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Gut microbiota mediate vascular dysfunction in a murine model of sleep apnea: effect of probiotics

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Abstract

Rationale: Obstructive sleep apnea (OSA) is a chronic prevalent condition characterized by intermittent hypoxia (IH) and is associated with endothelial dysfunction and coronary artery disease (CAD). OSA can induce major changes in gut microbiome (GM) diversity and composition, which in turn may induce the emergence of OSA-associated morbidities. However, the causal effects of IH-induced GM changes on the vasculature remain unexplored.

Objectives: To assess if vascular dysfunction induced by IH is mediated through GM changes.

Methods: Fecal microbiota transplantation (FMT) was conducted on C57BL/6J naïve mice for 6 weeks to receive either IH or room air (RA) fecal slurry with or without probiotics (VSL3). In addition to 16S rRNA amplicon sequencing of their GM, FMT recipients underwent arterial blood pressure (aBP) and coronary artery and aorta function testing, and their trimethylamine N-oxide

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Authors' contributions

M. Badran, A. Khalyfa, S. Bender and A. Ericsson performed experiments. M. Badran and A. Ericsson analyzed data. M. Badran wrote the paper. D. Gozal provided the conceptual framework and continuous guidance for the project, and critically reviewed and revised the manuscript. All authors reviewed and approved the contents of the manuscript.

(TMAO) and plasma acetate levels were determined. Finally, C57BL/6J mice were exposed to IH, IH treated with VSL3, or RA for 6 weeks, and assessed aBP and coronary artery function.

Results: GM taxonomic profiles correctly segregated IH from RA in FMT mice, and the normalizing effect of probiotics emerged. Furthermore, IH-FMT mice exhibited increased aBP and TMAO levels, and impairments in aortic and coronary artery function ($p<0.05$) that were abrogated by probiotic administration. Lastly, Treatment with VSL3 under IH conditions did not attenuate elevations in aBP or CAD.

Conclusions: Thus, GM alterations induced by chronic IH underlie, at least partially, the typical cardiovascular disturbances of sleep apnea, and can be mitigated by concurrent administration of probiotics.

Plain language summary:

Using a well-established mouse model of sleep apnea consisting of long-term intermittent hypoxia (IH), we show that the adverse cardiovascular effects of IH can be recapitulated in naïve mice receiving fecal material from IH exposed mice and that co-administration of probiotics markedly improves those cardiovascular outcomes. Thus, probiotics may serve as adjuvant treatment of sleep apnea to potentially mitigate sleep apnea-associated cardiovascular disease.

Take home summary:

intermittent hypoxia-induced gut microbiome alterations elicit cardiovascular disturbances such as hypertension and coronary artery dysfunction that are prevented by probiotics administration

INTRODUCTION

Obstructive sleep apnea (OSA) is a chronic and extremely frequent condition that has been estimated to affect nearly a billion people around the world [1]. OSA is characterized by recurrent partial or complete upper airway obstruction during sleep, and can result in intermittent hypoxia (IH) [2]. These recurrent events over long periods of time can induce and propagate pathological processes, including endothelial dysfunction, which is a critical effector of cardiovascular disease (CVD) [3, 4]. The prevalence of OSA is as high as 40–80% in patients with CVD, including ischemic stroke and coronary artery disease (CAD) [5]. Despite these strong associations, interventional trials based on continuous airway positive pressure (CPAP) as the treatment modality have inconsistently detected the anticipated improvements in CVD, suggesting the need for adjuvant therapies aimed at the core disturbances induced by the disease [6, 7]. To address these issues, multiple studies using animal models of OSA, especially consisting of IH exposures during the sleep period, reported the emergence and propagation of endothelial dysfunction and atherosclerosis in numerous rodent vascular beds [4, 8] including the coronary circulation [9].

The gut microbiome (GM) network plays many vital roles beyond digestion, including maintenance of structural integrity of the gut barrier [10]. Perturbations in the GM community can result in changes in diversity and proportion of commensal bacteria, and are attributed to various environmental factors including diet and drugs [11]. Recent evidence suggests that GM changes are associated with multiple diseases including CVD [12]

and OSA [13]. Indeed, studies involving fecal microbiota transplantation (FMT), specific GM-dependent pathways and metabolites have been shown to influence host metabolism and CVD [14]. For example, increases in GM-mediated systemic Trimethylamine N-oxide (TMAO) levels adversely impact CVD in animal models, and human studies have further corroborated such associations in CAD and hypertension [14–16]. As a corollary, interventional studies using pre- and probiotics improved cardiovascular outcomes in patients with CVD via multiple mechanisms involving gastrointestinal mucosal barrier protection, reducing systemic inflammation and TMAO levels, and increasing levels of short chain fatty acids (SCFA) [15, 16].

Similar to CVD, GM diversity and abundance are altered in OSA patients and in animals exposed to chronic IH exposures [16–19]. Indeed, fecal matter obtained from OSA patients revealed GM changes that varied with the severity of OSA, and suggested that they may underlie some of the cardiometabolic perturbations in such patients [13, 20]. Furthermore, we and others showed that in animals exposed to chronic IH pronounced alterations in gut microbiota were detected [18, 21, 22], and persisted even after IH cessation for six weeks [18]. The characteristics of IH-induced GM alterations further suggested that the changes in GM may mediate cardiometabolic disease [16, 23–25]. However, there are no studies exploring whether IH-mediated GM alterations can directly affect vascular function in the absence of concurrent IH. Therefore, we hypothesized that FMT from mice exposed to IH to naïve mice may alter their GM and impair coronary artery and aortic vascular function. Furthermore, we postulated that treatment with probiotics would prevent, or at least mitigate vascular dysfunction in naïve mice receiving IH-FMT and in mice exposed to IH.

MATERIALS AND METHODS

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Missouri (Protocols #9586 and #9720) and performed according to the Declarations of Helsinki conventions for the use and care of animals. Male C57BL/6J mice (8-week-old) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed in a controlled environment with 12 h light–dark cycles (07.00 h–19.00 h) at constant temperature (26 ± 0.2°C) with *ad libitum* access to water and food (normal chow). At the end of the experimental period, mice were euthanized using carbon dioxide (1 min) followed by cervical dislocation.

