting nearly 2 billion people by 2035 [2]. The human gut microbiota is a multifaceted biological network of bacteria, archaea, viruses, fungi, and protozoa that far outnumber the number of human "self" cells that pass through mammalian germlines. The human gut microbiota contains almost 100 times more genetic material than the human genome [3]. The modern concept of intestinal microbiota points out that the gut is not isolated. It is closely related to the body's immune system, nervous system, circulatory system, and endocrine system. The core functions of a healthy gut microbiota include the biodegradation of polysaccharides, the production of short-chain fatty acids, the enrichment of specific lipopolysaccharides, and the production of vitamins and some essential amino acids [4]. A healthy gut microbiota is highly diverse, and when its diversity decreases, it can lead to diseases such as obesity, and there is growing evidence that the gut microbiota plays a key role not only in the metabolism of nutrients and drugs and in the absorption of dietary fats, but also in the regulation of immunity, physiology, metabolism, and health maintenance [5] [6].

However, there have been no reports of Mendelian randomization to assess the causal relationship between childhood obesity and gut microbiota. Therefore, this study used Mendelian randomization and single nucleotide polymorphisms (SNPs) as instrumental variables (IVs) to explore the potential causal relationship between gut microbiota and childhood obesity, in order to provide new ideas for childhood obesity from the aspects of microbial markers and microbiota modification therapy.

2. Method

2.1. Genome-Wide Association Study (GWAS) Data Source

The IVs exposed to the intestinal microbiota were obtained from the study of Kuril Shikov *et al.* [7] in the MiBioGen consortium, with a total sample size of 18,340 cases, and a total of 24 cohorts (11 countries including Europe and North America) were included, with a total of 5,747,754 SNPs. Outcome data: Childhood obesity is available from IEU data (https://gwas.mrcieu.ac.uk/). The "childhood obesity" phenotype was used in this study, with a GWAS of 13,848 European participants with 5530 cases in the case group and 8318 in the control group, with a total of 2,442,739 SNPs. It should be noted that the effect allele frequencies are missing [8]. All analyses in this study are based on publicly available data and do not require additional ethical approval.

2.2. Two-Sample Mendelian Randomization Study Design

Two sample Mendelian randomization (TSMR) is one of several methods for Mendelian randomization (MR) analysis. Compared to other types of MR, TSMR can improve detection efficiency [9]. GWAS pooled data typically have large sample sizes, and these existing pooled data from large-scale genome-wide association studies make statistical power generally stronger. The large number of optional IVs can increase confidence in the genetic interpretation of the exposure instrumental variables. Based on the published GWAS summary data, 15 intestinal microbiota with unknown names were first excluded, and then a total of 182 intestinal microbiota (9 phyla, 16 classes, 20 orders, 33 families, and 116 genera) containing 5 biological taxonomic levels were analyzed using a two-sample Mendelian randomization method to analyze the potential causal relationship between childhood obesity. Finally, sensitivity analysis is performed to ensure the robustness of the causal relationship. This study followed the latest STROBE MR guidelines for Mendelian randomization analysis [10] and was conducted under three core instrumental variable assumptions: 1) association hypothesis: instrumental variables are strongly correlated with gut microbiota; 2) the independence assumption: the instrumental variables are independent from the confounders; 3) Exclusion hypothesis: instrumental variables can only have an effect on the outcome of childhood obesity through gut microbiota, as shown in **Figure 1**.

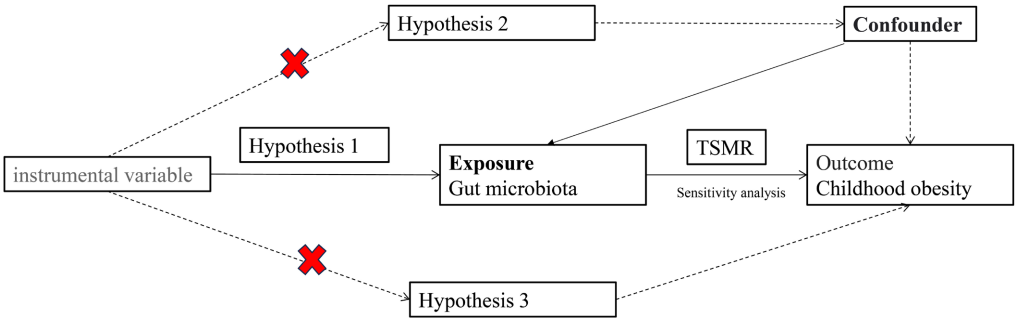


Figure 1. Three main assumptions of Mendelian randomization.

