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Chronic exposure to ambient traffic-related air pollution (TRAP) alters gut microbial abundance and bile acid metabolism in a transgenic rat model of Alzheimer's disease

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ABSTRACT

Background: Traffic-related air pollution (TRAP) is linked to increased risk for age-related dementia, including Alzheimer's disease (AD). The gut microbiome is posited to influence AD risk, and an increase in microbial-derived secondary bile acids (BAs) is observed in AD patients. We recently reported that chronic exposure to ambient TRAP modified AD risk in a sex-dependent manner in the TgF344 AD (TG) rat.
Objectives: In this study, we used samples from the same cohort to test our hypothesis that TRAP sex-dependently produces gut dysbiosis and increases secondary BAs to a larger extent in the TG rat relative to wildtype (WT) controls.
Methods: Male and female TG and age-matched WT rats were exposed to either filtered air (FA) or TRAP from 28 days up to 15 months of age (n = 5–6). Tissue samples were collected after 9 or 14months of exposure.
Results: At 10 months of age, TRAP tended to decrease the alpha diversity as well as the beneficial taxa *Lactobacillus* and *Ruminococcus flavefaciens* uniquely in male TG rats as determined by 16 S rDNA sequencing. A basal decrease in Firmicutes/Bacteroidetes (*F/B)* ratio was also noted in TG rats at 10 months. At 15 months of age, TRAP altered inflammation-related bacteria in the gut of female rats from both genotypes. BAs were more affected by chronic TRAP exposure in females, with a general trend of increase in host-produced unconjugated primary and microbiota-produced secondary BAs. Most of the mRNAs of the hepatic BA-processing genes were not altered by TRAP, except for a down-regulation of the BA-uptake transporter Ntcp in males.
Conclusion: In conclusion, chronic TRAP exposure produced distinct gut dysbiosis and altered BA homeostasis in a sex and host genotype-specific manner.

1. Introduction

Neurodegenerative disorders, which are commonly characterized by immature death of neuronal cells in the central nervous system due to accumulation of misfolded, neurotoxic proteins, are suggested to be the second leading cause of death worldwide [16,17]. Among various types of neurodegenerative diseases, AD is one of the most significant health issues worldwide. It was recently reported that approximately 6.5 million people aged 65 and older in the United States are living with AD ([2] 2021) and it is estimated that the number could grow to 13.8 million (2021; [51]).

A transgenic model of AD, the TgF344-TG rat, expresses both mutant human amyloid precursor protein (APP) and presenilin 1 (PS1ΔE9) genes and manifests signs with face validity to early onset of familial AD [29]. We recently characterized the effect of traffic-related air pollution (TRAP) on AD-relevant neuropathology in this TG rat model and wild-type rats [91]. We observed that TRAP increased amyloid plaque deposition, hyperphosphorylated tau levels, neuronal cell loss,

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microglial cell activation, and cognitive deficits in an age-, sex-, and host genotype-specific manner.

Various environmental factors play an important role in the incidence and progression of AD and related dementia. Air pollution is considered a potent risk factor for AD-relevant neuropathology [15], ranking 8th among the leading risk factors for mortality, accounting for 2.5% of all deaths in developed countries [18,41]. Several prior and recent observations noted the links between diesel exhaust (DE) and particulate matter smaller than 2.5 μm ($PM_{2.5}$) and ultrafine PM (particles smaller than 1 μm) and AD-associated dementia ([30,42]; Cacciottolo, 2017; Chen et al., [27]; Grande et al., [47]; Yuchi et al., [125]). A study on a large population cohort found that development of dementia is higher in individuals living close to heavy traffic [26] and a recently published epidemiological study again noted that elevated long-term exposure to $PM_{2.5}$ is associated with increased risk of dementia [102]. In a preclinical study, exposure to urban nanosized PM for 225 h over 15 weeks increased cerebral Aβ deposition in female AD mice [14].

During the last few years, an association between gut dysbiosis and the development of neurodegenerative diseases and neurologic dysfunction has been established (Chandra, 2020; Parker et al., [90]; Megur et al., [73]). The microbiota-gut-brain axis is a bi-directional communication system that is recognized as a factor in determining the health of the central nervous system [35,83]. Alterations in the gut microbial composition have been reported in several animal models of neurodegenerative diseases and neurologic dysfunction. For example, removal of the gut microbiome can prevent the development of relapsing-remitting demyelination in a mouse model of multiple sclerosis (MS) [10]. Oral ingestion of probiotics (*Lactobacillus* and *Bifidobacterium*) attenuates neuroinflammation in rats and mice [65,116]. Reduced fecal microbial composition has been reported in both autistic children and mouse models of autism [32,44]. In AD patients, reduced microbial richness in the feces has been correlated with increased glial cell activation [120]. In a germ-free (GF) APPS1 AD mouse model, there was a significant reduction in cerebral amyloid pathology along with reduced microgliosis in both young and old animals [48]. Further, fecal microbiome transplant (FMT) of GF-APPPS1 transgenic mice using feces from conventionally raised APPPS1 transgenic mice increased cerebral Aβ pathology, confirming a role for the gut microbiome in the progression of AD [48]. Studies using another mouse model of AD (5x FAD transgenic mice) revealed an altered microbial composition in feces of these mice, evident as an increase in the pro-inflammatory species *Clostridium leptum* [13,19]. In addition, increased permeability of gut barrier with age leads to the translocation of microbes and/or microbial components to the systemic circulation, and this has been suggested to induce local and systemic inflammation and altered brain function [35,58,68,7].

The gut microbiome can produce neuroactive microbial metabolites, such as short chain fatty acids (SCFAs), neurotransmitters (gamma-aminobutyric acid (GABA), histamine, acetylcholine, serotonin, melatonin, etc.), and BAs [101,104,54,6]. Some of these metabolites can reach the brain via the systemic circulation to modulate various cognitive functions [21]. While the neuroactive features of several microbially metabolites such as SCFAs have been well characterized in neurological disease [98], less is known as to whether and how microbial-derived secondary BAs are associated with environmental toxicant exposures and AD susceptibility.

BAs are amphipathic molecules derived from cholesterol in the liver that function in lipid metabolism; these host-derived BAs are called primary BAs [108]. Most of the primary BAs are conjugated with taurine or glycine in humans, or predominantly with taurine in mice and rats, before they are secreted into the intestine. In the intestine, BAs control bacterial proliferation and overgrowth; conversely, bacterial enzymes convert primary BAs to secondary BAs through deconjugation, dehydroxylation and epimerization [75,111].

Recently, BAs have emerged as important signaling molecules with systematic endocrine functions that are associated with metabolic and neuronal diseases [107,111,39,85]. Elevated secondary BAs deoxycholic acid (DCA), lithocholic acid (LCA), glycine conjugated deoxycholic acid (G-DCA) and glycine conjugated ursodeoxycholic acid (G-UDCA) have been reported in the plasma of patients with amnestic mild cognitive impairment (aMCI) and AD [1,67,71,86]. BAs, such as cholic acid (CA), chenodeoxycholic acid (CDCA) and DCA, have been detected in the brain of rats [40,69,70]. The BAs UDCA and its taurine-conjugated form (T-UDCA) have been shown to have a neuroprotective effect in patients with AD, PD and Huntington's disease, and systemic administration of these two BAs was neuroprotective in corresponding animal models [57,87,95]. Higher concentrations of toxic secondary BAs have also been detected in the brain of APPPS1 transgenic mice compared to WT controls [87].

Recent studies have shown that chronic TRAP exposure is associated with greater risk of metabolic disease such as type 2 diabetes [115,124,4]. Studies have also shown that air pollution is an important etiological factor in the development of neurodegenerative disorders, such as AD [30]. However, little is known regarding the effect of the crosstalk between TRAP and gut microbiome in individuals carrying genetic risk factors for AD, and whether TRAP causes gut dysbiosis and/or alters important microbial metabolites, such as secondary BAs. Therefore, in this study, we tested how chronic ambient TRAP exposure from a heavy commute corridor modulates the gut microbiome and microbial BA metabolism in a transgenic rat model of AD and WT controls.

