

Gut microbiome diversity and composition is associated with hypertension in women

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Objectives: Animal studies support a role for the gut microbiota in hypertension development, but large human studies are lacking. Here, we investigated the relationship between hypertension prevalence and gut microbial composition in two cohorts.

Methods: We included 871 unrelated TwinsUK women with faecal microbiome data (16s rRNA gene sequencing). Multivariable linear models adjusted for age, age² and BMI as well as MiRKAT models, were used to estimate the association of hypertension with alpha- and beta-diversity metrics. To identify taxa associated with hypertension, a generalized additive model for location, scale and shape was computed adjusting for covariates and multiple testing. Results were replicated in 448 women from PREDICT-1.

Results: We found that measures of alpha diversity are significantly lower in hypertensive cases [Beta(95% confidence interval, 95% CI) = -0.05 (-0.095 to -0.004), $P=0.03$] and a significant association between beta diversity and hypertension (FDR < 0.05). We identified and replicated two genera associated with hypertension. The genus, *Ruminiclostridium* 6 was less abundant in hypertension cases [meta-analysis (95% CI) = -0.31 (-0.5 to -0.13), $P=1 \times 10^{-3}$]. The uncultured microbe *Erysipelotrichacea-UCG003* was more abundant in hypertensive cases [meta-analysis (95% CI) = 0.46 (0.3–0.62), $P=1 \times 10^{-4}$]. We genomically analysed the 16s rRNA sequence and established a 100% identity match with the 16s rRNA sequence of the genus *Faecalibacillus*. We functionally annotated *Ruminiclostridium*, identifying 83 metabolic pathways, including pathways previously linked to blood pressure regulation.

Conclusion: In this large human observation, we show that gut microbiome diversity and composition are associated with hypertension. Our results suggest that targeting the microbiome may be a novel means to prevent or treat hypertension.

Keywords: diversity, gut microbiome, hypertension

Abbreviations: ASV, amplicon sequence variants; BEZI, zero-inflated beta distribution; BP, blood pressure; GAMLSS, generalized additive model for location, scale and shape; KEGG, Kyoto Encyclopedia of Genes and Genomes; MRCA, most recent common ancestor

INTRODUCTION

Hypertension is the most prevalent modifiable risk factor for cardiovascular morbidity and mortality affecting more than 1.3 billion people worldwide [1]. Given the burden of hypertension, any strategy that will improve blood pressure (BP) control will have major public health benefits [2]. The causation of hypertension is multifactorial [3], influenced by a host of genetic and environmental factors, including poor diet, obesity, inactivity and smoking, in addition to interactions between these factors [3]. The human microbiota comprises 10–100 trillion symbiotic microbial cells harboured by each person and is acquired from the environment starting at birth [4,5]. The gut microbiome, that is the community of microbes in the gastrointestinal tract, has been recently shown to be an important determinant of inflammation [6], obesity [7], type-2 diabetes [8] and arterial stiffness [9–11], all of which contribute to the risk of hypertension [12]. Moreover, animal studies suggest that gut microbes may act on downstream cellular targets, directly contributing to the pathogenesis of hypertension [9,13]. Recently, human studies have identified lower gut microbiome diversity [13–16] and specific gut microbes associated with hypertension [17,18]. In a recent review on gut microbiome composition and hypertension, Verhaar *et al.* [18] indicated the negative role of Gram-negative microbiota, including *Klebsiella*, *Parabacteroides*, *Desulfovibrio* and *Prevotella* and the possible neutral/protective role of *Ruminococcaceae*, *Roseburia* and *Faecalibacterium* [18]. Previous research into the gut microbial composition to hypertension also highlights

