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Effect of gut microbiota from children with autism spectrum disorder on behavior and ASD-related biological markers in germ-free mice

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Introduction

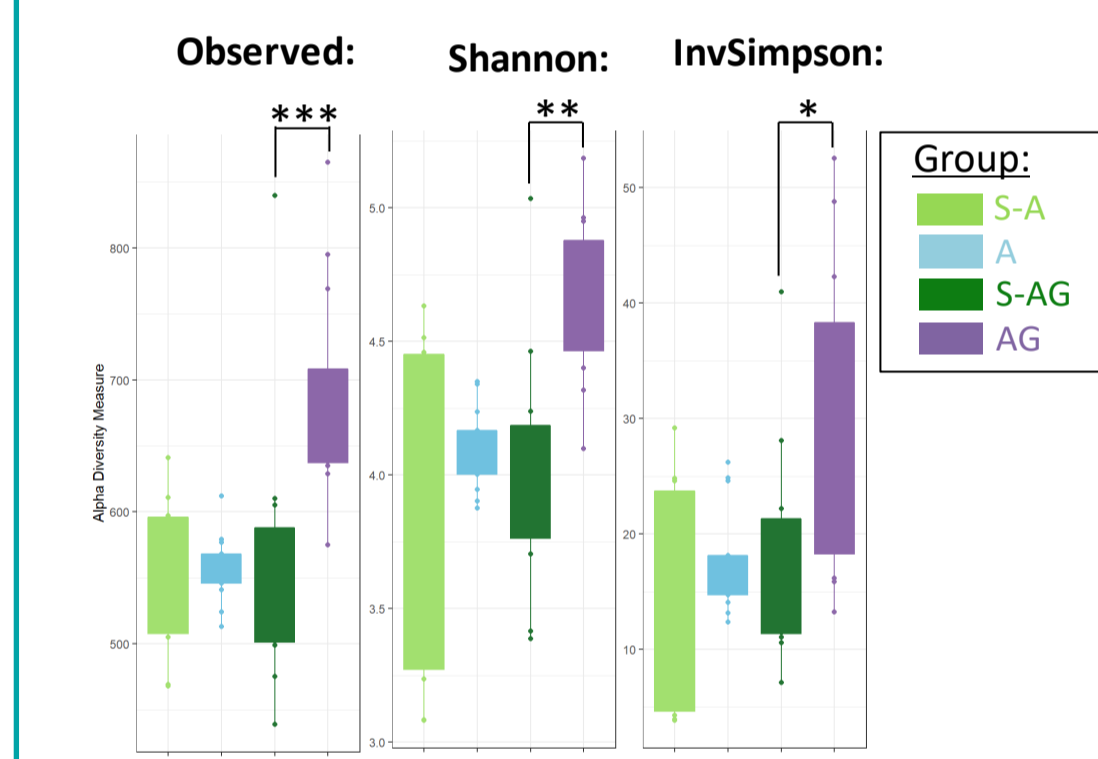
Autism spectrum disorder (ASD) is a neurodevelopmental disorder affecting 1 in 160 people in the world. Although there is a strong genetic heritability to ASD, it is now accepted that environmental factors can play a role in its onset. As the prevalence of gastrointestinal (GI) symptoms is four-times higher in ASD patients, the potential implication of the gut microbiota in this disorder is being increasingly studied. A disturbed microbiota composition has been demonstrated in ASD patients, accompanied by altered production of bacteria metabolites. This project aims to determine if the microbiota can influence behavior when transplanted to germ-free mice. Our hypothesis is that a pool of microbiota from patients with ASD, especially those with GI symptoms, will worsen behavioral phenotypes related to ASD in those mice as well as impact various ASD related biological markers, in comparison to GF mice transplanted with microbiota for the neurotypical siblings of those children. To ensure that the mice were exclusively exposed to donor microbiota, they were kept in isolators for the duration of the experiments. Experiments have been carried out on both BALB/c and C57BL/6 mice, but some analyses are still ongoing.

