

# Gut Microbiota Profiles in Myopes and Nonmyopes

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**PURPOSE.** To identify compositional differences in the gut microbiome of nonmyopes (NM) and myopes using 16S ribosomal RNA sequencing and to investigate whether the microbiome may contribute to the onset or progression of the condition.

**METHODS.** Faecal samples were collected from 52 adult participants, of whom 23 were NM, 8 were progressive myopes (PM), and 21 were stable myopes (SM). The composition of the gut microbiota in each group was analysed using 16S ribosomal RNA gene sequencing.

**RESULTS.** There were no significant differences in alpha and beta diversity between the three groups (NM, PM, and SM). However, the distributions of *Bifidobacterium*, *Bacteroides*, *Megamonas*, *Faecalibacterium*, *Coprococcus*, *Dorea*, *Roseburia*, and *Blautia* were significantly higher in the myopes (SM and PM combined) when compared with emmetropes. The myopes exhibited significantly greater abundance of bacteria that are linked to the regulation of dopaminergic signalling, such as *Clostridium*, *Ruminococcus*, *Bifidobacterium*, and *Bacteroides*. Individuals with stable myopia were found to have a significantly higher proportion of *Prevotella copri* than those with progressive myopia. *Bifidobacterium adolescentis*, a gamma-aminobutyric acid (GABA)-producing bacterium, was significantly higher in all myopes than in NM and, in the comparison between SM and PM, it is significantly higher in SM. *B. uniformis* and *B. fragilis*, both GABA-producing *Bacteroides*, were present in relatively high abundance in all myopes and in SM compared with PM, respectively.

**CONCLUSIONS.** The presence of bacteria related to dopamine effect and GABA-producing bacteria in the gut microbiome of myopes may suggest a role of these microorganisms in the onset and progression of myopia.

Keywords: gut microbiome, myopia, GABA, dopamine, 16S rRNA sequencing

The gut microbiome is an intricate ecosystem comprised of trillions of microorganisms,<sup>1</sup> including bacteria, yeasts, fungi, viruses, and protozoa. The microbiota plays an invaluable role in human health,<sup>2,3</sup> with 90% of the dominant gut microbial phyla belonging to Firmicutes and Bacteroidetes, as well as Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia phyla.<sup>4,5</sup> Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria make up the core microbiome required for optimal functioning of humans.<sup>6</sup> Any alteration to this core microbiome could result in dysbiosis and has been detected among those suffering from inflammatory diseases,<sup>7</sup> immune diseases,<sup>8</sup> and certain cancers.<sup>9</sup> These observations suggest that the gut microbiome is crucial in maintaining health.

The gut microbiome has also been shown to have a systemic impact on the host, with evidence suggesting links between the gut and other organs such as skin<sup>10</sup> and the brain.<sup>11</sup> Recent studies have even demonstrated the connection between the eyes and the gut microbiome and have illustrated how gut microbiota mediate/cross-talk via what is referred to as the gut-eye axis.<sup>12</sup> Indeed, dysbiosis of the gut microbiome has been found to play a role in several

ocular diseases, including glaucoma,<sup>13,14</sup> diabetic retinopathy (DR),<sup>15-17</sup> AMD,<sup>18,19</sup> keratitis,<sup>6</sup> uveitis,<sup>20</sup> and Sjogren syndrome-associated dry eye.<sup>21</sup> Data from these studies would also suggest that the make-up of the gut microbiome differs across these diseases.

A clinical study conducted in China comparing the composition of the gut microbiome of patients with POAG with a control group found that patients with POAG had an increase in the relative abundance of the bacterial family *Prevotellaceae* and unidentified *Enterobacteriaceae*.<sup>13</sup> In contrast, the bacterial genus *Megamonas* was more prevalent in the control group. Another study in Europe found an increased abundance of *Escherichia coli* species in patients with POAG and more *Bacteroides plebeius* in the control group.<sup>14</sup> Both *Prevotella* spp. and *E.coli* have proinflammatory properties. In AMD, two clinical studies conducted in Switzerland on patients with neovascular AMD found that the *Firmicutes* phylum was more prevalent in AMD patients compared with the control group.<sup>18,19</sup> The relative abundance of *Anaerotruncus* and *Oscillibacter* was increased in AMD patients compared with controls.<sup>18</sup> For DR, studies have been conducted in India and China. In the Indian study,



the abundance of the *Actinobacteria* phylum was lower in patients with DR.<sup>15</sup> In contrast, in the Chinese study, there was a decrease in the abundance of the *Firmicutes* phylum in patients with DR.<sup>16</sup> However, in another study conducted in India comparing diabetics with and without DR, no difference was found between the two groups.<sup>17</sup> Nevertheless, the study suggested that the relative abundance ratio of Bacteroides to Firmicutes (B/F ratio) could be considered as a DR developmental marker.<sup>17</sup> Altogether, these data suggest that the gut microbiome differs across diseases, although factors such as sample size, age, host genetics, and environmental factors, as well as independent cohorts, could contribute.

Relationships between the gut and eye may even exist at finer ocular structural level, rather than purely at the level or gut–eye axis as a whole, with the concept of the gut–retina axis being introduced in relation to retinal diseases.<sup>22</sup> Several studies have characterised the gut microbiome and its effects on dybiosis in healthy controls and individuals with ocular diseases such as DR,<sup>23</sup> POAG,<sup>13,24</sup> and AMD.<sup>25</sup> Myopia, however, has been little researched in this context, despite the known retinal structural consequences,<sup>26</sup> as well as the known importance of retinal image quality and function on normal ocular development.<sup>27,28</sup> Nonetheless, speculation may be growing in this area; recently, a review paper was published examining the Chinese and Western medicinal perspectives on the relationship between the gut microbiota and the pathological mechanisms of myopia in adolescents,<sup>29</sup> and one paper has characterised the ocular microbiome between low and high myopia, finding a higher relative abundance of Proteobacteria at the phylum level and *Acinetobacter* at the genus level in patients with high myopia.<sup>30</sup> In addition, studies on the gut microbiome of myopic individuals are limited. To date, only one study has been conducted using a myopic animal model.<sup>31</sup> The results of this study showed that the relative abundance of Firmicutes decreased while that of Actinobacteria increased significantly in myopic mice.

A review by Xi et al.<sup>29</sup> proposes a possible mechanism linking myopia to the gut microbiome that involves changes in dopamine production. Certain gut bacteria, such as enterococci, may possess homologues of enzyme genes similar to those found in mammals for the production of dopamine<sup>29,32,33</sup> via the existing phenylalanine–tyrosine–dopa–dopamine pathway.<sup>29,34</sup> Several case reports have reported that oral antibiotics have caused a transient effect of myopia,<sup>35–37</sup> and once the medication was stopped or changed to other medications, the effect had reversed. It could be speculated that antibiotics might have an effect on the dopamine-producing bacteria, because dopamine can be synthesised in the gut by these bacteria and released into the bloodstream and travels to the brain and other parts of the body to perform its functions.<sup>29</sup> Dopamine is a neurotransmitter in the retina, hence it has an important role in vision and therefore, its deficiency can lead to abnormal visual development and vision loss.<sup>38</sup> To this point, only a single supplement has been approved for clinical trials for myopia control interventions.<sup>39,40</sup> This supplement consists of 7-methylxanthine (7-mx), a caffeine metabolite. The Danish Medicines Agency has granted approval for the 7-mx tablet to be utilised in clinical trials within Denmark only as a therapeutic intervention for myopia control. Despite the lack of understanding regarding its precise mechanism of action, it is hypothesised that 7-mx modulates muscarinic acetylcholine or dopamine receptors, which

could potentially influence eye elongation and myopia progression.<sup>39</sup>

Given the suggestion that dopamine is linked to the gut microbiome and studies on supplements have implicated dopamine receptors in the mechanism of action, it is empirical to investigate whether individuals with myopia exhibit a different bacterial composition in their gut compared with those without myopia, particularly with respect to the bacteria involved in dopamine production. At present, no studies have characterised the gut microbiome of myopic and NM individuals. To investigate this notion, the present study aimed to identify the microorganisms in the faecal matter of individuals with and without myopia and to investigate whether the microbiome may play a crucial role in the onset or progression of the condition. The results will give a better understanding of the gut microbiome profiles of myopes and NM, which in turn may elucidate developmental mechanisms and serve as a basis for finding novel approaches to targeting the gut microbiome to develop new interventions for myopia control.