2. Materials and Methods

2.1. Chemicals and reagents

Fecal DNA isolation kits were purchased from QIAGEN. For bile acid extraction, liquid chromatography mass spectrometry (LC-MS) grade methanol, acetonitrile and ammonium acetate were purchased from Thermo Fisher Scientific. Deuterated internal standards (IS) used included: d4-DCA (CDN Isotopes; CAS No: 112076–61–6), d4-CDCA (CDN Isotopes; CAS No: 99102–69–9), d4-CA (TRC, Canada; Cat no #: C432603), d4-GCDCA (Iso Sciences, CAS No: 1201918–16–2) and d4-LCA (PubChem CID of LCA: 9903, Steraloids). Nineteen major BAs (Table S1) were purchased from either Sigma Aldrich (St. Louis, Missouri) or Steraloids (Newport, Rhode Island). ωMCA and T-ωMCA was a kind gift from Dr. Daniel Raftery's laboratory at University of Washington Northwest Metabolomics Research Center. Agilent ZORBAX Eclipse Plus C18 columns were purchased from Agilent Inc. All other chemicals and reagents, unless indicated otherwise, were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Animals and exposure details

Male and female Tg344-AD (TG) rats that overexpress the human mutant amyloid precursor protein (APPSW) and presenilin 1 (PS1ΔE9)) transgenes, and age-matched WT rats were housed in TRAP or FA exposure chambers beginning at postnatal day 28 and continuing for up to 14 months as described previously [91].

In brief, TRAP exposure groups were continuously exposed to unchanged real-time traffic air from a heavy commute tunnel. The air flow rate in the exposure chamber was 35 cubic feet per min [91]. FA air exposure group was exposed to background ambient air after several filtrations of residual air pollutants [38]. The exposure chambers were insulated for sounds to avoid significant sound related stress arising from the roadway and the vehicle noise [38]. For TRAP, the total particle number and PM2.5 mass concentrations fluctuate, mirroring traffic flow, with the mean 24 h PM2.5 levels being 15.6 ± 3.7 μg/m^3. Average particle diameters in TRAP were 33 ± 4 nm. Total particle numbers in the TRAP chambers were 10- to 100-fold higher than in FA chambers [91]. A subset of these animals was euthanized at 10 or 15 months of age (Fig. 1). Immediately after euthanasia, various tissues including serum,

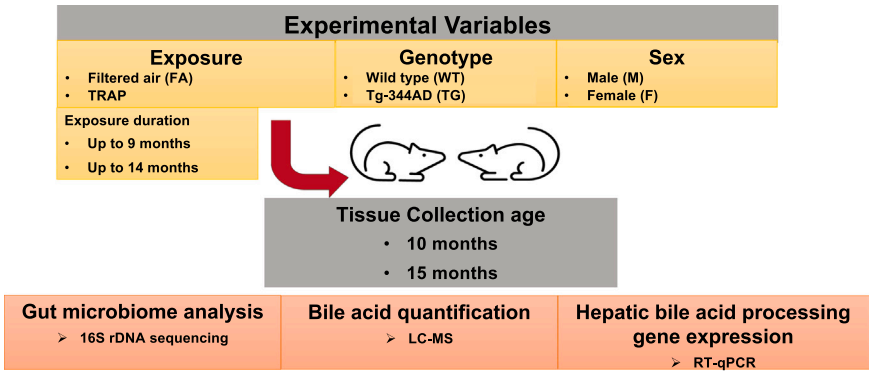

Fig. 1. A diagram illustrating the main hypothesis and experimental design of the study. Male and female rats in WT or TgF344-AD background were exposed to either filtered air (FA) or traffic related air pollution (TRAP) starting from postnatal day (PND) 28. Tissues were collected after 9 and 14 months of exposure while the age of those animals were 10 months and 15 months respectively. Microbial DNA from the large intestinal content (LIC) was quantified using 16 S rDNA sequencing. Bile acids (BAs) in serum, LIC, and liver were quantified using LC-MS/MS. Messenger RNA expression of host BA processing genes in liver and ileum was quantified using RT-qPCR.

liver and large intestinal content (LIC) were collected, weighed and frozen in liquid nitrogen and then stored at − 80 °C until further analysis. The animal studies were approved by the IACUC Committee at UC Davis and all experimentation with the live animals occurred at UC Davis.

2.3. Quantification of bacterial DNA and 16 S rDNA sequencing

Total DNA was isolated from LIC from both WT and TG rats using QIAGEN Genomic DNA Isolation Kits according to the manufacturer's protocol. The concentration of DNA was quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA). Bacterial DNA was sequenced using an Illumina HiSeq system (250 bp paired end; n = 5–6/group; Novogene, Sacramento, CA) for bacterial 16 S rDNA V4 amplicon sequencing. The paired-end sequence reads were joined and demultiplexed using QIIME [20]. Joined FASTQ files of all samples were labelled with a unique identifier. Chimera sequences generated from PCR amplification artifacts were removed using identify_chimeric_seqs.py, and reference-based chimera detection was performed using usearch61 against the Greengenes 13_8 operational taxonomic units (OTUs) database (99_otus.fasta) (http://qiime.org/home_static/dataFiles.html). OTU tables were then sorted, format-converted and taxonomy-summarized from level 3 (L3, class level) to L7 (species level). The α- and β-diversity were determined using alpha_refraction.py and jackknifed_beta_diversity.py in QIIME.

2.4. RNA isolation

Total RNA was isolated from frozen liver samples using RNAzol Bee reagent according to the manufacturer's protocol (Tel-Test Inc., Friendswood, Texas). RNA concentration was determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, Massachusetts) at 260 nm. Integrity of total RNA was confirmed by gel electrophoresis. Total RNA was reversed transcribed into cDNA using a high-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, California). The cDNAs were amplified by qPCR using SsoAdvanced Universal SYBR Green Supermix in a BioRad CFX384 Real-Time PCR Detection System (Bio-Rad, Hercules, California). The qPCR primers targeting the cDNAs of the host BA-processing genes were synthesized by Integrated DNA Technologies (Coralville, Iowa, USA) and the sequences are shown in Table S2. The ddCq values were calculated for each target gene and then normalized to the expression of the housekeeping gene, β-actin.

2.5. Bile acid quantification

2.5.1. Preparation of bile acid (BA) standard curve

One mg/mL stock solutions of individual BAs (for standard curve) and internal standards (ISs) were prepared in methanol and water (1:1). The 19 individual BA stock solutions were further diluted in 50% methanol to obtain 10 working standard solutions (0.05–10000 ng/mL). The 5 ISs were mixed to obtain a working IS solution.

2.5.2. BA extraction from Liver serum and LIC

BAs were extracted from frozen LIC samples (n = 5–6 per group) using a similar method as we described previously [34,45] with modifications. Briefly, 75–85 mg of feces were used for each sample, and were vortexed in sterile water (2.5 μl per mg of fecal mass) at 4 °C. Afterwards, 200 μl of well-homogenized fecal suspension was removed and mixed with 10 μl of IS mixture and equilibrated on ice for 5–10 min. 1.5 mL of ice-cold alkaline acetonitrile (5% ammonia in acetonitrile) was added to the homogenate, which was then vortexed vigorously and shaken continuously for 1 h at room temperature. The mixture was then centrifuged at 21,000 g for 15 min at 4 °C, and the supernatant was collected into 5 mL glass tubes. The pellet was re-suspended in 750 μl of methanol, shaken for 20 min, and centrifuged at 21,000 g for 20 min. The two supernatants obtained were combined, evaporated under vacuum (30 °C) for 4 h, and reconstituted in 100 μl of 50% methanol. The suspension was transferred into a 0.2 μm Costar Spin-X HPLC microcentrifuge filter (Corning Inc., Corning, NY), and centrifuged at 21,000 g for 10 min. 50 μl of this filtrate was injected into the ultra-performance liquid chromatography coupled with mass spectrometry in tandem (UPLC-MS/MS) for analysis. Calibrator and different quality control (QCs) samples were prepared by adding the appropriate amount of the different standard stock solutions and were extracted using the similar sample preparation procedure described above for fecal sample extractions.

