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Effects of psychosocial stress on laryngeal microbiology and epithelial barrier integrity

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Psychosocial stress and laryngeal physiology are linked. However, the biological mechanisms of psychosocial stress on voice have not been studied. This study delineated the effects of psychosocial stress on laryngeal microbiota composition and vocal fold epithelial integrity. We hypothesized that stress would result in reduced microbial diversity and abundance in laryngeal microbiota, and reduced vocal fold epithelial barrier integrity, with more pronounced differences in females and with increased duration of stress. One hundred and eight, conventionally-raised, C56BL/7 mice (8–10 weeks of age, 54 males, 54 females) were allocated to short stress, prolonged stress or control groups. Psychosocial stress involved restraint stress for 7 days (short stress) and 14 days (prolonged stress). Laryngeal microbiota profiles were compared across stress groups using 16S rRNA sequencing (N = 66). Outcome measures of alpha and beta diversity, differentially abundant taxa were obtained. Independently, stress-altered epithelial targets were delineated using RT-qPCR (N = 24) and immunofluorescence (N = 18).We found that prolonged stress, but not short stress, altered measures of alpha, beta diversity, indicating distinct laryngeal microbiota composition compared to control samples. Prolonged stress samples were dominated by Firmicutes phyla, whereas, short stress and control groups by Actinobacteria, and Proteobacteria phyla. Within genera, prolonged psychosocial stress decreased relative abundance of *Corynebacterium* and increased *Streptococcus*. Laryngeal microbial differences were more pronounced in females following psychosocial stress, as hypothesized. In addition, short and prolonged psychosocial stress downregulated gene and/or protein expression of inflammatory cytokines, sensory receptors, adherens and tight junction (E cadherin, Zo-1), TLRs and mucins (MUC2) within the larynx, with more severe effects in the prolonged stress group. Short and prolonged psychosocial stress alters laryngeal microbiota composition and vocal fold epithelial barrier integrity. Future studies should delineate causal host epithelial-microbiome interactions in the larynx in response to stress.

Keywords Laryngeal microbiome, Psychosocial stress, Laryngeal epithelial barrier integrity

Psychosocial stress and laryngeal pathology are inextricably and circularly linked[1,2]. While 25% of patients with voice problems report increased levels of self-perceived psychosocial stress[3], a single stressful event may sufficiently cause negative acoustic voice changes[4,5]. Despite an associative relationship between psychosocial stress and laryngeal pathology[6], an underlying biological mechanism has not been previously elucidated.

Stress-induced gut dysbiosis has deleterious consequences on social behavior, acting via the microbial gut-brain axis[7,8]. Across mucosal organs, such as gut, vagina, brain, stress-altered microbial dysbiosis is characterized by reduced microbial diversity and abundance[9–12]. However, laryngeal microbiota composition is distinct[13] with a relatively higher proportion of aerobic, gram-negative, and facultative pathogenic bacteria is found in the larynx compared to the gut. How this distinct bacterial composition of the laryngeal microbiome is influenced by psychosocial stress remains unknown. If psychosocial stress alters laryngeal microbiota, this could potentially provide a biological basis for psychosocial stress-induced changes in laryngeal function.

The duration of the stressor is an important factor in delineating stress-altered microbial changes in the gut. There is overwhelming evidence to support a gut microbial compositional shift (i.e. changes in measures of beta diversity) with stress[14–19]. However, studies with a shorter duration of stress exposure—2–10 h single exposure to restraint stress—can have minimal effects on gut microbial composition[20,21]. As a result, we investigated the effects of two stress protocols of varying durations on laryngeal microbiota to determine if the same may be

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true of the larynx. Literature on the influence of sex on stress-altered microbial dysbiosis across mucosal organs remains scarce, but females may have more pronounced microbial composition differences in response to stress in the gut and report a greater prevalence to voice disorders than their male counterparts[22,23]. Thus, we included females and males, to report potential sex-related variation in stress-altered laryngeal microbiota.

Stress-induced microbial dysbiosis can increase epithelial permeability in mucosal organs, increase bacterial translocation via toll-like receptors, and lead to chronic inflammation[20,24–27]. In fact, psychosocial stress increases epithelial permeability, known in "leaky" membrane, in the gut[28–30]. Stress-altered barrier integrity in the gut is characterized by reduced mucus thickness and downregulation of mucin genes[20,27,31], decreased epithelial tight junction integrity[20,24–27], and altered expression of visceral sensory receptors[32]. If replicated in the larynx, stress-altered epithelial barrier dysfunction can increase laryngeal susceptibility to noxious environmental, systemic and mechanical stimuli, and stymie recovery from injury.

The overarching aim of this study was to determine the effects of psychosocial stress on laryngeal microbiota composition and host epithelial barrier integrity in the larynx. Two murine stress paradigms of varying durations, hereafter termed short and prolonged stress, were employed in mice of both biological sexes. We hypothesized that psychosocial stress exposure would reduce microbial diversity and abundance of laryngeal microbiota and decrease laryngeal epithelial barrier integrity, characterized by reduced gene/protein expression of mucins, epithelial tight junctions, increased expression of toll-like receptors and inflammatory cytokines, with more pronounced differences with increased duration of stress exposure as well as in female mice.

Methods

All procedures and protocols were approved by the University of Wisconsin Madison Institutional Animal Care and Use Committee, (IACUC, Protocol #M006632. All methods details below were performed in accordance with relevant guidelines and regulations.

Animals

One hundred and eight, conventionally-raised, adult C56BL/7 mice from the same lineage (8–10 weeks of age, 54 males, 54 females) completed the experiment. Animals were housed in the same room since birth, 30–70% humidity and 20–24 °C temperature with a 12-h light/12-h dark cycle, 1/8″ corn cob bedded cages. The following precautions were taken to minimize stress prior to the experiment; social group housing of up to 5 animals/cage, provision of environmental enrichment, ad libitum access to a standard diet of food and water – standard 2920 × rodent diet, acidified water—and minimal handling. Females were housed together to coordinate estrus cycles and minimize hormonal variation, to induce the Whitten effect.

Group allocation

Animals were equally allocated to short stress, prolonged stress or control groups, resulting in 36 animals per group, 18 males and 18 females. Mice of a certain sex and group were housed together and separate from mice of other conditions to minimize potential microbial cross-contamination.

Experimental stress protocol

Mice in the short stress group (N = 36, Fig. 1) underwent restraint stress; they were placed in a customized 50 ml Eppendorf tube with holes for 6 h a day at the same time each day, for 7 days. Mice in the prolonged stress group

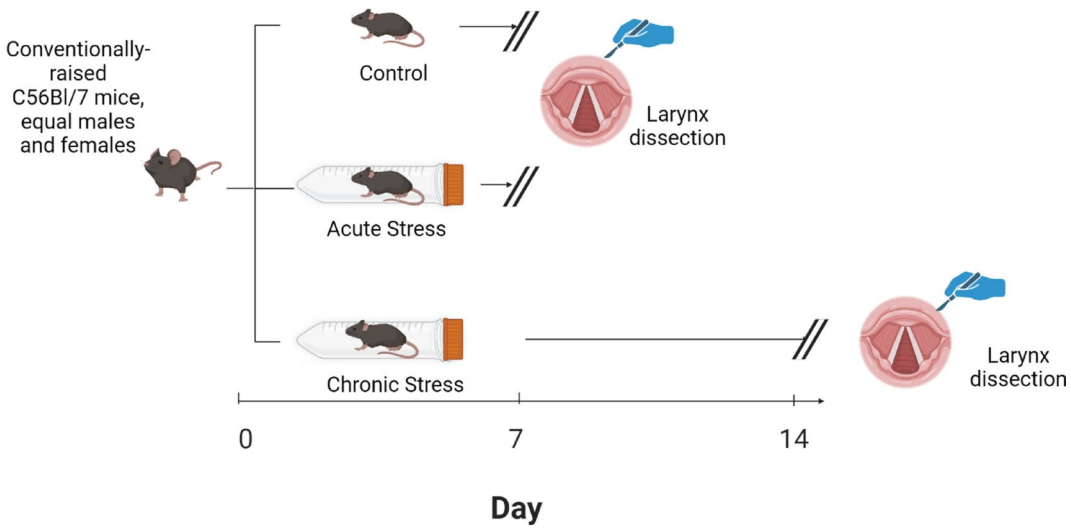

Fig. 1. Experimental Protocol: Animals were equally allocated to short, prolonged stress or control groups (N = 36, 18 males, 18 females/group). Short stress animals underwent a 7-day restraint stress protocol and prolonged stress animals underwent a 14-day restraint stress protocol. Following corresponding psychosocial stress protocols, larynges were dissected for downstream analyses.