Intermittent hypoxia exposures and probiotic treatment

The IH exposure protocol used has been described in detail previously [26]. Briefly, intermittent hypoxia (IH) mice were subjected to IH for 6 weeks while room air (RA) control mice were housed in standard housing conditions and exposed to normoxic gas (n = 10/group). IH exposures included alternating 21% F_{IO2} and 6% F_{IO2}, 20 cycles h^{-1} for 12 h day^{-1} during daylight (07:00 h – 19:00 h) using a commercially available commercial system (80 × 50 × 50 cm; Oxycycler A44XO, BioSpherix, Redfield, NY, USA). The exposures recapitulate nadir oxyhemoglobin saturations in the range of 68–75%, which are the primary correlate of moderate to severe OSA in humans [27]. The mice were in normoxic conditions (21% F_{IO2}) for the rest of the day (dark period from 19.00 h–07.00 h).

Mice were treated concomitantly with the probiotic VSL3 In drinking water (4×10^9 colony forming units). VSL3 is a commercial probiotic containing eight bacterial strains: four strains of *Lactobacillus* (*Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus delbrueckii* subspecies *bulgaricus*), three strains of *Bifidobacterium* (*Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium infantis*), and one strain of *Streptococcus* (*Streptococcus salivarius* subspecies *thermophilus*).

Fecal Microbiota Transplantation (FMT) in Naïve Mice

Fecal pellets from mice exposed to 6 weeks of IH or RA were collected on ice daily at noon (ZT-5) for one week then transferred to −80°C until use (n=10/group). FMT was performed by oral gavage of a fecal slurry into naïve mice three times a week at ZT-5 (male C57BL/6, 8 weeks old, Jackson Lab, n=10/group) as previously described [17, 28]. To prevent cage-related effects, recipients were randomly selected from different cages and housed with non-recipient mice and the experiments were repeated twice (n=5 in each experiment) to demonstrate reproducibility. Recipient mice were fasted for 2 hours prior to FMT, and the fecal slurry was obtained daily from fecal pellets of 5 donor mice suspended by vortexing in 1 mL PBS per 100 mg of fecal matter. Fecal mixtures were then centrifuged at 500*g* for 5 min and the supernatants were collected for FMT. Each recipient mouse received 100 µl of fecal slurry by oral gavage three times a week for 6 weeks with or without VSL3 probiotics (Alfasigma, Covington, LA) in all groups: i) RA-FMT, ii) IH-FMT, iii) RA-FMT-PRO, IV) IH-FMT-PRO. VSL3 was administered with FMT and in drinking water (4×10^9 colony forming units).

16S rRNA amplicon sequencing of gut microbiota

Fecal matter from mice corresponding to IH-FMT, RA-FMT, IH-FMT-PRO, and RA-FMT-PRO conditions were collected at ZT-5 on dry ice then processed using PowerFecal kits (Qiagen, Germany) according to the manufacturer's instructions [29]. Briefly, bacterial 16S rRNA amplicons were constructed via amplification of the V4 region of the 16S rRNA gene with universal primers (U515F/806R), flanked by Illumina standard adapter sequences [30]. The final amplicon pool was evaluated using the Advanced Analytical Fragment Analyzer automated electrophoresis system, quantified using quant-iT HS dsDNA reagent kits (Invitrogen, Carlsbad, CA, USA), and diluted according to Illumina's standard protocol for sequencing on the MiSeq instrument (Ilumina, San Diego, CA, USA) as 2×250 bp paired-end reads. Primers were designed to match the 5' ends of the forward and reverse reads. Cutadapt (https://github.com/marcelm/cutadapt) was used to remove the the primer from the 5' end of the forward read. If found, the reverse complement of the primer to the reverse read was then removed from the forward read as were all bases downstream. Thus, a forward read could be trimmed at both ends if the insert were shorter than the amplicon length. The same approach was used on the reverse read, but with the primers in the opposite roles. Read pairs were rejected if one read or the other did not match a 5' primer, and an error-rate of 0.1 was allowed. Two passes were made over each read to ensure removal of the second primer. A minimal overlap of three bp with the 3' end of the primer sequence was required for removal. The QIIME2 DADA2 plugin (version 1.10.0) was used to denoise, de-replicate, and count ASVs (amplicon sequence variants), incorporating the following parameters: 1) forward and reverse reads were truncated to 150 bases, 2) forward

and reverse reads with number of expected errors higher than 2.0 were discarded, and 3) Chimeras were detected using the “consensus” method and removed. A feature table rarefied to 44,960 features per sample was used for all 16S rRNA microbiome analyses. Taxonomies were assigned to final sequences using the Silva.v138 database, using the classify-sklearn procedure. Differential abundance testing was performed using analysis of composition of microbes (ANCOM) within QIIME2 v2021.8 and ALDEx2 within R v3.6.2 [31, 32]. An EMPress plot was generated using QIIME2 v2021.8. R v3.5.1 and Biom version 2.1.7 were used in QIIME2 [33].

Blood pressure measurements

Heart rate and arterial blood pressure (aBP) were measured using the tail-cuff method by volume pressure recording (CODA system—Kent Scientific, Torrington, CT, USA) in conscious animals between ZT-5 and 6. The tail-cuff method is reliable and comparable to telemetry measurements using an aortic catheter [34]. Mice were placed in a cylindrical holder over a warmed blanket. After 30 min of habituation, at least 8 recordings were obtained, each separated by 5 min. The mean of the lowest five values for systolic, diastolic and mean blood pressure were retained for analyses [35].