2.3. Screening of Instrumental Variables (IVs)

1) IVs must be strongly correlated with the intestinal microbiota of the exposure factor. In order to ensure the authenticity and accuracy of the causal link between gut microbiota and childhood obesity, the following steps were used to select SNPs strongly associated with exposure factors: a) The number of intestinal microbiota SNPs was small, and only a small number of SNPs could reach the threshold result of genome-wide statistical significance ($P < 0.05$), so the threshold was adjusted to after referring to relevant literature ($P < 0.01$) to obtain complete and reliable results [11]. and b) The results may be biased due to linkage disequilibrium (LD). So set the LD coefficient $R^2 = 0.001$, a width of 10,000 kb to exclude the influence of gene pleiotropy on the results as much as possible. SNPs with the lowest P value associated with the gut microbiota were selected. 2) Calculate the F-statistic to

evaluate the intensity of SNPs. Using F = 10 as the criterion, if F ≥ 10, the selected instrumental variable is not biased [12]. In addition, the Mendelian method of randomized pleiometric residuals and outliers (MRPRESSO) was used to detect outliers in MR analysis and remove outliers to provide corrected MR estimates to eliminate the effect of horizontal pleiotropy. 3) In order to satisfy the independence hypothesis of one of Mendel's three major hypotheses and eliminate possible confounding factors, this study is also published on the GWAS Catalog website (https://www.ebi.ac.uk/gwas/) performed a manual search for each SNP that was screened for relevance to the gut microbiota.

2.4. Mendelian Randomization Analysis

In this study, Mendelian randomization randomized random-effects inverse variance weighted (IVW), weighted median, and MR Egger regression were used to verify the causal relationship between gut microbes and childhood obesity in 182 taxa [13]. IVW is a commonly used Mendelian randomization analysis and is currently the mainstream analysis method to obtain an overall causal effect estimate [14]. This approach mimics the randomization process based on mutations in gene expression or function by SNPs to group subjects into groups to avoid collinearity between genetic variation and other potential confounders, thereby improving the power of causal inference. The weighted median method is the median of the distribution function obtained by the weighted ranking of all individual SNP effect sizes, and when at least 50% of the information comes from valid instrumental variables, the weighted median method can obtain a robust estimate. Even with more than half of the invalid instrumental variables, causality can still be estimated consistently [15]. MR Egger regression is most commonly used to solve horizontal pleiotropy problems. The weighted median method has a lower mean standard error and is generally more powerful than the IVW method [16]. Moreover, considering that MR Egger regression analysis will be used to test for level pliotropy in subsequent sensitivity analyses in this study. Therefore, in Mendelian randomization analysis, the results of the study were mainly based on the statistically significant IVW method and the weighted median method, and MR Egger regression was supplemented.

2.5. Sensitivity Analysis

In order to ensure the robustness and reliability of Mendelian randomization results, and to detect potential bias and the effect of instrumental variables on outcome variables, a series of sensitivity analyses and quality control were performed in this study, including tests for heterogeneity, horizontal pleiotropy, and leave one out ("leave one out"). The test for heterogeneity was quantified by Cochran's Q statistics, and the test result P > 0.05 concluded that there was no significant heterogeneity between the instrumental variables, and the bias of heterogeneity to the results could be ignored. The horizontal pleiotropy test was performed using MR Egger regression analysis, which provided an intercept term that could be

used to detect bias due to gene pleiotropy. The intercept term egger intercept and 0 were statistically tested, and if $P > 0.05$ was not statistically different, it could be considered that there was no horizontal pleiotropy. Detection The MR PRESSO method was used to detect the outliers, which estimated the corrected results by eliminating abnormal SNPs, and tested whether there was any difference between the pre- and post-calibration results. Finally, the "one retention" method was used to assess stability, and the meta-effect of the remaining SNPs was calculated by deleting individual SNPs sequentially to observe whether there would be significant changes in the results after the removal of specific SNPs [16]. Ideally, after removing any SNPs one by one, the results remain relatively stable and the overall error bars vary in a small range. In this study, the linkage disequilibrium analysis, MR analysis, and sensitivity analysis were performed using R packages such as TwoSample MR version 0.5.6 (The MR-Base platform supports systematic causal inference across the human phenome) and MR PRESSO version 1.0 in R 4.3.2 software.

