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Original research

Large-scale shotgun metagenomic analysis of gut bacteria–phage interactions in IBS

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ABSTRACT

Background IBS is a multifactorial disorder affecting approximately 10% of the global population, yet its underlying mechanisms are unclear. Alterations in gut bacterial composition have been reported in IBS, but findings are inconsistent and the phage component of the gut microbiome remains underexplored.

Objectives To characterise gut bacterial and phage alterations in IBS and assess bacteria-phage interactions in a large population-based cohort with replication.

Design We analysed shotgun metagenomes from 109 females with IBS and 592 controls from TwinsUK. Bacterial taxonomic and functional profiles were generated using MetaPhlAn4 and HUMAnN2, while viral operational taxonomic units (vOTUs) were identified by de novo assembly. Covariate-adjusted logistic mixed models with false discovery rate correction assessed associations. Differences in bacteria–vOTU correlation structure were evaluated using permutation-based approaches. Bacterial findings were replicated in 304 females from ZOE Personalised REsponses to DIetary Composition Trial (PREDICT)-1.

Results Bacterial richness and Shannon diversity were lower in IBS. 24 bacterial species were associated with IBS and replicated in ZOE PREDICT-1. These included *Erysipelatoclostridium ramosum*, previously linked to dysbiosis and serotonin regulation, *Eisenbergiella massiliensis,* previously associated with fibromyalgia and several butyrate producers. We identified 50 IBS-associated vOTUs, many predicted to infect IBS-relevant bacterial hosts. While phage diversity was unchanged, IBS-associated vOTUs differed in replication cycle compared with non-IBS-associated vOTUs. Bacteria–vOTU correlation structures also differed between IBS and controls.

Conclusion IBS is characterised by bacterial compositional changes and altered bacteria–phage interactions, suggesting microbiome alterations in IBS may extend beyond individual taxa to transkingdom correlation structure.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ IBS is associated with alterations in the gut microbiome, including reduced bacterial diversity and changes in bacterial taxa involved in short-chain fatty acid production and tryptophan metabolism, while emerging evidence suggests that viral alterations may also occur in IBS.

WHAT THIS STUDY ADDS

⇒ Replication of 24 IBS-associated bacterial species in a secondary cohort including novel taxa (eg, *Butyricicoccus* sp. AM29-23AC, *Eisenbergiella massiliensis* and *Candidatus* Cibiobacter qucibialis) with reported roles in butyrate production and tryptophan metabolism.

⇒ Identification of 50 IBS-associated viral operational taxonomic units (vOTUs) in the largest shotgun-based phageome analysis in IBS to date, many predicted to infect bacterial genera relevant to IBS.

⇒ IBS-associated vOTUs differed in predicted replication cycle compared with non-IBS-associated vOTUs and encoded auxiliary metabolic genes related to carbohydrate metabolism and the immune system, while global bacteria–vOTU correlation structure differs between IBS and controls, with nominal differences in network topology.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our findings support ecosystem-level approaches to investigating functional GI disorders and suggest that microbiome-based biomarker strategies in IBS may benefit from integrating viral as well as bacterial components.

INTRODUCTION

IBS is a common GI disorder affecting approximately 10% of the global population.[1] Symptoms vary in type and severity between individuals but include frequent abdominal pain along with altered bowel movement consistency and frequency, and extra-GI symptoms are common.[2] IBS reduces quality of life and is associated with substantial economic productivity losses and increased absenteeism.[3]

The aetiology and pathological mechanisms of IBS are poorly understood.[1] Reported alterations

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include altered GI motility, visceral hypersensitivity and disrupted gut–brain signalling, with risk factors including early-life trauma, depression and stress.[1] However, the exact contribution of each of these and the interplay between them remain unclear.

Diagnosis relies on the Rome criteria, a questionnaire-based tool assessing abdominal pain/discomfort and bowel habits. These criteria form the basis for subtypes: diarrhoea-dominant, constipation-dominant, mixed and unclassified.[4] Nevertheless, definitive diagnosis can take months owing to the need to exclude more serious pathology, during which patients often experience stress and uncertainty.[5] Symptom overlap with other GI disorders can make diagnosis challenging.[1]

Reliable biomarkers of IBS could greatly improve diagnosis. Additionally, biological signatures related to IBS could shed light on pathological mechanisms and potentially lead to improved treatment options.[1]

The gut microbiome has been a major focus in the search for IBS biomarkers.[6] Large cohort studies have reported reduced alpha diversity and short-chain fatty acid-producing bacteria in IBS, as well as an elevated abundance of pathogenic bacteria (including *Escherichia coli* and *Ruminococcus gnavus*) and bacteria related to neurotransmitter production, among others.[7–10] Despite this, results are inconsistent and much remains unknown. Most studies have focused on bacterial composition alone, often in relatively small or clinic-based cohorts, and rarely replicate findings. Additionally, technological advances now enable deeper gut microbiome profiling, including underexplored components like phages (bacteriophages, bacteria-infecting viruses), which are linked to health outcomes.[11]

Phages shape bacterial community structure through predation, lysogeny and horizontal gene transfer, and may therefore influence health outcomes not only via changes in phage abundance but also through reorganisation of bacteria–phage interaction networks.[11]

The microbiome's diagnostic potential, combined with the unmet need for IBS biomarkers, underscores the value of further research. We hypothesised that IBS is associated not only with altered bacterial composition but also with reproducible shifts in phage composition and differences in bacteria–phage interactions, representing an additional, underappreciated dimension of IBS dysbiosis. Using deep population-based shotgun metagenomics in TwinsUK, a UK registry of adult twins recruited from the general population, with replication of bacterial associations in ZOE Personalised REsponses to DIetary Composition Trial (PREDICT)-1, a UK-based nutritional interventional study, we characterised IBS-associated bacterial species, viral operational taxonomic units (vOTUs) and transkingdom ecological structure.

METHODS

Study sample

Discovery cohort

Participants were drawn from the TwinsUK Registry, a national cohort of adult twins recruited from the general population without selection based on disease status or traits.[12] We included data from 701 female twins who had responded to the Rome III criteria questions as part of their general health screening questionnaire and provided stool samples as part of general microbiome studies, which underwent shotgun metagenomic profiling.

IBS status definition

IBS classification in TwinsUK was based on questionnaire items derived from the Rome III criteria; however, available response categories did not exactly match the ≥3 days/month definition, and a weekly threshold was used as an approximation (online supplemental table S1). The Rome III criteria were administered at two time points. Participants meeting the criteria at either time point were classified as IBS cases; those never meeting the criteria and never self-reporting IBS were defined as controls. Individuals first meeting the criteria after their stool collection were excluded.

