SUPPLEMENTARY MATERIAL

Drinking pattern and sex modulate the impact of ethanol consumption on the mouse gut microbiome

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Material and Methods

Analysis of the gut microbiota by real-time quantitative PCR (qPCR)

To evaluate the maltodextrin effects on the gut microbiota, we used feces samples from control mice, collected before and 9 hours after intragastric gavage with maltodextrin [9 $g.kg^{-1}$ p.c., 45% solution (m/v)] (Infinity Pharma®, Nova Iguaçu, RJ, Brazil). DNA extraction was performed according to the recommendations of the DNeasy® PowerSoil® Pro Kit (QIAGEN, Germany). DNA quantity and quality were assessed using NanoDrop 2000 device (Thermo Fisher Scientific, USA). Then the samples were immediately frozen at −20°C until molecular analysis. For PCR analysis, 10 ng of DNA and 1 μM of forward and reverse primers [*Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, *Tenericutes*, and *Eubacteria* (normalizer gene)] were used. Specific primers sequences for all bacteria phyla are reported in Table S2. Differences (ΔCT) between *Eubacteria* cycle threshold (CT) values and the evaluated phyla were used to obtain normalized levels of each bacteria phylum ($2^{-\Delta\Delta CT}$) (1). The fecal samples collected before the maltodextrin exposure was used as a normalizer to define the relative abundance of each phylum.

REFERENCE

1. **da Silva JL**, **Barbosa LV**, **Pinzan CF**, **Nardini V**, **Brigo IS**, **Sebastião CA**, **Elias-Oliveira J**, **Brazão V**, **Júnior JC do P**, **Carlos D**, **Cardoso CR de B**. The Microbiota-Dependent Worsening Effects of Melatonin on Gut Inflammation. *Microorganisms* 11, 2023. doi: 10.3390/microorganisms11020460.

LEGENDS FOR SUPPLEMENTARY FIGURES

Figure S1. Experimental protocol used in the current study. Male and female C57BL/6J mice were randomly divided into four groups: control, chronic ethanol, binge, and chronic plus binge ethanol. Mice from the chronic ethanol and chronic plus binge groups were submitted to one-week-adaptation period with 5% (v/v) ethanol solution. Mice then had free access to 10% (v/v) ethanol in the drinking water for 10 days. Mice from the control and binge groups had free access to water during the treatment period. Mice from the binge and chronic plus binge groups, received an oral gavage with a high dose of ethanol (5 $g.kg^{-1}$) on day 11. Mice from control and chronic ethanol groups received an oral gavage with water on day 11. Blood samples and fecal pellets were collected 2 or 9 hours after the oral gavage, respectively.

Figure S2. Heatmap showing shifts in the gut microbiota induced by maltodextrin in male and female mice. Fecal samples were collected before and 9 hours after intragastric gavage with maltodextrin (9 $g.kg^{-1}$). Gut microbiota composition (phyla category) was evaluated by RT-qPCR. *Compared to fecal samples collected before oral gavage ($p<0.05$, Student's t test).

Figure S3. Rarefaction curves plotted using alpha diversity metric, Shannon index against number of sequencing depth. (A) C57BL/6J mice were randomly divided into four groups: control, chronic ethanol, binge, and chronic plus binge ethanol. Fecal pellets were collected 9 h after the oral gavage. Rarefaction curves for all gut microbiota samples sequenced from male mice groups. (B) Rarefaction curves for all gut microbiota samples sequenced from female mice groups. Each colored line represents one biological sample of male or female mice groups (n=5 per group). FB: female from binge groups; FC: female from control group; FE: female from chronic ethanol group; FEB: female from chronic plus binge ethanol group; MB: male from binge groups; MC: male from control group; ME: male from chronic ethanol group; MEB: male from chronic plus binge ethanol group.

Figure S4. Ethanol-induced changes in the relative abundance of major gut microbiota phyla in male and female mice. C57BL/6J mice were randomly divided into four groups: control, chronic ethanol, binge, and chronic plus binge ethanol. Fecal pellets were

collected 9 h after the oral gavage. Bar plots illustrate the relative abundance of major bacterial phyla in male (A) and female (B) groups. Results are expressed as the mean ± S.E.M. (n=5 per group).

Figure S5. Top 30 detected pathways across the samples. C57BL/6J mice were randomly divided into four groups: control, chronic ethanol, binge, and chronic plus binge ethanol. Fecal pellets were collected 9 h after the oral gavage. FB: female from binge group; FC: female from control group; FE: female from chronic ethanol group; FEB: female from chronic ethanol plus binge group; MB: male from binge group; MC: male from control group; ME: male from chronic ethanol group; MEB: male from chronic ethanol plus binge group.

Figure S6. Predicted functional profile of male mice microbiota. C57BL/6J mice were randomly divided into four groups: control, chronic ethanol, binge, and chronic plus binge ethanol. Fecal pellets were collected 9 h after the oral gavage. Functional profiling of the gut microbiome was performed using PICRUSt2. The Kruskal-Wallis non-parametric test was used to determine statistically significant differences among the groups ($p<0.05$). PICRUSt2: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2.

Figure S7. Predicted functional profile of female mice microbiota. C57BL/6J mice were randomly divided into four groups: control, chronic ethanol, binge, and chronic plus binge ethanol. Fecal pellets were collected 9 h after the oral gavage. Functional profiling of the gut microbiome was performed using PICRUSt2. The Kruskal-Wallis non-parametric test was used to determine statistically significant differences among the groups ($p<0.05$). PICRUSt2: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2.

LEGENDS FOR SUPPLEMENTARY TABLES

Table S1. Pathways identified in the mouse fecal microbiome.

Table S2. Primers sequences used in real-time PCR.