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the enrichment of *Eubacterium* [19], *Lactobacillus* [14], *Megasphaera* [14] and *Roithia* [14], and the depletion of *Bacteroides* [19], *Enterococcus* [20], *Oscillibacter* [16]. Moreover, a large study on 7928 individuals showed the periodontal microbiome, including *Campylobacter rectus*, *Veillonella parvula* and *Prevotella melaninogenica*, to be involved in BP regulation, suggesting the possible prohypertensive role of bacteria and bacterial products throughout the gastrointestinal tract and the mouth [21,22]. However, human studies are typically small and therefore underpowered, lack independent replication and there is a paucity of population-based evidence. Here, we first investigated the association between hypertension, and gut microbiome composition. We looked at the relationship between hypertension and the loss of microbiome composition and with specific genera in 871 people from TwinsUK. We then replicated our results in an independent population and further genomically characterise the hypertension-associated microbes using their 16s rRNA sequences.

MATERIALS AND METHODS

Design and study population

Discovery cohort

Study participants were female-unrelated individuals enrolled in the TwinsUK registry, a national register of adult twins recruited as volunteers without selecting for any particular disease or traits [23]. Here, we analysed data from 871 unrelated women from TwinsUK [23]. Twins provided informed written consent and the study was approved by St. Thomas' Hospital Research Ethics Committee (REC Ref: EC04/015). Data relevant to our analysis includes BP, antihypertensive drug use, BMI, age and gut microbiome composition assessed using 16s rRNA, as described below.

Replication cohort

The replication cohort consisted of an independent sample of 448 women from the UK-based PREDICT 1 study with analogous data. The PREDICT-1 study [24] was a single-arm nutritional intervention conducted between June 2018 and May 2019. Study participants were healthy individuals (thus eliminating potential confounders brought about by the presence of infections or other comorbidities) aged between 18 and 65 years recruited from the TwinsUK registry [23], and the general population using online advertising. Participants attended a full day clinical visit consisting of test meal challenges followed by a 13-day home-based phase, as previously described [24].

Measurements and variables

Blood pressure

BP was measured by a trained nurse using either the Marshall mb02, the Omron Mx3 or the Omron HEM713C Digital Blood Pressure Monitor (Omron Healthcare, Hoofddorp, Netherlands) performed with the patient in the sitting position for at least 3 min. At each visit, the cuff was placed

on the individual's arm so that it was approximately 2–3 cm above the elbow joint of the inner arm, with the air tube lying over the brachial artery. The individual's arm was placed on the table or supported with the palm facing upwards, so that the tab of the cuff was placed at the same level of the heart. Triplicate measurements were taken with an interval of approximately 1 min between each reading, with mean of second and third measurements recorded.

Participants were classified into two groups based on their BP level, age and use of BP lowering drugs: hypertension cases (<60 years of age, with SBP \geq 140 mmHg, or DBP \geq 90 mmHg, or currently using antihypertensive drugs, or started using before 60 years); and controls (if age > 50 years, SBP \leq 120 mmHg and DBP \leq 80 mmHg, and not on BP-lowering medication, or if age \leq 50, SBP \leq 115 mmHg and DBP \leq 80 mmHg and not on BP-lowering medication).

Gut microbiome composition

Gut microbiome composition was determined by 16S rRNA gene sequencing as previously described [4]. Briefly, the V4 region of the 16S rRNA gene was amplified and sequenced on Illumina MiSeq. 16S sequences were demultiplexed in QIIME. Amplicon sequence variants (ASV) were then generated using the DADA2 package in R using the pipeline described elsewhere [25]. Observed ASVs and beta diversities were computed using the R packages 'vegan' [26] and 'microbiome' [27].

Statistical analysis

Statistical analysis was done using R 4.0.2 (R Foundation, Vienna, Austria).