(N = 36, Fig. 1) underwent restraint stress for a longer duration, i.e., 6 h a day, at the same time each day, for 14 days. Food and water were removed for all groups, including control, for the duration wherein stressed mice were restrained. Mice in the control group were otherwise left undisturbed for the same duration (N = 36, Fig. 1). Restraint stress is a feasible psychosocial stress protocol that prevents microbial cross contamination. Microbial cross contamination in other stress protocols with water (forced swim test), between animals of different groups (social defeat, changed housing), or species (predator odor or exposure). Translationally, restraint stress increases corticosterone and activates fear-assocated brain regions similar to the human psychosocial stress response[33–35].

Euthanasia and tissue processing

Twenty-four hours following the short or prolonged stress protocol (± 1 h), all mice were euthanized via CO_2 inhalation. Control mice were euthanized after 7 days, at the same time as the short stress mice. Whole larynges were dissected with sterile tools and prepared for downstream processing.

Confirming stress induction

Before and following short or prolonged psychosocial stress and just prior to euthanasia, 200 uL blood was collected from all animals via the maxillary vein to measure corticosterone plasma levels. University of Wisconsin Madison Assay Services performed corticosterone plasma assays via liquid chromatography with triple quadrupole mass spectrometry. Average change in corticosterone plasma levels were compared between animals across all three groups.

Microbial compositional analysis

Sample processing

Sixty-six larynges were hemisected, and minced using sterile tools in a sterile petri dish. To recover maximal bacteria, minced tissue was suspended in a 2 ml tube containing sterile Dulbecco's phosphate-buffered saline (DPBS, Ca/Mg-free) and washed 2 times at max speed for 5 min using Genie 2 Vortex Mixer (Scientific Industries Inc., Bohemia, New York, USA) on a horizontal microtube holder (LABRepCo, Horsham, Philadelphia, USA). Bacterial cell pellets were harvested from the cell suspension by centrifuging at 15,000 rpm for 10 min and removing the supernatant. Bacterial cell pellets were stored in − 80 °C until bacterial DNA extraction. Bacterial DNA extraction was conducted with DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions, and eluted in 35 uL of low TE buffer 10 mM Tris/0.1 mM EDTA). Concentration of bacterial DNA was quantified with Qubit® Fluorometer (Invitrogen, San Diego, CA, United States). Approximately, 10–40 ng/uL of bacterial DNA was recovered from each mouse larynx and stored at 4 °C prior to 16S rRNA gene amplification.

The V3-V4 region of the 16S rRNA gene was amplified, in a reaction containing DNA-free Platinum Taq Polymerase (Invitrogen, Waltham, Massachusetts). Amplification used a 25 uL PCR reaction with 20 ng of bacterial DNA and 400 uM of previously-validated 515F/806R primers [13]. An extraction negative control, and positive control (mouse fecal DNA) was included in each test PCR run. Thermocycling parameters of the PCR reactions were as follows: 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, 72 °C for 5 min. Presence of PCR amplicons were confirmed with 1.5% agarose gel, and amplicon concentration was quantified with a Qubit® Fluorometer (Invitrogen, San Diego, CA, United States). Five samples from removed from subsequent analysis (1 control female, 2 short stress female, 1 prolonged stress female, 1 prolonged stress male) as PCR amplicons could not be confirmed (due to low bacterial yields). The Oral Microbiome Whole Cell Mix (ATCC, Manassas, VA) was used as positive control, processed with tissue samples to test data collection/analysis effectiveness. PCR products from subsequent biological replicates and positive control were pooled by each stress group into equimolar libraries. These pooled amplicons were run on a 1.5% agarose gel. Visualized bands were extracted Zymoclean Gel DNA Recovery Kit (Zymo research, Irvine, CA) was used to obtain a final purified DNA library (containing 10 ng of DNA/sample). UW Madison Biotechnology center completed library sequencing on Illumina MiSeq platform (Illumina, San Diego, CA) using 250-bp paired-end sequencing chemistry.

Analysis

Demultiplexed sequences were analyzed in QIIME2 (v2022.11). QIIME2 is a commonly-used, plug-in based platform for microbiome compositional analysis[36]. Following quality-filtering and denoising, a DADA-2 pipeline (from open-source q2-dada2 plugin (v2017.20) identified de novo amplicon sequence variants (ASVs). Alignment of ASVs were completed with mafft using q2-alignment plug-in (v3.6)[37]. Shared ASVs across groups and sexes were visualized. *Classify-sklearn* was used to identify unique taxa (against Greengenes 13_8 99% references sequences (open-source))[38,39]. Then, microbial compositional with taxa-level specificity was obtained with the QIIME2 *taxa-collapse* function.

Alpha-diversity, beta-diversity and subsequent measures detailed below were obtained using Rstudio (version 3.3.0), at a rarefaction depth of 2108 sequences per sample. One short stress male sample was removed due to the rarefaction depth. For unweighted and weighted beta-diversity analysis, microbial community differences across stress and sex groups were assessed with permutational analysis of variance (PERMANOVA) and visualized with principal coordinate analysis (PCoA). Dominant taxa and for each stress group were obtained in R. Differential abundances of bacterial taxa across stress and sex groups were obtained using linear discriminant analysis (LDA) effect size (LEfSe) in Galaxy/Hutlab open source software[40]. Bacterial taxa having a LDA score > 4 and p value < 0.05 were deemed enriched for that group. LDA analysis is easy to implement and interpret while allowing for comparisons across other studies in the murine laryngeal microbiota[13,90].

Measures of host laryngeal epithelial barrier structure and function

Gene expression analysis of epithelial markers

Larynges from eight mice per group (4 females and 4 males per group X 3 groups [short stress, prolonged stress, control] = 21 mice) were extracted and stored at 20 °C in 100 uL of RNA*later*™ stabilization solution (Invitrogen™, Thermo Fisher Scientific Inc., Carlsbad, CA). Primers for target genes are shown in Table 1. Five hundred nanogram of total mRNA from each sample was used for cDNA transcription. Transcription of cDNA in a 10 uL final reaction was completed with GoScript RT-PCR Promega, Madison, US). SYBR Green PCR Master Mix (Thermofisher, MA, USA) was used for RT-qPCR reaction with CFX Connect Real-Time PCR Detection System (Applied Biosystems, Thermo Fisher). Total mRNA was obtained from each larynx using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Transcription of cDNA from 500 ng of total mRNA per sample was completed using GoScript RT-PCR in a 10 uL final reaction (Promega, Madison, US). SYBR Green PCR Master Mix (Thermofisher, MA, USA) was used in the final RT-qPCR reaction in CFX Connect Real-Time PCR Detection System (Applied Biosystems, Thermo Fisher). Thermal cycling conditions; 95 °C for 3 min, 40 cycles at 95 °C for 10 s, 55 °C for 30 s, 95 °C for 10 s and 65–95 °C, and increment 5 °C. Within the 96-well plate, each sample was run in triplicate for all genes including the housekeeping gene (*β-actin).* Quantification of relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method and was expressed as fold change relative to the control group.

Histology and immunofluorescence

Larynges from six mice per group (3 females and 3 males per group X 3 groups [short stress, prolonged stress, control] = 18 mice) were collected in 4% paraformaldehyde for 24 h, then transferred to 70% ethanol. UW Madison Department of Surgery Histology Core processed these samples to obtain Formalin-Fixed Paraffin Embedded (FFPE) slides visualizing 5 µm-width, coronal sections of the larynx. Slides with mid-membranous vocal fold portions were identified within each sample and stained for Hematoxylin and Eosin (H&E) via validated procedures.

Following confirmation of laryngeal morphology and presence of mid-membranous vocal fold region, consecutive FFPE slides were stained for E-cadherin and Zonula occludens 1 (Zo-1). Staining of one slide from each sample resulted in triplicates per sex per group. Following deparaffinization, slides were processed for antigen retrieval (i.e., boiling 10 mM citrate buffer in a water bath for 2 h, pH 6). Each slide was stained for E-cadherin and Zo-1 using validated immunofluorescence procedures. Antibody validity was confirmed with mouse dermal epithelium (positive control) and a no-primary antibody negative control (mid-membranous coronal section of the larynx). Slides were incubated with primary antibody at 4 °C overnight (1:100 concentration, rabbit monoclonal anti-E-cadherin antibody (#3195, Cell Signaling Technologies): 5% goat serum or 1:200 concentration, mouse monoclonal, (#33–9100, Fisher Scientific): 5% goat serum). Secondary antibody (1:500, Alexa Flour 488, anti-rabbit IgG (A27034, ThermoFisher): 5% goat serum for E cadherin or 1:500, Cy3, goat anti-mouse (#115-166-003, Jackson labs, Zo-1) was applied to slides at room temperature for 1 h. Floromount with DAPI (Fisher) was used for mounting slides. Slides were then cured in the dark for 24 h at room temperature. Images were taken at 10X and 60X with Nikon Eclipse Ti2 inverted microscope, Nikon DS-Ri2 camera and visualized using NIS Elements Software. All samples were imaged at the same time. For all both immunofluoroescnce assays, epithelial localization and relative intensity were described qualitatively.