3. Results

3.1. Tool Variable Selection Results

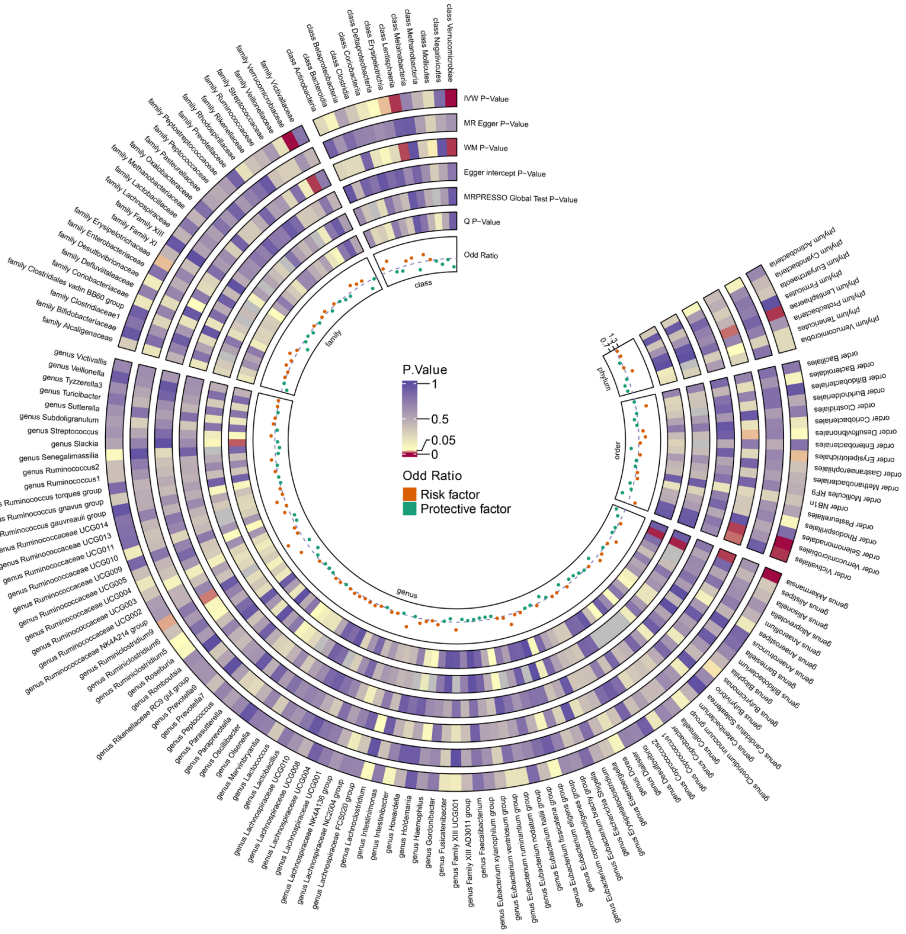


Figure 2. MR results of 182 intestinal microbiotas.

A total of 182 intestinal microbiota were screened at $P < 0.01$. A total of 874 independent SNPs were screened, including five taxonomic levels, including phylum, class, order, family and genus. The linkage imbalance was removed, and the LD coefficient $R^2 = 0.001$ was set to 10,000 kb, and the instrumental variables representing 182 intestinal microbiota were obtained, and each instrumental variable met the $F > 10$ (Figure 2).