Aortic and Coronary artery function

After euthanasia, the heart was excised, and the left anterior descending (LAD) coronary artery was micro-dissected and mounted for isometric tension recordings (Danish Myo Technology, Model 630MA, Aarhus, Denmark). In the subset of mice exposed to FMT experiments, the aorta was concurrently harvested and prepared for the myograph experiments. Data were analyzed using PowerLab software (AD Instruments). Excised vessels were normalized to a tension equivalent to that experienced by the vessels in vivo at 90mmHg pressure as previously described [36] in tissue baths of warmed (37 °C), aerated (95% O2, 5% CO2), in a physiological solution (118.99 NaCl; 4.69 KCl; 1.17 $MgSO_4$; 0.03 EDTA; 2.5 $CaCl_2$; 25 $NaHCO_3$; 1.18 KH_2PO_4; 5.5 glucose). Vessel viability was assessed by exposure to 80mM KCl. Vasoconstrictor responses were assessed to incremental concentrations of thromboxane A2 analog U46619 (10^{-9}–10^{-5} M). For relaxation studies, vessels were pre-constricted with U46619 (0.1–0.3 μM), before administration of Acetylcholine (ACh) (10^{-9}-10^{-5}M) and sodium nitroprusside (SNP) (10^{-9}-10^{-5}M) [36]. Constrictor responses are presented as a percent of the max response to 80mM KCl and the vasodilator responses are presented as a percent of maximal dilation from the pre-constricted tension.

Plasma TMAO determination

Plasma levels of TMAO (50μl) were prepared and analyzed as described previously [37] using liquid chromatography-mass spectrometric multiple reaction monitoring (LC-MS MRM) (Acquity Ultra Performance Liquid Chromatography coupled with TQ-S triple-quadrupole mass spectrometry; Waters, PA) at The University of Missouri Metabolic Core. Serum (50 ul) was mixed with 350 ul methanol and 25 ul solution of labelled internal standard (10 ug/mL) in methanol. After vortex, the samples were centrifuged at 13000 g for 5 min. The clear supernatant (~350–400 uL) was recovered and analyzed on a Water TQ MS. A series of standard solutions were prepared by diluting a master solution (5 ug/mL)

with methanol containing internal standard labeled TMAO solution. The final concentrations were: 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0 and 5.0 ug/mL. The concentration C13 labeled internal standard is aa ug/mL. A linear calibration curve was obtained by plotting area under curve against concentration of standard solutions. LC-MS MRM analyses were performed on a Waters Xevo TQ MS coupled to a Waters Acquity UPLC system (Waters, PA). Separations of amino acids were achieved on a Waters High Strength Silica (HSS) C18 column (2.1 × 150 mm, 1.7-μm particles) using a linear gradient of mobile phase A (A: 0.1% formic acid) and B (B: methanol). The gradient condition was: B increased from 5% to 46% over 19 min, then to 90% in 0.1 min and held at 90% for 1.9 min, returned to 5% for equilibrium in 0.1 min where it was hold for another 3.9 mins. The flow rate was 0.375 mL/min and the column temperature was 40 °C. Under the current LC conditions, the retention time of TMAO and TMAO-13C3 were found to be 0.85 min. MRM analyses were performed on the Waters Xevo TQMS in positive electrospray ionization mode for TMAO/TMAO-13C3. Two transitions, i.e., one qualitative and one quantitative transition, were monitored. The transitions were auto-optimized using Waters Masslynx Intellistart program. The two transitions for TMAO were (exact mass: 75.06) were: m/z 76.1 -> m/z 42.17 (cone voltage: 26 V, collision energy: 44 eV), m/z 76.1 -> m/z 57.40 (cone voltage: 26 V, collision energy: 16 eV). For TMAO-13C3 (exact mass: 78.08), m/z 79.1 -> m/z 41.13 (cone voltage: 24 V, collision energy: 46 eV), and m/z 79.1 -> m/z 63.09 (cone voltage: 24 V, collision energy: 14 eV) were used. MS MRM data were processed using Waters TargetLynx software. Transition m/z 76.1 -> m/z 42.17 was used as the quantitative transition for TMAO and transition m/z 79.1 -> m/z 63.09 was used for TMAO-13C3.

Plasma acetate determination

Plasma acetate levels were determined using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer protocol (Abcam, Cambridge, UK).

Statistical analysis

Data analysis was performed using MetaboAnalyst 5.0, Past4.04, Prism 9 (GraphPad, San Diego, Ca, USA), and R version 3.6.2 statistical software. One-way and Two-way ANOVA with repeated measures and Tukey *post-hoc* test, two-way PERMANOVA, and differential abundance testing were used as appropriate. Data were tested for normality using Shapiro-Wilk test and expressed as mean ± SD. A p value < 0.05 was considered as statistically significant.

RESULTS

Changes in gut microbiome composition in naïve mice subjected to FMT from mice exposed to RA and IH and treated with VSL3

To assess the causal relationship between IH-associated changes in the fecal microbiome, FMT experiments were performed. IH was associated with changes in the microbiome similar to those seen previously, and several features of the microbiome were successfully transferred via FMT (Fig. S1). The PCoA plots shown (Fig. 1A, 1B) reveal significant differences in fecal bacterial composition in all four groups using weighted and unweighted UniFrac distances. As in other mammalian hosts, Bacteroidetes and Firmicutes were

the predominant phyla, with phylum Bacteroidetes dominated by class Bacteroidia, order Bacteroidales, and phylum Firmicutes being dominated by class Clostridia, order Clostridiales (Fig. S2). The relative abundance of bacterial taxa in IH-FMT group shows higher abundance of *Lachnospiraceae* and *Ruminococcaceae* from the phylum Firmicutes and *Prevotellaceae* and *Muribaculaceae* from the phylum Bacteroidetes, when compared to the other three groups (Fig. S3, Table S1). Two separate tools, ANCOM and ALDEx2, were used in tandem to test for differences in ASV relative abundance between groups. Fig. S1 shows a cladogram of all ASVs detected among the four groups, with concentric outer circles indicating the strength of the differences detected using those methods. ASVs identified as differentially abundant between groups included two taxa present in VSL3 (Fig. 1C). Treatment with VSL3 resulted in a higher abundance of *Lactococcus* and *Bifidobacterium* species, which were noted in both RA-FMT-PRO and IH-FMT-PRO groups (Fig. 1C, Table S1) relative to IH-FMT. Thus, FMT procedures using different fecal GM as obtained from IH-exposed and RA-exposed mice altered the GM of naïve mice to recapitulate the previously documented differences in GM induced by IH [17]. Furthermore, treatment with VSL3 prevented the selective increases in bacteria abundance induced by FMT from IH mice, and increased abundance of the putatively beneficial bacteria.