## MATERIALS AND METHODS

### Recruitment of Participants and Consent

This study was approved by the University Research Ethics Committee 2 of The University of Manchester (Ref: 2020-9547-16047) and adhered to the tenets of the Declaration of Helsinki. Participants were recruited via institutional and ethics-approved channels and were self-selecting. Interested participants were consulted to rule out whether they met the inclusion and exclusion criteria based on their self-reported medical history, including information on preexisting conditions such as high blood pressure. The inclusion criteria were: age between 18 and 45 years, visual acuity of 0.00 logMAR or less, astigmatism of 1.00 DC or lower, and anisometropia of 1.00 D or lower in each eye. Participants were excluded if they had any of the following: a history of ocular or systemic pathology, a history of refractive/ocular surgery, a history of myopia management intervention (including orthokeratology, multifocal spectacles, or contact lenses or atropine therapy), a history of medication use known to affect growth and/or retinal function or of taking antibiotics in the 8 weeks prior to the study, or a history of a previous clinical diagnosis of depression or mental health conditions.

Participants who passed the initial consultation were given a participant information sheet, where they were thoroughly informed about the study objectives and their eligibility to be a study participant. Informed consent was given by each participant, acknowledging that they had been through the participant information sheet. As part of the study protocol, participants with a known history of high blood pressure were excluded based on their self-reported medical history. Our exclusion criteria were designed to identify individuals with preexisting conditions that could impact the focus of our research. Participants who decided to participate after going through the participant information sheet were given an appointment date to attend a visit to the research laboratory for clinical measurement. Informed consent was obtained before any measurements were made.

### Clinical Measurement

After receiving consent, participants were again asked about their general and ocular health history to make sure they met

the inclusion and exclusion criteria. In this study, myopia is defined as a mean spherical equivalent (MSE) refractive error of  $-0.50$  diopters (D) or less. The threshold used is based on international consensus on quantitative definitions of myopia.<sup>41</sup>

For the purpose of this project, refraction of MSE  $-0.50$  D or less, which has not altered in the preceding 3 years, is referred to as stable myopia. Refractions of MSE  $-0.50$  D or lower, which have progressed by more than  $0.25$  D in the preceding year, are referred to as progressing myopia. Nonmyopia refers to MSE refractive errors of more than  $-0.50$  D and less than  $+0.50$  D, which have not altered in the preceding 3 years.<sup>41</sup> Refer to the supplementary file for details of procedures and instrumentation.

## Sample Collection and Processing

**Sample Collection and Storage.** The kit used in this study was a faeces sample collection kit BASIC, containing Fe-Col Faeces collection paper, a sterile sample tube, mailing pack, a mailing envelope and instructions for use (Diagnostic Products, Alpha Laboratories, Eastleigh, UK). After the participant returned the faecal sample to the researchers, the sample was transported from the collection site to the storage site on the same day. The sample was then weighed to  $200$  mg, transferred to  $2$ -mL Eppendorf tubes (Starlab, Milton Keynes, UK) and frozen at  $-80^{\circ}\text{C}$  until needed for DNA extraction.

**DNA Extraction and Quantification.** DNA was extracted using a DNeasy PowerSoil Pro extraction kit according to the manufacturer's instructions (Qiagen, Manchester, UK). For DNA extraction,  $200$  mg of the sample was added to the kit. The DNA concentration was measured using a Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

**DNA Amplification.** Polymerase chain reaction (PCR) was used to amplify the extracted DNA. The primers used to target the V4 region of the bacterial 16S small-subunit ribosomal gene were V4515FB: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGC MGCCGCGGTAA-3' and V4806R: 5'-GTCTCGTGGGCTCGGA GATGTGTATAAGAGACAGGGACTACNVGGGTWCTAAT-3'. The MasterMix for the PCR was prepared by adding  $2.5$   $\mu\text{L}$  of extracted DNA,  $5$   $\mu\text{L}$  of 515F primer,  $5$   $\mu\text{L}$  of 806R primer, and  $12.5$   $\mu\text{L}$  of KAPA HiFi HotStart ReadyMix (Roche, London, UK). The total volume of the PCR reaction was  $25$   $\mu\text{L}$ . PCRs were run for 30 cycles on a thermal cycler, Biometra TONE (Analytik Jena, Germany). The following PCR cycle conditions were used: 3 minutes of initial denaturation at  $95^{\circ}\text{C}$  followed by 30 denaturation cycles at ( $30$  s at  $95^{\circ}\text{C}$ ), annealing ( $30$  s at  $62^{\circ}\text{C}$ ), elongation ( $30$  s at  $72^{\circ}\text{C}$ ), and a final elongation ( $5$  min at  $72^{\circ}\text{C}$ ).

**Agarose Gel Electrophoresis.** To check proper amplification was completed, the PCR product was run for 50 minutes at  $90$  V on 1% agarose gels in agarose gel electrophoresis (PowerPac Basic, BioRad, UK). The gels were made by dissolving  $0.4$  g type I agarose in  $40$  mL of  $1\times$  Tris Acetate EDTA (TAE) buffer ( $\text{pH} = 8.0$ ). TAE buffer was made up of ( $20$  mL EDTA ( $0.5$  M);  $4.84$  g Tris-HCl; and  $1.14$  mL glacial acetic acid, which was diluted from a  $50\times$  TAE stock solution ( $40$  mM Tris base,  $20$  mM glacial acetic acid, and  $1$  M EDTA,  $\text{pH} = 8.0$  at  $25^{\circ}\text{C}$ ) with distilled water. GelRed™ DNA stain (from  $10,000\times$  stock solution) (Biotium, Fremont, CA, USA) was added to the agarose after the mixture had cooled, resulting in a final dilution of

$1:10,000$ . The mixture was then poured into Mini gel tanks (Bio-Rad, Hemel Hempstead, UK) and allowed to set up with the comb (for creating wells) in place. We mixed  $5$   $\mu\text{L}$  of the PCR product and  $1$   $\mu\text{L}$  of loading dye (both from Thermo Fisher Scientific) and pipetted into the wells. All samples were run in comparison with  $1$  Kb DNA ladder ( $5$   $\mu\text{L}$ ) (Bioline Ltd, London, UK). The gel was immersed in approximately  $400$  mL of TAE buffer. Bands formed on gels were viewed under  $312$  nm UV transilluminator (UVP, Upland, CA, USA). The samples were then sent to the DeepSeq laboratory at the University of Nottingham for sequencing on the Illumina MiSeq platform (Illumina, San Diego, CA, USA). Refer to Supplementary file for details of library generation, quality control, and sequencing.

## Microbiome Analysis

The data were analysed using QIIME 2.0,<sup>42</sup> with DADA2<sup>43</sup> as the denoising step. The mean number of reads in the experimental samples after quality filtering was  $30,080$ . There were  $13$  reads in a negative control of purified water after denoising, which was deemed comparatively negligible. Microbiome data was processed using the Phyloseq R package,<sup>44</sup> version 1.3.6. To avoid bias caused by sequencing depth during diversity analysis, data were rarefied to  $11,604$  reads (the lowest read depth). Beta diversity was calculated using weighted UniFrac<sup>45</sup> and plotted using principle coordinates analysis with the 'plot\_ordination' function in the Phyloseq package. Jaccard Index values were also calculated amongst all samples and plotted as nonmetric multidimensional scaling graphs, checking to ensure convergence in all cases. A scree plot was generated using the dimcheck-MDS function in the 'goevig' R package,<sup>46</sup> version 0.5.1, to ensure that stress values were below the  $0.2$  acceptability threshold.<sup>47</sup>

The relative abundance of each taxon was calculated at each taxonomic level. Taxa that made up less than  $0.01\%$  of the total abundance for phyla, class, and order, less than  $0.02\%$  for family, and  $0.003\%$  for genera were merged into one group. Differential abundance between taxa was calculated using the DESeq2 R package,<sup>48</sup> version 1.32.

Random forest analysis was performed with the 'randomForest' R package, version 4.6, using relative abundances based on identified genera. Out of bag error values were obtained and used to estimate the predictive accuracy of the model in finding associations between the microbiome and the different groups. To validate these associations, taxon abundance and samples were randomised to create a 'negative control' model, to see whether these associations were simply due to chance. Taxa most important for making distinctions were identified based on their 'MeanDecreaseAccuracy' values.

## Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 9.3.1 (GraphPad Software Inc., California) for descriptive analysis of the participants' characteristic and continuous variables. Normality tests were performed using the Shapiro-Wilk test. Differences between groups for alpha diversity (observed, evenness and Shannon) were calculated using one-way ANOVA or Kruskal-Wallis tests. For beta diversity, differences between groups were calculated with permutational multivariate ANOVA using the Adonis function of the 'vegan'<sup>49</sup> R package, version 2.5.7. Differential

abundance analysis was performed with the calculation of geometric means before estimation of size factors in R, using the DESeq2 package and the DESeq function. Significantly different taxa were defined as taxa with a  $P$  value of less than 0.01 and were then corrected using the Benjamini–Hochberg test for multiple testing and an adjusted  $P$  value ( $P_{\text{adj}}$ ) of less than 0.01 was accepted as significantly different. Correlation analysis between the right eye (RE) MSE (Spearman correlation), axial length (Pearson correlation), and significant bacterial genus abundance from DESeq2 was performed using GraphPad Prism. This analysis was conducted to determine the relationship between these variables, with  $r$  serving as the correlation coefficient and a 95% confidence interval. The threshold value considered significant was a  $P$  value of less than 0.05. Significant differences between predictive accuracy values using random forest, was calculated using the Student  $t$  test in R.