Statistical analysis

Prior to study commencement, a priori power analyses were conducted to determine adequate sample size for obtaining a sufficient power = 0.80, with an alpha level of 0.05 and an assumed difference between groups of similar sample size, using data from published research[13,41–43]. We determined that 11 mice/group were required for 16S rRNA sequencing, 4 mice/group were required for qPCR, 3 mice/group were required for Immunofluorescence and 16 mice/group were required for corticosterone plasma level comparisons. We included these number of male and female animals for each above-mentioned outcome.

Following data collection and analysis for microbial measures, Kruskal Wallis and Dunn's multiple pairwise comparison was used to compare alpha diversity measures, relative abundance of top bacterial genera across stress and sex groups as per prior literature using R studio (version 3.6.0+)[13,44]. For gene expression data and corticosterone plasma levels, SPSS (Version 22, IBM SPSS Statistics) was used for statistical analyses. Normality and variance were assessed. A two-way ANOVA was conducted (factors: stress, sex), and no significant interaction effects of sex or stress or main effects of sex was found ($p > 0.05$). Post-hoc Tukey HSD comparisons

Primer	Accession #	FWseq	RVseq	Size	Animal
IL1β	NM_008361.4	5′-GAAATGCCACCTTTTGACAGTG 3′	5′-TGGATGCTCTCATCAGGACAG 3′	116	Mouse
TNF alpha	Y00467.1	5′-CTGTAGCCCACGTCGTAGCA 3′	5′-TGTGGGTGAGGAGCACGTA 3′	198	Mouse
IL6	BC138766.1	5′-GTCCTTCCTACCCCAATTTCC 3′	5′-GGTCCTTAGCCACTCCTTCTG 3′	72	Mouse
MUC2	NM_023566.4	5′-GTCCTGACCAAGAGCGAACA 3′	5′-ACAGCACGACAGTCTTCAGG 3′	103	Mouse
TLR2	NM_011905.3	5′-AAACCTCAGACAAAGCGTCA 3′	5′-CACACCCCAGAAGCATCACA 3′	138	Mouse
TLR4	NM_021297.3	5′-AGATCTGAGCTTCAACCCCTTG 3′	5′-ATTGTTTCAATTTCACACCTGGA 3′	102	Mouse
β -actin	NM_007393.5	5′-AGAGGGAAATCGTGCGTGAC 3′	5′-CAATAGTGATGACCTGGCCGT 3′	138	Mouse

Table 1. Quantitative PCR Primer Sequences.

were completed for significant main effects of stress to compare gene expression and corticosterone plasma level data across short, prolonged stress and control groups and are reported below. Alpha level for these analyses was set at $p \leq 0.05$.

Results

All results are reported in accordance with ARRIVE guidelines.

Confirming stress induction

Average corticosterone plasma levels were increased following short stress (Mean difference ± SE; + 71.06 ± 10.3 ng/ml, $p < 0.001$, Fig. 2) and prolonged stress (Mean difference ± SE; + 90.034 ± 8, $p < 0.001$, Fig. 2) compared to controls.

Microbial compositional analysis—effects of stress

Phylum and genus-level variation

At phylum level, short stress and control groups are dominated by Actinobacteria, and Proteobacteria and prolonged stress was dominated by Firmicutes (Fig. 5A). There was a decrease in Actinobacteria and Proteobacteria and an increase in Firmicutes for prolonged stress group compared to the control and short stress groups (Fig. 5A). There were no other significant differences across groups.

At the genus level, there was an increase in *Streptococcus* ($p = 0.022$, Fig. 6A) in prolonged stress, compared to short stress. There was a decrease in *Corynebacterium* ($p < 0.001$) and *S24_7* ($p < 0.005$, Fig. 6A) in the prolonged stress group compared to controls. There were no other significant differences across groups. There was one enriched taxon identified via LEfSe analysis for controls (Betaproteobaceria, LDA score > 4, $p < 0.05$).

Alpha and beta diversity measures

Regarding measures of alpha diversity or within-sample microbial diversity, we found no differences in the number of ASVs, called Observed ASV Richness, between stress groups (H (2) = 2.562, $p = 0.278$, Fig. 3A). However, there was a significant decrease in Shannon Diversity Index (H (2) = 7.631, $p = 0.022$, Fig. 3A) with prolonged stress compared to short stress and untreated controls. There was also a significant decrease in Simpson Diversity Index (H (2) = 7.019, $p = 0.030$, Fig. 3A). with prolonged stress compared to controls.

PCoA of unweighted UniFrac distances via PERMANOVA analysis revealed distinct microbial composition amongst groups (pseudo-F = 3.785, $p = 0.001$, Fig. 4A). Pairwise comparisons show that microbial composition of prolonged stress group was significantly different from short stress (pseudo-F = 4.100, $p = 0.001$, q = 0.0015, Fig. 4A) and control groups (pseudo-F = 4.100, $p = 0.001$, q = 0.0015, Fig. 4A).

Similarly, PCoA of Weighted UniFrac distances via PERMANOVA analysis revealed distinct microbial composition amongst groups (pseudo-F = 33.03, $p = 0.001$, Fig. 4C). Pairwise comparisons show that microbial composition of the prolonged stress group was significantly different from short stress (pseudo-F = 39.83, $p = 0.001$, q = 0.0015, Fig. 4C) and control groups (pseudo-F = 71.72, $p = 0.001$, q = 0.0015, Fig. 4C).

Microbial compositional analysis—interactive effects of stress and sex

Phylum and genus-level variation

At the phylum level, short stress and control female and male groups are dominated by Actinobacteria, and Proteobacteria and prolonged stress females and males are dominated by Firmicutes (Fig. 5B). For both females and males, there was a decrease in Actinobacteria and Proteobacteria and an increase in Firmicutes for prolonged stress compared to control and short stress (Fig. 5B). There was a decrease in Fusobacteria for prolonged stress females compared to control females, but no change across male stress groups (Fig. 5B). There was no change in Bacteroidetes across groups (Fig. 5B).

At the genus level, there was a decrease in *Corynebacterium* ($p < 0.001$, Fig. 6B), *Lactobacillus* ($p = 0.004$) and an increase in *Streptococcus* ($p = 0.023$, Fig. 6B) and *S24_7* ($p < 0.001$, Fig. 6B) in prolonged stress females

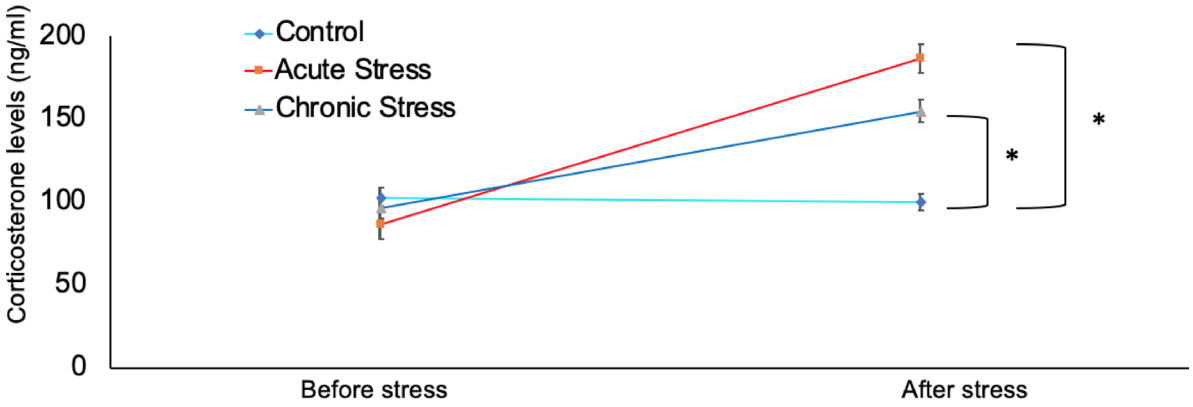

Fig. 2. Corticosterone plasma levels were increased in the short stress group ($p < 0.001$) and the prolonged stress group ($p < 0.001$) compared to control group.