VSL3 treatment normalizes elevated blood pressure in naïve mice receiving FMT from IH exposed mice

IH-FMT induced elevations of systolic (122 ± 10 mmHg), diastolic (93± 9 mmHg) and mean (102 ± 10 mmHg) blood pressure values, and such changes were abrogated by VSL3 treatment (systolic BP: 107 ± 8 mmHg, $p = 0.004$; diastolic BP: 83 ± 5 mmHg, $p = 0.02$; mean BP: 91 ± 6 mmHg, $p = 0.02$, all p values *vs.* IH-FMT; Fig. 2A–2C). aBP values in RA FMT and RA-FMT-PRO experimental groups were also significantly lower when compared to IH-FMT but were similar to those obtained in IH-FMT-PRO treated animals.

VSL3 treatment mitigates coronary artery dysfunction in naïve mice induced by IH-FMT

IH-FMT enhanced maximal coronary artery contractility responses to the thromboxane A2 analog U46619 (174 ± 20%) when compared to RA-FMT (141 ± 18%, $p < 0.0001$), and such effects were attenuated by VSL3 treatment (IH-FMT-PRO: 157 ± 13%, $p = 0.02$; Fig. 3A). IH-FMT impaired coronary artery endothelium-dependent relaxation responses to ACh (65 ± 9%) when compared to RA-FMT (85 ± 7%, $p < 0.0001$; Fig. 3B). Concurrent administration of VSL3 prevented the relaxation impairments induced IH-FMT (IH-FMT-PRO: 79 ± 9%, $p < 0.0001$). Similar to the coronary arteries, IH-FMT increased maximal aortic contractility responses to phenylephrine (166 ± 22%) when compared to RA-FMT (138 ± 14%, $p = 0.006$; Fig. S4A), while IH-FMT-PRO treated mice showed no significant differences compared to controls (IH-FMT-PRO: 131 ± 19%, $p = 0.0004$). Endothelium-dependent relaxation was also impaired in the aorta of the IH-FMT group (69 ± 22%) when compared to RA-FMT mice (85 ± 4%, $p = 0.003$; Fig. S4B) and VSL3 concurrent treatment mitigated such impairments (IH-FMT-PRO: 82 ± 4%, $p = 0.04$ vs. IH-FMT). Endothelium-independent relaxation responses to the nitric oxide donor sodium nitroprusside (SNP) were similar in all groups in both aortic and coronary arteries (Fig. 3C, S4C).

VSL3 treatment reduces TMAO levels in naïve mice receiving FMT from mice exposed to IH

Plasma levels of TMAO were significantly elevated in IH-FMT mice (0.43 ± 0.06 ng/ml) when compared to RA-FMT mice (0.19 ± 0.11 ng/ml, p = 0.008; Fig. 4A). Administration of VSL3 to IH-FMT-treated mice abrogated the increase in TMAO levels (IH-FMT-PRO: 0.27 ± 0.06 ng/ml, p = 0.02). However, acetate plasma concentrations were significantly lower in IH-FMT mice (1.1 ± 0.2 μM) when compared to RA-FMT mice (3.5 ± 1.3 μM, p = 0.01, Fig. 4B), but VSL3 treatment was not associated with significant improvements in acetate concentrations in IH-FMT-PRO treated mice (1.8 ± 0.6 μM, p = 0.6).

VSL3 administration does not attenuate mean BP elevations or coronary artery dysfunction in mice exposed to IH

After 6 weeks of IH exposures and co-administration of VSL3, mean BP was significantly elevated in mice exposed to IH (111 ± 6 mmHg) when compared to RA (92 ± 4 mmHg, p < 0.001) (Fig. 5A). Treatment with VSL3 had modest non-significant reductions of mean BP (IH-PRO: 105 ± 7 mmHg vs. RA, p = 0.0009). IH exposure enhanced maximal coronary artery contractility responses to the thromboxane A2 analog U46619 (182 ± 31%) when compared to RA (133 ± 14%, p < 0.0001, Fig 5B) that were not attenuated by VSL3 treatment (161 ± 28% vs. RA, p = 0.0015). Furthermore, maximal response to endothelium-dependent relaxation by ACh was abolished in coronary arteries of IH-exposed mice (63 ± 9%) in comparison with RA (85 ± 5%, p < 0.0001; Fig. 5C) and was not rescued with VSL3 administration (68 ± 13% vs. RA, p = 0.0002). Endothelium-independent relaxation responses to the nitric oxide donor sodium nitroprusside (SNP) were similar in all groups (Fig. 5D)

DISCUSSION

The present study uncovers several novel and unprecedented findings. First, we show that changes in the composition and diversity of the GM induced by IH exposures but in the absence of actual hypoxia elicits elevations in blood pressure levels and induces impairments in coronary and aortic blood vessel functions. Furthermore, such GM changes promote increases in TMAO levels in IH-FMT mice. Secondly, VSL3 probiotic administration prevents the emergence of the vascular phenotypes induced by IH-FMT and abrogates the increases in aBP. Lastly, supplementing mice exposed to IH with VSL3 does not attenuate aBP elevations nor mitigates coronary artery dysfunction. Taken together, current findings indicate that alterations in GM diversity caused by IH can singlehandedly elicit cardiovascular perturbations, even in the absence of environmental IH, and that such cardiovascular changes can be mitigated or abrogated altogether by probiotics such as VSL3. However, IH clearly incorporates additional mechanisms that are not represented exclusively by GM alterations and likely involve autonomic dysfunction, oxidative stress, and systemic inflammation.