3.2. Two-Sample Mendelian Randomization Analysis

Trait	Method	nSNP	P−Value	OR (95% CI)
class Erysipelotrichia	MR Egger	4	0.683	0.414 (0.011 − 15.939)
class Erysipelotrichia	Weighted median	4	0.223	0.654 (0.330 − 1.295)
class Erysipelotrichia	IVW	4	0.035	0.530 (0.293 − 0.958)
class Erysipelotrichia	Simple mode	4	0.446	0.666 (0.268 − 1.654)
class Erysipelotrichia	Weighted mode	4	0.429	0.669 (0.282 − 1.588)
class Verrucomicrobiae	MR Egger	4	0.611	0.184 (0.001 − 47.604)
class Verrucomicrobiae	Weighted median	4	0.008	0.490 (0.290 − 0.828)
class Verrucomicrobiae	IVW	4	0.001	0.475 (0.311 − 0.726)
class Verrucomicrobiae	Simple mode	4	0.172	0.535 (0.269 − 1.063)
class Verrucomicrobiae	Weighted mode	4	0.169	0.529 (0.265 − 1.057)
family Erysipelotrichaceae	MR Egger	4	0.683	0.414 (0.011 − 15.939)
family Erysipelotrichaceae	Weighted median	4	0.233	0.654 (0.325 − 1.315)
family Erysipelotrichaceae	IVW	4	0.035	0.530 (0.293 − 0.958)
family Erysipelotrichaceae	Simple mode	4	0.445	0.666 (0.269 − 1.653)
family Erysipelotrichaceae	Weighted mode	4	0.406	0.669 (0.295 − 1.516)
family Verrucomicrobiaceae	MR Egger	4	0.611	0.184 (0.001 − 47.950)
family Verrucomicrobiaceae	Weighted median	4	0.008	0.491 (0.291 − 0.827)
family Verrucomicrobiaceae	IVW	4	0.001	0.475 (0.311 − 0.726)
family Verrucomicrobiaceae	Simple mode	4	0.157	0.535 (0.278 − 1.028)
family Verrucomicrobiaceae	Weighted mode	4	0.171	0.529 (0.263 − 1.061)
genus Akkermansia	MR Egger	4	0.611	0.186 (0.001 − 46.496)
genus Akkermansia	Weighted median	4	0.006	0.490 (0.296 − 0.813)
genus Akkermansia	IVW	4	0.001	0.476 (0.311 − 0.726)
genus Akkermansia	Simple mode	4	0.172	0.534 (0.268 − 1.063)
genus Akkermansia	Weighted mode	4	0.176	0.528 (0.260 − 1.073)
genus Butyricimonas	MR Egger	5	0.414	0.540 (0.150 − 1.936)
genus Butyricimonas	Weighted median	5	0.163	0.700 (0.425 − 1.154)
genus Butyricimonas	IVW	5	0.044	0.682 (0.471 − 0.990)
genus Butyricimonas	Simple mode	5	0.154	0.545 (0.277 − 1.074)
genus Butyricimonas	Weighted mode	5	0.342	0.691 (0.353 − 1.355)
genus Ruminiclostridium9	MR Egger	3	0.757	2.694 (0.021 − 337.723)
genus Ruminiclostridium9	Weighted median	3	0.017	2.559 (1.182 − 5.542)
genus Ruminiclostridium9	IVW	3	0.031	2.051 (1.069 − 3.932)
genus Ruminiclostridium9	Simple mode	3	0.190	2.872 (0.995 − 8.289)
genus Ruminiclostridium9	Weighted mode	3	0.195	2.872 (0.978 − 8.433)
order Erysipelotrichales	MR Egger	4	0.683	0.414 (0.011 − 15.939)
order Erysipelotrichales	Weighted median	4	0.239	0.654 (0.322 − 1.327)
order Erysipelotrichales	IVW	4	0.035	0.530 (0.293 − 0.958)
order Erysipelotrichales	Simple mode	4	0.444	0.666 (0.270 − 1.646)
order Erysipelotrichales	Weighted mode	4	0.403	0.669 (0.297 − 1.506)
order Verrucomicrobiales	MR Egger	4	0.611	0.184 (0.001 − 47.604)
order Verrucomicrobiales	Weighted median	4	0.008	0.490 (0.289 − 0.833)
order Verrucomicrobiales	IVW	4	0.001	0.475 (0.311 − 0.726)
order Verrucomicrobiales	Simple mode	4	0.166	0.535 (0.273 − 1.048)
order Verrucomicrobiales	Weighted mode	4	0.161	0.529 (0.270 − 1.037)

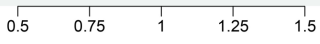


Figure 3. Results of MR analysis.