Medication use

Drug use was derived from self-reported questionnaires. Drugs potentially related to IBS were identified from the literature[13] and grouped by therapeutic class as previously described.[13 14] Only drugs used by ≥1% of the sample were considered. To minimise overadjustment, medication classes that differed between IBS cases and controls (χ^2, false discovery rate (FDR) <0.05[15]) were included as covariates in sensitivity analyses. Individuals reporting antibiotic use at questionnaire time points nearest to their stool collection date were excluded (n=6). Information on supplement use, including probiotics and prebiotics, was too sparse to permit reliable adjustment or subgroup analyses and was therefore not used as an exclusion criterion.

Habitual diet

Habitual dietary intake was assessed using a validated Food Frequency Questionnaire,[16] grouped into 20 food categories (animal fat, dairy products, eggs, fish, fruit juice, fruit, legumes, meat, other animal-based foods, nuts, potatoes, refined grains, sugar-sweetened beverages, sweets and desserts, tea and coffee, vegetable oils, vegetables, whole grains, margarine and alcohol)[17] and energy-adjusted via the residual method.[18] Outliers were defined as questionnaires with >10 items left unanswered and those for which the ratio of estimated kilocalories to the participant's basal metabolic rate (calculated via the Harris-Benedict equation) exceeded 2 SD from the mean. Outliers were excluded, and the remaining participants with missing data (3.4%) were imputed with group means.

Bristol Stool Score

Participants graded stool consistency using the Bristol Stool Score (BSS) within 2 weeks of sample collection. BSS scores of 7 were merged with 6 due to low frequency (n=5).

Replication cohort

Bacterial findings were replicated in a sample of 304 individuals (including 43 IBS cases) from the UK-based ZOE PREDICT-1 study (PREDICT-1).[19] The PREDICT-1 study was a nutritional intervention conducted between June 2018 and May 2019. Healthy adults aged 18–65 years were recruited from the TwinsUK registry[12] and the general population via online advertising. Participants completed a clinic visit involving standardised test meal challenges, followed by 13-day home-based monitoring, as previously described.[19] Stool samples were collected by participants at home prior to the meal challenge and processed as described below. IBS classification was based on questionnaire items derived from the Rome III criteria.

Shotgun metagenomic sequencing

Stool collection, processing, metagenomic sequencing and bacterial profiling were performed as previously described.[17] Briefly, deep shotgun metagenomic sequencing of stool samples was conducted by GenomeScan for TwinsUK[17] and in the bioBakery environment following general guidelines for PREDICT-1.[20] Bacterial taxonomic and functional profiles were generated

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using the YAMP pipeline (V.0.9.5.0), applying MetaPhlAn4 (V.4.beta.2)[21] and HUMAnN2,[22] respectively.

Phylogenetic annotation of unclassified bacterial taxa

To aid interpretation of IBS-associated bacterial taxa lacking species-level annotation, the closest characterised species was identified using phylogenetic distance. The reference phylogenetic tree generated by MetaPhlAn4 (V.4.beta.2)[21] was parsed from a Newick-formatted file using the Bio.Phylo module in Biopython (V.1.87) with Python (V.3.11.6). Pairwise phylogenetic distances were calculated between each unclassified taxon and all remaining taxa, and the nearest characterised species was defined as the species-level taxon with the smallest phylogenetic distance.

Phage community profiling

TwinsUK gut phageomes were profiled by de novo assembly of quality-controlled metagenomic reads.[23] See online supplemental figure S1.

Briefly, reads from each sample were assembled using MEGAHIT,[24] and viral sequences were identified using geNomad,[25] VirSorter2[26] and VIBRANT.[27] Viral sequences were combined, de-replicated using MMseqs2 (95% nucleotide identity and 85% alignment coverage) according to Minimum Information about an Uncultivated Virus Genome (MIUViG) recommendations[28] and quality assessed with CheckV to retain complete, high-quality and medium-quality viral genomes (≥5 kb). vOTUs were taxonomically classified using geNomad,[25] and replication strategies were inferred using BACPHLIP.[29] Reads were mapped to the curated vOTU catalogue using Bowtie2 with default settings, reporting a single best alignment per read. Following read mapping, vOTUs with <20% genome coverage or <0.5×mean sequencing depth were excluded. Abundances were quantified from samtools read counts.[30] Transcripts per million (TPM) values were calculated by first normalising read counts by viral sequence length and then scaling by the total reads-per-kilobase across all viral sequences, generating TPM-like relative abundances for downstream analyses. Auxiliary metabolic genes (AMGs) were identified using DRAM-v[31] and only high-confidence predictions (auxiliary score=1) were retained. Bacterial hosts of vOTUs were predicted using iPHoP[32] (confidence ≥90%) at the genus level. Bacterial species, vOTUs and functional pathways were centred log-ratio transformed before statistical analyses. Temperate phages were defined as vOTUs with predicted lysogenic potential, whereas virulent phages were defined as vOTUs predicted to be obligately lytic. vOTUs whose lifestyle could not be confidently assigned were classified as unknown and were not included in replication cycle analyses. We included bacterial species, functional pathways and vOTUs present in ≥10% of samples (with an additional relative abundance threshold of ≥0.001% for vOTUs). Bacterial species, functional pathways and vOTUs were centred log-ratio (CLR) transformed prior to analysis, using a pseudocount of one-tenth the lowest non-zero value in each dataset. After filtering, 561 bacterial species, 419 MetaCyc pathways, 261 Kyoto Encyclopedia of Genes and Genomes (KEGG) modules, 2584 UniProt enzymes and 6349 vOTUs were retained for downstream analyses.

Statistical analysis

An overview of the analysis pipeline is depicted in online supplemental figure S2. All statistical analyses were performed in R V.4.5.1, unless otherwise stated. Logistic mixed models were used to estimate associations between IBS and (1) bacteriome and phageome alpha diversity; and (2) bacterial species, vOTUs and functional pathway relative abundance. Models were adjusted for age, body mass index (BMI), batch effects and family relatedness (random effects). P values were adjusted for multiple testing using Benjamini-Hochberg FDR correction (FDR <0.05).

For visualisation purposes, log ORs from TwinsUK and PREDICT-1 were combined using random-effects inverse-variance meta-analysis implemented with the R *meta* package (V.8.5–0), with between-study variance estimated using restricted maximum likelihood.

Sensitivity analyses were performed by further adjusting for dietary intake (assessed using the 20 energy-adjusted food groups), medication use (antidepressants and anxiolytics, laxatives, antispasmodics and antimotility agents, analgesics and proton pump inhibitors) and stool consistency (BSS).