We used general linear models with a quasi-Poisson distribution to investigate associations between observed ASVs and hypertension. The association between beta-diversity metrics and hypertension was assessed via MiRKAT tests [28]. After grouping ASV at genus level, to identify taxa associated with hypertension, a generalized additive model for location, scale and shape (GAMLSS) fitted with the zero-inflated beta distribution (BEZI) was computed using the R package 'gamlss' [29]. The GAMLSS-BEZI model is a two-component mixture model including a zero-model accounting for excess zeros and a count model to capture the remaining component by beta regression, allowing for overdispersion effects. The first component of this mixture model is linked by the nu parameter that models the probability at zero, the second component is indexed by the mu and sigma parameters, respectively, the mean and precision parameters. Likelihood-ratio tests between the null model (covariates only) and full models (covariates and hypertension) were performed (FDR < 0.01). We adjusted for age, age², BMI and multiple testing using false discovery rate (FDR < 0.05).

We replicated significant genera (at $P < 0.05$) in PREDICT 1 and results were combined using an inverse variance random effect meta-analysis [30].

Genomic characterization of hypertension-associated microbes

All genomes isolated from the human gut belonging to the hypertension-associated microbes (*Ruminiclostridium* and

the *Erysipelotrichaceae*) families were downloaded from the Unified Human Gastrointestinal Genome catalogue [31] [and RefSeq data set (January 2021) for the *Erysipelotrichaceae* family]. CheckM v1.1.3 [32] was then ran using the 'lineage_wf' workflow to estimate genomic completeness and contamination. For *Erysipelotrichaceae* genera, we used high-quality genomes (<3% contamination, >95% completeness) of representative species. Whereas for the *Faecalibacillus* genus, we used the genomes of all existent species.

Evolutionary relationship of the *Erysipelotrichaceae* family

As *Erysipelotrichaceae* UCG003 was uncultured, we performed a genomic evolutionary analysis to investigate close relationships with other microbes. We predicted the 16S rRNA sequences of the representative *Erysipelotrichaceae* species using barrnap v0.9 (<http://www.vicbioinformatics.com/software.barrnap.shtml>). The predicted sequences together with the 16S rRNA sequence of *Erysipelotrichaceae* UCG-003 were then aligned using MUSCLE v3.8.1551 [33]. From which, we estimated the maximum-likelihood phylogeny with PhyML v3 [34] implemented in SeaView v5.0.4 [35], using the general time reversible (GTR) model of evolution, with optimized number of variant sites, nucleotide equilibrium frequencies and across site rate variation, and 100 bootstrap replicates. The constructed phylogenetic tree was then visualized using Interactive Tree Of Life (iTOL) [36]. In addition, we queried the 16S rRNA sequence of *Erysipelotrichaceae* UCG-003 in the RDP classifier [37] and BLASTn [38]. In BLASTn, the search was filtered by organism [*Erysipelotrichaceae* (taxid:128827)].

Prediction of the functional capabilities of *Ruminiclostridium*

To perform the functional analysis of *Ruminiclostridium*, we filtered genomes by a higher standard (>95% completeness, <1% contamination and <300 contigs). Missing sample accession numbers were obtained from the Sequence Read Archive (SRA) database of the NCBI, and the genomes from sample identifiers not found in the NCBI were discarded. Duplicated genomes were removed, keeping the genome with the highest N50 value. After filtering, we ran Prokka v1.12 [39] in 565 remaining *Ruminiclostridium* genomes while specifying the genus (parameter -genus) to retrieve the gff files. Specifically, enzyme commission

numbers were extracted to identify the Encyclopedia of Genes and Genomes (KEGG) [40] pathways using MiniPath (Minimal set of Pathways) (version: September 2020) [41]. All the KEGG identifiers related to environmental information processing and organismal systems were removed. For each metabolic pathway, the number of genomes that presented such pathway was calculated.

RESULTS

We included 871 unrelated women (397 cases, 474 controls) from TwinsUK, aged 56 (± 11.3) years and overweight, with an average BMI of 26 kg/m² (± 5) and we replicated our results in an independent sample of 448 women (57 cases, 391 controls) from the PREDICT-1 study, aged 44.8 (± 12.1) with an average BMI of 25.1 kg/m² (± 5.1). Descriptive characteristics of the study populations are summarized in Table 1.