First results on BALB/c mice

• No significant differences between groups on behavior, microglial profile, KYN/TRP ratio in serum, and gut barrier integrity

• Analysis of microbiota composition and fermentation activity

• Diversity and composition



• Short chain fatty acid (SCFAs) profile

Acetate, Butyrate, Propionate, Branched and long chain

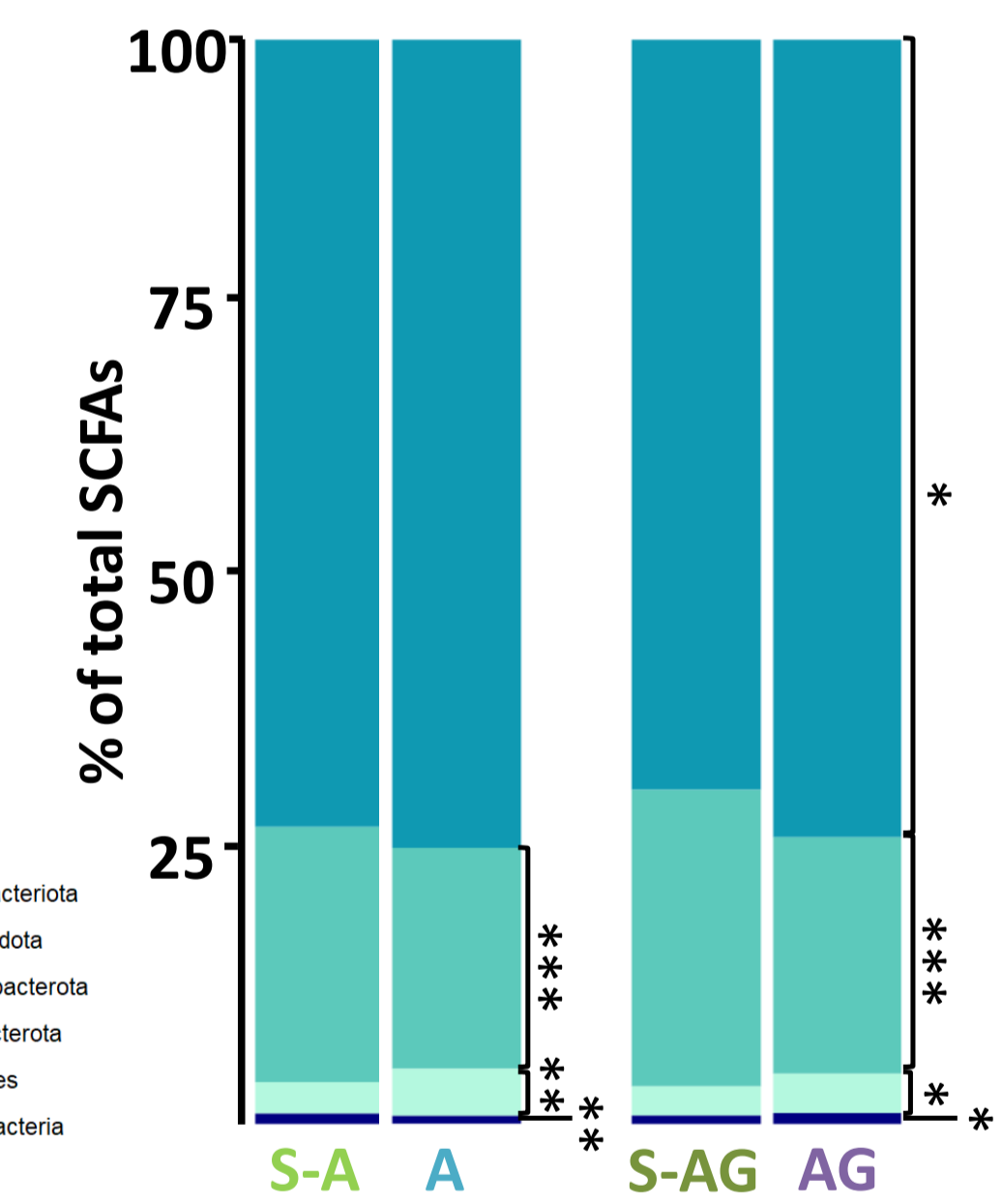


Fig 1: α -diversity measures of the microbiota (mice feces 6 weeks after FMT)