For serum BA extraction, 50 μl of serum samples were mixed with 10 μl of internal standard (IS) solution and vortexed for 5–10 min. 500 μl of ice-cold methanol was added to the serum samples and vortexed again to prep a homogenous mixture. The sample mixture was centrifuged at 21,000 g for 10 min at 4 °C. The supernatant was collected in a new tube and again 500 μl of ice-cold methanol was added to the pellet. The pellet was dissolved in methanol and again centrifuged following the same previous criteria. Each supernatant was combined and evaporate under vacuum (low temperature, 30 °C) for 2.5 h. The dried samples were then reconstituted using 100 μl 50% methanol. Before injecting the samples were again centrifuged for 10 min at 21,000 g and 50 μl of filtered samples was loaded in the autosampler vials for MS-analysis.

BA was extracted from frozen liver samples (n = 5–6) using a method similar to that described in our previous publications [34,36,45] with modifications. Approximately 50–60 mg of liver tissue was homogenized in Millipore water and 200 μl of this homogenate was mixed with 10 μl of IS solution. 1.5 mL acetonitrile containing 5% ammonium hydroxide was added to the sample for protein precipitation and placed in the shaker for one hour at room temperature. After one hour the samples were centrifuged at 21,000 g for 15 min and the supernatant was collected in a 5 mL glass vial. The methanol containing samples were again centrifuged at 21,000 g for 20 min and the supernatant was

carefully collected and combined with the previous one. The supernatant was then evaporated under vacuum for 4 h and reconstituted in 50% methanol for further analysis. The suspension was transferred into a 0.2 μm Costar Spin-X HPLC microcentrifuge filter (Corning Inc., Corning, NY), and centrifuged at 21,000 g for 10 min for purification. 10 μl of this filtrate was then injected into the instrument for further quantification.

2.5.3. UPLC-MS/MS method development for BA quantification

Agilent 1290 UPLC (ultra-high pressure liquid chromatography) system combined with an Agilent 6460 triple quadrupole mass spectrometer via an electrospray ionization interface, was used for analysis. The chromatographic separation was performed using a ZORBAX Eclipse Plus C18 analytical column (2.1X100mm; id: 1.8 μm). Samples were eluted using mobile phase A, consisting of 20% acetonitrile and 10 mM ammonium acetate in water, and mobile phase B, which consisted of 80% acetonitrile and 10 mM ammonium acetate in water, at a flow rate of 0.4 mL/min. The gradient profile details used for the LC pump are described in Table S3. The injection volume of the samples was 5 μl. The column temperature was set at 45 °C and the sample tray temperature was maintained at 9 °C. MS/MS spectra were produced using the negative ionization mode.

2.6. Data analysis

Data are represented as mean ± standard error (SE). Statistically significant differences among groups were determined by analysis of variance (ANOVA) followed by Tukey's post-hoc test ($p < 0.05$) in R. Three-dimensional Principal Coordinate Analysis (PCoA) plots (beta diversity) were generated using the weighted UniFrac diversity metric in Emperor (Gigascience). OTUs were visualized using stacked bar plots generated in SigmaPlot (Systat Software, Inc). Bar plots representing the bile acid concentration were prepared using SigmaPlot. Asterisks (*) represent significant differences between FA and TRAP exposure, hashes (#) represent significant differences between genotype (WT and Tg344-AD groups).

We have performed three-way ANOVA in the present study using a generalized linear model controlling for errors of multiple testing. The three factors were host genotype (wild type vs. transgenic), exposures (filtered air vs. TRAP), and sex (male vs. female). Our key question was to what extent TRAP modulates gut microbiome, and how sex and host genotypes modified the interactions between TRAP and gut microbiome at each time point.

3. Results

3.1. TRAP-mediated effects on the gut microbiome composition

After 9 months of exposure, the effect of TRAP on the alpha diversity (Chao1 index) which reflects the microbial richness in the gut of 10-month-old male and female WT and TG rats is shown in Fig. 2A. Although not statistically significant, TRAP tended to increase the richness of the gut microbiome in male WT rats, however, this trend was reversed in male TG rats, evidenced by an apparent decrease of richness in TRAP TG males. In female rats, the effect of TRAP on the gut microbiome was minimal in both host genotypes. Principal coordinate analysis (PCoA) showed that the beta diversity of the gut microbiome as calculated by the weighted Unifrac was not distinct across the eight experimental groups (Fig. S1A). At the phylum level, *Firmicutes/Bacteroides* (F/B) ratio, was significantly decreased in TRAP-exposed TG male rats compared to WT TRAP-exposed group (Fig. 2b). This observation supports the interaction between exposure and genotype in the alteration of gut associated microbial marker [117].

The effect of the 9-month TRAP exposure on the relative abundance of bacteria at the L7 species level is shown in Fig. S1B, and differentially regulated taxa are shown in Fig. 2 C and Fig. S1C. Most notably,

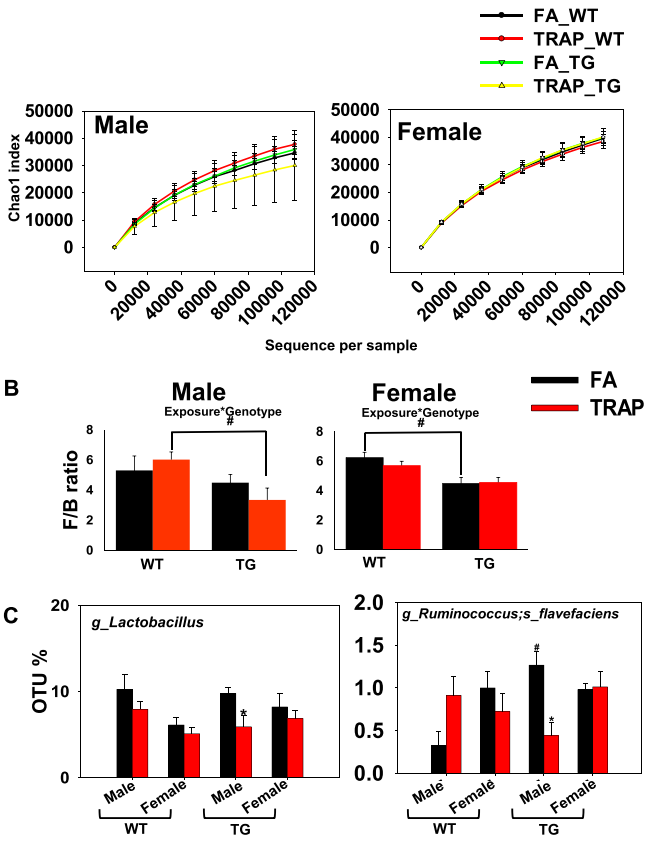

Fig. 2. (A) Alpha diversity of gut microbiota in LIC of male and female WT and TgF344-TG rats exposed to FA and TRAP for 9 months (n = 5–6 per group). The 16 S rDNA data were analyzed using QIIME. (B) *Firmicutes/Bacteroidetes* (*F/B*) ratio in male (left) and female (right) WT and TgF344-TG rats after 9 months exposure to FA and TRAP. Pounds (#) represent statistically significant differences between WT and TG of the same sex and exposure (two-way analysis of variance [ANOVA] followed by Tukey's post hoc test, $p < 0.05$). (C) Differentially regulated bacterial species (L7) after 9 months exposure to TRAP. Asterisks (*) represent statistically significant differences between FA and TRAP in rats of the same sex and genotype; pounds (#) represent statistically significant differences between WT and TRAP of the same sex and exposure (two-way analysis of variance [ANOVA] followed by Tukey's post hoc test, $p < 0.05$).