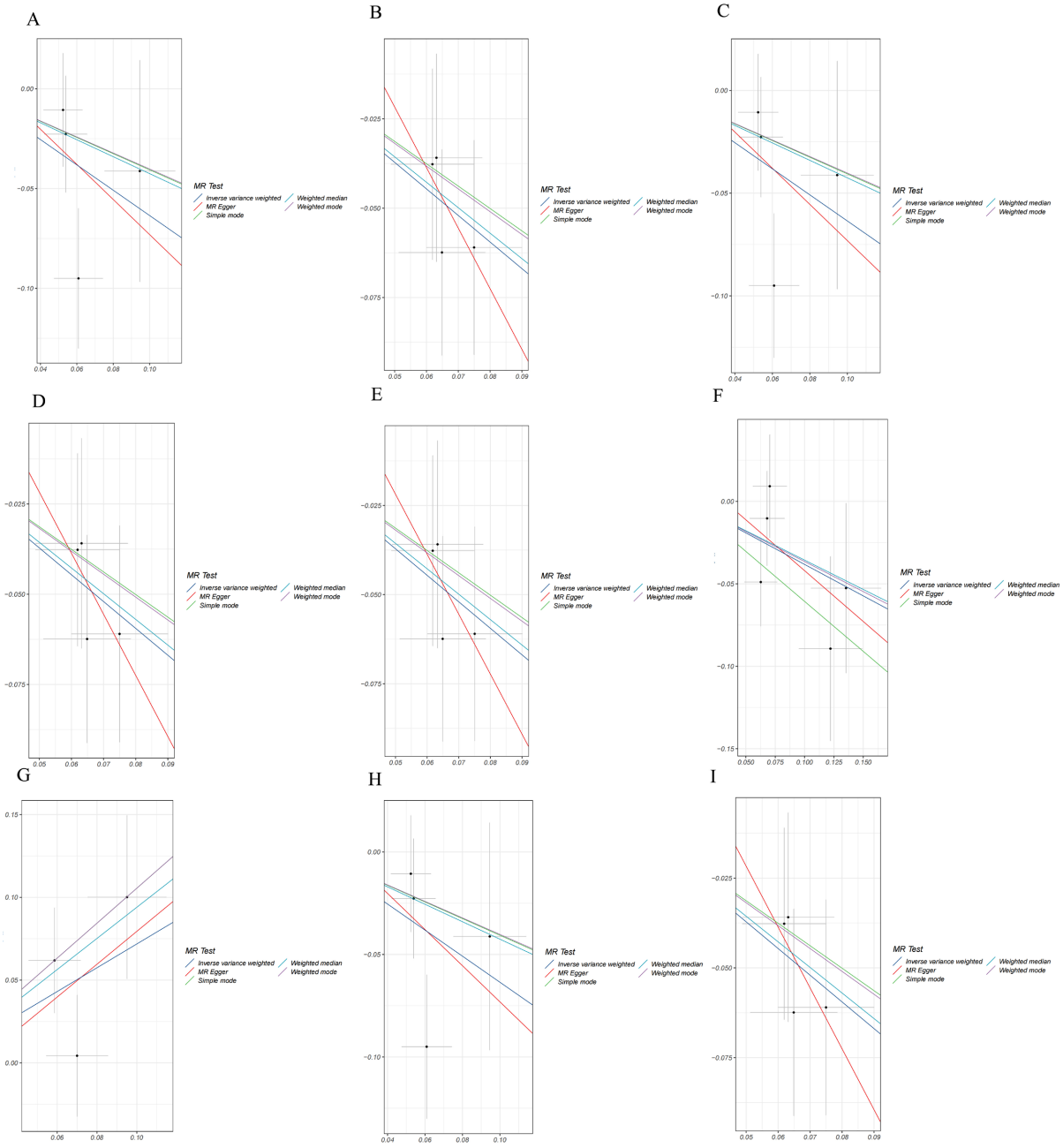


Figure 4. Scatter plot of the correlation between target microbiota and childhood obesity. A. Scatterplot of correlation between class Erysipelotrichia and childhood obesity; B. Scatterplot of correlation between class Verrucomicrobiae and childhood obesity; C. Scatterplot of correlation between class Bacteroidia and childhood obesity; D. Scatterplot of correlation between family Verrucomicrobiaceae and childhood obesity; E. Scatterplot of correlation between genus Akkermansia and childhood obesity; F. Scatterplot of correlation between genus Butyricimonas and childhood obesity; G. Scatterplot of correlation between genus Ruminiclostridium 9 and childhood obesity; H. Scatterplot of correlation between order Erysipelotrichales and childhood obesity; I. Scatterplot of correlation between order Verrucomicrobiales and childhood obesity.