## RESULTS

### Participants' Demographic and Clinical Characteristics

A total of 52 participants enrolled in this study, of whom 23 were categorised as NM, 8 as progressive myopes (PM), and 21 as stable myopes (SM) based on their RE MSE power. The mean age of the NM was  $31.96 \pm 7.595$ ; of the PM,  $32.25 \pm 4.862$ ; and of the SM,  $30.62 \pm 8.182$ . Of the myopes, 4 had a myopic father, 9 had a myopic mother, 6 had two myopic parents, and 10 had no myopic parents. There were no significant differences between the three groups in age, time spent outdoors, and time spent doing near work and in the age of myopia onset between PM and SM. Details of the participants' demographic and clinical characteristics are presented in Table and Supplementary Table S3.

There were no significant differences in RE and left eye MSE (Mann–Whitney  $U$  test;  $P = 0.865$ ) and axial length (unpaired  $t$  test;  $P = 0.917$ ), and both had a strong positive correlation (MSE: Spearman  $r = 0.96$ ;  $P \leq 0.001$ ; axial length, Pearson  $r = 0.99$ ,  $P \leq 0.001$ ). Therefore, only data from the RE were used for further analysis.<sup>50</sup> There were significant differences in RE MSE ( $P < 0.05$ ) and axial length ( $P < 0.05$ ) between the three groups. Tukey's post hoc tests showed that the NM were significantly different from the progressive and SM for RE MSE and axial length.

### Analysis of the Alpha Diversity and Beta Diversity of NM, PM, and SM

Alpha diversity was quantified to provide a measure of community richness (number of different taxa) using the observed (total number of unique bacteria) operational taxonomic units, evenness (spread of taxa abundance), and the Shannon Diversity Index. The Shannon Diversity Index takes into account both observed taxa and evenness.

There were no significant differences in the observed operational taxonomic units between the three groups ( $P = 0.872$ ) and between the SM and PM ( $P = 0.711$ ) (Fig. 1A). The median for NM and SM was approximately 150 observed operational taxonomic units, whereas that for PM was below 150. Analysis of evenness showed that the SM data spread was greater and reflected in the Shannon index, while the evenness for NM was lower. There were no significant differences of evenness among the three groups ( $P = 0.416$ ) and between the SM and PM ( $P = 0.518$ ) (Fig. 1B). Analysis of

the Shannon Diversity Index showed no significant differences among the three groups ( $P = 0.498$ ) and between the SM and PM ( $P = 0.615$ ) (Fig. 1C).

To examine large-scale patterns in the data, beta diversity was calculated using weighted UniFrac and plotted as principal coordinates analysis (Fig. 2). Visually, there were no differences in the microbial communities in the three groups (represented by different shapes) (Fig. 2A). Separation along the  $x$  axis accounted for 69.6% of the variation within the data. The samples were clustered closely together with one extreme outlier from the SM in the graph. Statistical comparisons using permutational multivariate ANOVA tests (999 permutations) revealed no significant differences among the three groups with weighted UniFrac ( $P = 0.747$ ). Analysis of combined SM and PM as a myopic group and comparison with NM (Fig. 2B) was performed, and sub analysis between SM and PM was also performed, both of which showed no significant differences ( $P = 0.634$ ;  $P = 0.685$ ).

### Relative Abundances and Differential Abundance

On average, the three most abundant phyla found across NM, PM, and SM were Firmicutes, Bacteroidetes, and Actinobacteria (Fig. 3A). Bacteroidetes were more abundant in PM than in NM and SM and Firmicutes were more abundant in SM than in NM and PM. Samples were filtered to more than 0.003%, and 29 genera were detected in all samples. *Collinsella* was among the three most abundant genera in all the three groups (Fig. 3B). *Prevotella* and *Bacteroides* were more abundant in PM, and *Bifidobacterium* was more abundant in SM. *Megamonas* were more abundant in NM and SM than in PM. For clarity, genera with an abundance of less than 0.003% were grouped into a single category.

When SM and PM were combined as one myopic group for analysis between myopes and NM, there were no differences in the average relative abundance between NM and myopes at the phylum level (Supplementary Fig. S1A). At the genus level, *Prevotella* and *Collinsella* were slightly more abundant in NM than in the myopes. A greater abundance of *Megamonas*, *Faecalibacterium*, *Ruminococcus*, and *Megasphaera* was observed in the NM, whereas a slightly higher abundance of *Bacteroides*, *Clostridium*, and *Coprococcus* and a higher abundance of *Bifidobacterium*, *Roseburia*, *Blautia*, and *Dorea* were observed in the myopes (Supplementary Fig. S1B). Relative abundances at the class, order, and family level were also plotted (Supplementary Figs. S2A–C, Figs. S3A–C), although no major differences were visualised.

To investigate whether any taxa displayed statistically significant differences in differential abundance between myopes and NM, the DESeq2 test was used. Myopes, comprising the SM and PM groups, were contrasted with the NM group. The results revealed considerable disparities, with myopes exhibiting five times higher relative abundance of the following genera belonging to the Firmicutes phylum: *Blautia*, *Coprococcus*, *Dorea*, *Faecalibacterium*, *Megamonas*, and *Roseburia*, compared with NM (Fig. 4A). Additionally, a higher relative abundance of *Bacteroides* from the Bacteroidetes phyla and *Bifidobacterium* from the 'Other'-phyla category was also observed in myopes than in NM (Figs. 4B–C) ( $P_{\text{adj}} < 0.01$ ). [*Eubacterium*] from the Erysipelotrichaceae family, *Catenibacterium*, *Lactobacillus*, *Phascolarbacterium*, and *Ruminococcus*, were four times less abundant in the myopes than in NM (Fig. 4A) ( $P_{\text{adj}} < 0.01$ ). The remaining taxa with significantly different

TABLE. Demographic and Clinical Characteristics of NM, PM, and SM Participants

Characteristic	NM (n = 23)	PM (n = 8)	SM (n = 21)	Total (n = 52)	F/H	P Value	Tukey's Post hoc Tests (Adjusted P Value)
Ethnicity	White = 2 Asian/Asian British = 16 Caribbean = 1 Other ethnic group = 4 (3 Arab, 1 Latin)	Asian/Asian British = 7 Other ethnic group = 1 (Turkish)	White = 4 Asian/Asian British = 15 Other ethnic group = 2 (Arab)	N/A	N/A	N/A	N/A
Age (years)	31.96 ± 7.595	32.35 ± 4.862	30.62 ± 8.182	31.46 ± 7.408	0.2254*	0.799	N/A
Age of myopia onset (years)	N/A	12.5 (10–16)	13 (11–17)			0.476†	N/A
Time spent outdoors (average h/day)	3 (1.5–4)	2 (1.25–3)	2 (1.25–4)	2 (1.5–4)	1.327‡	0.515	N/A
Time spent doing near work (average hr/day)	6.826 ± 2.674	9.125 ± 2.357	6.857 ± 3.321	7.192 ± 2.977	2.077*	0.136	N/A
RE MSE	0.08957 ± 0.3854	-4.201 ± 2.699	-3.147 ± 1.785	-1.878 ± 2.360	34.47*	<0.001	NM vs. PM (<0.001) NM vs. SM (<0.001) PM vs. SM (0.241)
Axial length	23.58 ± 0.5697	25.46 ± 1.316	25.05 ± 0.9547	24.46 ± 1.177	21.70*	<0.001	NM vs. PM (<0.001) NM vs. SM (<0.001) PM vs. SM (0.498)