Lactobacillus genus was uniquely downregulated by TRAP in male TG rats (Fig. 2 C). Several species of the *Lactobacillus* genus have been identified to improve AD in various research models through enhancing gut barrier functions, production of SCFAs, as well as having antioxidant and anti-inflammatory properties [114,5,82]. *Ruminococcus flavefaciens* was also down-regulated uniquely in male TG rats due to TRAP exposure (Fig. 2C). It is a well-known cellulolytic bacteria involved in the butyrate metabolic pathway [22]. Since butyrate is known to rescue the advanced stage of AD in a mouse model by cognitive improvement [46], the decrease in this bacterium may increase susceptibility to TRAP-induced neurotoxicity. Interestingly, the *Odoribacter* genus was eliminated by TRAP in all four groups of rats, indicating that it is a TRAP-targeted bacteria regulated independent of sex or host genotype (Fig. S1C).

In summary, at 10 months of age after 9 months of TRAP exposure, both host genotype and sex were modifying factors for susceptibility to TRAP-mediated effects on the gut microbiome, with male TG rats generally being the most susceptible to TRAP-induced gut dysbiosis.

At 15 months of age after 14 months of TRAP exposure, there was no marked difference in the alpha diversity among the eight experimental groups (Fig. S2A). However, with respect to beta diversity, while there was no distinct separation between FA and TRAP-exposed WT rats, there was a clear separation between FA and TRAP-exposed TG rats in both sexes (Fig. S2B), indicating that the AD genetic risk factor increases

susceptibility to chronic TRAP-induced gut dysbiosis. Overall, more taxa were differentially regulated after 14 months TRAP-exposure in both sexes and genotypes of rats, as observed to the earlier time point (Fig. 3). While the *F/B* ratio was not significantly altered by TRAP in any experimental group, at the phylum level (Fig. 3A), there was a significant increase in *Bacteroidetes* and *Tenericutes* uniquely in male WT rats exposed to TRAP, a significant decrease in *Proteobacteria* uniquely in male TG rats exposed to TRAP, and a significant increase in *Actinobacteria* uniquely in female TG rats exposed to TRAP (Fig. 3B). Increased richness of both *Bacteroidetes* and *Tenericutes,* as well as decreased richness of *Proteobacteria,* has been previously reported in AD mice (Harach, 2017; Park et al. [88]).

As shown in Fig. 3C and D, at the L7 species level, 14 months of TRAP exposure altered gut microbes in a sex- and host genotype-specific manner. Notably, TRAP was associated with a female-specific increase in *Turicibacter* and *Ruminococcus gnavus* in both genotypes. *R. gnavus* is known to produce inflammatory cytokines, whereas an increase in *Turicibacter* has been reported in diet-induced obesity (Henke, 2019; Liu, 2016). In male WT rats, an increased richness of *Paraprevotella* was noted after 14 months of TRAP exposure. This taxon has been associated with major depressive disorders (Jiang, 2015). Members of the family *Desulfovibrionacceae* and *Lachnospiraceae* decreased in both male and female TG rats after 14 months exposure. Lower abundance of the family *Lacnospiraceae* and *Ruminococcaceae* have been associated with lower BA and SCFA production [68] and are proposed to strengthen the integrity of the gut barrier [105,130,79]. Butyrate, which is a neuroprotective SCFA [62,76], has been reported to be decreased in the fecal samples of AD mice [128]. Another family, *Desulfovibrionaceae,* was decreased in 15-month-old male TG rats after 14 months of exposure to TRAP. Both *Desulffovibrio* and *Odoribacter sp* were reduced in a mouse model of autism and the authors reported a positive correlation of those taxa with impaired social behavior and a negative correlation with an anxiety phenotype [44]. *Odoribacter* is a known producer of SCFAs, decreased richness of *Odoribacter* in current study may affect host inflammation via less production of SCFA [43,78]. Interestingly, a recently published study documented the beneficial role of *Odoribacter* among the healthy centenarian population [99]. Interestingly, in the present study, *Odoribacter* was down-regulated by 14-months of TRAP exposure in all groups. It is possible that the depletion of *Odoribacter* may result in adverse health effects such as shortened lifespan and accelerated rate of aging-related diseases such as neurodegenerative disorders, although additional experiments are needed to establish a mechanist link. Collectively, these findings support a role of TRAP in gut dysbiosis and in accelerating the progression of neurodegenerative disease such as AD.

3.2. TRAP effects on BA homeostasis

To determine the effect of TRAP on BA homeostasis, targeted metabolomic analysis was done in liver, serum and LIC. In total, 19 primary and secondary BAs, and taurine-conjugated forms were quantified. Abbreviations for all BAs are provided in Supplementary Table S1. Individual BA concentrations among all tissue compartments (serum, liver and LIC) are provided in Supplemental Tables S4-S9.

At 10 months of age after 9 months of TRAP exposure, BAs in liver were not significantly altered by TRAP in any experimental group; and in general, the sum of all BAs, all primary BAs, and all secondary BAs were also not altered by TRAP (Fig. S3). However, interestingly, as shown in Fig. 4, the major primary conjugated BA, T-CA, was uniquely down-regulated by 9 months of TRAP exposure in both serum and LIC of male TG rats. The major primary unconjugated CA was significantly increased by TRAP in the serum of male WT rats and tended to be increased in male TG rats due to TRAP exposure. Interestingly, T-LCA, which is the conjugated form of a major secondary BA produced by intestinal bacteria, was increased by TRAP uniquely in LIC of male TG rats. In serum of TRAP-exposed female rats, the secondary BA ωMCA was higher in TG than in WT rats (Table S4B). The TRAP-mediated decrease in T-CA in serum and LIC, a trend towards increased CA in serum, and increased T-LCA in LIC (mostly observed in male TG rats), as well as higher ωMCA in TRAP-exposed female TG rats than TRAP-exposed female WT rats, suggest a TRAP-mediated increase in microbial deconjugation and dihydroxylation to promote secondary BA synthesis in AD genetic risk factor carriers at this time point.

At 15 months of age after 14 months of TRAP exposure, total BAs, total primary BAs, and total secondary BAs remained unchanged in liver, serum, and LIC across all experimental groups (Fig. S4). However, in serum, the primary conjugated BA T-αMCA and its unconjugated form αMCA were increased by TRAP uniquely in female TG rats (Fig. 5A). The other primary unconjugated CA, CDCA, and βMCA also tended to be increased in serum by TRAP in female rats of both genotypes, although a statistical significance was not achieved. In addition, the secondary conjugated BA T-DCA tended to be increased by TRAP in male TG rats although a statistical significance was not achieved (Fig. 5A).

In LIC, 14 months TRAP exposure increased the primary BAs, T-CA and CA, as well as the secondary BAs, T-ωMCA and T-HDCA in female WT rats. TRAP also increased CA in male TG rats (Fig. 5B).

Overall, after 14 months of TRAP exposure, distinct BAs were differentially altered by TRAP, with most upregulated. In addition, females appeared to be more susceptible than males to the effects of TRAP on BAs.

To determine the effect of TRAP exposure on host hepatic BA synthesis and disposition, we quantified serum C4 (7-αhydroxy-4-cholesten-3one), which is a stable intermediate in the rate-limiting pathway of BA biosynthesis in the liver [56]. C4 was not altered by TRAP at either time point in any of the experimental groups (Fig. S5). In addition, we quantified the mRNA expression of various BA synthetic enzymes (Cyp7a1, 7b1, 8b1, and 27a1) and the major BA uptake (Ntcp) and efflux (Bsep) transporters in liver (Fig. S6). Except for Ntcp, which was downregulated by TRAP in male WT rats at 10 months and in male TG rats at 15 months, none of the BA-processing genes were significantly regulated by TRAP (Fig. S6). Therefore, the TRAP-mediated changes in BAs may be more mediated by gut microbiome, rather than host liver.