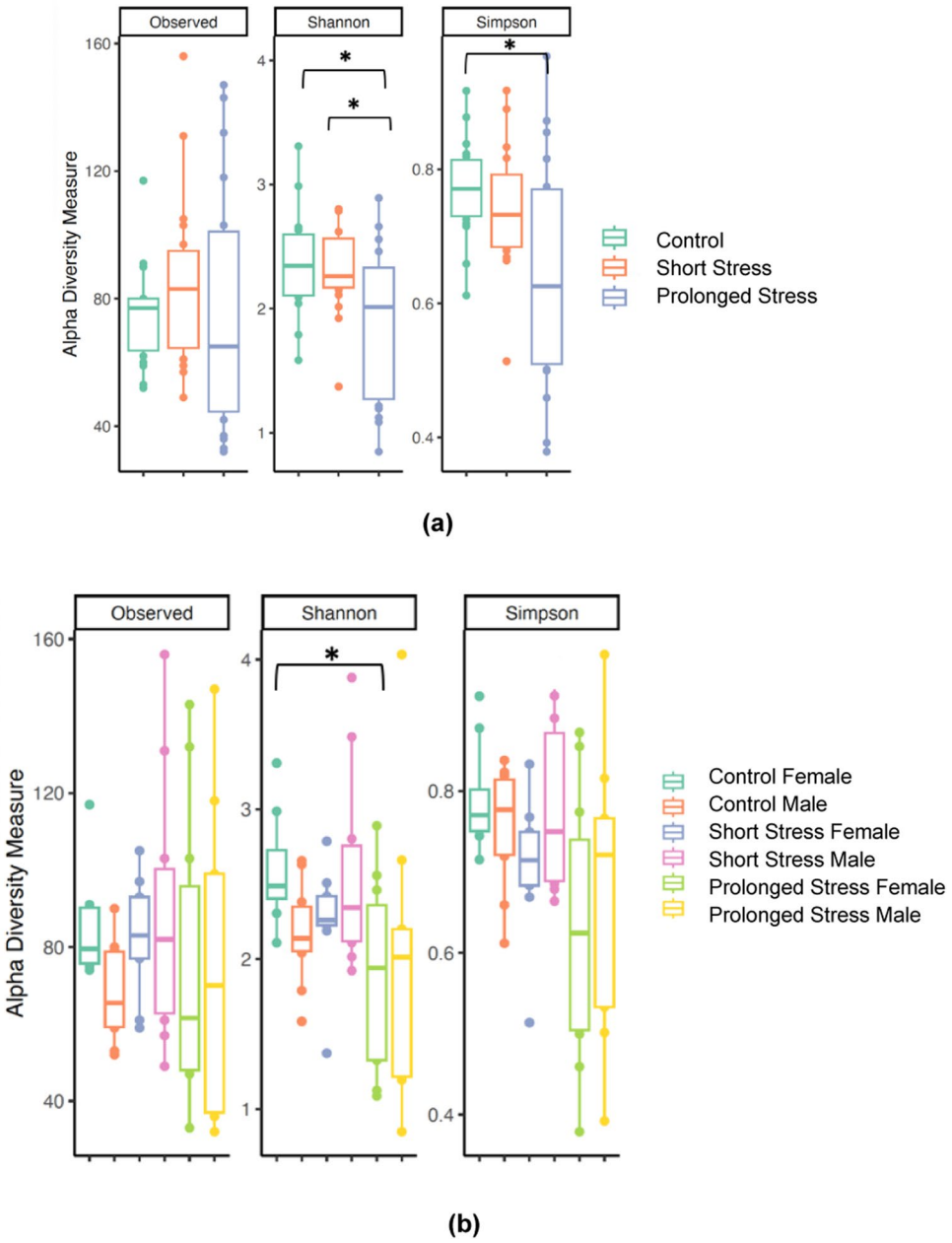

Fig. 3. Alpha Diversity measures across stress and sex groups. (**a**) There are no significant differences between groups ($p > 0.05$). (**b**) There was significant decrease in measures of Shannon diversity in prolonged stress female group compared to control female group ($p = 0.007$), but not in Simpson diversity and Observed ASVs.

compared to control females. There was a decrease in *Corynebacterium* ($p < 0.001$, Fig. 6B) in prolonged stress male group compared to controls. There were no other significant differences across groups. There were no enriched taxa identified via LEfSe analysis for stress and sex groups (LDA score > 4, $p < 0.005$).

Alpha and beta diversity measures

Regarding measures of within-sample microbial diversity (alpha diversity), there was no significant differences in the number of ASVs (Observed ASV Richness) across groups ($H(5) = 4.945$, $p = 0.423$, Fig. 3B). There was a significant decrease in Shannon Diversity index ($H(5) = 11.236$, $p = 0.047$, Fig. 3B) for prolonged stress female group, compared to control females ($p = 0.007$), but no other differences between stress groups of the same sex. Unlike Shannon Diversity Index, Simpson Diversity Index weights species relative to their abundance in the sample. There were no significant differences in Simpson diversity across groups ($H(5) = 9.243$, $p = 0.100$, Fig. 3B). Thus, less abundant bacteria was decreased in females following prolonged psychosocial stress.

PCoA of unweighted UniFrac distances via PERMANOVA analysis revealed distinct microbial composition amongst groups divided by both stress and biological sex (pseudo-$F = 2.708072$, $p = 0.001$, Fig. 4B). Pairwise comparisons show that microbial composition of the short stress male group was significantly different than

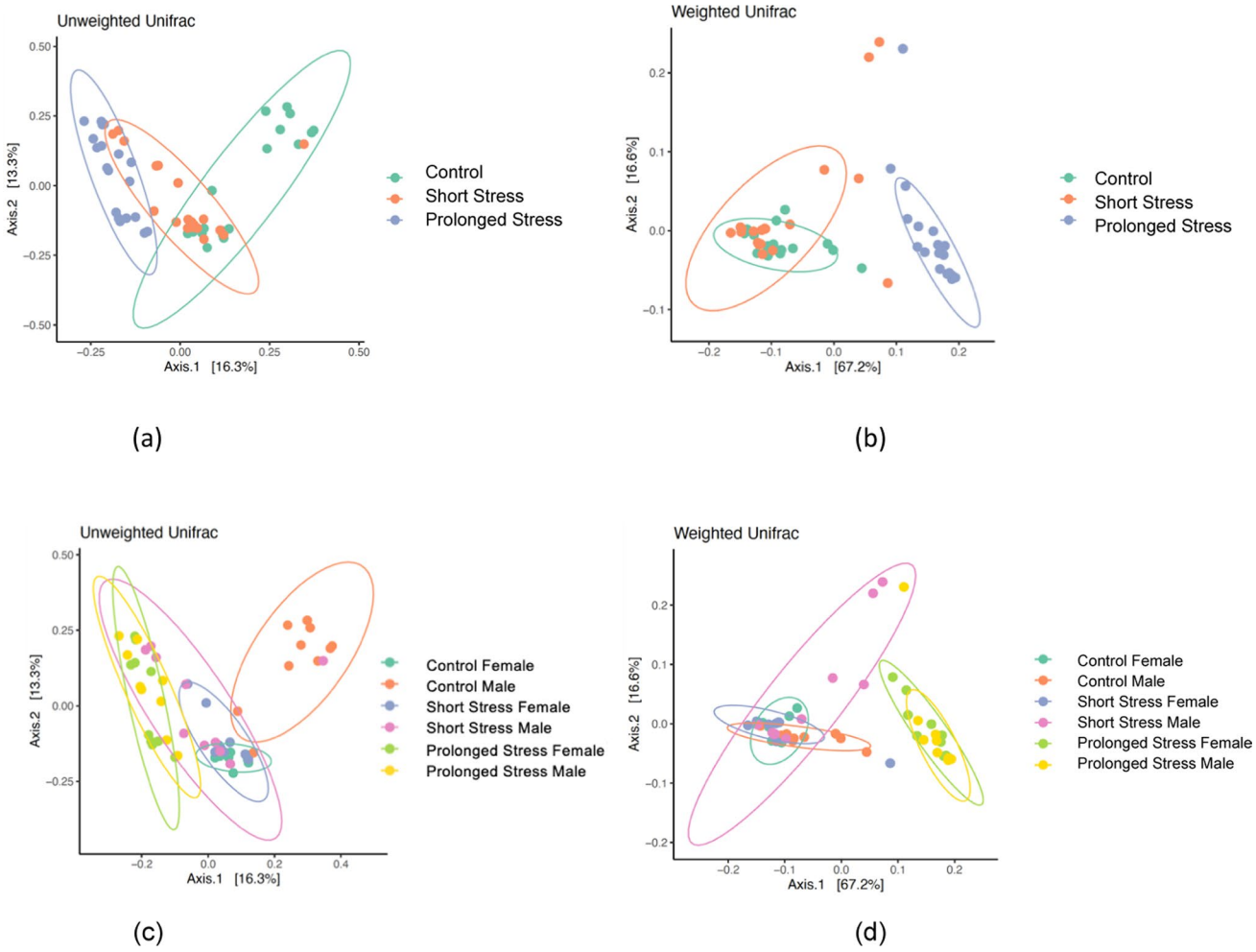

Fig. 4. Beta Diversity measures across stress and sex groups. PERMANOVA analysis revealed that prolonged stress group was significantly separated from short stress group and control group in (**a**) Unweighted and (**b**) Weighted unifrac analysis. Prolonged stress females and prolonged stress males are significantly separated from short stress female and male, and control female and male groups respectively on (**c**) Unweighted and (**d**) Weighted unifrac analysis.

control male group (pseudo-F = 2.055, $p = 0.015$, q = 0.02, Fig. 4B). However, there were no differences between the short stress female group and control female group (pseudo-F = 0.99, $p = 0.462$, q = 0.495, Fig. 4B). The prolonged stress female group was significantly different from short stress female group (pseudo-F = 3.50, $p = 0.001$, q = 0.002, Fig. 4B) and control females (pseudo-F = 5.49, $p = 0.001$, q = 0.002, Fig. 4B). Similarly, the prolonged stress male group was significantly different from short stress male (pseudo-F = 1.72, $p = 0.042$, q = 0.005, Fig. 4B) and male controls (pseudo-F = 3.37, $p = 0.001$, q = 0.002, Fig. 4B).