In TwinsUK, after adjusting for confounders, we identify significantly lower observed ASVs in hypertensive individuals [Beta (95% CI) = -0.05 (-0.095 to -0.004), $P = 0.03$] (Fig. 1). When considering community microbial composition as measures of beta-diversity, significant differences were also detected for hypertension with binomial, Jaccard, weighted and unweighted-UniFrac diversity after multiple-testing (FDR < 0.05) (Figure S1, <http://links.lww.com/HJH/B646>). GAMLSS models adjusted for covariates and multiple testing (FDR < 0.05) identified abundances of 10 genera significantly different in hypertensive cases compared with controls (Figure S2, <http://links.lww.com/HJH/B647>). These included *Coprococcus* 3, *Blautia*, *Dorea*, *Bifidobacterium*, *Erysipelotrichaceae* UCG-003, *Fusicatenibacter*, *Coprococcus* 1, *Ruminiclostridium* 6, *Subdoligranulum* and *Anaerostipes* (Figure S2, <http://links.lww.com/HJH/B647>). As fibre intake is recognized to influence gut microbial composition, we re-run our analysis adjusting for nonstarch polysaccharide intake and results were consistent. We then assessed whether these associations were robust by testing for association of these 10 microbes in 448 independent women from the PREDICT-1 study. Out of those, two genera *Ruminiclostridium* 6 and *Erysipelotrichaceae* UCG003 were nominally associated with hypertension ($P < 0.05$) in the replication cohort (Fig. 2). We then combined the results using inverse-variance random-effect meta-analysis [Fig. 2 (*Ruminiclostridium* 6 (meta-analysis, 95% CI = -0.31 -0.5 to -0.13, $P = 1 \times 10^{-3}$) *Erysipelotrichaceae* UCG003 [meta-analysis (95% CI) = 0.46 (0.3-0.62), $P = 1 \times 10^{-4}$].

TABLE 1. Descriptive characteristics of TwinsUK and PREDICT1 samples

	TwinsUK (n = 871)				PREDICT (n = 448)			
	Cases (n = 397)		Controls (n = 474)		Cases (n = 57)		Controls (n = 391)	
	n	%	N	%	N	%	n	%
Female	397	100	474	100	57	100	391	100
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age (years)	60.33	8.72	52.41	11.9	50.27	11.72	43.97	11.99
BMI (kg/m ²)	28.14	5.41	24.19	3.77	27.9	6.64	24.63	4.66
SBP (mmHg)	138.98	15.01	109.24	6.76	122.95	15.39	102.12	8.31
DBP (mmHg)	83.48	10.14	69.05	6.21	84.43	9.83	69.94	6.07

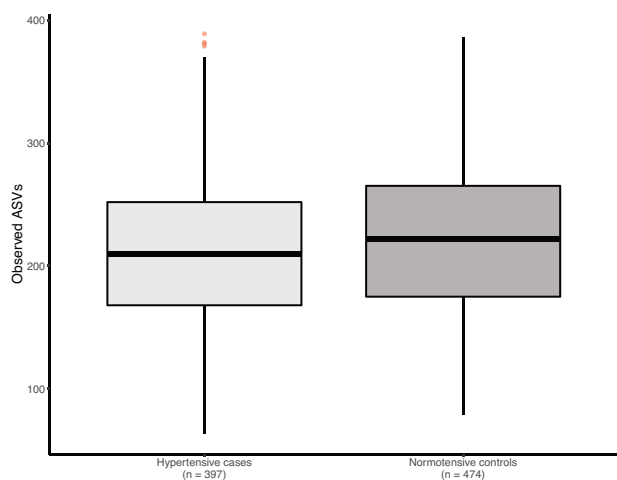


FIGURE 1 Comparison of observed ASVs within hypertensive cases and normotensive controls.