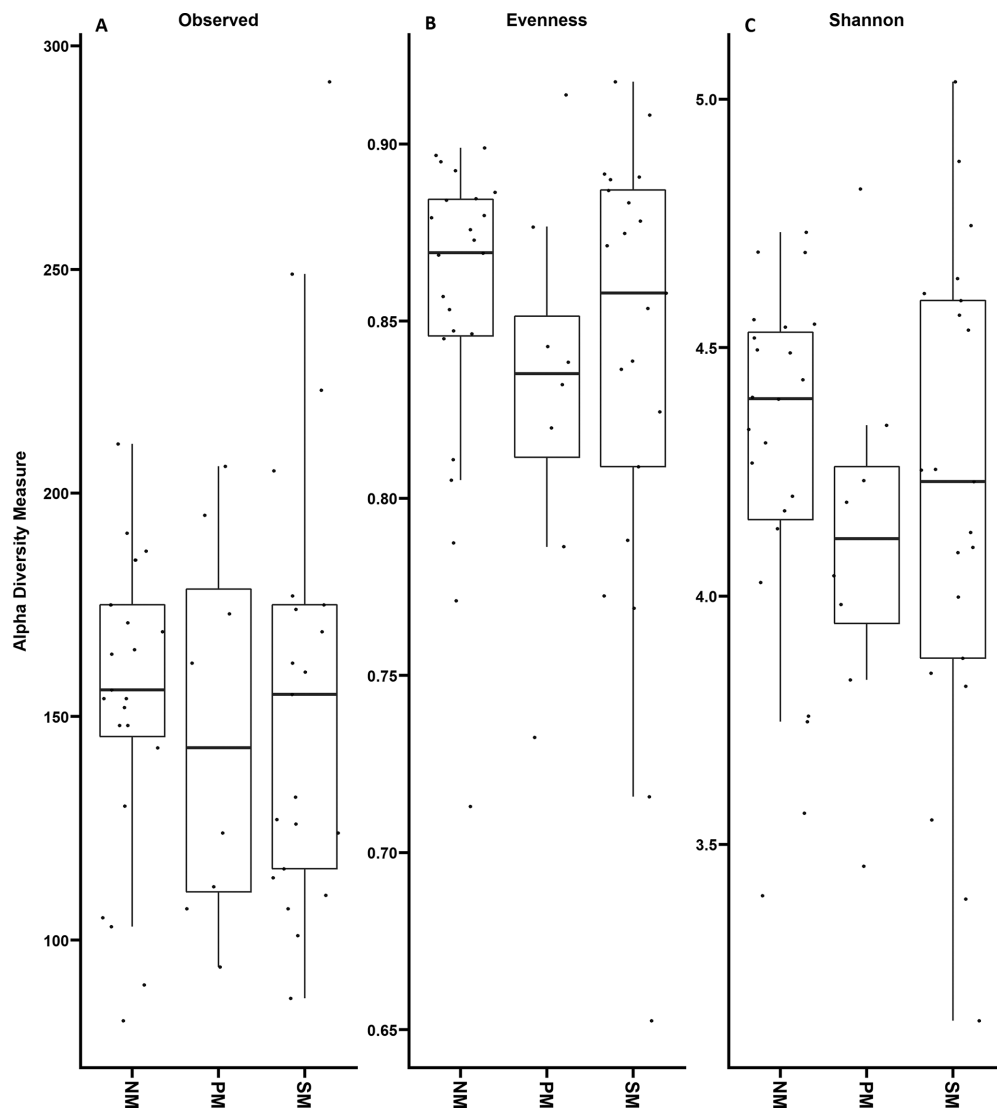
N/A, not applicable.

Significant difference threshold of  $P < 0.05$ .

\* F statistic, analysed using one-way ANOVA.

† Mann-Whitney statistic, analysed using the nonparametric Mann-Whitney test.

‡ Kruskal-Wallis statistic, analysed using the nonparametric Kruskal-Wallis test.



**FIGURE 1.** Alpha diversity analysis of gut microbiome data from NM, PM, and SM. Alpha diversity, in terms of observed taxa, evenness and Shannon Index was plotted comparing all groups. Individual data points are also shown on each graph. Graphs were generated using ggplot2 in R Studio version 3.4.2. No significant differences were identified ( $P > 0.05$ ) as determined by one-way ANOVA (observed) or Kruskal–Wallis test (Evenness and Shannon).

abundances are shown in [Figures 4A–C](#) and Supplementary Table S1.

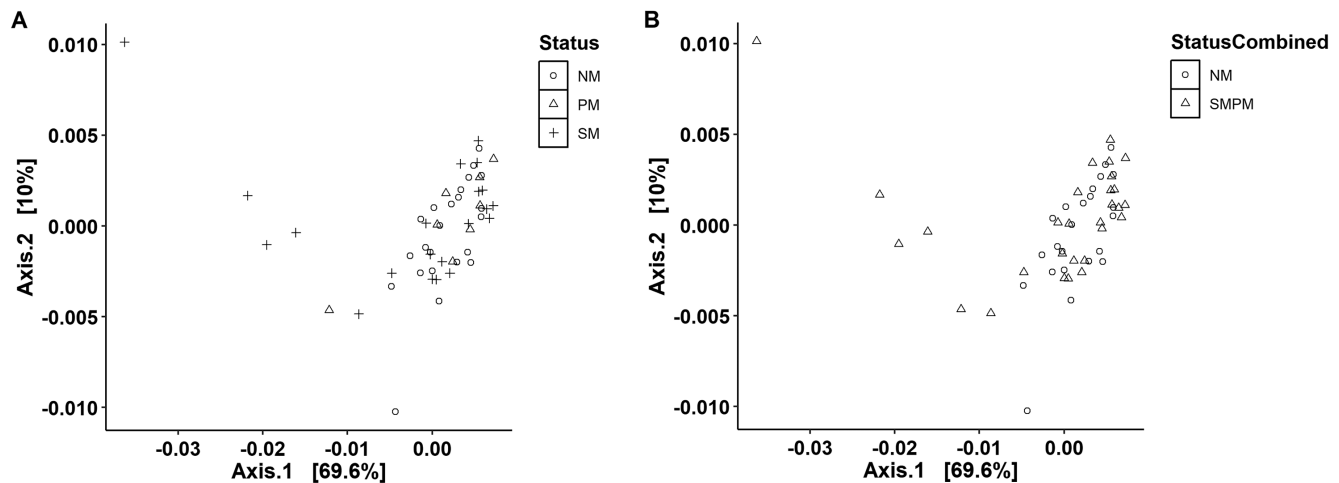
The difference in taxon abundance between SM and PM was also tested. The relative abundance graph showed that *Prevotella* was more abundant in the PM ([Fig. 3B](#)). However, this difference was not statistically significant after testing for differential abundances. When analysing *Prevotella copri*, it was found to be five times more abundant in the SM than in the PM ([Fig. 5B](#)) ( $P_{\text{adj}} < 0.01$ ). Similar results were observed for [*Clostridium*] from the family Peptostreptococcaceae and *Blautia*, both from the Firmicutes phyla ([Fig. 5A](#)) and *Bifidobacterium* ([Fig. 5C](#)) ( $P_{\text{adj}} < 0.01$ ). Additionally, *Clostridium* from the family Clostridiaceae and *Coprococcus* from Firmicutes phyla, *Barnesiella*, and *Bacteriodes* from Bacteroidetes phyla and *Slackia*, *Desulfovibrio* from ‘Other’ phyla were also found to be relatively less abundant in the SM ([Figs. 5A–C](#)) ( $P_{\text{adj}} < 0.01$ ). The remaining taxa with significantly different abundances are shown in [Figures 5A–C](#) and Supplementary Table S2.

### Correlation Analysis

A correlation was performed between the abundance of significantly different genera from DESeq2 and RE MSE. There was no significant correlation between all genera and RE MSE, except for the correlation between RE MSE and *Catenibacterium*. There was a positive correlation between the two variables (Spearman  $r = 0.29$ ;  $P = 0.041$ ) ([Fig. 6](#)). A correlation with the axial length was also performed and no significant differences were found. Supplementary Figure S4 shows a heat map plot of the correlation between axial length and the significant bacteria genus abundance.

### Random Forest Analysis

Machine learning was used to determine whether random forest analysis could accurately classify individuals according to their vision status (NM, PM, or SM) based on the composition of their gut microbiome. It was found that the



**FIGURE 2.** Principle coordinates analysis (PCoA) of gut microbiome data from NM, PM, and SM. PCoA was plotted using the weighted UniFrac method, comparing three groups (A) and NM vs combined SM and PM as myopic group (B). No significant differences ( $P > 0.05$ ) were identified, as determined by permutational multivariate ANOVA. The different groups of myopes are shown by shape (legend, right).

‘real’ model performed comparably to the negative control model ( $P > 0.05$ ), with only approximately 30% accuracy, whereas the negative control model had approximately 40% accuracy, suggesting that there was no significant association between the microbiome and vision status, based on this model (Fig. 7A). However, the most important bacteria in distinguishing between the three groups were identified by the model, as [*Clostridium*], [*Ruminococcus*], *Bacteroides*, *Streptococcus*, and *Subdoligranulum* (Fig. 7B). The results for the SM and PM groups, when combined, are shown in Supplementary Figures S5A–B.