IH- mediated hypertension and coronary artery dysfunction

Heart disease remains the main cause of death and disability in the United States according to the 2020 Heart Disease And Stroke statistics [38]. OSA is a well-recognized risk factor for CVD, independent of other commonly associated risk factors such as sex, age, obesity

and hypertension [39]. Moreover, approximately 35% of OSA patients have hypertension, while an estimated 50% of patients with hypertension suffer from concomitant OSA [40, 41].. Inevitably, untreated patients with severe OSA are 2.6 times more likely to suffer incident CAD [42]. Unfortunately, the beneficial effects of current OSA therapies such as CPAP on CVD outcomes are inconsistent and fraught with scientific controversy [6, 43]. Clinical and experimental studies denote chronic IH as the most detrimental perturbation in OSA-induced hypertension and CVD [2, 8]. Indeed, IH can induce sympathetic activation, oxidative stress, systemic inflammation, dyslipidemia and insulin resistance, all of which can contribute to elevated blood pressure, endothelial dysfunction and atherosclerosis [3, 4, 44, 45]. Multiple studies so far have reported evidence of endothelial dysfunction in animals exposed to IH, manifesting as reduced nitric oxide bioavailability, enhanced vasoconstriction, and impaired vasodilation in multiple vascular beds, including aorta, and cerebral, femoral or carotid arteries [46–49]. Only recently, our lab has demonstrated that IH can also impair coronary artery function (left anterior descending) and reduce flow velocity reserve [9]. Thereby, there is an established link between IH mimicking OSA and CAD. However, more mechanistic studies are required to elucidate the pathways underlying the effects of IH on coronary structure and function.

IH-induced GM changes

In an effort to identify potential contributors to IH-induced vascular dysfunction, we opted to explore the GM as a causal determinant of the vascular phenotype in OSA. Indeed, it is now well established that the stability and equilibrium of the GM ecosystem are essential for maintaining health, but that perturbations leading to GM alterations can induce and propagate detrimental health consequences [11]. Recent evidence suggests that OSA is associated with GM alterations in adults and children [13, 19, 50] where most of these findings reported higher Firmicutes to Bacteroides ratio and significantly lower microbial diversity and richness. Findings in mice exposed to IH for 6 weeks revealed significant alterations in GM profiles, with increases in obligate anaerobes, such as *Prevotella*, *Lachnospiraceae* and *Desulfovibrio* [51], suggesting that fluctuations in oxygen partial pressures in the gut drive such changes. Increased presence of *Desulfovibrio* has been linked to increased mucin degradation, while *Prevotrella* is strongly linked to systemic inflammation through the generation of lipopolysaccharides (LPS) [51].

Evidence from different studies shows a controversial role played by *Lachnospiraceae*, but it is plausible that the increases in are likely a response to IH as an environmental stressor [52]. In accordance with our results, other studies using IH and hypercapnia in low-density lipoprotein receptor-deficient mice ($Ldlr^{-/-}$) found more than 80 microbial different features, with the largest including *Lachnospiraceae* and *Clostridiaceae* families [53]. In previous work, we showed that naïve mice kept in normoxic conditions and receiving FMT from animals exposed to IH led to GM changes that were remarkably similar to those observed in mice exposed to IH, along with corresponding increases in the abundance of the aforementioned bacteria. Our current results concur with such earlier findings, indicating that FMT from IH-exposed donors is accompanied by reproducible and consistent GM changes in the recipient mice. In mice exposed to 4 weeks of sleep fragmentation (SF), another hallmark characteristic of OSA, there were GM changes that were predominantly

reflected by the growth of *Ruminococcaceae and Lachnospiraceae,* and such changes were accompanied by impaired insulin sensitivity and white adipose tissue inflammation [29]. Indeed, increased GM abundance of *Ruminococcaceae and Lachnospiraceae* is associated with atherosclerotic lesions in apolipoprotein E knockout mice ($ApoE^{-/-}$) fed a western diet [54]. Although these bacteria can produce SCFAs such as butyrate [55], they also contain bile acid inducible genes that encode enzymes involved in converting host primary bile acids to secondary bile acids and act on farnesoid X receptor (FXR) and G-protein-coupled receptor (TGR5) that have been implicated in atherosclerosis [56]. *Muribaculaceae* bacterium are a dominant family in the gut and are capable of degrading complex carbohydrates [57]. Although it has been reported that the timing of fecal material collection and the cyclical effects of IH and intermittent hypercapnia (IC), another hallmark of OSA, can cause dyssynchrony of the microbiome and metabolome [58], we standardize our procedures to minimize any potential factors that introduce variance and we found relative stability of the GM after 2–4 weeks of IH exposures in our previous work. The relative changes in GM are modest from week to week if the IH exposure continues and even after 6 weeks of IH cessation we noted an incomplete recover of the GM [18]. The overall net effect of such GM changes on the intestinal permeability of IH-FMT recipients was not examined, but clearly warrants future studies, since the overall metabolomic composition of the GM may facilitate the translocation of CAD-inducing metabolites in IH-exposed and IH-FMT mice.

IH-induced GM changes and cardiovascular disturbances

Recent evidence suggests an obligatory role of microbiota in BP homeostasis. Indeed, the absence of microbiota in germ-free rats resulted with relative hypotension accompanied by marked reduction is vascular reactivity, and both were restored by the introduction of microbiota to germ-free rats [59]. The evidence linking an association between GM alterations and hypertension is much more robust in both human and animal studies [16]. Apparent differences are consistently detected between the GM of normotensive and hypertensive mice and patients [60]. Collectively, there is less microbial richness and diversity, lower abundance of SCFA-producing bacteria, all of which play a role in inducing hypertension [61]. Indeed, SCFA (e.g., acetate, butyrate) are essential for maintaining gut barrier integrity, decreasing gut wall inflammation, and most importantly, reducing aBP. Previous studies using FMT from hypertensive mice and humans to normotensive mice resulted in increased aBP levels [60]. A recent review summarized the major mechanisms underlying GM induced-cardiovascular complication of OSA being decreased abundance of SCFAs, mucin-degradation and increased inflammation [62]. In animals, following FMT from rats exposed to a procedure aimed a reproducing OSA (repetitive tracheal balloon inflations during sleep) coupled with a high fat diet (HFD), recipient rats fed normal chow developed hypertension along with detectable changes in the GM diversity and abundance of SCFA-producing bacteria [63]. In the present study, we show that FMT from animals exposed to IH elicited elevations in systolic, diastolic, and mean arterial aBP. Furthermore, acetate levels were reduced in IH-FMT mice, which may account for the GM-induced elevations in aBP, since chronic acetate infusion in the cecum of rats exposed to a model of OSA for 2 weeks prevented the emergence of inflammation and hypertension, implicating acetate as a key player in OSA-induced hypertension [63].