PCoA of Weighted UniFrac distances via PERMANOVA analysis revealed distinct microbial composition amongst groups (pseudo-F = 14.65, $p = 0.001$, Fig. 4D). Pairwise comparisons show that microbial composition of the short stress female group was not significantly different than control female group (pseudo-F = 0.99, $p = 0.462$, q = 0.495, Fig. 4D). Similarly, short stress male group was not significantly different than control male group (pseudo-F = 1.93, $p = 0.13$, q = 0.16, Fig. 4D). Laryngeal microbial composition of prolonged stress female group was significantly different from control female group (pseudo-F = 50.97, $p = 0.001$, q = 0.001, Fig. 4D) and short stress female group (pseudo-F = 42.98, $p = 0.001$, q = 0.001, Fig. 4D). Similarly, the prolonged stress male group was significantly different from short stress male (pseudo-F = 1.72, $p = 0.042$, q = 0.005, Fig. 4D) and male controls (pseudo-F = 28.87, $p = 0.001$, q = 0.001, Fig. 4D).

Host Laryngeal Epithelial Barrier Structure and Function

Gene expression analysis

There were significant differences in relative gene expression between short stress and untreated control larynges, for the following target genes; *IL1β* (Mean difference + SE = 0.923 ± 0.29, $p = 0.018$, 95% CI (0.15, 1.68), Fig. 7), *TNFalpha* (Mean difference + SE = 0.64 ± 0.194, $p = 0.012$, 95% CI (0.14, 1.1), Fig. 7), *and MUC2* (Mean difference + SE = 0.766 ± 0.259, $p = 0.047$, 95% CI (0.093, 1.44), Fig. 7), *TRPV1* (Mean difference + SE = 0.695 ± 0.21, $p = 0.012$, 95% CI (0.14, 1.24), Fig. 7), *Piezo 2* (Mean difference + SE = 1.11 ± 0.147, $p = 0.01$, 95% CI (0.74,

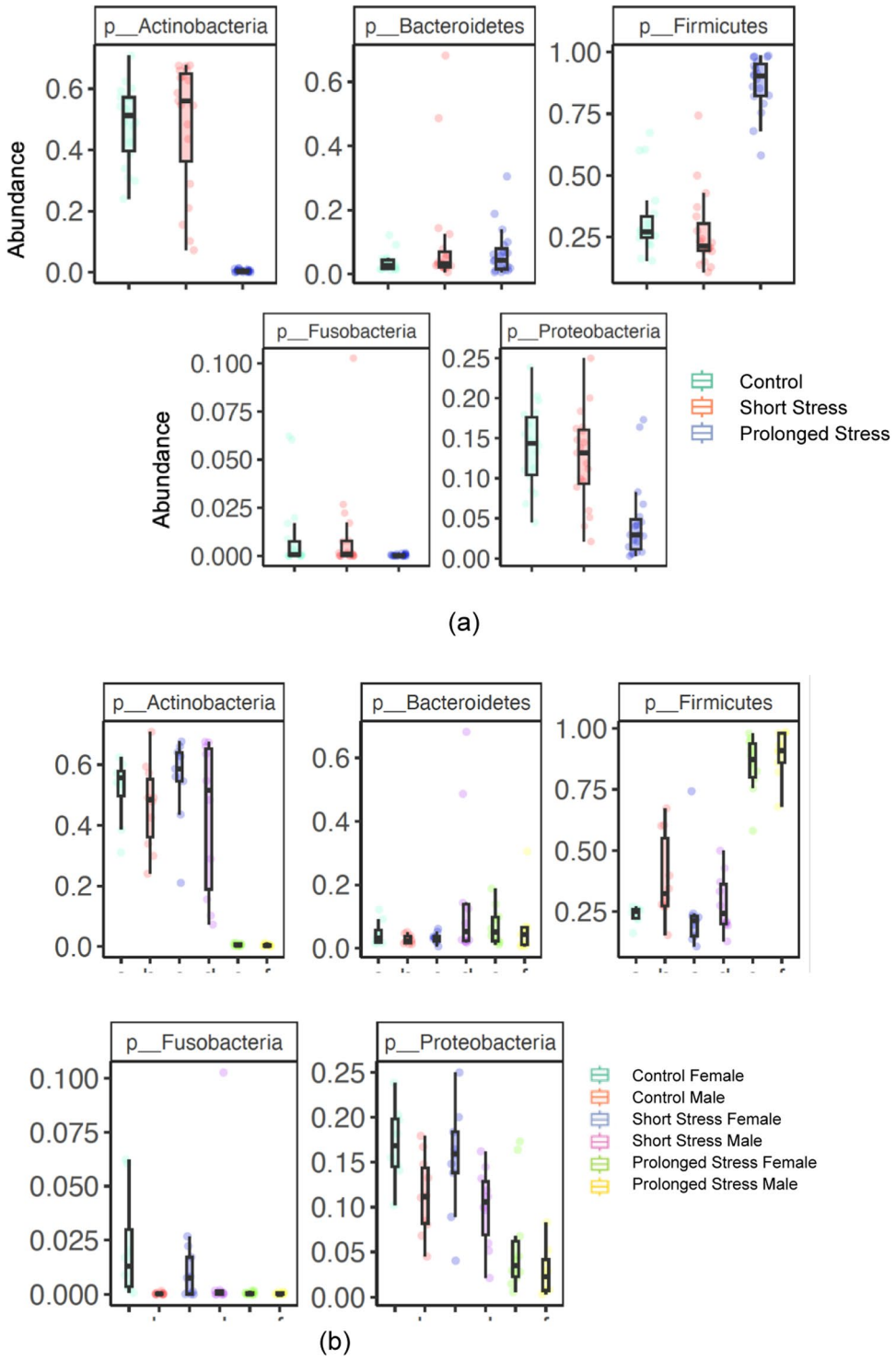

Fig. 5. Relative Abundance of Top 5 Phyla across stress and sex groups. (**a**) There is a decrease in relative abundance of Actinobacteria and Proteobacteria and an increase in Firmicutes in prolonged stress group compared to control group and short group. (**b**) For both females and males, there was a decrease in relative abundance Actinobacteria and Proteobacteria and an increase in Firmicutes for prolonged stress group compared to control and short groups. There is a decrease in Fusobacteria for prolonged stress females compared to control females.

1.4), Fig. 7). Significant differences in relative gene expression between prolonged stress and untreated controls were measured in the following target genes; *IL1β* (Mean difference + SE = 0.91 ± 0.28, $p = 0.001$, 95% CI (0.18, 1.64), Fig. 7), *TNF alpha* (Mean difference + SE = 0.624 ± 0.21, $p = 0.026$, 95% CI (0.7, 1.17), Fig. 7), *TLR2* (Mean difference + SE = 0.978 ± 0.26, p = 0.006, 95% CI (0.2837, 1.665), Fig. 7), and *MUC2* (Mean