## DISCUSSION

Myopia is understood to be caused by a multitude of factors and is often defined as the result of an interaction between genetics and the environment.<sup>51</sup> One of the mechanisms of interest in myopia development and progression is dopamine. Research using animal models, such as guinea pigs,<sup>52</sup> tree shrews, and chicks,<sup>53–55</sup> suggests a possible link between dopamine signalling and myopia, and eye growth regulation.<sup>38,56</sup> In the present study, *Bacteroides*, *Bifidobacterium*, *Clostridium*, and *Ruminococcus* were found to be significantly more abundant in all myopes (SM and PM) and *Prevotella copri* was found to be higher in SM than in PM. *Bacteroides uniformis* was three times more abundant in all myopes. All of these bacteria are known to play a role in regulating dopaminergic signalling.<sup>57</sup>

*Bacteroides* and *Prevotella*, which belong to the phylum Bacteroidetes, can produce metabolites and are associated with dopamine function via dopaminergic synaptic cleft activity modulation.<sup>57</sup> Hartstra et al.<sup>58</sup> showed via faecal microbiota transplantation in individuals with metabolic disorders that dopamine transporter concentration in the brain increased significantly when increased *Bacteroides uniformis* was detected in the faeces, but not with *Prevotella copri* species, in which an inverse relationship was observed.<sup>57</sup> Dopamine transporter is a protein that exists in the presynaptic membrane of dopaminergic terminal and plays an important role in regulating both synaptic and extracellular dopamine.<sup>57</sup> Although the sample size in the study by Hartstra et al.<sup>58</sup> was small and the exact mecha-

nism of the bacteria involved needs to be elucidated further, this is an indication of how and which bacteria can influence dopamine. A review by Hamamah et al.<sup>57</sup> indicated that, in addition to the bacteria already mentioned, *Lactobacillus* and *Enterococcus* also affect dopamine. However, our study found that the abundance of *Lactobacillus* was significantly lower in myopes than in NM and was not significant when analysed in SM versus PM. The abundance of *Enterococcus* was not significantly different between the three groups.

*Bacteroides*, which are abundant within the human gastrointestinal tract, have been linked to the synthesis of gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter in both the central nervous system and periphery.<sup>59,60</sup> The present study, using differential abundance, showed that the most abundant genus in the myopes was *Bacteroides* and they were mainly from *Bacteroides plebeius* species. There were GABA-producing *Bacteroides*,<sup>59</sup> of which *B. uniformis* was present in relatively high abundance in all myopes and *B. fragilis* was present in relatively high abundance in SM, which can have a significant effect on the synthesis and regulation of GABA. Studies have shown that GABA, along with dopamine, is associated with the development of myopia, where the balance between dopaminergic and GABAergic pathways are critical.<sup>61,62</sup> These mechanisms can be likened to a brake and accelerator system, where elevated levels of dopamine serve as a brake, retarding the progression of myopia, and increased levels of GABA act as an accelerator, hastening the development of this condition. Glutamate and glutamine were found to be the precursors of GABA production.<sup>59</sup> According to a study in a myopia-induced animal model, the metabolic pathway that was most affected was associated with glutamine and glutamate metabolism, with an increase in GABA levels observed in myopic mice.<sup>31</sup> However, because this animal model study was conducted in mice, where the gut microbiota may differ from that of humans, future studies could investigate glutamate and glutamine levels in both myopic and NM human subjects. This finding is important, because GABA production is influenced by both factors.

*Bifidobacterium* was another bacterium found in significantly greater relative abundance in myopes in the present

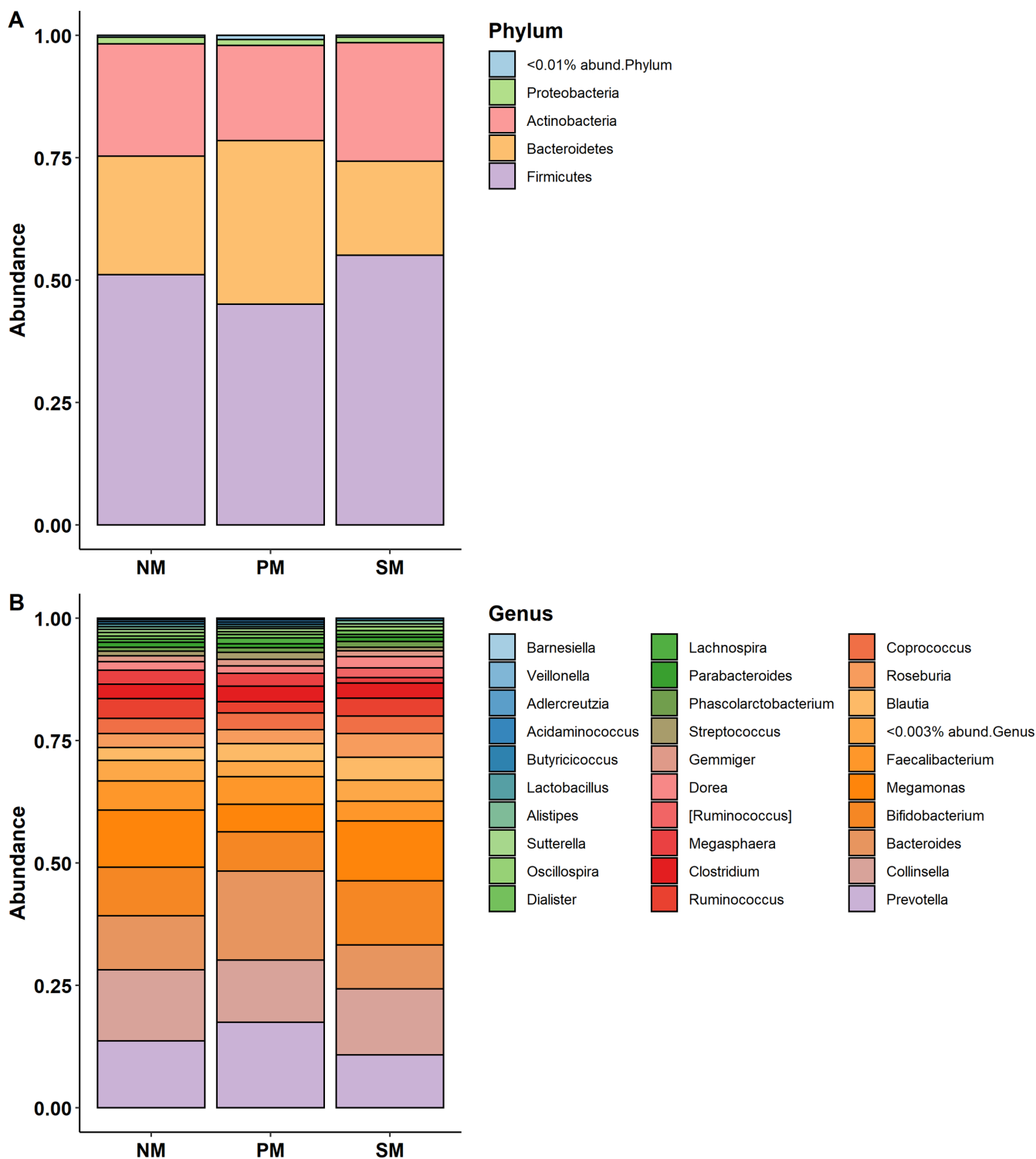
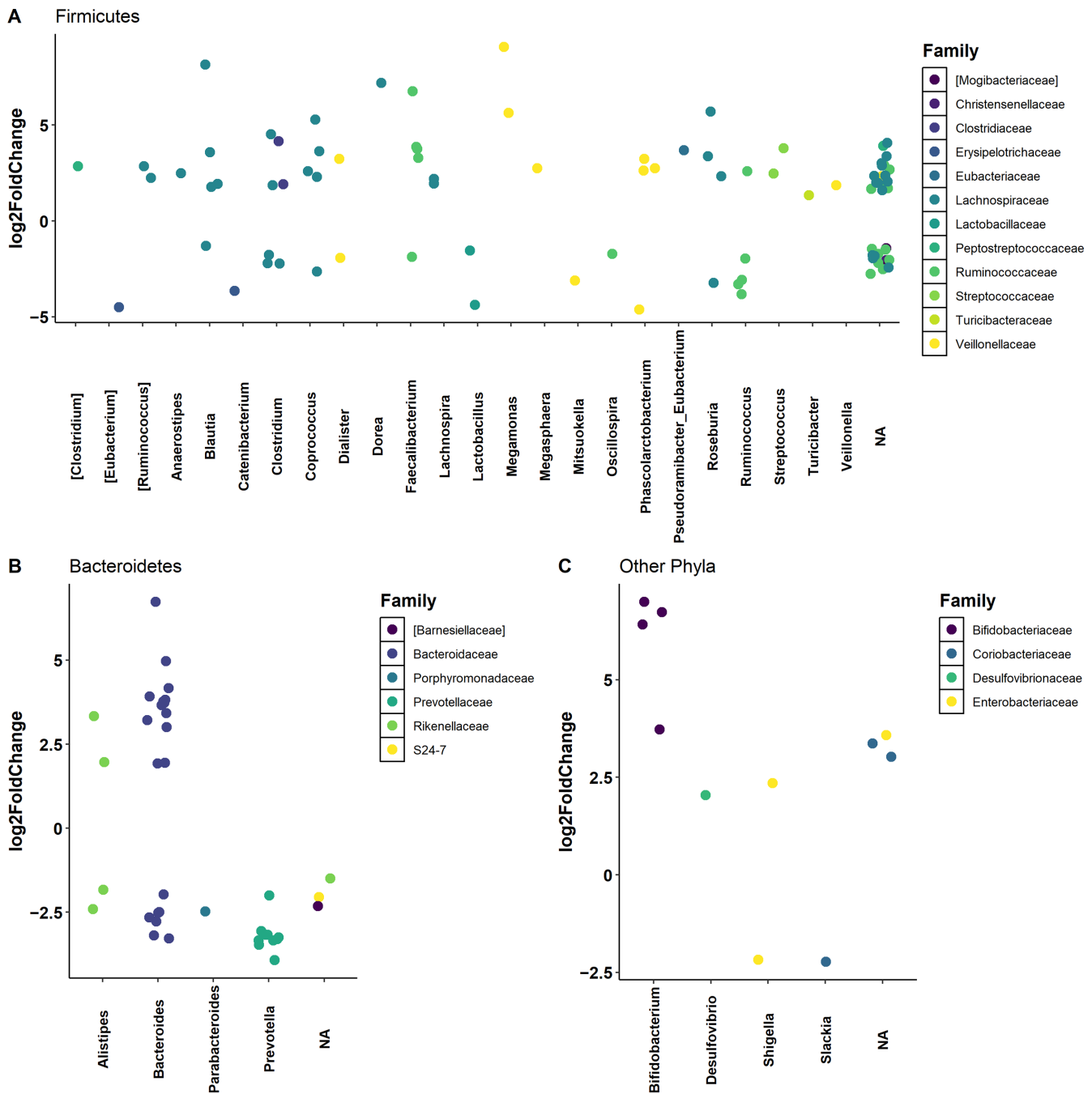