Most CVD risk factors, including OSA, can induce GM alterations. The associated intestinal inflammation and intestinal barrier damage can facilitate the translocation of microbial structural components and metabolites, including TMAO, to promote the development of CVD [14]. TMAO is a product of gut microbial metabolism of TMA-containing nutrient precursors (i.e., choline) using TMA-lyases. Following transport to the liver via the portal vein, TMA is metabolized by flavin monooxygenases into TMAO [64]. Senthong *et al.* found that elevated TMAO levels were an independent predictor of diffuse CAD atherosclerotic lesions, even after adjustment for traditional risk factors [65]. Initial functional studies in mice consistently show that GM-derived TMAO increases atherosclerosis susceptibility [14]. Indeed, dietary supplementation of choline in $ApoE^{-/-}$ mice enhanced atherosclerotic lesion formation [64]. However, not all TMAO precursor feeding studies have shown similar results, suggesting that differences in host microbial composition have a substantial influence the phenotype observed [66]. Furthermore, a recent study showed that IH and IC can modulate atherosclerosis progression differently in distinct vascular beds (aorta, pulmonary artery) in $ApoE^{-/-}$ mice where IH promotes an atherosclerotic luminal gut environment [67]. Thus, it is critical to take in consideration that various models of OSA may induce changes in gut metabolome and microbiome that can interact differently with distinct vascular beds. A study conducted by the same group showed that treatment with 3,3-dimethyl-1-butanol (DMB), an inhibitor microbial TMA lyase, reduces the size of atherosclerotic lesions enhanced in pulmonary arteries of mice exposed to IH and IC [68]. In our study, naïve mice receiving FMT from IH-exposed mice developed aortic and coronary artery endothelium-dependent relaxation impairments and enhanced vasoconstrictive responses, in addition to elevated TMAO plasma levels, thereby confirming the detrimental role of IH-induced GM alterations on endothelial function in the absence of IH. Furthermore, the elevated levels of TMAO despite normal chow diet suggests increased abundance of TMA-producing bacteria. Other modulators of atherosclerosis impacted by GM alterations are bile acids [69]. Several bile acids have proinflammatory and proatherogenic activities such as deoxycholic acid (DCA) and tauro-β-muricholic acid (TβMCA) and have been shown to increase in animal models of OSA [58, 67]. However, whether endothelial dysfunction in IH-FMT mice resulted from elevated TMAO levels, aBP elevation, or a combination thereof remains to be explored and the role of bile acids should be explored.

Probiotics effects on GM-mediated cardiovascular disturbances

Probiotics refer to species of live bacteria that confer beneficial health effects on the host when ingested in adequate amounts can exert a wide range of beneficial effects, such as inhibiting colonization by pathogenic bacteria and reinforcing the mucosal barrier [70]. The relatively insufficient effectiveness of pharmaceutical interventions for the management of atherosclerosis and CVD, combined with the recent advances in understanding and recognizing GM - host interactions have generated substantial interest in probiotics as a potential add-ons therapy for CVD [71]. Supplementation with probiotics significantly improved markers of CAD, including nitric oxide, inflammation and oxidative stress [72]. One study reported that supplementation with *Lactobacillus plantarum* 299v ameliorated vascular endothelial dysfunction and inflammation in men with CAD [73]. In animals, probiotic supplementation with VSL3 or *Lactobacillus plantarum* ZDY04 reduced

high HFD-induced lesion development in ApoE$^{-/-}$ mice along with reduced vascular inflammation, adhesion molecules, plasma TMAO, and TMAO-induced atherosclerosis [74], [75]. VSL3 supplementation attenuated oxidative stress-mediated endothelial dysfunction in rat mesenteric arteries following bile duct ligation [76]. These data suggest that probiotics, such as VSL3, have the potential to ameliorate vascular dysfunction in atherosclerotic disease. In OSA rats fed HFD, administration of SCFA replenishing probiotic *C. butyricum* and the prebiotic Hylon VII increased the relative abundance of SCFA-producing bacteria and reduced elevated BP [63]. Another study in OSA rats fed HFD treated with *Lactobacillus rhamnosus* probiotic showed that TMAO levels, inflammatory cytokines, and hypertension severity were all reduced [77]. Lastly, mice exposed to IH and fed HFD with high fructose diet for 12 weeks had significant cardiac morphological changes, cardiac dysfunction, cardiac collagen accumulation and increased cardiac inflammation and oxidative stress that were all prevented by administration of *Lactobacillus rhamnosus GG* [78]. In our study, VSL3 supplementation prevented the elevations in aBP and TMAO levels and attenuated aortic and coronary artery endothelial dysfunction in naïve mice receiving FMT from IH-exposed animals. Thus, targeted probiotic supplementation exerts protective effects against GM alteration-induced CAD in the context of IH mimicking OSA. However, despite treating mice exposed to IH with VSL3, only modest decreases in aBP were noticed with no improvements in coronary artery function. Despite the positive results in our FMT experiments and previous pre- and probiotic treatments in other murine models of OSA, it is predictable that VSL3 treatment was insufficient to protect against the plethora of adverse pathological stimuli induced by chronic IH including excessive sympathetic innervation, oxidative stress, inflammation, and metabolic dysregulation [4, 79].

Our study has several limitations; firstly, only young lean C57Bl/6 mice were studied, and the effect of age, sex and obesity were not evaluated despite having great clinical relevancy. Secondly, recent evidence points to potential role of upper and lower respiratory microbiota modulation in OSA and its correlation with multiple pathologies. This study lacks the characterization of this microbiota which may have potentially influenced the outcomes [80–82]. Thirdly, the study lacks comprehensive metabolomic assessments that may reveal microbial metabolic pathways and identifying specific metabolites involved in IH-induced CAD. However, the study of the microbiome in the context of OSA is still in the early stages and our study contributes to the field of OSA and CVD.