Three TSMR methods were used to analyze 182 intestinal microbiota. In the IVW method, this study found that 9 intestinal microbiota met the requirements $P < 0.01$, and after the weighted median method was integrated as required [17], the two Mendelian randomization analyses of IVW and weighted median method

were selected to be meaningful ($P < 0.05$), five intestinal microbiota associated with childhood obesity were finally obtained (Figure 3). It is worth mentioning that although IVW and weighted median methods were used to screen the intestinal microbiota in this study, the analysis results were mainly based on the IVW method. The results of IVW showed that class Erysipelotrichia (OR = 0.530, 95% CI: 0.293 - 0.958, P = 0.035), family Verrucomicrobiaceae (OR = 0.475, 95% CI: 0.311 - 0.726, P = 0.001), genus Akkermansia (OR = 0.476, 95% CI: 0.311 - 0.726, P = 0.001) and order Verrucomicrobiales (OR = 0.475, 95% CI: 0.311 - 0.726, P = 0.001) were protective factors for the occurrence of childhood obesity, while genus Ruminiclostridium 9 (OR = 2.051, 95% CI: (1.069 - 3.932, P = 0.031) was a potential risk factor for childhood obesity (Figure 2). The scatter plots of the three MR analyses showed that genus ruminiclostridium9 was positively correlated with childhood obesity, while class Erysipelotrichia, family Verrucomicrobiaceae, genus Akkermansia, and order Verrucomicrobiales were negatively correlated (Figure 4). In order to eliminate possible confounders and exclude the effect of level pleiotropy on causality, this study used the GWAS website (https://www.ebi.ac.Theuk/gwas/) query did not find that a total of 19 SNPs associated with these 5 gut microbiotas were associated with confounding factors.

3.3. Sensitivity Analysis

Table 1. Sensitivity analysis of MR.

Classify	Strains		Cochran's Q		MR-Egger Regression			MR-PRESSO	
		method	Cochran's Q	P	MR-Egger Intercept	SE	P	RSSobs	P
Class	Verrucomicrobiaceae	MR Egger	0.400163973	0.8186636	2.8383748	0.0629484	0.7691383	0.887734676	0.923
		IVW	0.513494405	0.9159169					
Family	Verrucomicrobiaceae	MR Egger	0.401768656	0.818007	2.8358555	0.0630945	0.7684261	0.886286593	0.93
		IVW	0.514363905	0.9157246					
Genus	Akkermansia	MR Egger	0.401171052	0.8182515	0.2661259	0.0622335	0.7700541	0.884820926	0.931
		IVW	0.512825027	0.9160649					
Genus	Ruminiclostridium 9	MR Egger	2.339717759	0.1261124	0.405806	-0.0196857	0.9285184	-	-
		IVW	2.369465413	0.3058279					
Order	Verrucomicrobiaceae	MR Egger	0.400163973	0.8186636	2.8358555	0.0630945	0.7684261	0.886286593	0.937
		IVW	0.513494405	0.9159169					

The sensitivity analysis of the four intestinal microbiotas screened in TSMR was carried out, and the heterogeneity was tested by Cochran's Q statistic, the level pleiotropy was detected by MR Egger regression analysis, and the outlier values were detected by MR PRESSO, and the results were all $P > 0.05$ was not statistically significant, indicating no significant heterogeneity and level pleiotropy among the pooled instrumental variables (Table 1). In addition, the stability of the MR

results was assessed by the leave one out method, although there were cases where a single SNP crossed "0", the final summary result "All" did not cross "0", and there was no single SNP that had a large impact on the stability of the results (**Figure 5**), so the causal relationship between the analyzed gut microbiota and childhood obesity was considered stable.