Alpha and beta diversity

Alpha diversity metrics—observed richness and Shannon diversity—were calculated from raw counts using the vegan package (V.2.7.1)[33] and standardised to mean 0 and 1 SD. Beta diversity between IBS cases and controls was assessed using multivariable permutational analysis of variance (adonis2 function, vegan V.2.7.1) with 999 permutations on the Aitchison distance matrix, including age, BMI and batch effects as covariates, with family ID accounted for as a blocking factor. To test the robustness of the model, analyses were rerun in randomly sampled unrelated subsets.

vOTUs–bacteria interactions

To assess global differences in bacteria–vOTU relationships, we compared the structure of vOTU–bacterial correlation matrices between IBS and controls. For each group, Spearman correlation matrices were computed between all filtered vOTUs and bacterial species. Frobenius norm was used to quantify dissimilarity between matrices. Statistical significance was evaluated by calculating an empirical null distribution derived from 5000 permutations by shuffling IBS labels while preserving twin IBS status concordance and the case-control ratio. Empirical p values were defined as the proportion of permuted norms exceeding the observed value.

Network analysis

Network analyses were performed to investigate associations among bacterial species and vOTUs using the netConstruct function from the NetCoMi package (V.1.2.0),[34] with Sparse Inverse Covariance Estimation for Ecological Association Inference (SPIEC-EASI) (V.1.1.2)[35] used for network inference. To minimise the influence of low-prevalence taxa, only bacterial species and vOTUs present in at least 20% of both cases and controls were included. An additional more stringent relative abundance threshold of 0.01% was applied to vOTUs for visualisation purposes. Prior to network construction, count data were CLR transformed using a pseudocount of 1 and residuals adjusted for age, BMI and sequencing batch were used to account for potential confounding effects.

Separate association networks were constructed for cases and controls. Adjacency matrices were extracted from the inferred networks, and vOTU–vOTU interactions were set to zero to retain only bacteria–bacteria and bacteria–vOTU associations. Network properties were compared between cases and controls using the netAnalyze function in NetCoMi.

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The statistical significance of differences in network statistics was assessed using permutation testing. IBS case–control labels were randomly permuted 999 times while preserving twin concordance and the proportions of concordant and discordant twin pairs. Network statistics were recalculated for each permuted dataset, and empirical p values were obtained by calculating the proportion of permuted statistics that were as extreme as or more extreme than the corresponding observed statistic. Nodes with the top 2% of eigenvector centrality values in each network were considered hubs.

Patient and public involvement

The TwinsUK voluntary advisory panel at KCL, a 24-member group representing the registry, advises on twin engagement, research protocols, grant submissions and ethical issues. Members serve three years and the VAP is co-chaired by the Programme Manager and a TwinsUK representative. They are regularly consulted (email, online and surveys) and attend biannual meetings at KCL.

RESULTS

Sample characteristics

We included 701 female participants from TwinsUK (109 IBS cases and 592 controls) and 304 females from PREDICT-1 (43 IBS cases and 261 controls). The descriptive characteristics of the study population are reported in table 1 and stratified by IBS subtype in online supplemental table S2. Due to the limited sample size for each IBS subtype, we did not perform subgroup analysis. IBS cases had a slightly higher BMI and more frequently used antidepressants or antianxiolytics, laxatives, antispasmodic or antimotility drugs, analgesics and proton pump inhibitors compared with controls (FDR <0.05).

Bacterial alterations in IBS

Alpha and beta diversity

In TwinsUK, bacterial richness (OR (95% CI)=0.58 (0.43 to 0.80); $p=2.74\times10^{-4}$) and Shannon diversity (0.59 (0.43 to 0.81); $p=3.73\times10^{-4}$) were lower in IBS cases compared with controls after adjusting for covariates (figure 1) and when further adjusting for medication and diet (online supplemental table S3). Results were similar for PREDICT-1 (richness: 0.55 (0.36 to 0.83); p=0.001), though Shannon diversity was not significant (0.78 (0.55 to 1.1); p=0.16).

Beta diversity differed between cases and controls in TwinsUK (R^2=0.37%; p=0.001; figure 1), including after adjusting for diet and medication use or using alternative dissimilarity metrics (online supplemental figure S3). In PREDICT-1, the proportion of variance in beta diversity explained by IBS was higher but did not reach statistical significance (p=0.25), potentially owing to the smaller sample size.

IBS-species associations

Of 561 bacterial species, 45 were found to associate with IBS after adjusting for covariates in TwinsUK (FDR <0.05; online supplemental table S4). Of these, 24 were replicated in PREDICT-1 (p<0.05; figure 2; online supplemental table S5). For replicated taxa lacking species-level annotation, phylogenetic mapping to the nearest characterised species is provided in online supplemental table S6 to facilitate biological interpretation.

Results were consistent when further adjusting for diet and medication, though three species (GGB9712 SGB15244 (Ruminococcaceae), *Anaerostipes caccae* and *Candidatus* Cibiobacter qucibialis) were no longer significant after adjusting for BSS (online supplemental figure S4). Given the reduced sample size in BSS-adjusted models (n=509), this attenuation may reflect limited statistical power.

12 of the replicated IBS-associated species belonged to Ruminococcaceae and 5 to Lachnospiraceae (figure 2). Additional associations included two Clostridiaceae, two unclassified Clostridia and one from Erysipelotrichaceae, Aeromonadaceae and unclassified Firmicutes.

20 species were lower in individuals with IBS, including *Blautia* sp OF03 15BH (OR (95% CI)=0.91 (0.86 to 0.97); FDR=0.038) and *Butyricicoccus* sp AM29 23AC (0.92 (0.87 to 0.98); FDR=0.044). Conversely, four taxa were associated with increased odds of IBS, including *Clostridium* sp AT4 (1.19 (1.08 to 1.31); $FDR=2.23\times10^{-3}$) and *Eisenbergiella massiliensis* (1.10 (1.03 to 1.16); FDR=0.038).

Functional pathways analysis revealed no significant differences in MetaCyc pathways (online supplemental table S7). Among KEGG modules and UniProt enzymes, only an uncharacterised carbon–nitrogen ligase (EC 6.3.5.-) was identified in TwinsUK and replicated in PREDICT-1 (p<0.05), with suggestive evidence for hydroxypyruvate reductase (EC 1.1.1.81; p=0.054) (online supplemental tables S8–S10).

Gut phageome alterations in IBS

De novo assembly and viral identification generated approximately 6.2 million initial viral predictions, which were reduced to 1.8 million candidate viral sequences after dereplication according to MIUViG recommendations and to 116 740 viral genomes following CheckV quality filtering. Of these, 6349 vOTUs met the prevalence (≥10% of samples) and relative abundance thresholds for downstream analyses. Most were predicted to be temperate (n=4430; 69.8%), while 1905 (30.0%) were predicted to be virulent and 14 (0.2%) could not be predicted.

No difference was observed in the overall relative abundance of temperate versus virulent vOTUs between IBS cases and controls. Phageome alpha and beta diversity (figure 3A and B) also did not differ significantly by IBS status.