4. Discussion

Characterizing both WT and transgenic rats expressing AD risk genes, we found that chronic ambient TRAP exposure produced gut dysbiosis and altered BA metabolites in a host genotype- and sex-specific manner. As summarized in Fig. 6, after 9 months TRAP exposure, we observed genotype- and sex-specific changes in TRAP-induced gut dysbiosis and BAs, with most changes observed in male TG rats. The male TG-specific alterations include 1) a tendency of decreased alpha diversity; 2) decreased beneficial taxa such as *Lactobacillus sp.,* and *R. flavefaciens*; as well as 3) reduced T-CA and increased CA in serum, reduced T-CA and increased T-LCA in LIC – together indicating increased secondary BA synthesis. As reported in [91], at the 9-month time point, male TG rats had elevated neuroinflammation markers (CD68 +/IBA1 + levels), and there was increased neuronal cell loss of TG rats. In addition, sex is an important variable in contextual fear conditioning, where male rats' performance was worse than females. After 14 months of TRAP exposure, there were more prominent TRAP-induced gut dysbiosis and BA changes than the earlier time point. Specifically, gut microbiome and BAs of both sexes and host genotypes were impacted by TRAP, and the AD-related phenotypes were also impacted by both sex and the genotype variations.

The transgenic AD rats were used in the study to understand the gene vs. environment interaction in the progression of gut dysbiosis associated with the reported susceptibility of AD-like phenotype. All neurological endpoint parameters (e.g., amyloid beta accumulation, inflammatory marker of brain microglia, behavioral changes) associated with TRAP exposure in both transgenic and wild-type rats have been shown in our collaborators' recent publication [91] using the same

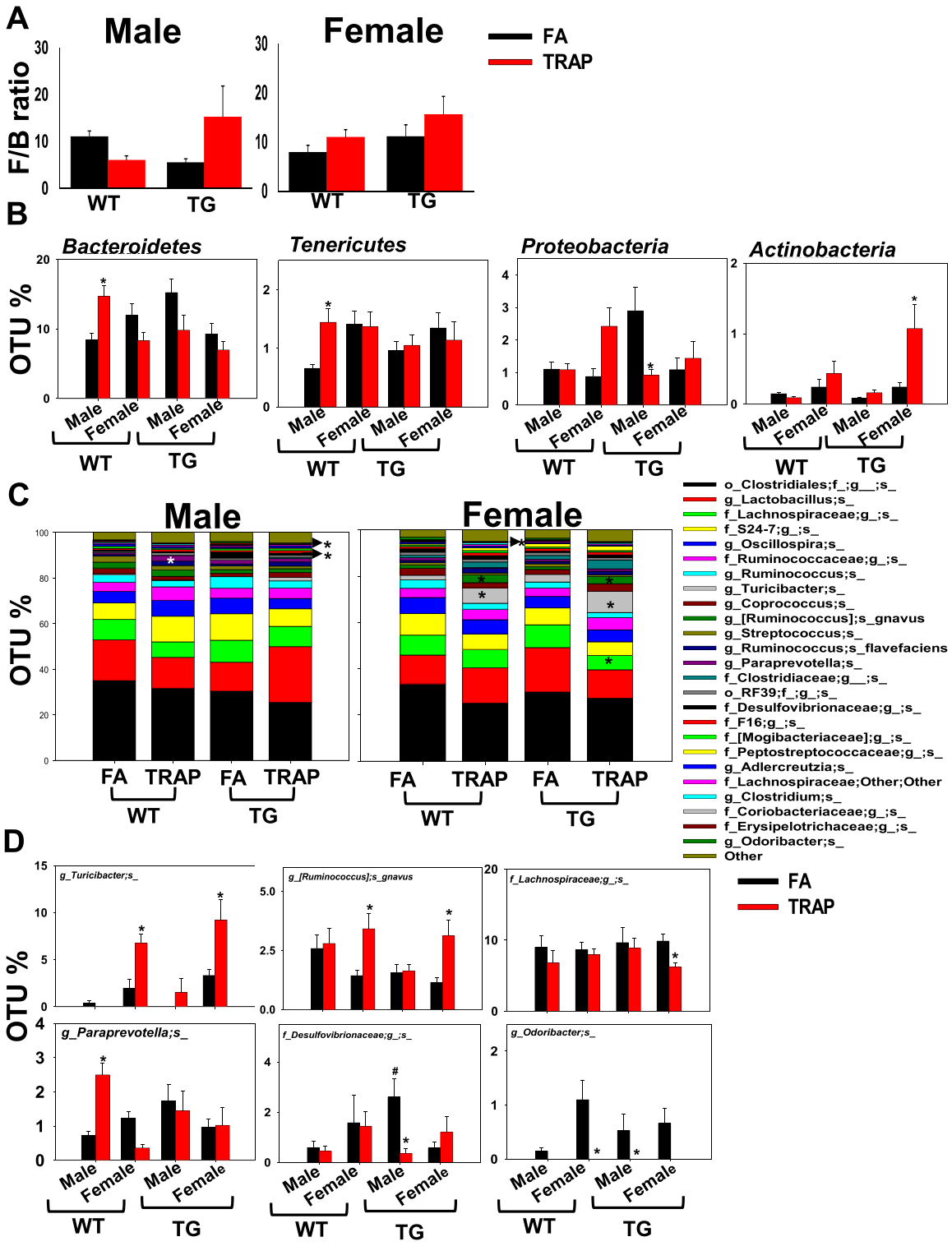

Fig. 3. (A) *F/B* ratio in male (left) and female (right) WT and TgF344-TG rats after 14 months exposure to FA or TRAP (n = 5–6 per group). (B) Differentially regulated bacterial phyla (L2) by TRAP after 14 months exposure (asterisks [*] represent statistically significant differences as compared to the FA-exposed group of the same sex and genotype, $p < 0.05$). Statistical significance was determined using two-way ANOVA followed by Tukey's post hoc test. (C) Proportion of bacterial species (L7) observed in FA and TRAP exposed male and female rats. (D) Differentially regulated bacterial species (L7) in female and male rats after 14 months exposure to TRAP. Asterisks (*) represent statistically significant differences when compared with different exposure of similar sex and genotype ($p < 0.05$) whereas pound (#) symbolize statistically significant differences among different genotypes of similar sex and exposure ($p < 0.05$). Data was analyzed using two-way ANOVA followed by Tukey's post hoc test.

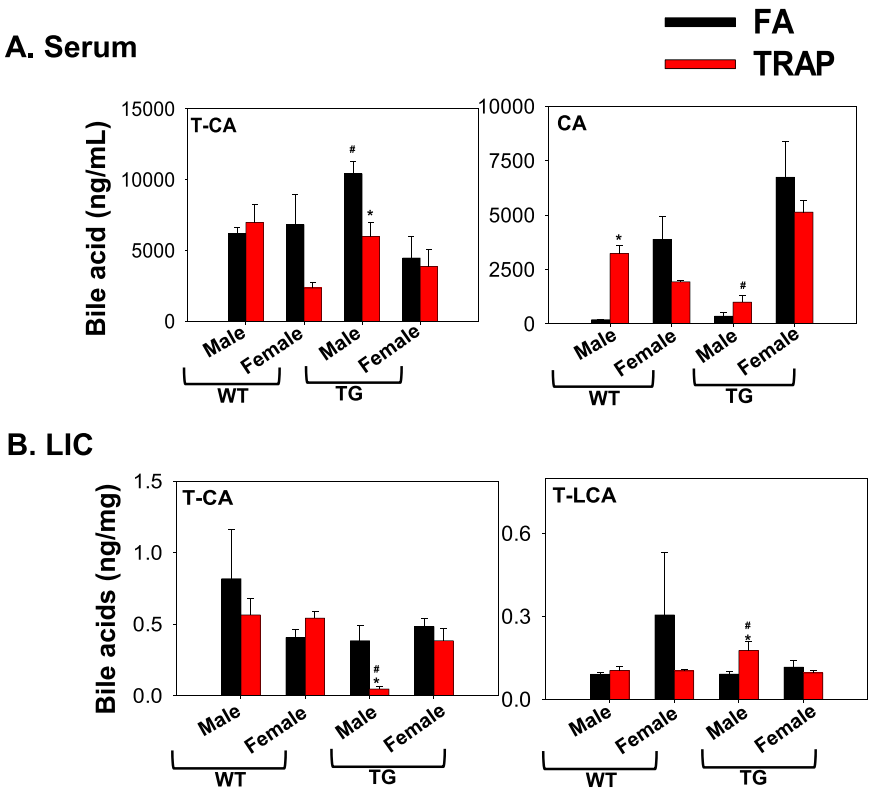

Fig. 4. LC-MS quantification of individual BAs from serum (A) and LIC (B) of 9 months FA and TRAP-exposed rats. The differentially regulated BAs are shown. Asterisks (*) represent statistically significant differences between exposure groups (FA vs TRAP) and pounds (#) represent statistically significant differences between genotypes ($p < 0.05$). Data is represented as means ± SE. All data has been analyzed using two-way ANOVA followed by Tukey's post hoc test.

cohort. Specifically, neural cell loss is a pathological hallmark of AD, and this was observed at 10 months in the TgF344 transgenic AD rats with no sex-specific effects [91]. At 10 months the higher neural cell loss in the TgF344 rats was not TRAP-specific however, at 15 months TRAP played a significant role and exacerbated neural cell loss in both WT and TgF344 rats.