In conclusion, FMT from mice exposed to IH simulating OSA into naïve mice recapitulates IH systemic aBP elevations and vascular perturbations in naïve recipient mice in the absence of IH exposures. Thus, a causal link emerges between IH-induced GM alterations and vascular dysfunction affecting both aortic and coronary arteries. Furthermore, probiotic administration prevents the detrimental cardiovascular phenotype in IH FMT recipient mice despite the modest attenuation observed in IH-exposed mice. The data indicates an integral role of GM as a one modulator, but not the only modulator of OSA-induced CAD and suggests probiotics as a form of targeted adjuvant therapy in patients with OSA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data and materials availability

Custom script used to analyze 16S rRNA amplicon sequencing data are available at [https://github.com/ericsson-lab/IH_RA_FMT]. All sequencing data have been uploaded to the NCBI Sequence Read Archive (SRA), and a SRA BioProject accession number is pending

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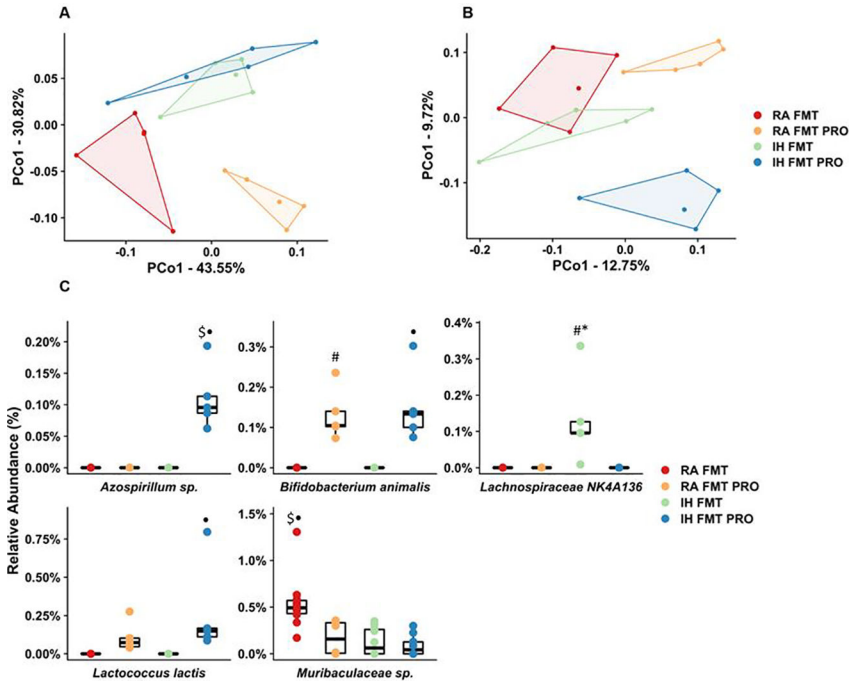

Fig.1: Alterations in fecal bacterial composition between IH and RA FMT with or without probiotic treatment.

Principal coordinate analysis plots ordinated using weighted UniFrac (**A**) and unweighted UniFrac (**B**) distances. Relative abundance of the five distinct ASVs found differentially abundant by ANCOM and ALDeX2 (**C**). FMT: fecal matter transplantation, IH: intermittent hypoxia, PRO: probiotic, RA: room air. Statistical analysis was done using two-way ANOVA followed by Tukey post-test •$p<0.05$ vs. IH-FMT, *$p < 0.05$ vs. IH-FMT-PRO, $^{\#}p < 0.05$ vs. RA-FMT, $^{\$}p < 0.05$ vs. RA-FMT-PRO. FMT: fecal matter transplantation, IH: intermittent hypoxia, PRO: probiotic, RA: room air, VEH: vehicle.

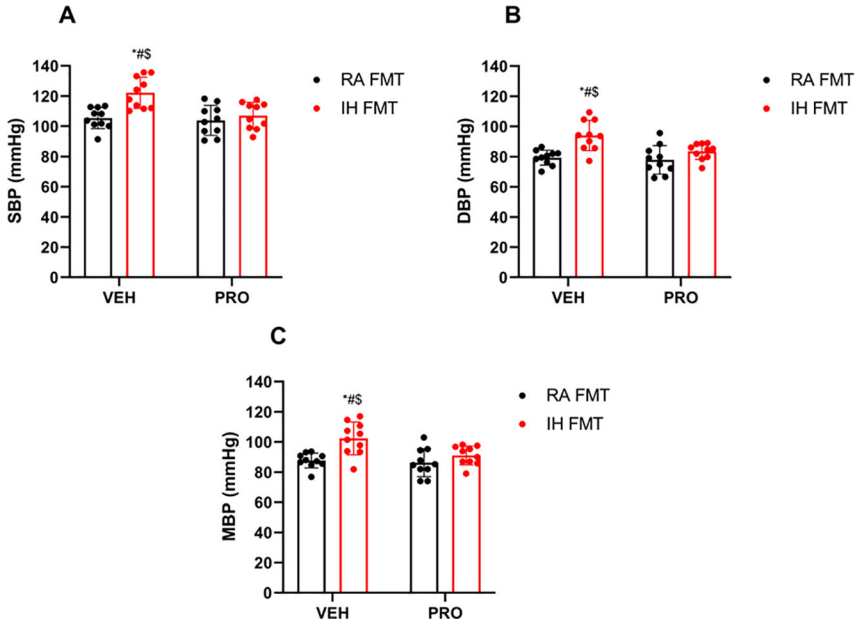

Fig. 2: FMT from IH-exposed mice elevates BP in naïve mice, and such BP changes are prevented by VSL3 probiotic treatment.

Systolic blood pressure (SBP) (**A**), diastolic blood pressure (DBP) (**B**), mean blood pressure (MBP) (**C**). Values are displayed as mean ± S.D (n = 10) mice. Statistical analysis was done using two-way ANOVA followed by Tukey post-test *p < 0.05 vs. IH-FMT-PRO, $^{\#}$p < 0.05 vs. RA-FMT, $^{\$}$p < 0.05 vs. RA-FMT-PRO. FMT: fecal matter transplantation, IH: intermittent hypoxia, PRO: probiotic, RA: room air, VEH: vehicle