FIGURE 3. Relative abundances of dominant bacterial phyla and genera observed in the gut samples from NM, PM, and SM. (A) Average abundance of bacteria at the phyla level. (B) Abundance of bacteria at the genus level observed in three groups. Each phylum and genus is shown in an individual colour. The graphs were generated using ggplot2 version 3.4.2 package in R Studio.

study. *Bifidobacterium* also affects dopamine and GABA levels. We found a greater abundance of *Bifidobacterium adolescentis* in myopes. However, *B. adolescentis* was only significantly higher in abundance in SM when compared with PM. *B. adolescentis* has long been recognized as a probiotic micro-organism.<sup>63</sup> However, evidence from

Duranti et al.<sup>64</sup> has shown that *B. adolescentis* plays a crucial role in the biosynthesis of GABA within the human gut microbiome. They found that *B. adolescentis* has the highest prevalence of *gad* genes in their genomes. *B. adolescentis* and *gad* genes were completely absent in rats' cecum, suggesting that they are unique to the human gut and would



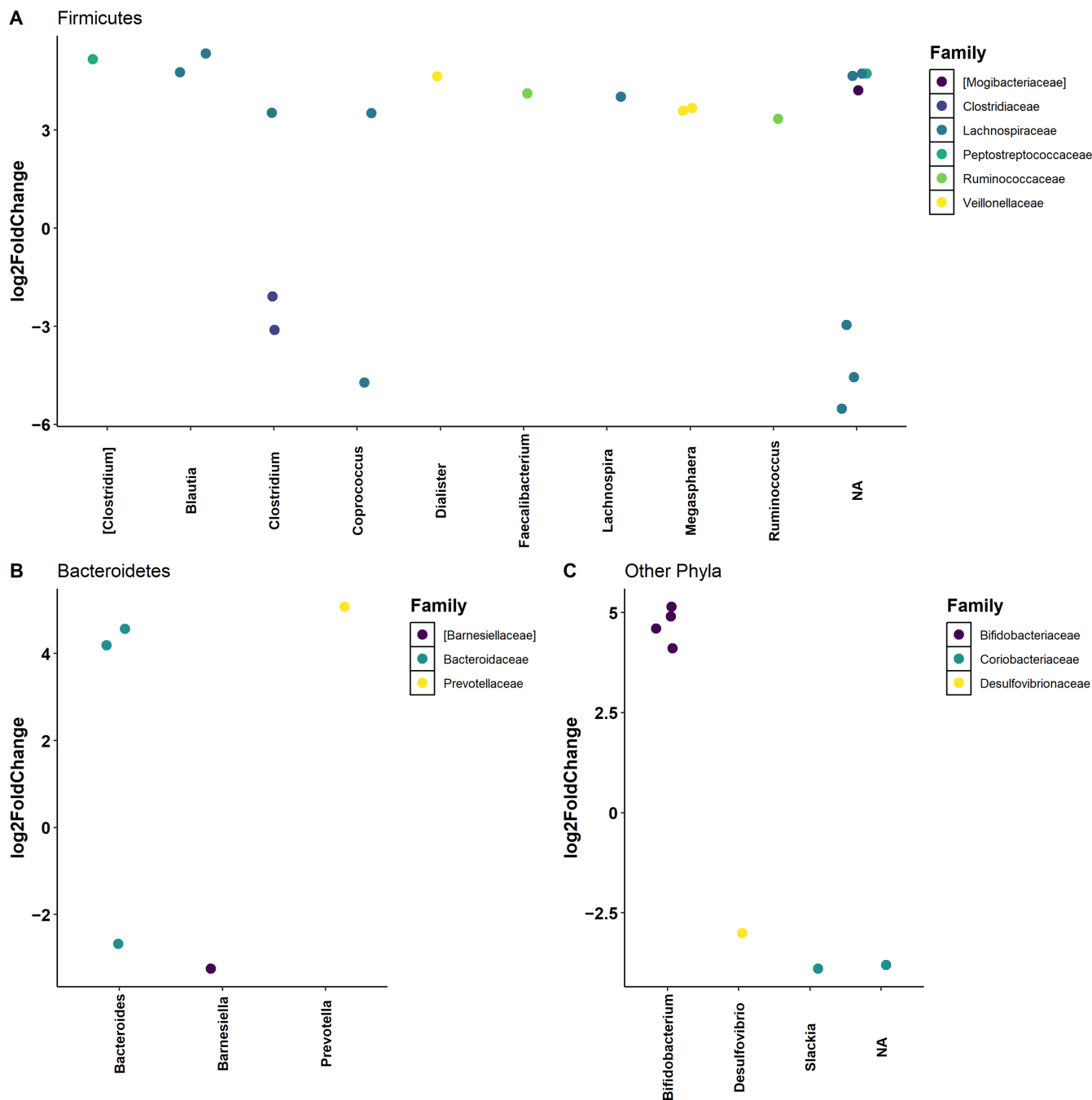


**FIGURE 4.** Differential abundance of bacterial composition in the gut microbiome according to visual status (myopes vs NM). The gut microbiome was analysed from stool samples obtained from healthy volunteers (NM) and myopes (comprising SM and NM). The differential abundance of taxa between the groups was calculated using the DESeq2 R package version 1.32. Taxa are classified as *Firmicutes* (A), *Bacteroidetes* (B), or from other phyla (C). The y axis is the degree of differential abundance and the x axis represent the taxa. Dots above 0 represent bacteria with higher relative abundance in myopes, whereas dots below 0 represent bacteria with lower relative abundance in myopes compared with NM. Statistical significance was determined using the Wald test and corrected for multiple testing using the Benjamini–Hochberg test, with an adjusted  $P$  value ( $P_{adj}$ ) of  $<0.01$  defined as significant.

be a perfect model for the GABA-producing gut environment.<sup>64</sup> Furthermore, in the Duranti et al. study, *B. adolecentis* was found to be a high GABA producer, as 23% of 82 bifidobacterial strains of *B. adolecentis* tested were able to effectively convert 65% of the precursors to GABA, namely monosodium glutamate.<sup>64</sup> In the myopia-induced mice study, *Bifidobacterium* was found to be a dominant

genus in myopic mice.<sup>31</sup> Li et al.<sup>31</sup> also found that *Bifidobacterium* was positively correlated with axial length in the myopic mice. These findings suggest that the possibility of *Bifidobacterium* playing a role in the mechanism of myopia warrants further investigation.