In the current study, we have demonstrated how TRAP accelerates gut dysbiosis, which in turn may alter gut-brain axis secondary to disrupting the gut-liver interaction. A systematic comparison between the neurobehavioral study [91] and the gut microbiome investigations (current study) follows:

Patten et al. demonstrated that over a time course of ambient TRAP exposure, the most prominent neurotoxicity was observed at 10 and 15 months of age (which were after 9 and 14 months TRAP exposure, respectively) [91]. Similarly, in our current study we have noted prominent gut related dysbiosis at 10 and 15 months of age (which is 9 months and 14 months of TRAP exposure). Therefore, in the current manuscript, for our investigations on the gut-brain axis, we focused on these two time points. At 10-months, interestingly, TRAP exposure increased the Aβ42: Aβ40 ratio, which is a predictive biomarker for impaired cognitive status and AD pathology [11], uniquely in the cortical tissue from male TG rats but not in the other groups [91]. Correspondingly, in the present study, we observed a TRAP-mediated decrease in *Lactobacillus* and *Ruminococcus flavefaciens*, uniquely in male TG rats at the same time point (Fig. 2C). These two taxa are considered as beneficial taxa and studies have reported their neuroprotective potential in several animal's model [114,22,5,82]. Specifically, as discussed above, certain *Lactobacillus* species can improve AD in laboratory models via enhancing gut barrier function, SFCA production, and having antioxidant and anti-inflammatory effects, whereas *Ruminococcus flavefaciens* is also known to produce the neuroprotective SCFAs. We also observed an overall tendency of reduced gut microbial alpha diversity uniquely in male TG rats following TRAP exposure at the 10-months age (Fig. 2A). Interestingly, in AD patients, it has been shown that gut microbiome microbial alpha diversity is also reduced and is compositionally distinct from control age- and sex-matched individuals (Vogt, 2017). Therefore, the observed tendency in reduction of the alpha diversity by TRAP uniquely in male TG rats is consistent with the literature report on the positive associations between AD phenotype and gut dysbiosis in humans, and provides additional evidence that TRAP is a contributing factor for the onset of AD, and such toxicity is likely further modified by sex and the host genotype. In addition to changes in intestinal bacteria, at 10 months, TRAP increased the microbial derived T-LCA in LIC uniquely in male TG rats (Fig. 4A). Serum T-LCA and G-LCA (the human-enriched LCA conjugate) have been shown to positively associate with the AD biomarkers fibrillary tau and phosphorylated tau in the cerebrospinal fluid of AD patients [81]. In addition, an increase in serum secondary DCA has been detected in AD patients (MahmoudianDehkordi, 2019). However, we did not observe significant changes in the secondary BAs concentration in serum. In summary, the association among the TRAP-mediated increase in the cortical Aβ42: Aβ40 ratio, the TRAP-mediated decrease in certain neuroprotective intestinal bacteria, and the TRAP-mediated increase in potentially toxic microbial derived secondary BA T-LCA in LIC, all of which were uniquely observed in male TG rats suggest that the gut dysbiosis may serve as a contributing factor for the sex- and host genotype-specific susceptibility to TRAP-induced neurotoxicity.

While we observed a positive correlation between Aβ42:Aβ40 ratio and gut dysbiosis that likely explain TRAP-mediated neurotoxicity in male TG rats at 10 months, there are several other neurotoxic biomarkers that do not entirely associate with the microbial or metabolic signatures. For example, at 10 months, TG rats of both sexes had more participles in hippocampus and more neuronal cell loss following TRAP exposure [91], whereas changes in gut microbiome and secondary BAs in female TG rats were minimal at this time point. At the 15-months' time point, both TRAP-mediated neurotoxicity and the microbial-related

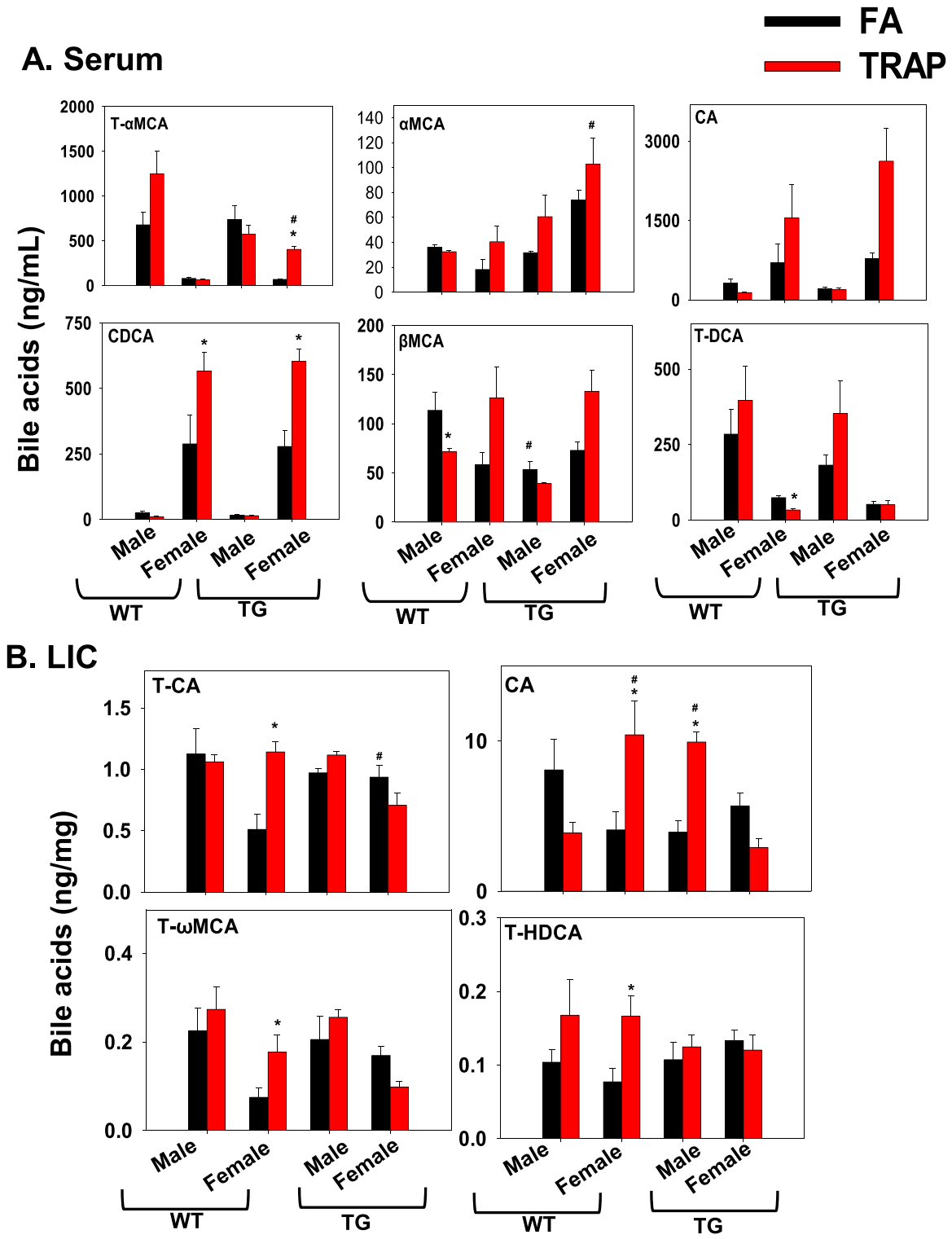

Fig. 5. LC-MS quantification of individual BAs from serum (A) LIC (B) of 14 months FA and TRAP exposed rats. The differentially regulated BAs are shown. Asterisks (*) represent statistically significant differences between exposure groups (FA vs TRAP) ($p < 0.05$). Data has been represented as means ± SE. All data were analyzed using two-way ANOVA followed by Tukey's post hoc test.