IBS-associated vOTUs

After adjusting for covariates, 50 vOTUs were associated with IBS (FDR <0.05; online supplemental table S11). 40 of these were depleted in IBS compared with controls, whereas the remaining 10 were enriched (figure 3C). Taxonomy was known for all but one of these phages, with 48 belonging to *Caudoviricetes* and one belonging to *Cirlivirales*.

We next investigated whether the proportion of temperate and virulent vOTUs differed between IBS-associated and non-IBS associated vOTUs. The proportion of virulent vOTUs was significantly lower among IBS-associated vOTUs than among non-IBS-associated vOTUs (8/50 (16.0%) vs 1897/6299 (30.1%), χ^2 test with Yates' continuity correction, p=0.043) (figure 3D), suggesting phage replication cycle balance may be altered in IBS.

To evaluate whether IBS–vOTU associations were primarily driven by changes in predicted host abundance, we performed sensitivity analyses further adjusting for the relative abundance of predicted host genera. Associations were largely unchanged, indicating that changes in host genus relative abundance alone did not fully account for the observed IBS–vOTU relationships (figure 3E; online supplemental table S12).

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Table 1 Descriptive characteristics of the cases and controls in TwinsUK and PREDICT-1

	TwinsUK			PREDICT-1		
Phenotype	IBS (n=109)	Controls (n=592)	P value	IBS (n=43)	Controls (n=261)	P value
IBS subtype, n (%)						
IBS-C	31	–	–	15	–	–
	(28.4)			(34.9)		
IBS-D	66	–	–	26	–	–
	(60.6)			(60.5)		
IBS-M	12	–	–	2	–	–
	(11.0)			(4.7)		
Mean sequencing reads	32 565 760	31 910 046	0.50	57 957 946	58 997 634	0.64
	(9 180 959)	(9 722 498)		(13 293 125)	(15 140 682)	
Age, years	64.3	65.7	0.24	51.6	49.6	0.27
	(10.8)	(11.3)		(11.0)	(11.3)	
BMI, kg/m²	27.2	25.9	0.01	26.0	25.4	0.54
	(5.0)	(4.7)		(6.2)	(5.2)	
Microbiome diversity						
Bacterial richness	276.8	308.0	2.74×10^{-4}	278.5	314.5	1.00×10^{-3}
	(88.0)	(77.4)		(78.3)	(66.6)	
Bacterial Shannon diversity	3.7	3.9	3.73×10^{-4}	4.1	4.2	0.16
	(0.6)	(0.6)		(0.4)	(0.4)	
Phage richness	3282.5	3369.1	0.35	–	–	–
	(1958.9)	(2034.2)				
Phage Shannon diversity	6.8	6.90	0.25	–	–	–
	(1.4)	(1.29)				
Medication use, n (%)						
Antidepressants/anxiolytics	22	58	3.00×10^{-3}	6	16	0.13
	(20.2)	(9.8)		(14.0)	(6.1)	
Laxatives	7	13	0.03	0	1	1.00
	(6.4)	(2.2)		(0.0)	(0.4)	
Antispasmodics/antimotility	12	7	4.13×10^{-8}	1	0	0.30
	(11.0)	(1.2)		(2.3)	(0.0)	
Analgesics	21	39	3.16×10^{-5}	0	3	1.00
	(19.3)	(6.6)		(0.0)	(1.2)	
Proton pump inhibitors	38	93	4.64×10^{-6}	5	8	0.30
	(34.9)	(15.7)		(11.6)	(3.1)	
Diet						
Participants with dietary data, n	106	571	–	39	239	–
Energy intake (kcal/day)	1707.9	1684.5	0.66	1650.2	1664.9	0.83
	(498.3)	(513.1)		(384.4)	(459.9)	
Alcohol consumption, n (%)	78	450	0.29	34	208	1.00
	(73.6)	(78.8)		(87.2)	(87.0)	
BSS						
Participants with BSS data, n	83	426	–	41	245	–
1–2, n (%)	17	84	0.99	12	22	5.51×10^{-4}
	(20.5)	(19.7)		(29.3)	(9.0)	
3–4, n (%)	45	256	0.38	23	205	1.16×10^{-4}
	(54.2)	(60.1)		(56.5)	(83.7)	
5–7, n (%)	21	86	0.37	6	18	0.21
	(25.3)	(20.2)		(14.6)	(7.4)	

Values are presented as mean (SD) unless otherwise indicated.
Phage diversity was assessed only in the discovery (TwinsUK) cohort.
BMI, body mass index; BSS, Bristol Stool Score; IBS-C, constipation-predominant IBS; IBS-D, diarrhoea-predominant IBS; IBS-M, mixed IBS; PREDICT, Personalised REsponses to DIetary Composition Trial.

Host prediction and functional potential

16 IBS-associated vOTUs were predicted to infect the unclassified genus UBA11524 within the family Aristaeellaceae, whereas CAG-170 (Oscillospiraceae), CAG-103 (Oscillospiraceae) and SFHK01 (Aristaeellaceae) were infected by one phage each (figure 3F). vOTUs predicted to infect UBA11524