We then investigated the functional capacity of *Ruminiclostridium* by calculating the percentage of 16s *Ruminiclostridium* genomes that presented metabolic or genetic information processing KEGG pathways. This prediction of the functional capabilities of *Ruminiclostridium* revealed that this genus might be involved in 84 KEGG pathways, 83 of which relate to metabolism (Fig. 3), including lipid, amino acid, carbohydrate, cofactors and vitamin metabolism, among others (Fig. 3).

We then performed a genomic analysis to uncover the *Erysipelotrichaceae* genus' evolution as it was uncultured. After constructing a maximum-likelihood phylogenetic tree using the 16S rRNA sequences of *Erysipelotrichaceae* UCG-003 and the different genera within the *Erysipelotrichaceae* family, we identify that *Erysipelotrichaceae* UCG-003 is closely related to existent species of the *Faecalibacillus* genus, namely, *F. intestinalis*, *F. sp. H12* and *F. faecis*, sharing the most recent common ancestor (MRCA) (Fig. 4). To further interpret these results, we conducted a search using the 16S rRNA sequence of *Erysipelotrichaceae* UCG-003 as a query in the RDP classifier and in BLASTn search. The RDP classifier obtained a unique match to the *Faecalibacillus* genus with 100% identity. BLASTn search reported an E-value of 2×10^{-117} , and a 100% identity and query and query cover with sequences from the species *Faecalibacillus intestinalis* and *Faecalibacillus faecis*.

DISCUSSION

In this large-scale human study investigating the association between gut microbiome composition and hypertension with an independent replication, we report that gut microbiome diversity is inversely associated with hypertension in women (Fig. 1, Figure S1, <http://links.lww.com/HJH/B646>). We also identify two genera, an uncultured genus of the *Erysipelotrichaceae* family, whose relative abundance is higher in hypertensive cases and that of