parameters displayed a more diverse pattern and were modulated by both sex and the host genotype. Regarding the contribution of sex, at 15-months, TRAP increased *Turicibacter* and *R. gnavus* in female rats of both WT and transgenic genotypes, whereas these taxa remain unchanged in male rats (Fig. 3D). To note, both *Turicibacter* and *R. gnavus* are well known for their pro-inflammatory effects, which are implicated in the pathogenesis of neurodegenerative disorders [27,52]. Studies reported that *R. gnavus* can employ mucin as a carbon source and might be directly associated with the breakdown of gut barrier function [113, 31,93], which in turn allows the translocation of inflammatory lipopolysaccharide (LPS) from the gut. *R. gnavus* is also known to produce and secrete a complex glucorhamnan polysaccharide molecule, which can induce secretion of the inflammatory cytokine TNFα from dendritic cells [52]. *Turicibacter* has also been found to increase in diet-induced

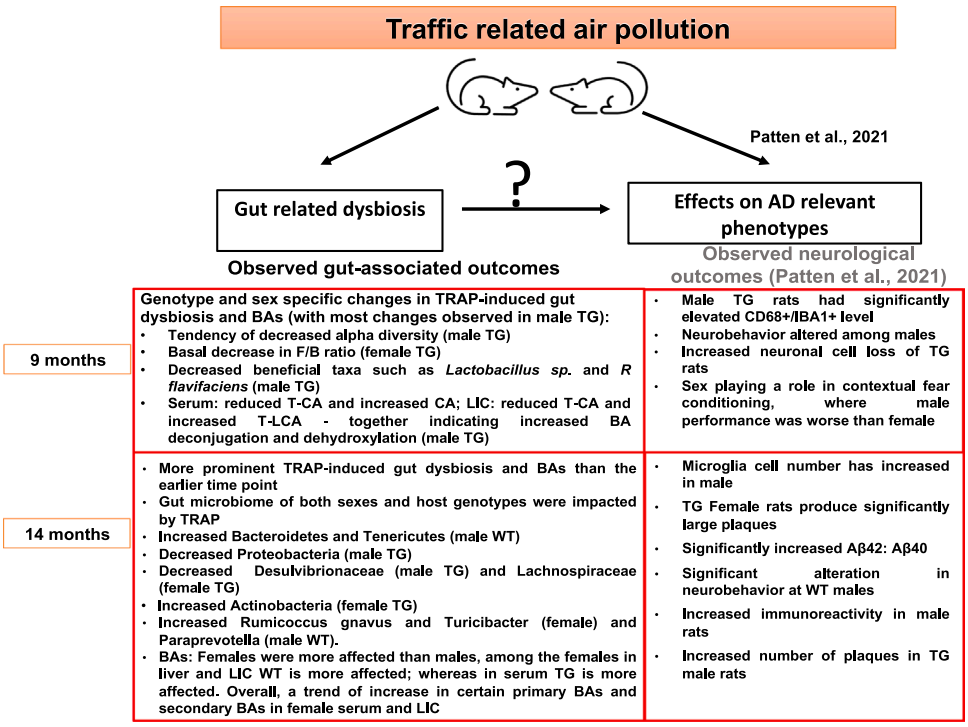

Fig. 6. Summary of major findings in the current study and comparison with the AD-related phenotypes in the same cohort as observed by [91].

obesity [63], which is a known risk factor for AD [74,94]. Studies have revealed that ketogenic diets are able to improve neurovascular function in mice by reducing the abundance of *Turicibacter* [66]. However, despite the TRAP-mediated increase in these pro-inflammatory taxa in the gut of female rats of both genotypes, the CD68 + /IBA1 + microglial cells were decreased by TRAP in female rats at 15 months [91], indicating a reduction in neuroinflammation and neuronal loss. This discrepancy may be due to other confounding factors, including basal differences in microglia cells (the AD females have higher density of cells that are IBA1-positive [a biomarker for microglia] than WT females, regardless of exposure) [91], and a rise in serum T-αMCA (Fig. 5A), which was shown to be inversely (yet weakly) associated with brain atrophy of AD patients as evidenced by MRI [81]. Another bacterial genus, *Paraprevotella* increased significantly in 15-month-old male WT rats after 14 months of TRAP exposure. Increased abundance of *Paraprevotella* seems to be associated with major depressive disorder [55]. A decrease in *Paraprevotella* in fecal samples was observed in AD patients [50]. Despite this observation, TRAP exposed WT males had impaired recognition of the fear conditioning context in comparison with FA WT males [91], suggesting that the fecal *Paraprevotella* levels are not necessarily a specific biomarker for neurotoxicity.

In summary, a comparison of the neurological outcomes (Pattern et al.) and the gut microbiome data (present study) in the same cohort of animals at least partially supports the hypothesis that gut dysbiosis (evidenced as a reduction in neuroprotective bacteria and an increase in pro-inflammatory bacteria) and elevated microbial BA metabolism contribute to the promotion of AD-relevant phenotypes by exposure to chronic ambient TRAP in both WT and TG animals (Fig. 6). However, the discrepancies between the neurotoxicity study [91] and the present study on gut microbiome indicate that gut dysbiosis is likely a contributing factor but does not entirely explain the TRAP-induced neurotoxicity. Additional intervention studies involving manipulation of the gut microbiome are needed to further confirm the mechanistic involvement of gut microbiome in TRAP effects on AD.

GI inflammation and impaired gut barrier functions are increasingly recognized risk factors for neurological diseases [24]. Under basal conditions, a high *F/B* ratio is associated with increased energy harvest from diet [117]. The *F/B* ratio is also a well-known marker of gut dysbiosis, and a low ratio is associated with inflammatory bowel disease (IBD) [109]. In our study, after 9 months of TRAP exposure, the basal *F/B* ratio was significantly reduced in TG rats. We also observed a TRAP-mediated decrease in taxa that are important for maintaining gut barrier functions and preventing inflammation. Permeability of the gut membrane is known to increase with age, which in-turn allows bacteria and their products (such as LPS) to translocate through the disrupted barrier and reach peripheral organs [97,126]. Other groups have reported the presence of LPS in the parenchyma and blood vessels of brain samples from aged and AD patients [127,129] and in the plasma of AD transgenic mice [112]. Memory impairment, Aβ aggregation and astrocyte activation have also been reported in mice after administration of LPS [61]. In the present study, we saw a TRAP-mediated increase in the abundance of inflammation-producing taxa such as *Ruminicoccus gnavus,* [52] and taxa associated with depression such as *Turicibacter, Paraprevotella* [8], along with decrease in the abundance of beneficial taxa such as *Lactobacillus, R. flavefaciens, Odoribacter* and *Desulfovibraceae* in both male and female rats. From these observations in the present study, we conclude that long term TRAP exposure is associated with gut dysbiosis and exacerbates brain-associated pathogenesis regardless of host genotype. Lower abundance of the family *Lachnospiraceae* and *Ruminococcaceae* have been reported to be associated with lower BA and SCFA production [68] and enhanced integrity of the gut barrier [105,130,79]. Similarly, in our current study, we have observed a TRAP-mediated decrease of family *Lachnospiraceae in* TG female rats after 14 months exposure. Therefore, our present study suggests that TRAP may negatively impact the gut integrity. Additional studies including quantifying serum LPS or bacterial DNA, quantifying intestinal tight junctional proteins etc., will be needed to verify our hypothesis in the future.