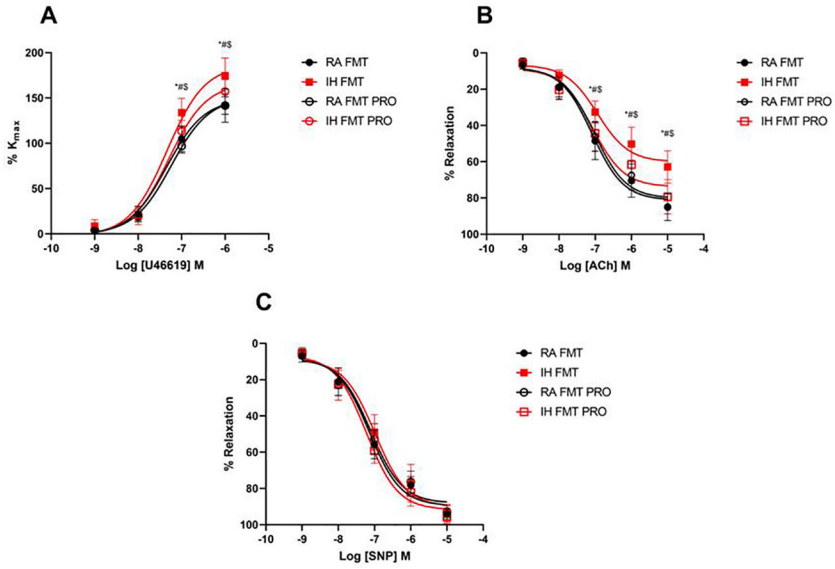

Fig. 3: FMT from IH-exposed mice impairs coronary arteries function in naïve mice and VSL3 probiotic administration prevents such effects.

Cumulative concentration response curve of U46619 (**A**), acetylcholine (ACh) (**B**), and sodium nitroprusside (SNP) (**C**). Values are displayed as mean ± S.D (n = 9 −10) mice. Statistical analysis was done using two-way ANOVA followed by Tukey post-test *$p < 0.05$ vs. IH-FMT-PRO, $^{\#}p < 0.05$ vs. RA-FMT, $^{\$}p < 0.05$ vs. RA-FMT-PRO. FMT: fecal matter transplantation, IH: intermittent hypoxia, PRO: probiotic, RA: room air, VEH: vehicle

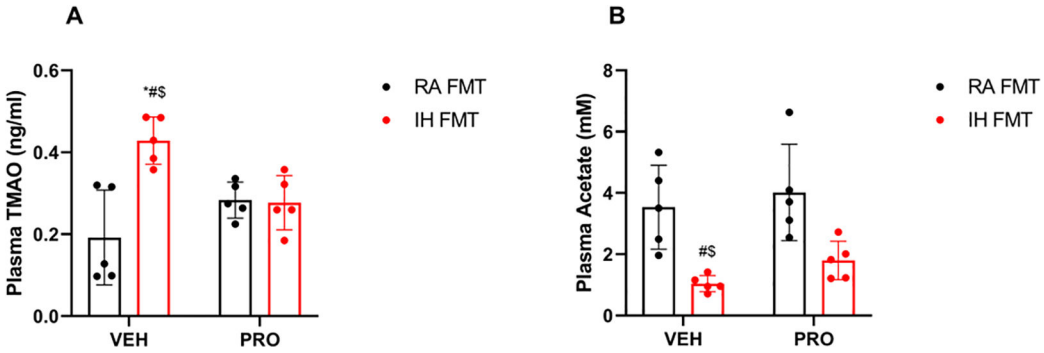

Fig. 4: FMT from IH exposed mice increases TMAO plasma levels and decreases acetate plasma levels, and VSL3 probiotics restore TMAO but not acetate plasma concentrations.

Plasma TMAO levels (**A**), Plasma acetate concentrations (**B**). Values are displayed as mean ± S.D (n = 5) mice. Statistical analysis was done using two-way ANOVA followed by Tukey post-test *$p < 0.05$ vs. IH-FMT-PRO, $^{\#}p < 0.05$ vs. RA-FMT, $^{\$}p < 0.05$ vs. RA-FMT-PRO. FMT: fecal matter transplantation, IH: intermittent hypoxia, PRO: probiotic, RA: room air, TMAO: trimethylamine N-oxide, VEH: vehicle

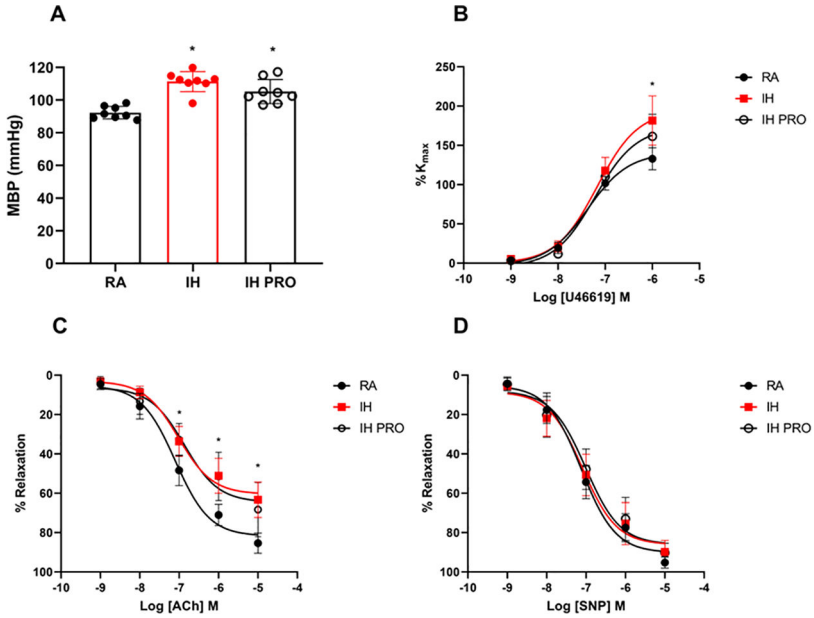

Fig. 5: VSL3 probiotic administration does not lower elevated BP or prevent coronary artery dysfunction in mice exposed to IH.

Mean arterial blood pressure (MBP) (**A**), Cumulative concentration response curve of U46619 (**B**), acetylcholine (ACh) (**C**), and sodium nitroprusside (SNP) (**D**). Values are displayed as mean ± S.D (n = 8–10) mice. Statistical analysis was done using two-way ANOVA followed by Tukey post-test *p < 0.05 vs. RA. IH: intermittent hypoxia, PRO: probiotic, RA: room air