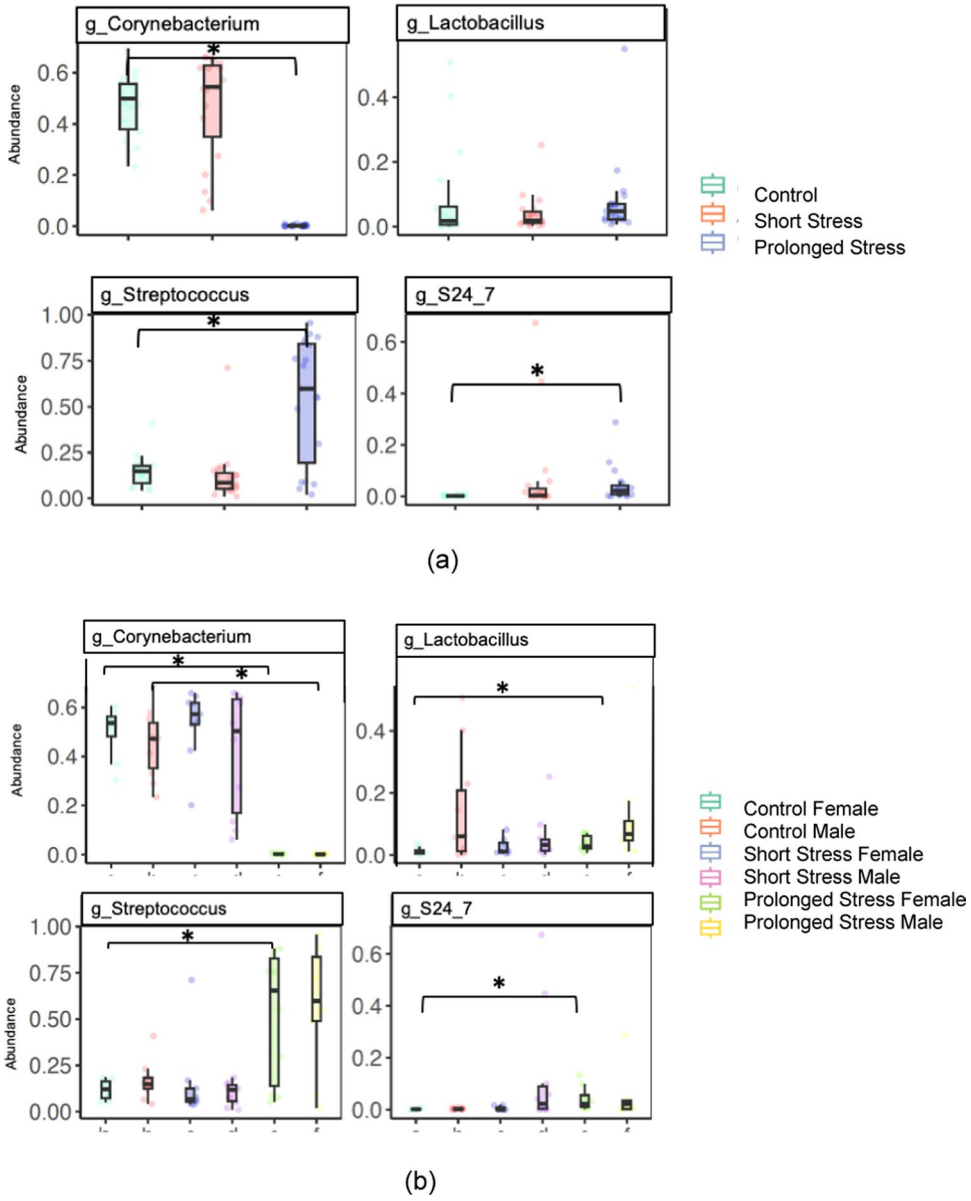

Fig. 6. Relative Abundance of Top 4 general across stress and sex groups. (**a**) There was a decrease in *Corynebacterium* and an increase in *Streptococcus* in prolonged stress group compared to both control and short stress groups ($p < 0.001$). There was also an increase in *S24_7* in short stress and prolonged stress groups compared to control group ($p < 0.001$), but no change between stress groups ($p > 0.05$). There was no change in *Lactobacillus* between groups ($p > 0.05$). (**b**) There was a decrease in *Corynebacterium* ($p < 0.001$), *Lactobacillus* ($p = 0.016$) and an increase in *Streptococcus* ($p = 0.043$) and *S24_7* ($p < 0.001$) in prolonged stress female group compared to control female group. There was a decrease in *Corynebacterium* ($p < 0.001$) an increase in *Streptococcus* ($p = 0.028$) in prolonged stress male group compared to control group.

difference + SE = 0.741 ± 0.281, $p = 0.047$, 95% CI (0.098, 1.47), Fig. 7), *TRPV1* (Mean difference + SE = 0.677 ± 0.21, $p = 0.015$, 95% CI (0.1222, 1.252), Fig. 7), *Piezo 2* (Mean difference + SE = 1.03 ± 0.147, $p = 0.01$, 95% CI (0.6653, 1.4), Fig. 7). There were no significant differences for other target genes between short and prolonged stress groups and when comparing these stress groups with the control group; *IL6* ($F(2,23) = 0.386$, $p = 0.686$, Fig. 7) and *TLR2* ($F(2,23) = 7.147$, $p = 0.007$, Fig. 7), *TRPV2* ($F(2,23) = 3.908$, $p = 0.066$, Fig. 7).

Histology and Immunofluorescence

H&E staining was successfully completed to visualize coronal laryngeal sections containing mid-membranous vocal fold regions in each animal (N = 6 animals per group, 3 females, and 3 males, panels A-F, Fig. 8). Consecutive FFPE slides within each animal were successfully stained for E-cadherin and Zo-1. There was either absent or decreased protein expression of E-cadherin and Zo-1 in the short stress and prolonged stress groups, regardless of sex, compared to the control group, seen in Fig. 8. Specifically, E-cadherin protein expression

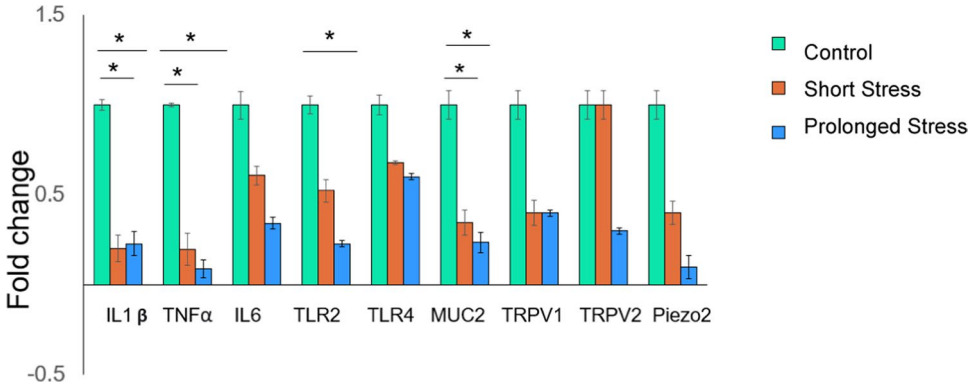

Fig. 7. Relative gene expression of target genes revealed significant differences in target genes between short stress and control group; *IL1β* ($p = 0.018$), *TNF alpha* ($p = 0.012$), *and MUC2* ($p = 0.047$). Significant differences between control and prolonged stress were measured for *IL1β* ($p = 0.001$), *TNF alpha* ($p = 0.026$), *TLR2* ($p = 0.006$), and *MUC2* ($p = 0.047$).

was not consistently noted throughout the mid-membranous portion of the vocal fold epithelium of short and prolonged stress groups (panels G,H,I, Fig. 8). Decreased protein expression of Zo1 was consistently observed across the mid membranous portion of the vocal fold epithelium in short and prolonged stress groups, compared to control (panels J,K,L, Fig. 8). This decrease in Zo1 protein expression was inconsistent across the length of vocal fold epithelium, with greater protein expression noted in the inferior vocal fold epithelium compared to the superior vocal fold epithelium in short and prolonged stress groups, across samples (panels J,K,L, Fig. 8).

Discussion

This study investigated the effects of two stress protocols of varying durations, termed short and prolonged, on laryngeal microbiota composition and host epithelial barrier integrity. We found that prolonged, but not short, psychosocial stress results in distinct laryngeal microbiota composition, with altered relative abundance of dominant bacteria. This indicates that increased duration of stress exposure resulted in more pronounced laryngeal microbiota compositional changes. In addition, females had more stress-induced microbial differences compared to male mice exposed to prolonged psychosocial stress, as hypothesized. Regarding host epithelial barrier integrity, both short and prolonged psychosocial stress exposure alter gene and/or protein expression of epithelial tight junctions, TLRs, mucins and inflammatory cytokines within the larynx, compared to the unstressed animals, with no pronounced differences across the sexes.

Following short and prolonged psychosocial stress, microbial evenness and richness remained relatively stable. In literature on restraint stress-induced changes in gut and oropharynx, microbial richness and evenness remain mixed[45,46], with most studies reporting relative stability in these measures[14,47–49]. We found microbial richness of less abundant bacteria was decreased in females following prolonged psychosocial stress, but not males. Although prior research has indicated relative stability in laryngeal microbiota between the biological sexes[44], estrogen is known to have an impact on the stressed gut microbiome, particularly in measures of microbial evenness /richness[50]. Thus, natural hormonal variation in estrogen could potentially impact laryngeal microbial compositional changes in response to stress.

There was a significant microbial compositional shift within the larynx following prolonged stress compared to the control group, in both sexes. Prior research overwhelmingly similarly reports a significant microbial shift following prolonged psychosocial stress exposure in the rodent gut[14–19]. Significant microbial shifts are noted in the oral microbiome following similar prolonged restraint stress protocols in rodents[45,51]. Commensal bacteria composition is thought to be altered following interaction with neurotransmitters, metabolites and hormones involved in the physiological stress response[52]. However, studies with a single stress exposure, i.e. 2–10 h single exposure to restraint stress, do not report changes in beta diversity within the gut microbiota[20,21]. This indicates that duration of the stressor is an important factor in delineating stress-altered microbial changes in the gut[53]. In the larynx, we see a similar result. Our short stress protocol did not result in significant changes in beta diversity within the larynx.