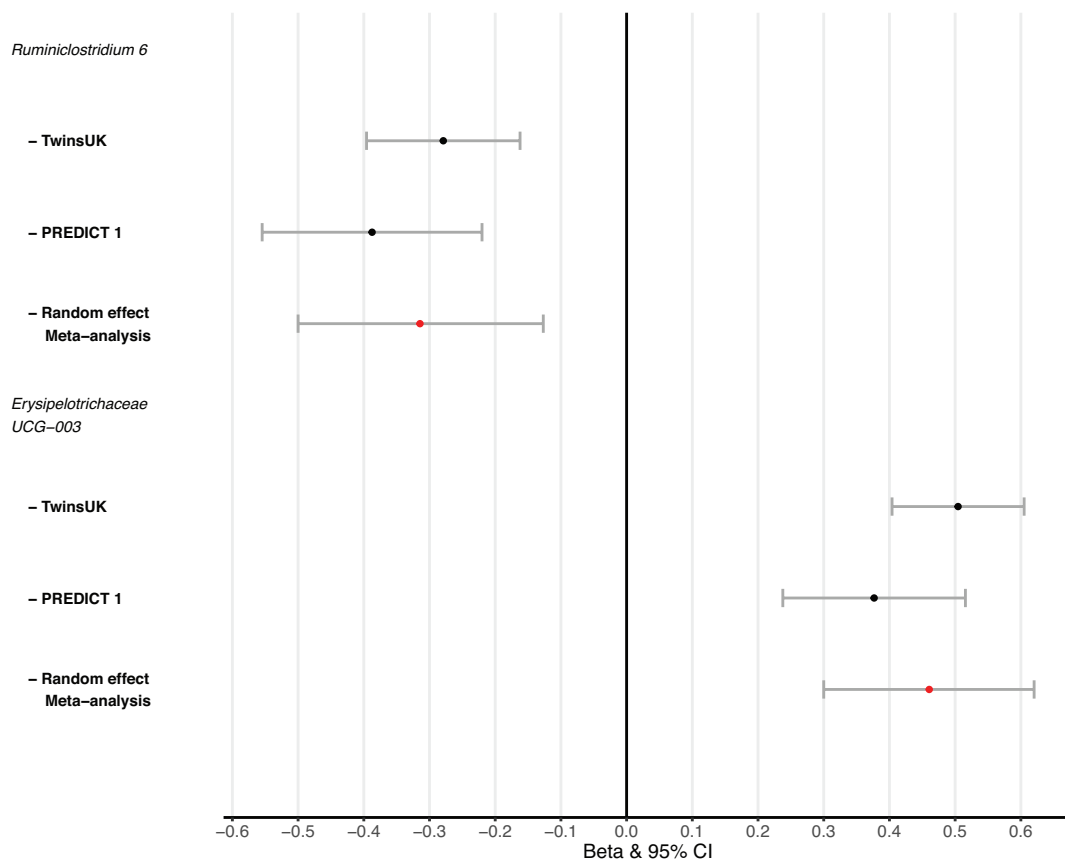


FIGURE 2 Inverse variance random effect meta-analysis of *Erysipelotrichaceae*_UCG-003 and hypertension from TwinsUK and PREDICT 1.