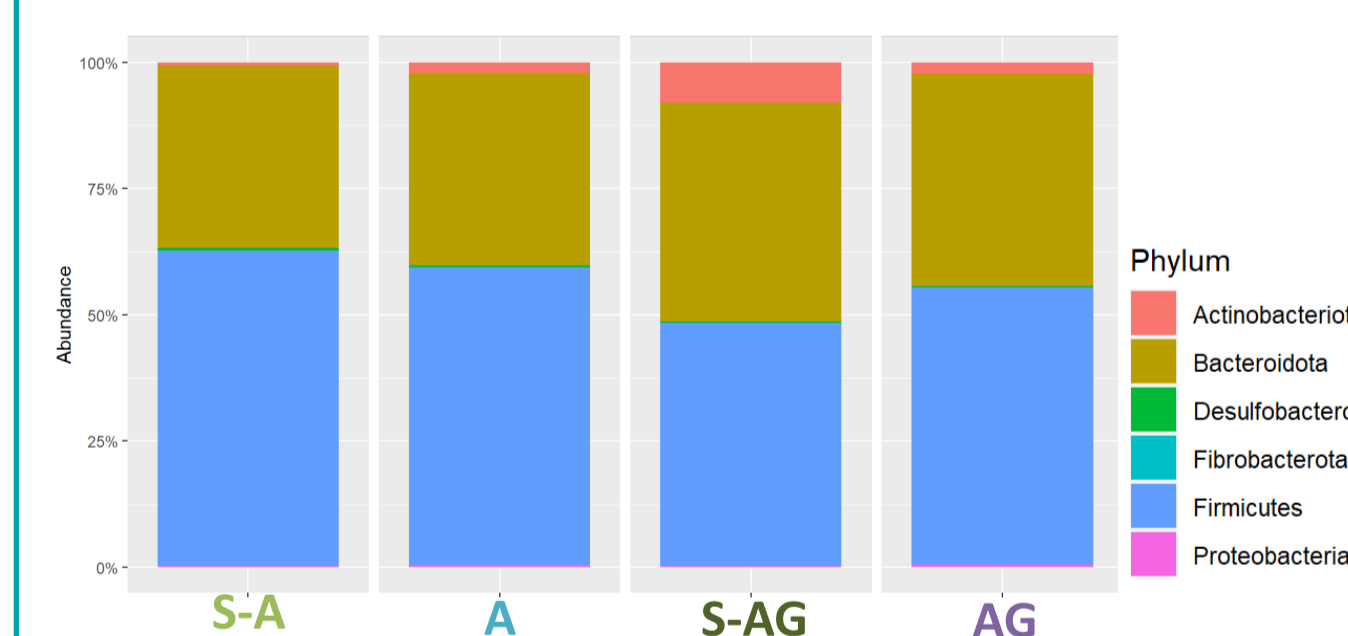


Fig 2: Relative abundance at Phylum level in the microbiota (mice feces 6 weeks after FMT)

Fig 3: Proportion of SCFAs in mice caecal content (analysed by gaz chromatography)

Results: 16S rRNA sequencing analysis showed an increase in diversity in the AG group compared to S-AG according to three different indexes (Richness observed $p=0.0007$; Shannon $p=0.0011$; Inversed Simpson $p=0.013$) (Fig.1), accompanied by an increase in the relative abundance of the Desulfobacterota and Proteobacteria phyla ($p=0.0459$ and 0.0219 respectively) (Fig2). No difference of diversity was found between the S-A and A groups, however, the relative abundance of the Actinobacteria phylum was increased in the A group ($p=0.0386$) (Fig2). Many differences were also observed at lower phylogenetic levels, with more than 100 OTUs differing between each groups (not shown).