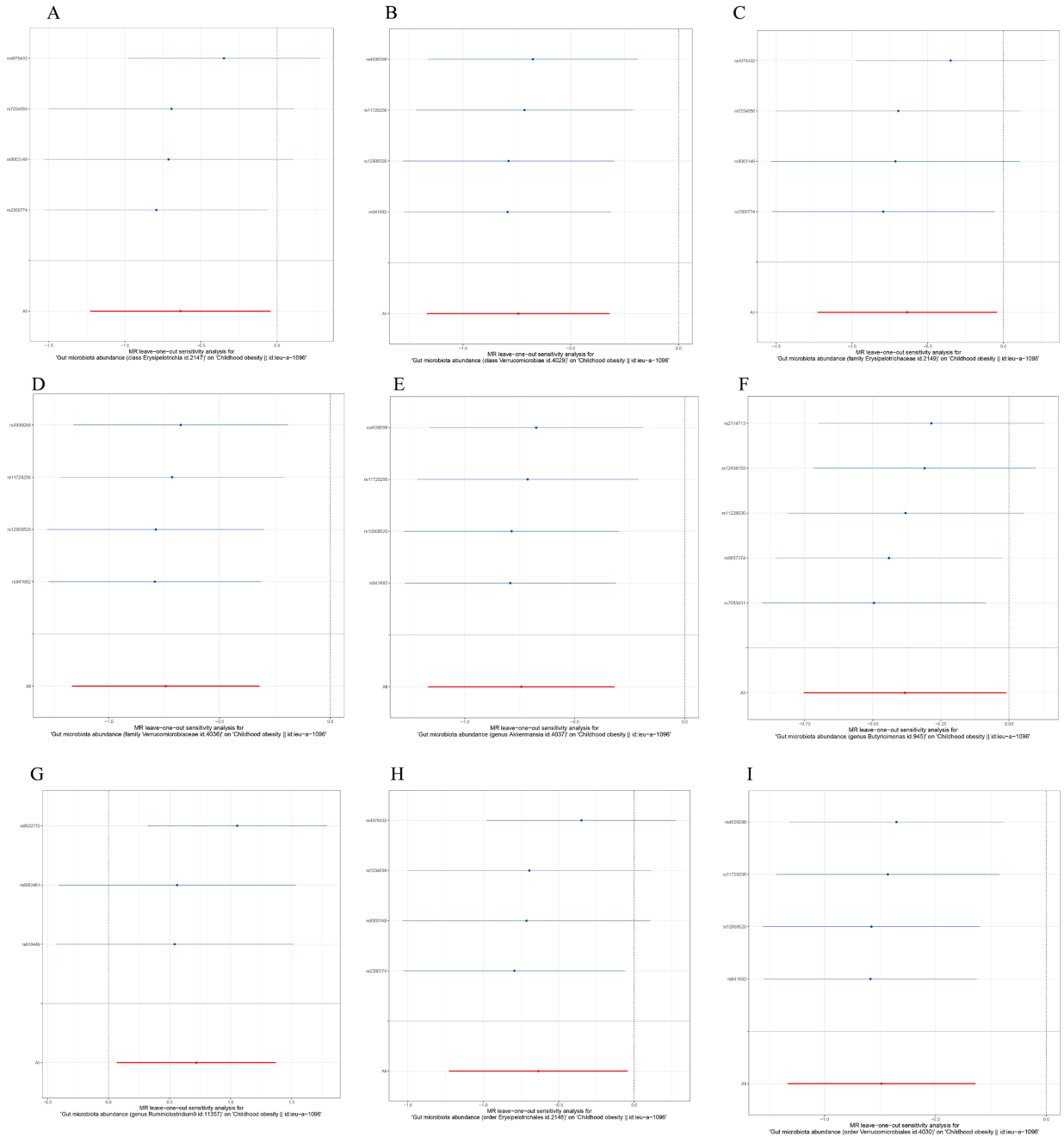


Figure 5. Retention and sensitivity analysis of the correlation between target microbiota and childhood obesity.