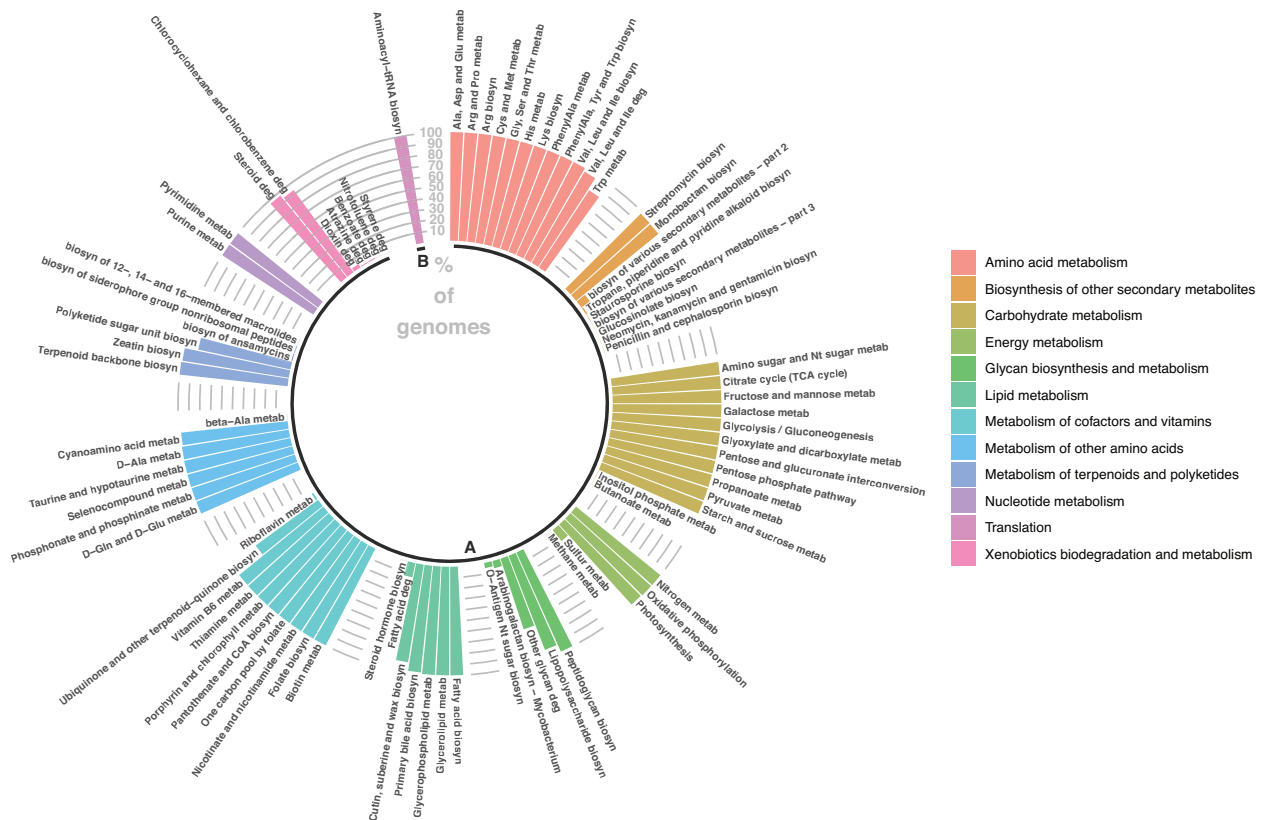


FIGURE 3 Functional genomic annotation of *Ruminiclostridium* genomes. Each bar represents the % of *Ruminiclostridium* genomic sequences that presented each KEGG pathway. The clusters are presented in the figure legend and groups symbolise processes: (a) Metabolism; (b) Genetic information processing.

Ruminiclostridium 6, whereby abundance was lower in hypertensive individuals (Fig. 2).

Functional annotation of *Ruminiclostridium* suggests its involvement in 84 pathways (Fig. 3), almost all of which related to pathways in metabolism. Moreover, the majority of the metabolic pathways were present in all genomic sequences (Fig. 3), implying a high homogeneity in the metabolic functional capabilities among the different species of *Ruminiclostridium*. A number of these pathways have been previously implicated in BP regulation. For instance, tryptophan biosynthesis and metabolism has been linked to SBP [3], while thiamine metabolism has also been correlated to hypotension in murine models [42] and thiamine supplement studies [43] (Fig. 3).

Through evolutionary analysis of the uncultured *Erysipelotrichaceae* genus, we identify a close relationship, and shared MRCA with *F. sp. H12*, *F. faecis* and *F. intestinalis*, species of *Faecalibacillus* (Fig. 4). Implying that *Erysipelotrichaceae UCG-003* belongs to or has evolved from *Faecalibacillus*. Subsequent results obtained from the RDP classifier and BLASTn search support that *Erysipelotrichaceae UCG-003* does in fact belong to *Faecalibacillus* with a 100% identity match and E-value of 2×10^{-117} .

Faecalibacillus is a Gram-positive genus [44], first isolated from healthy South Korean subjects [45]. Due to the

novelty of this genus and lack of phenotypic characterization, it was not included in the latest SILVA database, from which we assigned taxonomic ranks [45,46].

Animal models and case studies of patients have shown that members of the *Erysipelotrichaceae* family are significantly increased in a number of inflammatory conditions [47], such as irritable bowel syndrome [48], colorectal cancer [47] and rheumatoid arthritis with anticitrullinated protein autoantibodies [49], suggesting a pro-inflammatory effect of this bacterial family on the host. Previous studies report an association between abundance of *Erysipelotrichaceae* family members and host lipidemic profile, particularly relating to cholesterol levels [47]. However, after adjusting our analysis for levels of total cholesterol, the association between hypertension and *Faecalibacillus* remained statistically significant.

Our study has some limitations. Firstly, the study was based on middle-aged white female twins and hence may not be generalizable to other ethnic groups or to men. Although the characteristics of these women are representative of the general UK female population [23], clearly, studies in men and in other ethnic groups are needed. Secondly, the cross-sectional nature of our data does not allow us to infer causality. Third, although our genomic analysis highlights the functional capabilities of *Ruminiclostridium*, it does not