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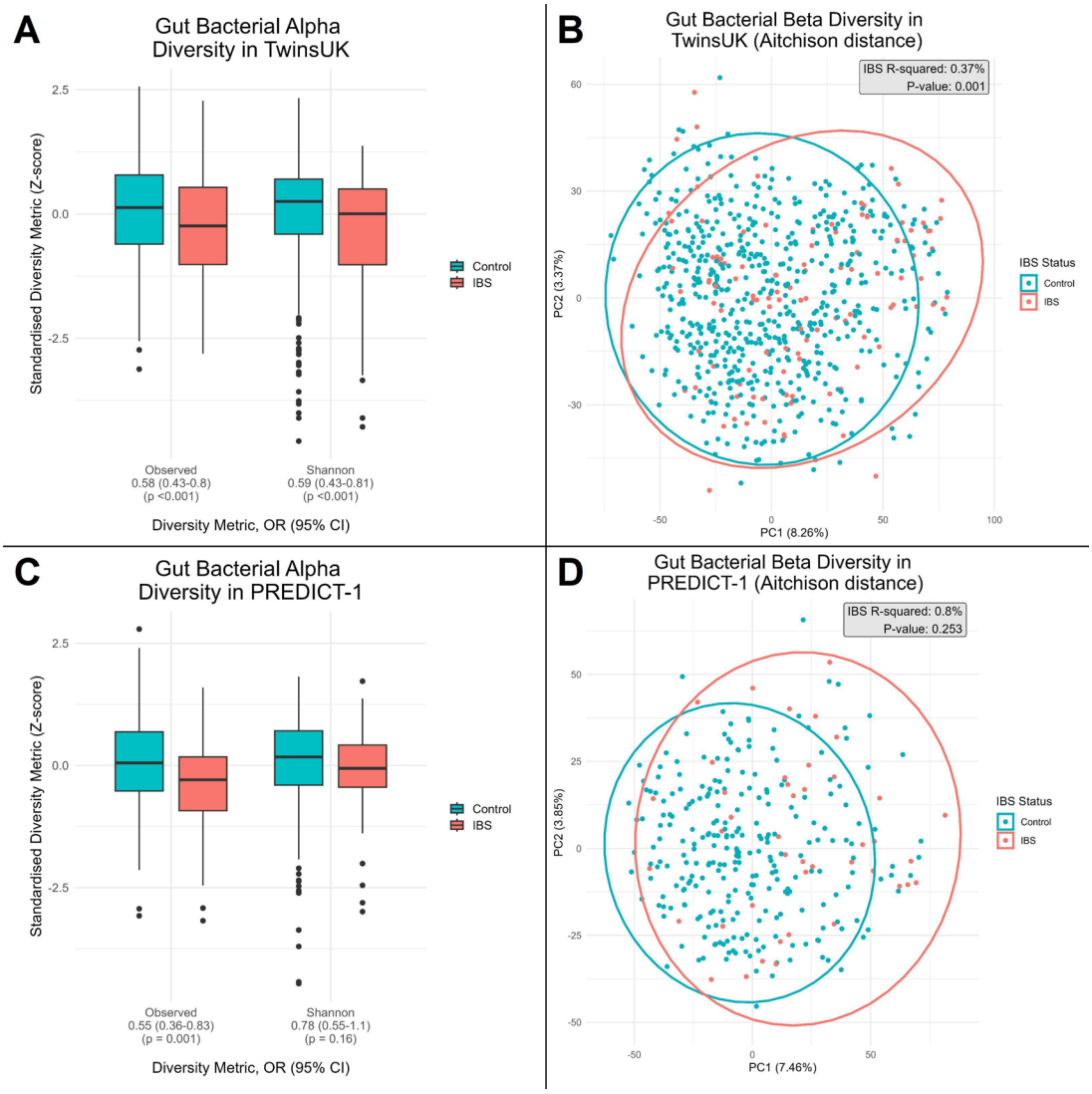

Figure 1 Gut microbiome diversity in IBS and controls. (A) Gut bacterial alpha diversity (observed richness and Shannon diversity) between IBS cases and controls in TwinsUK. (B) Gut bacterial beta diversity between IBS cases and controls in TwinsUK. (C) Gut bacterial alpha diversity (observed richness and Shannon diversity) between IBS cases and controls in PREDICT-1. (D) Gut bacterial beta diversity between IBS cases and controls in PREDICT-1. CI, confidence interval; OR, odds ratio; PC, principal component; PREDICT, Personalised REsponses to DIetary Composition Trial.

were strongly correlated (ρ=0.66 – 0.91) with GGB9758 SGB15368, a Clostridia species that was negatively associated with IBS in TwinsUK and approached significance in PREDICT-1 (p=0.05). The vOTU predicted to infect SFHK01 was also correlated with an unknown Clostridia species (GGB9787 SGB15410; ρ=0.66), which was associated with IBS in TwinsUK and PREDICT-1. The vOTU predicted to infect CAG-170 was positively associated with an unknown Ruminococcaceae (SGB15236; ρ=0.71) and negatively associated with various species in Lachnospiraceae (ρ=−0.59 – −0.67), including some that were associated with IBS in TwinsUK or PREDICT-1, such as *Blautia caecimuris*, *Clostridium* sp. AT4 and *Enterocloster asparagiformis*. In contrast, the vOTU predicted to infect CAG-103 was not strongly correlated with any of the bacterial species (maximum absolute ρ=0.29).

Predicted hosts included genera identified as IBS-associated in our bacterial analysis in TwinsUK (*Faecalibacterium*, *Blautia*, *Mediterraneibacter* and *Enterocloster*), as well as genera previously linked to IBS (eg, *Streptococcus*, *Bacteroides*, *Prevotella*, *Gemmiger*, *Alistipes*) and others not previously reported (eg, *Vescimonas* and *Ventricola*).

Potential AMGs were assessed only among the 50 IBS-associated vOTUs. Two of these encoded high-confidence AMGs. One was an endopeptidase that cleaves the heavy chain of human immunoglobulin A1 (IgA1) and is tightly associated with the bacterial cell surface. The other was categorised as a glycoside hydrolase involved in starch backbone cleavage.

Phage–bacteria interactions in IBS

Among IBS-associated vOTUs and IBS-associated bacteria, 1708 of 2250 pairwise combinations (75.9%) showed significant correlations (FDR <0.05; online supplemental figure S5). Extending analyses to all filtered vOTUs and bacterial species revealed a significant difference in the global vOTU–bacterial correlation structure between IBS cases and controls (permutation p=0.002).

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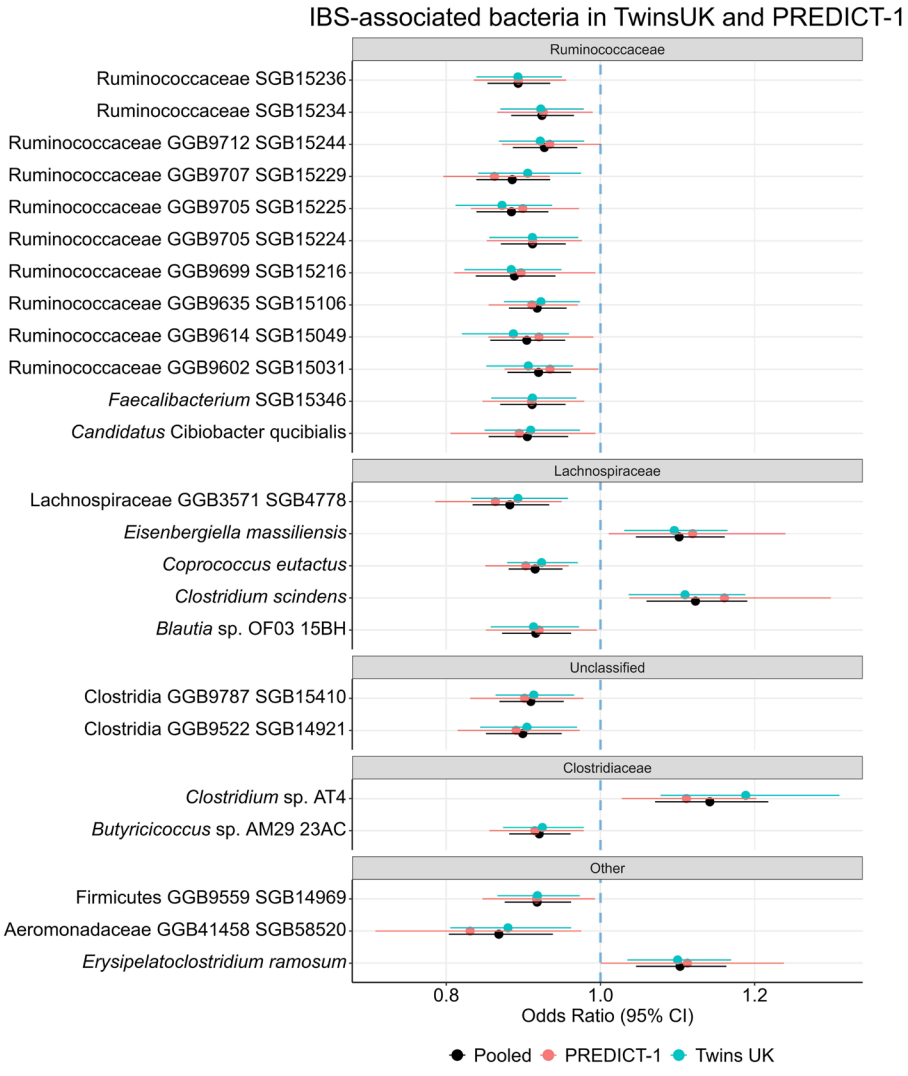