4. Discussion

In this study, TSMR analysis was carried out, and after a series of stringent quality

control and sensitivity analysis, four intestinal microbiota (genus Ruminiclostridium 9, class Erysipelotrichia, family Verrucomicrobiaceae, genus Akkermansia, order) were finally obtained Verrucomicrobiales) and the risk of childhood obesity. These intestinal microbiotas can affect the occurrence and development of childhood obesity by changing energy intake and regulating lipid metabolism. Erysipelotrichia belongs to a class under the phylum Firmicutes and is a gram-positive bacterium. The importance of erysipelas in the human gut microbiota is less explored. Erysipelas has been associated with higher dyslipidemia in MR studies [17]. Another clinical study showed that adipose tissue, adipocytes, and lipid transport-related proteins are strongly expressed in rheumatic mitral valves, suggesting that adipose tissue formation may be one of the important pathways in rheumatic heart disease pathology. In addition, adipose tissue and adipocytes are also involved in the inflammatory process of rheumatic heart disease [18]. This abnormal inflammatory response may be one of the causes of childhood obesity. It is worth mentioning that in the MR analysis of this study, there was no statistical significance for the screening of erysipelas using two statistical methods, IVW method and weighted median method. However, if the screening conditions were relaxed and only the mainstream IVW method was used, the class Erysipelotrichia (OR = 0.530, 95% CI: 0.293 - 0.958, $P < 0.05$), there has been no significant positive correlation between erysipelas and the risk of childhood obesity, and there are few studies on Erysipelotrichia, so the mechanism of erysipelotrichia increasing the risk of childhood obesity can be further explored in the future, and it can be used as a key microbial marker to provide new ideas for the prevention and treatment of childhood obesity. *Akkermansia muciniphila* (*A. muciniphila*, AKK), belonging to the phylum Verrucous Microbacteria, was originally isolated from the feces of healthy adults and is a gram-negative anaerobe, detected in more than 90% of healthy adults. At the same time, it is also one of the most abundant commensal bacteria in the intestine [19]. Previous studies have shown a negative correlation between the abundance of *A. muciniphila* and obesity. Animal experiments have shown a 100-fold reduction in the abundance of *A. muciniphila* in the cecum of mice after 8 weeks of administration of a high-fat diet (60% fat) [19]. In diet-induced obese mice, changes in the abundance of *A. muciniphila* were significantly correlated with the expression of markers of lipid metabolism and inflammation, as well as some circulating markers, such as glucose, insulin, triacylglycerol, and leptin [20]. Some clinical data have also shown that the abundance of *A. muciniphila* is reduced in overweight or obese individuals, and a decrease in the abundance of *A. muciniphila* has been demonstrated during the subclinical phase of obesity [21]. A recent study of dietary interventions for obesity and diabetes showed that the abundance of *A. muciniphila* in overweight and obese subjects was inversely correlated with fasting blood glucose, waist-to-hip ratio, and fat cell diameter; Subjects with high abundance of *A. muciniphila* also showed healthier metabolic status, particularly in fasting blood glucose, triacylglycerol, and body fat profile; Six weeks after the calorie-restricted diet, subjects with a high abundance

of *A. muciniphila* experienced more significant improvements in blood glucose and lipids [22]. Many studies have shown that the abundance of *A. muciniphila* represents the degree of improvement in obesity levels, and the more important finding is that the abundance of *A. muciniphila* is more stable than other intestinal commensal bacteria. Existing studies have shown that obesity-induced microbiome alterations persist over a long period of time and accelerate weight regain after a second high-fat diet [23], so *A. muciniphila* may be the key to weight regain after weight loss. As a result, obesity rates remain high.

In this study, TSMR was used for the first time to explore the causal relationship between intestinal microbiota and childhood obesity by using gut microbiota as an exposure factor, and strict quality control conditions and analysis methods were used in the process, and the results were reliable and robust. However, there are some limitations: 1) all data in this study are from European populations, and the generalization of results to other populations may be limited; 2) The 182 intestinal microbiota did not include all the intestinal microbiota, and the analysis was not specific to the species or strain; 3) In order to obtain sufficient instrumental variables of intestinal microbiota, the screening criterion P of this study was lower than that of the traditional screening tool variables less 5×10^{-8}; 4) The purpose of this study was to find as many potentially positive gut microbiota as possible, although the combination of IVW and weighted median analysis methods was used to strictly screen the gut microbiota, but no multiple test correction was performed. In summary, this study revealed the potential causal relationship between gut microbiota and childhood obesity, and screened out five groups of gut microbiota associated with childhood obesity, which may become new biomarkers, provide new insights into the pathogenesis of childhood obesity from the perspective of genetic exposure interaction, and help deepen the understanding of obesity mechanism.

Conflicts of Interest

The authors declare no conflicts of interest.

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