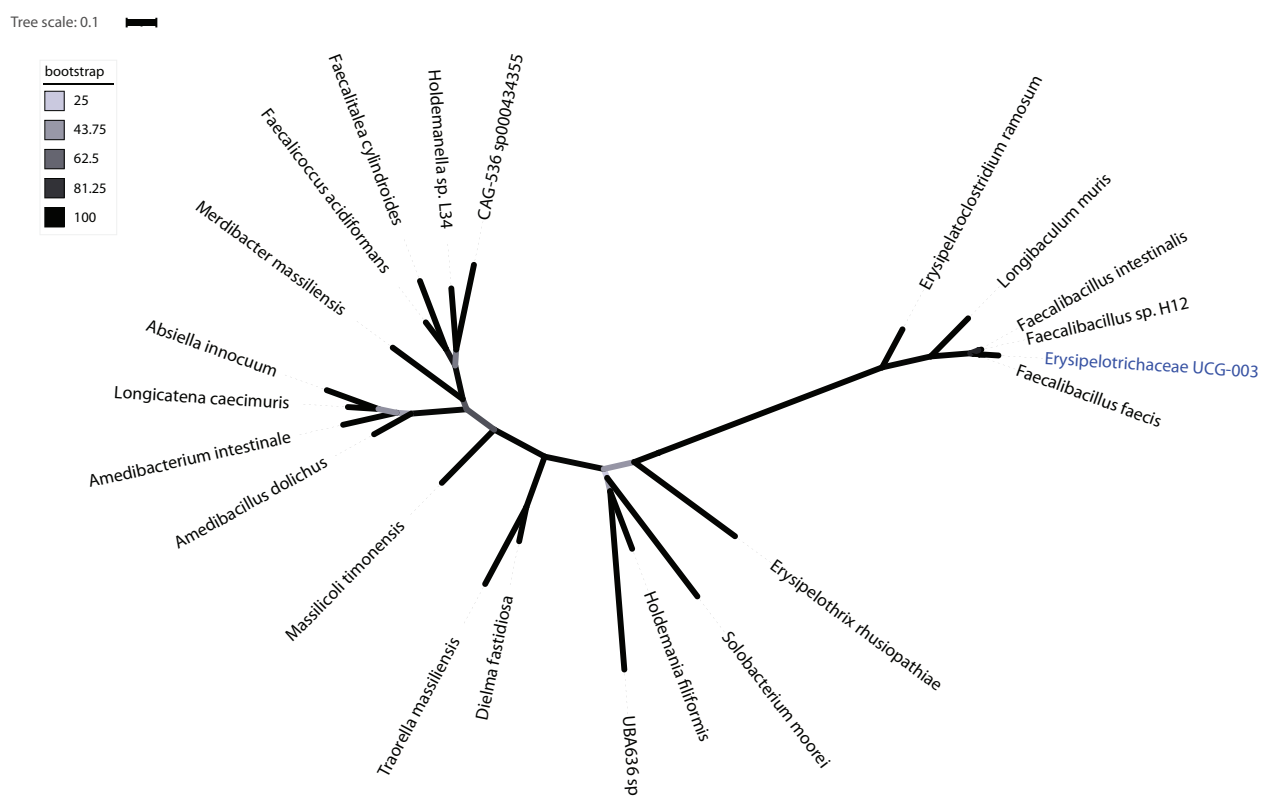


FIGURE 4 Unrooted maximum-likelihood phylogenetic tree of the 16S rRNA sequences showing the relationships of *Erysipelotrichaceae* UCG-003 and the genera within the *Erysipelotrichaceae* family. Branch colour and bar scale indicate the bootstrapping values and the average number of substitutions per site, respectively.

provide information on which pathways are actually active. Therefore, more research is required to comprehensively understand the mechanisms by which *Ruminiclostridium* can influence hypertension. However, our study does benefit from our large sample size, facilitating the analysis of numerous microbes with sufficient power and an independent replication.

In conclusion, we find that gut microbiome diversity and composition are associated with hypertension. Our results suggest that targeting the microbiome may be a novel way to prevent or treat hypertension. Foremost, more research is necessary to further corroborate correlations between the gut microbiome and hypertension and provide insights into mechanistic capacity of the gut microbiome to control BP.

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Conflicts of interest

T.D.S is co-founder of Zoe Global. A.M.V., F.A and N.S are consultants to Zoe Global. All other authors declare no competing financial interests.

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