With regards to specific phyla, there was an increase in Firmicutes and no change Bacteroidetes with prolonged stress, compared to the control group. Firmicutes are short chain fatty-acid producing bacteria. They are responsible for maintaining immunological homeostasis[37]. Increased abundance of Firmicutes, in the oral microbiota, is associated with high levels of life stress[54]. Our finding is in contradiction to prior literature in the gut, wherein stress-altered gut dysbiosis is associated with reduced Firmicutes and a lower Firmicutes/Bacteroidetes ratio[48,52,53,55]. However, psychosocial stress can also be associated with increased relative abundance of certain Firmicutes bacteria in the gut, such as pathogen, *Staphylococcus*. The larynx has a distinct microbial composition, with increased relative abundance of pathogenic bacteria compared to the gut[13]. Thus, high abundance of Firmicutes in the larynx—in response to psychosocial stress—may be due to the specific metabolic and immunological needs of the larynx. There was a decrease in relative abundance of Fusobacterium in prolonged stress females when compared to control females. Fusobacteria is an opportunistic pathogen, found

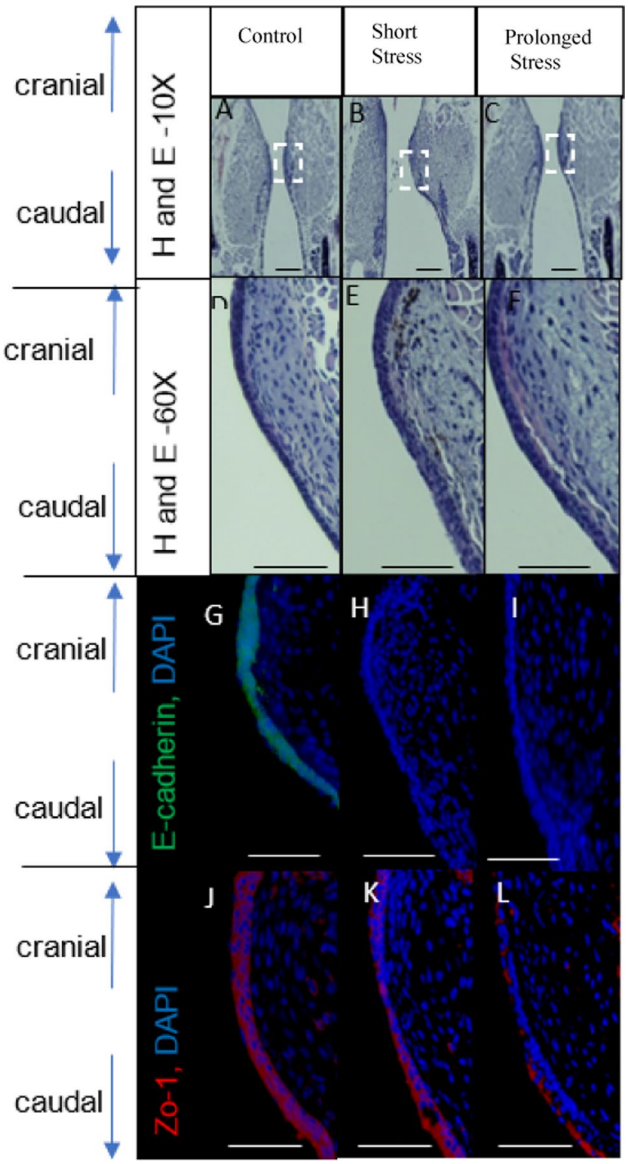

Fig. 8. Representative histology and Immunofluorescence vocal fold images for E cadherin and Zo-1-staining. Hematoxylin and Eosin-stained coronal sections of murine vocal fold region (10X [Figures **A**–**C**, black scale bar represents 100 um] and 60X [middle panel, Figures **D**–**F**, black scale bar represents 100 um]). Black box labelled within images in the top panel (Figures **A**–**C**) represent the portion (vocal fold region) that is magnified at 60 X (middle panel, Figures **D**–**F**). Mid-membranous vocal fold region stained with E-cadherin and DAPI (60X-Figures **G**–**I**, white scale bar represents 100 um) reveal no protein expression of E-cadherin across the length of the mid-membranous vocal fold epithelium of animals in short and prolonged stress groups, compared to control group (60X, panels **G**–**I**, white scale bar represents 100 um). Similarly, mid-membranous vocal fold region stained with Zo-1 and DAPI (60X, panels **J**–**L**, white scale bar represents 100 um) reveal decreased expression of Zo-1 in short and prolonged stress groups, compared to control group.

in the healthy oral microbiota, implicated in diseases of the head and neck[56]. Literature on the stress- and sex-specific differences of Fusobacteria in other mucosal microbiota are scarce and require further study[50].

Actinobacteria and Proteobacteria are responsibility for maintaining immunological and metabolic homeostasis, and maintaining an anaerobic environment in the gut[57,58]. Following psychosocial stress, these bacteria were decreased in the larynx, with betaproteobacteria identified as an enriched bacteria in the unstressed group via LEfSe. In prior literature, Actinobacteria and Proteobacteria are increased in the gut in response to psychosocial stress, but literature is inconsistent across studies[48,52]. The larynx has increased relative abundance of aerobic not anaerobic bacteria compared to the gut[13], thus, the function of these bacteria in the larynx may differ from that of the gut. Laryngeal microbiota enhances host cellular defense pathways including those associated with regulating inflammation, neutrophil-mediated immunity[59]. The role of members of Actinobacteria and Proteobacteria in maintaining laryngeal immunological homeostasis following psychosocial stress should be investigated with whole genome shotgun sequencing in future research.

At the genus level, there was an increase in *Streptococcus*, in the prolonged stress female compared to the control female animals. Similarly, in prior literature, *Streptococcus* has increased relative abundance in the gut in response to stress[48,52,53,55]. *Streptococcus* genera represent a group of opportunistic pathogens that have also been found in a number of oral diseases and benign vocal fold lesions[60–62]. Future work should identify the specific strain of *Streptococcus* influenced by psychosocial stress exposure, as certain strains of this genera may have both protective effects (*S. salivarius*) or deleterious effects on (*S. pseudopneumoniae*) laryngeal epithelial barrier integrity[63]. Specifically, in an in vitro human vocal fold mucosa model, exposure to *S. pseudopneumoniae* resulted in vocal fold epithelial barrier dysfunction (decreased tight junction integrity and increased epithelial permeability), while exposure to *S. salivarius* reduced host inflammation and provided a protective effects against these deleterious host epithelial effects of *S. pseudopneumoniae*[63]. We also found a decrease in *Corynebacterium* in the prolonged stress animals of both sexes, compared to the control animals. Similar decreases in *Corynebacterium* are observed in oral microbiome following prolonged restraint stress[46]. Consequently, *Corynebacterium* has increased or decreased abundance in the gut depending on the duration of psychosocial stress[64,65]. Decreased abundance of *Corynebacterium* can be associated with resilience to stress[64,65]. The 14-day duration of our prolonged stress protocol, may have led to habituation.

With respect to sex-related genera-level differences, we found increased *S24_7* in prolonged stress compared to control females. Increased abundance of *S24_7* is associated with certain types of murine stressors[66]. Although, *S24_7* is not abundant in the conventionally-raised murine larynx, mice engage in coprophagy, otherwise known as ingestion of feces. This behavior may increase in response to psychosocial stress, and within the confines of the restraint tube. Sex-related differences in abundance of *S24_7* have been reported in the gut microbiota in response to psychosocial stress[67]. We also found increased abundance of *Lactobacillus* with prolonged stress compared to control female animals, but not males. A number of studies have reported that females are more prone to stress-altered abundance of *Lactobacillus* in the gut[48,52,53,55]. Literature reporting stress-induced changes in Lactobacillus in the oropharynx remain scarce[45]. Murine estradiol levels are higher in proestrus females and males compared to other female estrus stages, and estradiol has long to interact with cortisol to protect against stress-associated functional memory deficits in females[68]. We did not measure estradiol levels in mice via blood plasma as additional blood collected from mice would augment stress and increase chance of attrition. Females were housed together to coordinate estrus cycles and minimize hormonal variation across female animals. Both of our stress protocols exceeded the duration of a normal estrus cycle (4–5 days).