In the context of dopamine, *Bifidobacterium* appears to have a greater influence on dopamine due to its probiotic



**FIGURE 5.** Differential abundance of bacterial composition in the gut microbiome according to visual status (SM vs PM). The gut microbiome was analysed from stool samples obtained from SM and PM. The differential abundance of taxa between the groups was calculated using the DESeq2 R package version 1.32. Taxa are classified as *Firmicutes* (A), *Bacteroidetes* (B), or from other phyla (C). The y axis is the degree of differential abundance and the x axis represent the taxa. Dots above 0 represent bacteria with higher relative abundance in SM, whereas dots below 0 represent bacteria with lower relative abundance in SM compared with PM. Statistical significance was determined using the Wald test and corrected for multiple testing using the Benjamini–Hochberg test, with an adjusted  $P$  value ( $P_{adj}$ ) of  $<0.01$  defined as significant.

effect and has been studied for the stress response.<sup>65,66</sup> For example, a study on a maternal separation rat model subjected rats to a forced swim test, as dysregulation of dopamine beta-hydroxylase can stimulate stress response.<sup>66</sup> *Bifidobacterium infantis* was administered to study the rats' dopamine-related behaviour, monoamine concentrations in the brain, and central and peripheral hypothalamic–pituitary–adrenal responses. The maternal separation rats

in the control group were immobile in the forced swim test, had decreased noradrenaline levels, increased IL-6, and elevated amygdala corticotropin-releasing factor mRNA levels. Treatment with probiotic reversed the behavioural deficits, normalised immune responses and reduced noradrenaline levels. The results of this study support the hypothesis that *B. infantis* affects neuronal function and the activity of neurotransmitters particularly dopamine.<sup>57,66</sup>

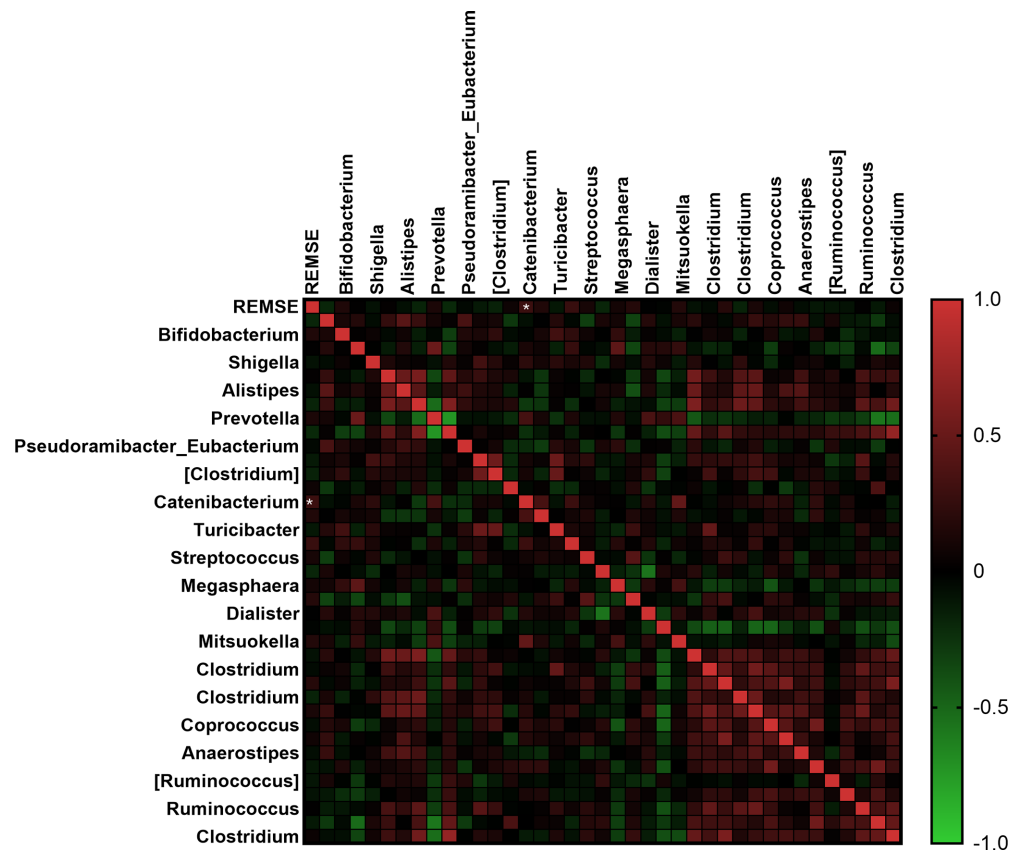


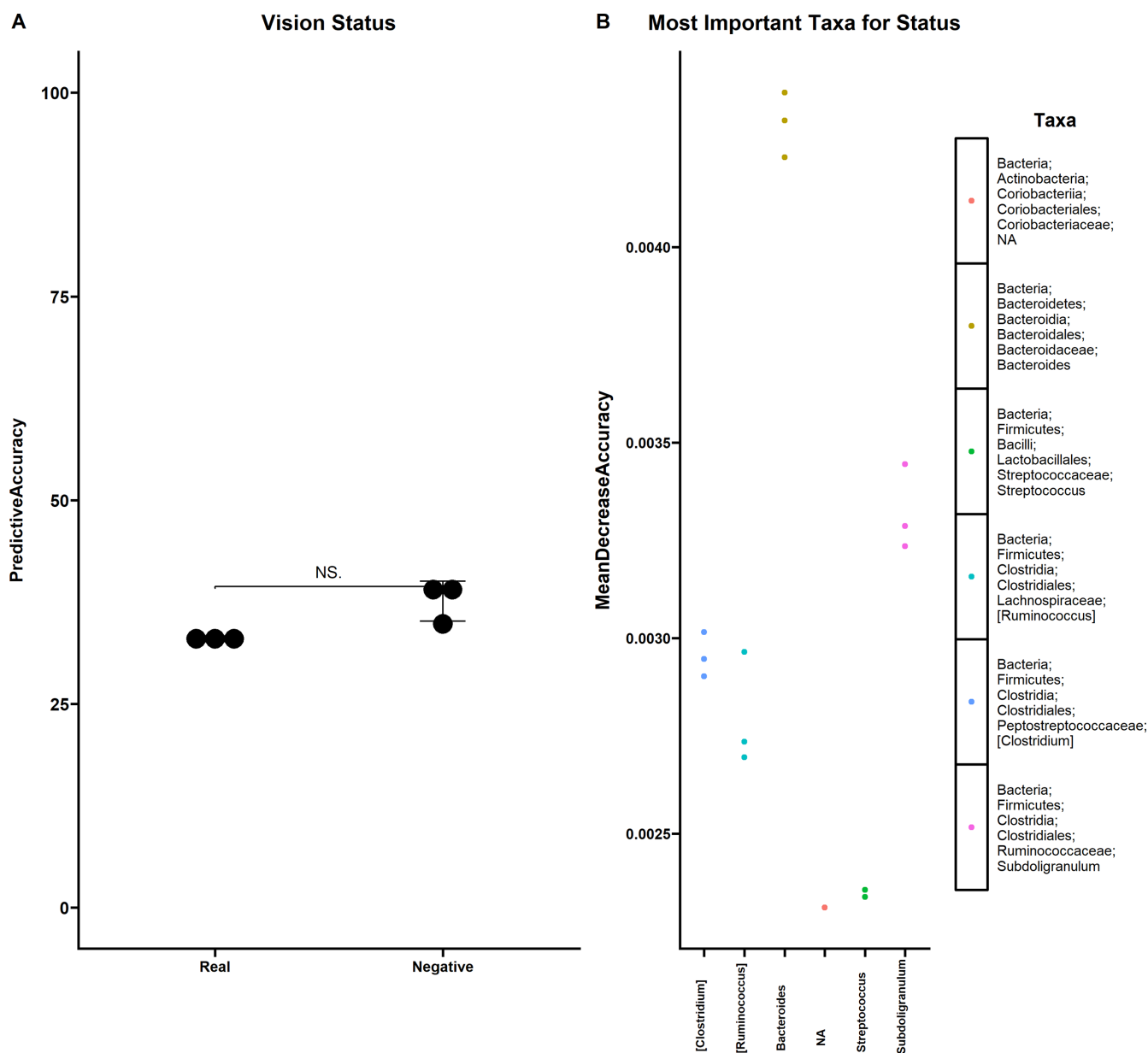
FIGURE 6. Correlation between RE MSE and significant bacteria genus abundance. Spearman correlation was computed and there was a positive correlation between RE MSE and *Catenibacterium* ( $r = 0.29$ ,  $P = 0.041$ ). Red indicates positive correlation and green indicates negative correlation.

However, we did not detect *B. infantis* in any of the groups we studied.

Many of the bacteria highlighted in the differential abundance analysis in the present study, that were significantly more abundant in myopic subjects were among the butyrate-producing gut bacteria, *Blautia*, *Roseburia*, *Faecalibacterium*, *Dorea*, and *Coprococcus*. Butyrate produced by these bacteria can affect signalling between the gut and brain by altering neurotransmitter levels via its intrinsic histone deacetylase inhibitor activity.<sup>57</sup> Additionally, butyrate can activate the G protein-coupled receptor (GPR41), also known as free fatty acid receptor 3 (FFA3R), which can protect dopaminergic neurons from toxicity caused by salsolinol, a selective neurotoxin that targets dopaminergic neurons.<sup>57,67</sup> In a review by Shivaji<sup>63</sup> on the relationship between gut dysbiosis and ocular disease found that these butyrate producing bacteria, which may have an anti-inflammatory effect in uveitis, are decreased in abundance. Aside from dopamine and the anti-inflammatory effect of butyrate-producing bacteria, an interesting finding was found in a study of patients with POAG, whereby *Blautia* was negatively correlated with mean VA.<sup>13</sup> This finding suggests that the presence or dysbiosis of butyrate-producing bacteria is not only important for maintaining gastrointestinal health, neurotransmitter concentrations, and gut-brain communication, but may also affect the eye's function via their actions on dopamine, anti-inflammatory properties and effect on vision: this requires further investigation.