Figure 2 Replicated bacterial species associated with IBS. OR and 95% CI in TwinsUK (blue), PREDICT-1 (red) and pooled (black) for each bacterium associated with IBS in logistic mixed models adjusting for age, BMI, (batch effects in TwinsUK) and family ID as random effects. P values were adjusted for multiple testing using the Benjamini-Hochberg FDR. Bacteria are separated vertically by family. BMI, body mass index; FDR, false discovery rate; PREDICT, Personalised REsponses to DIetary Composition Trial.

Network analysis

SPIEC-EASI network analysis suggested differences in bacteria–bacteria and bacteria–vOTU network organisation between IBS and controls (figure 4). Modularity was higher among controls compared with IBS cases at the nominal level (0.34 vs 0.17, p=0.02, FDR=0.17; online supplemental table S13). Hub composition differed between networks (online supplemental table S14). In IBS, prominent hubs included *Roseburia intestinalis*, *Streptococcus thermophilus*, *Blautia obeum* and *Phocea massiliensis*. Control network hubs were enriched for several replicated IBS-associated species, including *Clostridium scindens*, two unclassified Ruminococcaceae (GGB9712 SGB15244 and GGB9705 SGB15224), an unclassified Clostridia (GGB9787 SGB15410), an unclassified Firmicutes (GGB9559 SGB14969) and *Mediterraneibacter glycyrrhizinilyticus*.

DISCUSSION

In this large UK-based cohort study, we used shotgun metagenomic sequencing to characterise the gut microbiome in IBS. Our bacterial analysis identified both novel associations and confirmed taxa with established links to IBS, while our phageome analysis, to the best of our knowledge the largest shotgun metagenomic characterisation of the gut phageome in IBS to date, identified 50 IBS-associated vOTUs. Two of these vOTUs encoded potential AMGs related to carbohydrate metabolism and IgA1 cleavage, suggesting functional relevance. Predicted hosts of IBS-associated vOTUs included both bacterial genera associated with IBS in our bacterial analysis and genera not previously implicated in IBS. We identified a significant difference in the global bacteria–vOTU correlation structure between IBS and controls. Complementary SPIEC-EASI network analysis provided nominal evidence for differences in network topology. Together, these findings highlight the importance of studying bacteria and phages in concert and suggest that IBS-associated dysbiosis extends beyond changes in individual taxa to encompass transkingdom ecological organisation.

Among predicted hosts of IBS-associated vOTUs were genera identified as IBS-associated in our bacterial analysis

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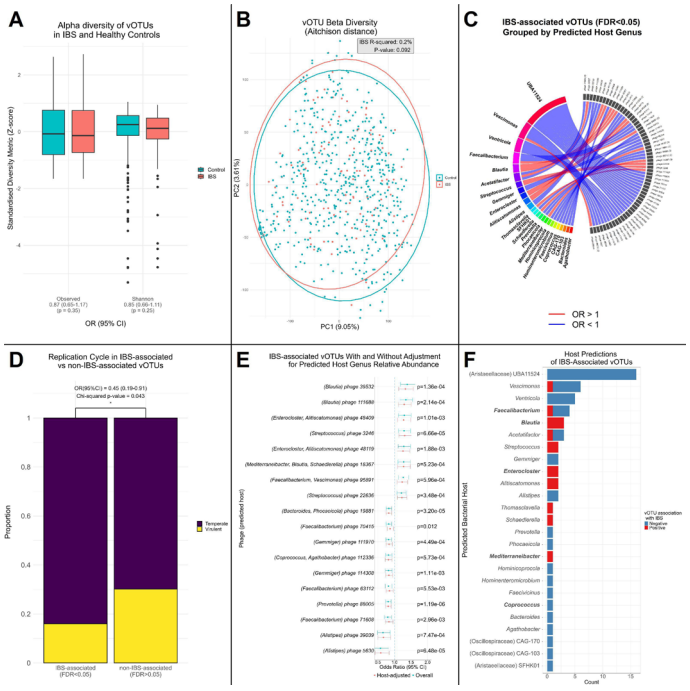

Figure 3 Gut phageome alterations and phage–host associations in IBS. (A) Gut phage alpha diversity (observed richness and Shannon diversity) between IBS cases and controls in TwinsUK. (B) Gut phage beta diversity between IBS cases and controls in TwinsUK. (C) vOTUs (right) associated with IBS in logistic mixed models adjusting for age, BMI, batch effects and family ID as random effects, and their predicted host genera (left). P values were adjusted for multiple testing using the Benjamini-Hochberg FDR. Positive associations are represented in red, negative in blue. (D) Proportion of vOTUs annotated as virulent versus temperate in IBS-associated vOTUs (left) compared with those not associated with IBS (right). (E) OR and 95% CI before (blue) and after (red) adding host genus relative abundance to the logistic mixed models among IBS-associated vOTUs with hosts present in the bacterial dataset. P value after adjustment for host genus relative abundance is represented in the text on the right-hand side. (F) The number of each predicted host genus among IBS-associated vOTUs. Bars are colour-coded based on the proportion of vOTUs that are negatively (blue) and positively associated with IBS (red). BMI, body mass index; FDR, false discovery rate; vOTUs, viral operational taxonomic units.

(*Faecalibacterium*, *Blautia*, *Mediterraneibacter* and *Enterocloster*), as well as genera previously linked to IBS (eg, *Streptococcus*, *Alistipes*, *Gemmiger*, *Phocaeicola* and *Agathobacter*).[9 36–38] Similar overlap between IBS-associated phages and bacterial hosts has been reported previously,[39–42] consistent with ecological coupling between the two microbiome components. In contrast, several predicted hosts, including *Vescimonas*, *Hominicoprocola*, *Schaedlerella*, *Faecivicinus* and *Ventricola*, have not previously been associated with IBS, suggesting that phageome analysis may identify additional bacterial taxa relevant to the condition.