Stress-altered commensal bacteria could have a deleterious impact on downstream immunopotentiation[52]. The mucosal microbiome is essential for maintaining epithelial permeability in other mucosal organs, such as the gut[69]. Psychosocial stress and other stress-related disorders have been associated with increased epithelial permeability in the gut, and nasal epithelium regardless of the duration of stressor exposure[69,70]. Toll like receptors 2 and 4 (*TLR 2, 4*)—essential for communicating and recognizing distinct microbial ligands on the epithelial surface[71,72]—are involved in potentiating neuroinflammation associated with psychosocial stress exposure[73]. Gene expression of TLRs are generally upregulated with psychosocial stress[73]. TLR2 is distributed evenly throughout the supraglottic and subglottic laryngeal epithelium, whereas TLR4 is mainly found in the supraglottis[72]. We found downregulation of *TLR2* in response to prolonged psychosocial stress only. Firstly, these results support our 16S rRNAseq findings, wherein short psychosocial stress exposure was insufficient to alter laryngeal microbiota composition. Additionally, TLR2 and TLR4 recognize different microbes on the epithelial surface in mucosal organs, including the larynx[72,74]. Research is required to identify role of TLRs in stressed host-microbe interactions in the larynx.

Psychosocial stress exposure has also been associated with mucus thinning and downregulation of mucins in the gut epithelium[75]. We found a similar downregulation of MUC2 gene expression in the larynx following short and prolonged psychosocial stress. Regardless of the duration of stress exposure—both short or prolonged—psychosocial stress downregulates gene/protein epithelial tight and adherens junctions in intestinal mucosa, thereby altering epithelial barrier integrity[76–78]. We similarly found a downregulation of protein expression in E cadherin and Zo-1 in the vocal fold epithelium following short and prolonged psychosocial stress, when compared to unstressed animals. This may leave the larynx vulnerable to exposure from noxious elements[79,80], as seen in the gut. Limited literature exists on characterizing vocal fold epithelial barrier integrity in laryngeal inflammatory conditions such as vocal fold nodules and polyps. Current literature suggests similar downregulation of epithelial tight junctions, and disruption of the basement membrane by loss of anchoring structures[81,82].

With regards to laryngeal sensory receptors in the epithelium, receptors of the TRPV family (Transient Receptor Potential Cation Channel Subfamily V) are mainly responsible for detecting harmful stimuli, such as heat, pain, chemical, pollutants[83–88]. We found that gene *TRPV1* , but not TRPV2 was downregulated with short and prolonged psychosocial stress, contrary to our hypothesis. These sensory receptors are generally increased in the colon in response to stress. Reduced expression of TRPV1 are associated with aberrant pathogenic defense and reduced ATP release in response to mechanical stimuli[86,89]. TRPV1 has been implicated in the development of laryngeal sensitivity[90], with TRPV channels used as a therapeutic targets for treatment of chronic cough[91]. These data lay the groundwork for elucidating a role for stress in conditions of laryngeal sensitivity.

Piezo 1/2 are a mechanically-gated ion channels, located in the laryngeal epithelium[87], that are implicated in somatosensation, proprioception, mechanotransduction, cytokeratin differentiation and epithelial renewal[32,87,88,92–95]. We found that relative gene expression of *Piezo2* in the larynx, was downregulated in response to short and prolonged stress. In the colon, changes in Piezo2 levels are correlated with somatosensory changes[32]. In addition, stress-induced changes in *Piezo2* have specifically been associated with pain hypersensitivity[96]. Regarding laryngeal somatosensation, prior research has hypothesized a specific role of Piezo2 in the laryngeal adductor reflex (LAR), an airway protective response[97], triggered laryngeal sensory receptors. Whether stress augments LAR, requires further study.

We found a downregulation of pro-inflammatory cytokines in the larynx of short and prolonged stress mice, compared to unstressed mice. However, compromised epithelial barrier integrity is associated with increased—rather than decreased—inflammation in the gut. In cases of repeated stress exposure (like restraint stress), studies have shown that inflammatory mediators experience a habituation of stress over time (leading to potential downregulation or decreased pro-inflammatory cytokine responses with repeated stress)[80,98–100]. Habituation to stress is generally associated with a gradual decrease in cortisol levels over time with repeated stress[98–100]. In our data, although elevated from baseline, there is a muted or decreased corticosterone response to stress in the prolonged stress group compared to the short stress group. This may provide an explanation for the discrepant findings between compromised vocal fold epithelial barrier integrity and pro-inflammatory gene expression. In spite of potential habituation to stress, the potential severity of the short and prolonged stress paradigms could have had a lasting effect on vocal fold epithelial barrier integrity. Prolonged stress is associated with compromised epithelial barrier integrity and underlies the pathogenesis of numerous systemic diseases.

Taken together, we have shown that prolonged psychosocial stress alters laryngeal microbiota composition, however, both short and prolonged stress altered host epithelial barrier integrity in the larynx. The enteric nervous system plays a role in stress-induced intestinal changes, acutely responding to elevated levels of cortisol[100,101]. We found elevated levels of corticosterone following both short and prolonged stress.

Limitations and future directions

There are few limitations to the study that require further elaboration. We used a murine model to elucidate stress-induced effects on the laryngeal microbiota, rather than human subjects. Although research on the laryngeal microbiota remains relatively new, the C56BL/7 murine model has been used extensively to characterize the laryngeal microbiota[13,59]. Using germ-free mouse models, prior research has demonstrated the selective nature of the larynx in microbial colonization[13], and elucidated that microbiota has a significant impact on the host laryngeal immune system[59]. Likewise, a future direction of this research is the incorporation of germ-free mouse models to determine if microbial-mediated mechanisms are the primary drivers of compromised vocal fold epithelial barrier integrity in the larynx.

Bacterial DNA for 16S rRNA sequencing was obtained from the entire murine larynx, rather than vocal fold tissue. The bacterial biomass of the murine larynx is already relatively low [10–40 ng/ul] compared to the murine gut [> 600 ng/ul]. The vocal fold surface itself would not have sufficient bacterial biomass to perform 16S rRNA sequencing. In addition, it would be difficult to practically sample the vocal fold surface in the murine model, without contamination from adjacent laryngeal sites. Combining inoculate from multiple conventionally-raised mice may not elucidate individualistic differences that may occur following exposure to stress. Prior research has found similar microbial diversity and abundance of core bacterial taxa between the larynx and adjacent anatomical sites including trachea, esophagus and base of tongue, thus there may be minimal variation across sites with the larynx[13].

We investigated the effects of psychosocial stress on gene/protein expression of select laryngeal epithelial markers. This small set of genes/proteins does not allow for thorough investigation of stress-altered epithelial barrier integrity in the larynx. Investigating stress-altered epithelial transcriptome dynamics via single cell and/or spatial RNA sequencing could reveal cell-specific transcriptome changes in response to stress and their location within the larynx. Recent evidence suggests that the laryngeal microbiota plays a role in regulating host immunological defense and epithelial barrier integrity[59], However, our study does not allude to any causal effects of the laryngeal microbiota on host epithelial barrier integrity in response to stress.

We noted biological sex differences in the microbial responses to prolonged stress and the interaction of psychosocial stress and estrogen are highly complex. The murine estrus cycle is 4–5 days, thus, future studies should incorporate periodic monitoring of estradiol throughout the length of short or prolonged restraint stress to determine if the estrus stage at time of larynx collection contributed to sex differences in stress-altered laryngeal microbiota composition.

Our study employed two psychosocial stress protocols, and found that 7 days of restraint stress did not sufficiently impact laryngeal microbiota composition. The differences between "short" and "prolonged" stress protocols indicate that the duration of stress has an impact on laryngeal microbiota composition. The duration of our short and prolonged stress protocols were determined by prior literature describing significant shifts in gut microbial composition in rodents following 7 and 14 days of restraint stress[48,102]. Future research can vary the duration of the stressor, incorporate intermittent stress exposure with adequate time for recovery, and investigate potential persistent effects of stress in the larynx.

Conclusion

In our study, prolonged but not short, psychosocial stress has the potential to alter the laryngeal microbiota composition. As hypothesized, females have a more pronounced effect to prolonged psychosocial stress in the laryngeal microbiota. Within host epithelial barrier integrity in the larynx, psychosocial stress,short and prolonged, downregulates the relative gene and/or protein expression of inflammatory cytokines, adherens and tight junctions (E cadherin, Zo1) and mucins. Prolonged psychosocial stress further downregulates gene expression of toll like receptors. Future studies should investigate the potential relationship between stress-altered microbiota and host epithelial barrier integrity within the larynx. Overall, this study lays the groundwork for delineating the potential role of psychosocial stress in the development of laryngeal pathologies.

Data availability

Sequence data that support the findings of this study have been deposited to SRA GeneBank with the primary accession code PRJNA1239093.

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A.V.—conceptualization, data collection, analysis, writing K.J, J.B—data collection R.A—conceptualization, supervision F.R—conceptualization, supervision, manuscript editing S.T.—conceptualization, supervision, writing, manuscript editing.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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