Myopes exhibited a significant abundance of *Megamonas* bacteria. This finding is similar to Bai et al.<sup>68</sup> in their research on gut composition and ocular diseases, where an increase in *Megamonas* levels was detected in the DR group versus healthy controls. However, in contrast with patients with DR, a study on patients with POAG revealed a decrease in *Megamonas* levels compared with healthy controls, which was believed to offer protection against the disease.<sup>13</sup> Nevertheless, the precise protective effects of *Megamonas* on glaucoma remain unclear.<sup>13</sup> Moreover, Gong et al.<sup>13</sup> observed that the abundance of *Megamonas* was inversely related to the visual outcome, as evidenced by their findings.<sup>12,69</sup> This finding could potentially clarify the rationale behind the higher relative abundance of *Megamonas* in the myopes in this study.

The identification of *Prevotella copri* in the SM is a noteworthy observation, despite not being significantly different from its presence in PM via differential abundance analysis. In a study of bacterial keratitis in an Indian cohort *Prevotella copri*, which possesses proinflammatory properties, was found to be increased.<sup>63,70</sup> *Prevotella* has also been linked to various ocular diseases, including autoimmune uveitis,<sup>71</sup> AMD,<sup>72</sup> and POAG.<sup>73,74</sup> In patients with AMD, an increased number of *Prevotella* species were observed,<sup>72</sup> whereas in patients with POAG, the gut's bacterial flora showed altered *Prevotella*.<sup>73,74</sup> *Prevotella* exerts its inflammatory effect via stimulating the epithelial cell production of IL-8 and IL-6, as well as CCL20, leading to mucosal Th17 immunity and neutrophil recruitment. The resulting



**FIGURE 7.** Random forest analyses for vision status determining the associations between gut microbiome and vision status. Random forest was used to find associations between the relative abundance of identified genera and the accuracy of the model (**A**) and the bacterial taxa with highest importance to microbiota differences (**B**). No significance (NS.) was found, as determined by the Student's *t* test. Individual data points are shown  $\pm$  SEM.

*Prevotella*-mediated inflammation of the mucosa could lead to the systemic spread of inflammatory mediators and bacteria, which could impact systemic outcomes.<sup>75</sup> These findings suggest that certain *Prevotella* strains could be clinically significant pathobionts, which can contribute to human disease through chronic inflammation.<sup>75</sup> In a review by Xi et al.,<sup>29</sup> it was suggested that inflammation could be one of the mechanisms by which the gut microbiome affects myopia. Although not abundant, the presence of *Prevotella copri* in SM may indicate that the gut microbiome plays a role in myopia.

In this study, we also performed a correlation analysis between significant bacterial genus and the RE MSE, as well as axial length. Only *Catenibacterium* was observed to

be significant and had a positive correlation with RE MSE. This finding may be explained with the results of Liu et al. who found an increased risk of allergic conjunctivitis, resulting from decreased level of *Catenibacterium*.<sup>76</sup> Children with allergic conjunctivitis have a 2.35-fold higher incidence and risk of myopia compared with children without allergic conjunctivitis.<sup>77</sup> Nonetheless, there is limited research on *Catenibacterium* in relation to the eye. One study on patients with DR did find that *Catenibacterium* was detected in the DR group when compared with healthy controls.<sup>68</sup> Further studies are needed to learn more about the effects of this bacteria on the eye.

At any taxonomic level, changes in the gut microbiome are often linked with functional effects on the host.<sup>7,78</sup> In

the present study, the taxonomic profile at the phylum level closely resembled a typical gut profile,<sup>5</sup> characterised by a predominance of Firmicutes and Bacteroidetes, accompanied by a significant presence of Actinobacteria and a smaller fraction of Proteobacteria phyla. The ratio of Bacteroidetes to Firmicutes has been used as a biomarker in DR studies.<sup>17</sup> Because there has been no study to date on myopia, perhaps the ratio of Firmicutes to Bacteroidetes can be suggested for further studies in relation to the onset or progression of myopia, as both DR and myopia affect the retina.

A diversity analysis was conducted using alpha and beta diversity. Alpha diversity measures the complexity of a community's composition at a given site, which is directly proportional to the number of species present and their relative abundance<sup>79,80</sup>; beta diversity measures the taxonomic differences between pairs of samples. Species abundance is typically not considered, and data on species presence and absence are employed to determine which species are shared among samples and which are not.<sup>80</sup> In this study, even though the alpha and beta diversity was not significant, the more widespread alpha diversity in SM indicates much greater variation, and the lower evenness in PM may indicate that the PM was dominated by one or two bacteria with high abundance. High evenness means that there are more bacteria with relatively similar abundance, whereas low evenness means that there are fewer bacteria, but they may be highly abundant.<sup>79,80</sup> This might be due to the small sample size of PM compared with SM.

The differences in the gut microbiome of myopes and NM indicate that it is possible for the gut microbiome to play a role in myopia development and progression. However, the study included only a small number of PM samples. It would be beneficial to incorporate a larger number of samples, particularly from the PM, to comprehensively represent the gut microbiota profiles of stable and progressing myopes. Moreover, functional analysis of metabolites such as glutamate and GABA could augment the strength of such a study. Nonetheless, the present study provides an outset for future longitudinal studies to monitor alterations that could unveil a potential causal relationship between the gut microbiome and myopia. Furthermore, Mendelian randomization using large-scale genetic data may be a more appropriate method for investigating the causal relationship between the gut microbiome and myopia, as demonstrated in previous studies that examined the connections between the gut microbiome and AMD and glaucoma.<sup>81,82</sup>

Although we provided the fundamental results of the gut microbiota profiles in myopes and NM, the limitation of this study was the small sample size. A larger sample size is required to make the study more generalizable. Furthermore, it is also noteworthy to bear in mind that our study did not measure ocular haemodynamics, which is hypothesised to be related to the gut microbiome and myopia via hypoxia-activated pathways.<sup>29</sup> Oxygen dynamics have recently been demonstrated to play a crucial role in maintaining homeostasis in the intestine and exhibit a bidirectional regulatory relationship with the gut microbiota.<sup>83</sup> The intestine relies heavily on adaptive pathways triggered by hypoxia with hypoxia-inducible factor (HIF) particularly HIF-1 $\alpha$ , which is a critical hypoxic factor. Therefore, HIF-1 $\alpha$  is inseparably connected to the intestinal flora. Consequently, the gut microbiota may weaken scleral structure by elevating HIF-1 $\alpha$  levels, leading to myopia development.<sup>29</sup> Dysbio-

sis of the intestinal flora contributes to the development of ischaemic stroke or exacerbates cerebral ischaemia by enhancing systemic inflammation.<sup>84</sup> As such, future studies can incorporate the measurement of ocular haemodynamics and determine whether there are any differences in the gut microbiota profiles of myopes and NM under these circumstances.

Another limitation of this study was that we did not measure the participants' blood pressure and glucose levels and relied on the participants' self-reported medical history. Despite this limitation, the study still provides valuable insights into the gut microbiota profiles of myopes and NM. Future studies could consider the measurement of blood pressure and blood glucose levels.

## CONCLUSIONS

To our knowledge, this is the first study to explore the gut microbiota profiles of individuals with myopia compared with those without myopia. Our results showed that there are differences in the composition of the gut microbiota of myopic and NM individuals, with myopic individuals having a higher abundance of bacteria associated with the regulation of dopaminergic signalling and GABA production. Given the important role that dopamine and GABA play in the development and progression of myopia, these findings provide new insights into the existing knowledge of myopia mechanisms that could potentially contribute to a better understanding of the role of the gut microbiome in the development and progression of myopia. Dissecting this role may assist in the formulation of novel therapies for myopia control targeting the gut microbiome in myopes.

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**Author Contributions:** WEWO did the data collection, laboratory work, and analysis, made figures and wrote the first draft of the manuscript. GS facilitated the laboratory work, performed the microbiome analysis, made figures and critically reviewed and edited the manuscript. AJM supervised the microbiome component of the study and critically reviewed and edited the manuscript. FC supervised the clinical component of the study and critically reviewed and edited the manuscript. HR designed and supervised the clinical component of the study and critically reviewed and edited the manuscript. All authors contributed to the manuscript and approved the submitted version.

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