To understand the impact of long-term exposure to TRAP on the gut-liver axis, we quantified BAs in different tissue compartments of rats. BAs are not only digestive detergents or metabolites of cholesterol catabolism but are also recognized as important microbial metabolites that can influence host health and disease pathogenesis ([Anonymous] 2019). In brief, host liver-derived primary BAs can be modified into

more polar secondary BAs by dehydroxylation reactions catalyzed by the gut microbiome. Any alteration in gut microbiome composition can alter BA homeostasis in the host. Altered BA profiles are known to be associated with the development of diseases such as obesity, IBD, cancer, and AD [123,50,60,81,84].

Recent studies have implicated BAs in the progression of neurodegenerative disease such as AD. Cytotoxic BAs, such as LCA and DCA are increased in the serum of AD patients [67,72,86]. A neuroprotective effect of BAs, such as from UDCA and T-UDCA, has also been reported, reducing Aβ-induced apoptosis in primary rat cortical neurons [106]. In our study, we saw a significant decrease in UDCA levels in the liver of WT female rats after 14 months exposure to TRAP. It has also been reported that UDCA can reduce the production of ROS and decrease sodium nitroprusside-induced cytotoxic cell-death [28]. A decrease of UDCA in both WT and Tg344-AD female rats and increased *R. gnavus* population in our study aligns with the findings of [64] where UDCA treatment led to a decrease in *R. gnavus* in men [92]. Also, the concentrations of conjugated secondary BAs such as T-DCA, T-LCA, T-ωMCA, T-HDCA were altered in the serum and LIC of TRAP-exposed rats, supporting the hypothesis of TRAP-induced gut-dysbiosis. Increased CA concentrations are known to directly alter gut microbial composition, increasing the abundance of opportunistic pathogens and decreasing the abundance of beneficial microbes [122]. Regarding the host contribution of BA levels, we were unable to see any significant alteration in the gene expression of BA synthesis genes and transporter in the liver after TRAP exposure. Only the hepatic BA uptake transporter Ntcp expression was reduced significantly in male rats after long-term exposure to TRAP. This finding suggests enhancing concentration of gut produced secondary BA in the serum, which might be able to reach brain and accelerate neurodegeneration. Hence, our observations suggest that it is likely that TRAP did not impact gut-liver homeostasis but instead impacted gut microbial abundance, which in turn modified the gut-brain axis.

While the present study focused on BAs, which have recently been recognized to have neuroactive functions [77], other microbial metabolites may also contribute to TRAP effects on the brain. *Lactobacillus* spp. can produce neurotransmitter molecules such as acetylcholine and gamma-aminobutyric acid (GABA) [33,37,9]. Modulation in the signaling of neurotransmitters, such as acetylcholine and GABA, are associated with depression, and impaired synaptogenesis, which subsequently influence brain function and behavior [23,33].

In the present study, TRAP significantly reduced the abundance of *Odoribacter* in both male and female rats exposed for 9 or 14 months. *Odoribacter* is also a known producer of SCFA, a decreased abundance of which may increase host inflammation [43,78]. It has also been reported that *Odoribacter* is responsible for the production of the sulfonolipid molecule “sulfobacin B” [121], which is able to suppress acute inflammation in mice [25]. *Odoribacter* spp. *was* reduced in abundance in a mouse model of autism and authors reported a positive correlation of those taxa with social behavior and negative correlation with anxiety phenotypes [44]. Therefore, the decreased abundance of *Odoribacter* spp. in our study might be at least in part responsible for increased inflammation in the brain of TRAP-exposed animals.

Members of the family *Lachnospiraceae* appeared to be decreased significantly in TG female rats after 14-months exposure to TRAP. Members of the family *Lachnospiraceae* is known to associated with BAs synthesis [68]. SCFA is an important bacterial metabolite that has been reported to be altered in the AD transgenic mice [12,128]. Butyrate is a neuroprotective SCFA [62,76] that has been reported to be decreased in the fecal samples of AD mice [128]. Another family, *Desulfovibrionaceae* seems to be decreased significantly in male TG rats after 15-months exposure to TRAP. *Desulfovibrio,* a member of the *Desulfovibrionaceae* family was reduced in a mouse model of autism, and it was correlated with social behavior and inversely correlated with anxiety phenotype [44]. Hence, these additional observations support the role of TRAP in gut dysbiosis and accelerate the progression of neurodegenerative disease such as AD.

One limitation of the present study is that while we have established for the first time the associations between the gut dysbiosis and TRAP exposure in a sex and host genotype-specific manner using a rat model that is genetically prone to AD, we did not fully address how gut dysbiosis mechanistically contributes to the sex- and genotype-specific neurotoxicity induced by TRAP. The current study, with a primary focus on characterizing the gut microbiome changes, is a follow up of the study by [91], which focused on the neurological outcomes of the same cohort of rats after long-term TRAP exposure. To fully address the necessity of gut dysbiosis in the onset of TRAP-induced neurotoxicity and identify specific microbes that promote or mitigate the pathogenesis of AD are among our ongoing research goals and need to be clarified in future studies. The effect of TRAP and its interactions with host genetics, metabolites, and neurological parameters appear far more complex than we initially hypothesized. While a clear trend was not observed for all endpoint parameters to conclude that gut microbiome mechanistically contributes TRAP-induced neurotoxicity with unique specificity towards one sex or host genotype, it is clear that the TRAP-induced gut dysbiosis and alterations in bile acids is highly sex- and host-genotype dependent. A similar trend in behavior changes was also reported using the same cohort of rats [91]. It is important to acknowledge that additional studies need to be conducted to further confirm our findings.

The use of gnotobiotic models, fecal transfer experiments, or specific microbe inoculations may confirm the mechanistic contributions of gut microbiome in the development of TRAP-mediated neurodegenerative disorders including AD. Studies have reported that FMT is an effective measure to alleviate AD phenotypes in mice models and human patients [110,49,59,89]. Several studies have reported the role of FMT and its promise in the treatment of neurodegenerative disorders including Parkinson disease and autism spectrum disease [103,118,3,53]. As the current study is an initial notion of TRAP induced gut dysbiosis, we expect the exact association between TRAP and gut dysbiosis will effectively be explored using gnotobiotic model animals, fecal transplant experiments, and specific microbe inoculations.

The current study was designed to consider sex as a critical variable for TRAP-induced risks to neurotoxicity. While epidemiological studies have suggested that the incidence rate of AD is higher in women [100, 80,96], it was suggested that higher longevity and other sex-specific factors could serve as potential contributors [119]. In addition to TRAP, many other environmental and genetic risk factors contribute to the pathogenesis of AD, thus the sex-differences may be context specific. In addition, differences between rats and humans may serve as another confounding factor in the sex-specific response to toxic exposures. Additional experiments need to be performed to systematically compare the TRAP-induced neurotoxicity between humans and animal models to better assess the feasibility of using the transgenic rats to recapitulate AD risks in humans.

5. Conclusion

In conclusion, in the current study we demonstrated for the first time the impact of chronic exposure to ambient TRAP on gut dysbiosis. In brief, our data indicate that TRAP exposure increases the abundance of pro-inflammatory taxa and decreases the abundance of beneficial taxa in the gut. Further, the modified *F/B* ratio observed in TRAP-exposed animals (regardless of their genotype and sex) supports the hypothesis that long-term TRAP exposure promotes gut dysbiosis. Significantly increased AD-associated phyla *Bacteroidetes and Ternicutes,* and significantly decreased *Proteobacteria* in TRAP-exposed animals suggest a mechanism by which TRAP promotes the development of neurodegenerative disease. In the current study, we also noted TRAP exposure also altered the abundance of beneficial taxa and increased toxic secondary BA metabolites particularly in genetically susceptible male rats compared to females. We observed gut microbiome alteration after long term exposure to TRAP and this dysbiosis is present in both male and

female regardless of their genotype, although TG male rats show TRAP-induced dysbiosis early in comparison to the age- and genotype-matched female rats. Brain and behavior alteration due to TRAP exposure reported by [91] again supports our gut-associated findings. These findings altogether support the potential of gene environment interaction in the pathogenesis of neurological dysfunction through the alteration of gut homeostasis.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.03.003.

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