Previous studies have reported inconsistent findings for viral alpha and beta diversity in IBS. Viral alpha diversity has been reported to be reduced in some studies,[41 43] whereas other studies found no significant differences between IBS and controls.[40 42 44] Consistent with the latter, we observe no difference in phage alpha diversity between IBS cases and controls. Similarly, phage beta diversity did not differ between groups, consistent with previous reports[42 44] but in contrast to other studies that identified significant alterations in viral community composition in IBS.[40] Differences in sequencing approaches, virome profiling pipelines, diversity metrics and statistical methods likely contribute to these inconsistencies and highlight the need for standardised analytical approaches.

Most detected phages were predicted to be temperate, which may partly reflect the greater recovery of integrated prophages by bulk shotgun metagenomic sequencing relative to virus-like particle (VLP)-enriched virome approaches.[45] Temperate lifestyles were over-represented among IBS-associated vOTUs; however, as most were reduced in abundance, this finding should be interpreted with caution. Although altered phage lifestyles have been implicated in IBD,[46 47] a previous IBS study reported no difference in the ratio of lytic to lysogenic phages.[41]

Two IBS-associated vOTUs encoded putative AMGs involved in starch hydrolysis and IgA1 heavy-chain cleavage, suggesting potential interactions with host carbohydrate metabolism and mucosal immune processes. Alterations in gut microbiome-mediated carbohydrate metabolism have previously been observed in IBS and may contribute to pathophysiology through changes in microbial fermentation and related luminal and sensory effects.[1 9] IgA is an important component of mucosal host–microbe interactions; therefore, IgA1 cleavage may potentially influence mucosal immune function. Consistent with our findings, previous IBS virome studies have also reported alterations in viral AMGs and their associated functional pathways.[42] Although functional consequences cannot be inferred from genomic annotation alone, these findings raise the possibility that phages contribute to metabolic modulation within the gut ecosystem.

vOTU–IBS associations beyond host abundance

When adjusting for the relative abundance of predicted host genera, vOTU–IBS associations were largely preserved, suggesting that vOTU-IBS associations were not fully explained by shifts in host genus relative abundance alone. Potential alternative

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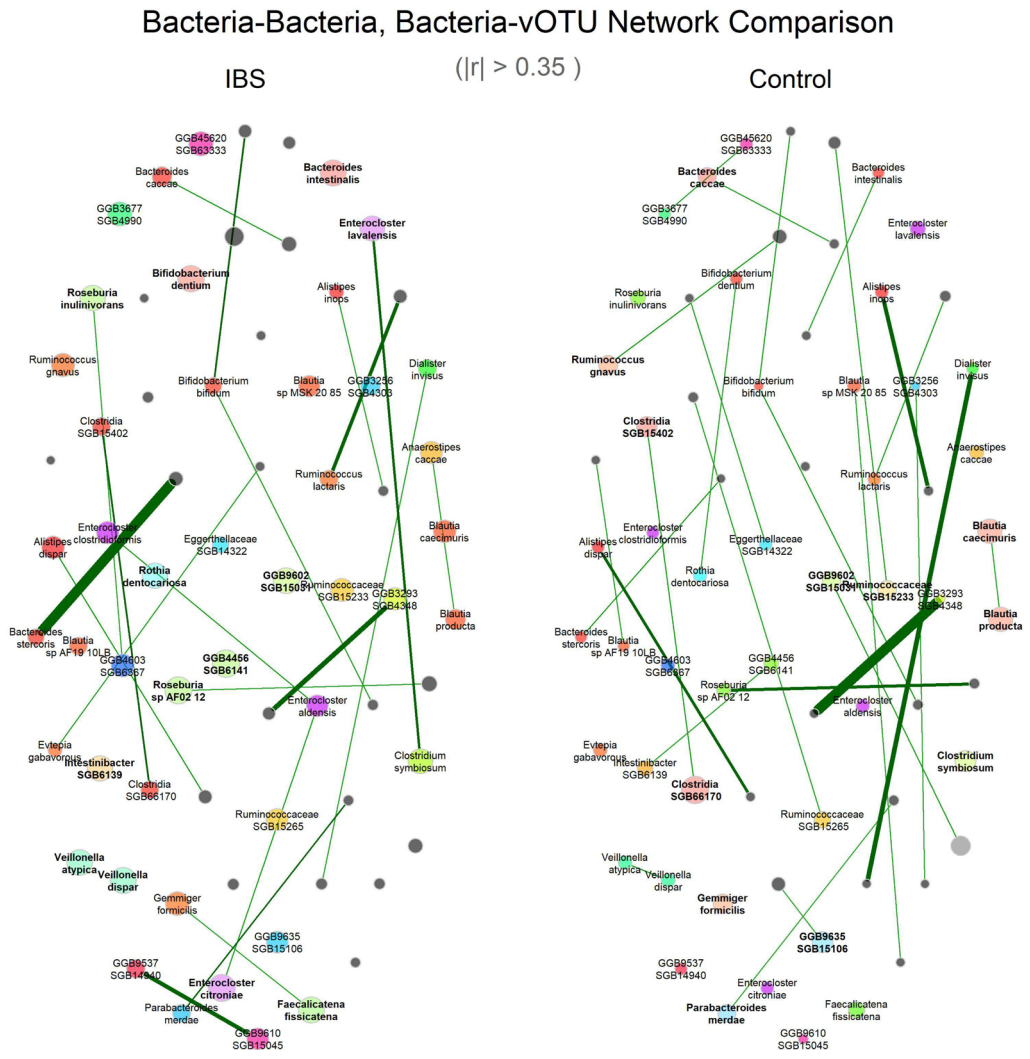

Figure 4 Bacteria–phage interaction networks in IBS and controls. Networks including interactions between bacterial species and bacteria and vOTUs in IBS (left) and controls (right). Only nodes with absolute correlation coefficients exceeding 0.35 in one of the networks are shown. Nodes are scaled by eigenvector centrality. Bacterial nodes are coloured by genus. vOTUs are unlabelled and grey. Bold labels indicate nodes whose eigenvector centrality ranks in the top 10% within each network for graphical labelling only; hubs were defined separately as nodes in the top 2% of eigenvector centrality values. Edge darkness denotes correlation strength. All edges remaining after correlation threshold filtering were positive (green). vOTUs, viral operational taxonomic units.

mechanisms mediating these associations could include modulation of bacterial metabolism via phage AMGs, horizontal gene transfer or effects on host–microbe immune interactions.[48]

Reorganisation of bacteria–phage interaction networks

Most IBS-associated vOTUs were correlated with IBS-associated bacteria, consistent with previous observations in IBS.[49] Importantly, we identified a significant difference in the global correlation structure between vOTUs and bacteria in IBS compared with controls. Network analysis further suggested altered connectivity patterns, including reduced modularity in IBS. Greater modularity has been associated with greater stability in microbiome networks, as distinct modules are less easily influenced by changes in other parts of the network.[50]

Interestingly, taxa previously linked to IBS, including *Roseburia intestinalis*, *Blautia obeum* and *Streptococcus thermophilus*,[51 52] were also identified as key hubs in the IBS network despite modest changes in relative abundance. However, confirmation of these hub taxa is required in datasets with more balanced case–control sample sizes. These findings suggest that IBS-associated microbial disruption may involve rewiring of ecological relationships rather than solely expansion or depletion of individual taxa.

Novel bacterial associations in IBS

We validated 24 IBS-associated bacterial species in a replication cohort. At least five of these have not, to the best of our knowledge, been previously linked to IBS. These included *Butyricicoccus* sp. AM29 23AC, *Eisenbergiella massiliensis*, *Candidatus* Cibiobacter qucibialis, *Blautia* sp. OF03 15BH and *Clostridium* sp. AT4. Several of these taxa belong to bacterial lineages with established roles in butyrate production or tryptophan metabolism, pathways relevant to inflammation, epithelial integrity and gut–brain signalling.[53 54]

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Consistent with prior literature, we observed depletion of butyrate-producing taxa (eg, Ruminococcaceae, *Faecalibacterium*, *Butyricicoccus* and *Coprococcus eutactus*).[10 55 56] We also observed enrichment of taxa previously associated with bile acid metabolism and IBS-D (*Clostridium scindens* and *Erysipelatoclostridium ramosum*),[57 58] as well as others with potential links to vitamin B12 biosynthesis pathways, including *Clostridium scindens*, *Candidatus* Cibiobacter qucibialis, Clostridiales bacterium KLE1615, *Blautia producta* and *Blautia hydrogenotrophica*.[59 60] Changes in vitamin B12 may influence not only microbial cross-feeding and host metabolic signalling but may also be related to IBS outcomes via cholinergic signalling.[61]

A reduction in bacterial alpha diversity in IBS is consistent with most prior work.[8 10 62] Despite these taxonomic links with potential downstream functional consequences, our functional pathway analysis revealed limited differences at the community level, aligning with some prior studies[10] but differing from others.[8] This relative functional stability despite taxonomic shifts is consistent with ecological redundancy within the gut microbiome. However, future work should aim to resolve conflicting findings regarding functional alterations of the gut microbiome in IBS.[8 10]

Several limitations should be acknowledged. First, the small number of participants within each IBS subtype precluded subtype-specific analyses. Second, the study included only female participants, limiting generalisability. Diagnostic investigations (eg, colonoscopy and CT) were unavailable, so organic GI disease could not be excluded and unmeasured comorbidities may have contributed to residual confounding and IBS misclassification. Third, approximation of Rome III criteria because of questionnaire response categories may affect comparability with standard definitions. Fourth, although we adjusted for habitual diet, residual dietary confounding cannot be excluded because food groupings were limited. Information on supplement use, including prebiotics and probiotics, was too sparse for reliable adjustment or subgroup analyses and could not be used to define a robust exclusion criterion; therefore, residual confounding related to probiotic or prebiotic use cannot be excluded. Fifth, the medication questionnaire did not distinguish between non-use and missing data. Sixth, the smaller PREDICT-1 cohort limited replication. Moreover, the modest effect sizes observed suggest that individual microbial features are unlikely to have sufficient diagnostic value in isolation. Although IBS-associated vOTUs may represent candidate biomarkers, their predictive performance and clinical utility require validation in larger, independent and geographically diverse cohorts. Finally, host prediction was limited to the genus level, and functional inference was based on genomic annotation rather than experimental validation. The cross-sectional design precludes causal inference, and shotgun metagenomic sequencing is likely to underrepresent low-abundance viral populations compared with VLP-enriched virome approaches.[45]

Consequently, low-abundance viral populations may have been under-represented despite the deep sequencing depth. Future longitudinal and experimental studies incorporating dedicated virome sequencing are needed to establish causal relationships and validate the functional relevance of gut phages in IBS, including their relationship with symptom severity.

CONCLUSION

We identified 24 replicated IBS-associated bacterial species and 50 IBS-associated vOTUs, many of which were predicted to infect bacterial genera relevant to IBS. We also demonstrated altered bacteria–vOTU correlation structures and potential differences in network topology in IBS. These findings suggest that IBS-associated microbiome alterations extend beyond changes in individual taxa towards reorganisation of transkingdom ecological interactions. By integrating phages and bacteria, our results highlight the phageome as a structured component of the gut ecosystem whose interaction architecture may be involved in IBS pathophysiology.

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Competing interests TDS is a cofounder of ZOE. NS, FA, SEB and TDS are consultants to ZOE. TDS, NS and FA have received options from ZOE. All other authors declare no competing interests.

Patient and public involvement Patients and/or the public were involved in the design, or conduct, or reporting, or dissemination plans of this research. Refer to the Methods section for further details.

Patient consent for publication Not applicable.

Ethics approval This study was carried out under TwinsUK BioBank ethics, approved by North West—Liverpool Central Research Ethics Committee (REC reference 19/NW/0187), IRAS ID 258513. This approval supersedes earlier approvals granted to TwinsUK by the St Thomas' Hospital Research Ethics Committee, later London—Westminster Research Ethics Committee (REC reference EC04/015, approved on 24 May 2019), which have now been subsumed within the TwinsUK BioBank. Ethical approval for ZOE PREDICT-1 was obtained from St Thomas Hospital research ethics committees. All individuals provided informed written consent (IRAS 236407) and the trial was registered on ClinicalTrials.gov (registration number: NCT03479866).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. The raw metagenomic sequence data used in this study have been deposited in the European Bioinformatics Institute European Nucleotide Archive database (TwinsUK

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accession code: PRJEB98467; ZOE PREDICT1 accession code: PRJEB39223). All data relating to TwinsUK samples have been deposited to the TwinsUK BioResource data management team. These data and non-metagenomic data for ZOE PREDICT1 are available by application to the Twin Research Executive Access committee (TREC) at King's College London. The TwinsUK BioResource is managed by TREC, which provides governance of access to TwinsUK data and samples. TwinsUK data users are bound by data sharing agreement set out in the data access application form, which includes responsibilities with respect to third party data sharing and maintaining participant privacy. Further responsibilities include a responsibility to acknowledge data sharing. All scripts used for data processing and statistical analyses are publicly available on GitHub: https://github.com/daniel-kirk-325/ibs_microbiome_twinsuk_scripts.git. Metagenomic data analysis was carried out using the YAMP pipeline (V.0.9.5.0) 19 (https://github.com/alesssia/YAMP), with taxonomic profiling and species level microbial relative abundances computed using MetaPhlAn (V.4.beta.2) 20 (https://github.com/biobakery/MetaPhlAn).

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