In parallel, analysis of the relative proportions of SCFAs in caecal content of the same animals (collected at sacrifice), showed a higher % of acetate in the AG group compared to S-AG ($p=0.0156$) as well as higher butyrate in both A and AG groups compared to S-A ($p=0.0010$) and S-AG ($p=0.0225$) respectively. A decrease of propionate was also observed in both A ($p=0.0008$) and AG ($p=0.0004$) groups compared to their respective sibling groups. Finally, branched and long chain SCFAs were decreased in A compared to S-A, ($p=0.0066$) but increased in AG compared to S-AG ($p=0.253$) (Fig.3)

• Immunohistochemistry analysis of serotonergic neurons in the raphe nuclei and serotonin positive cells in the ileum

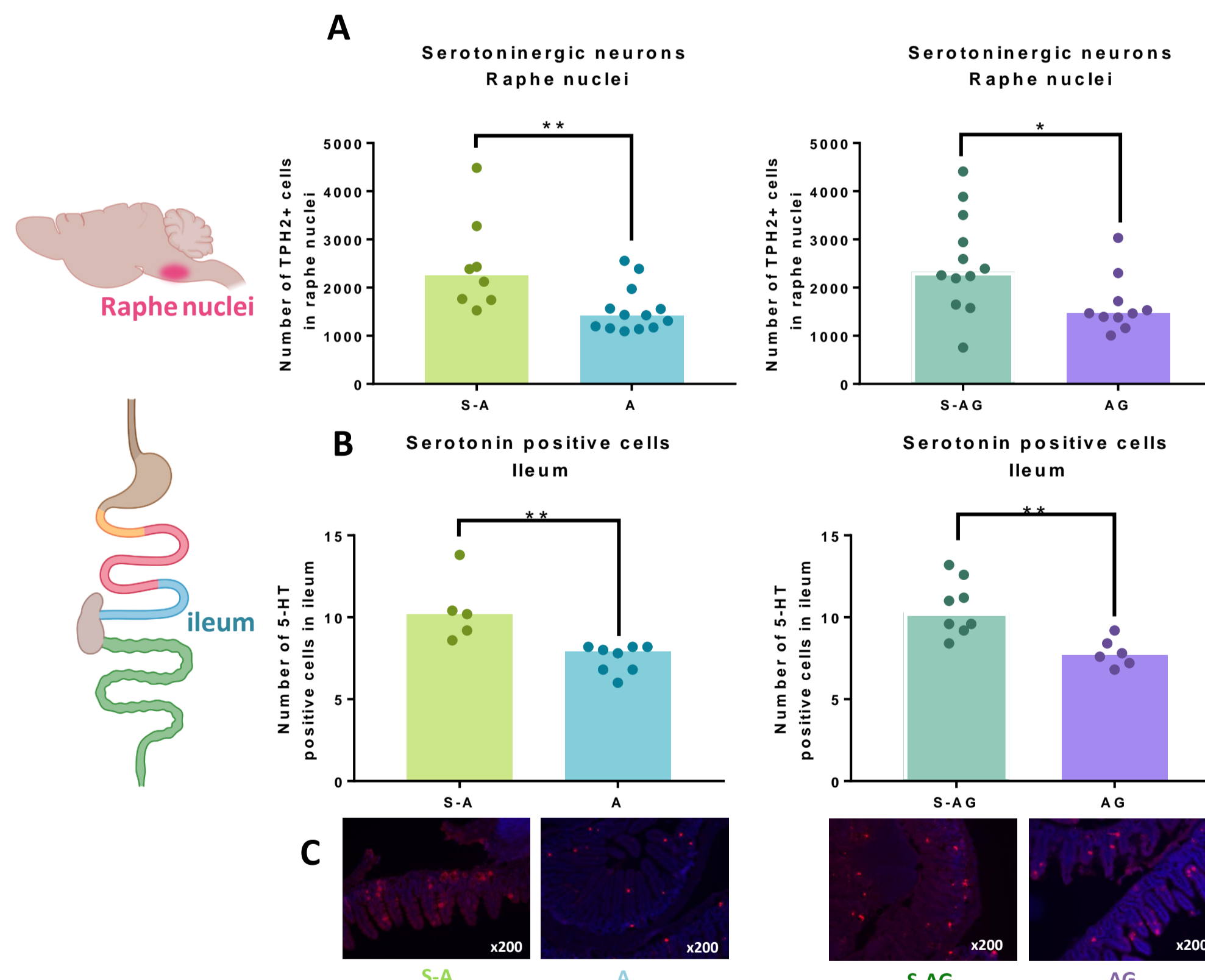
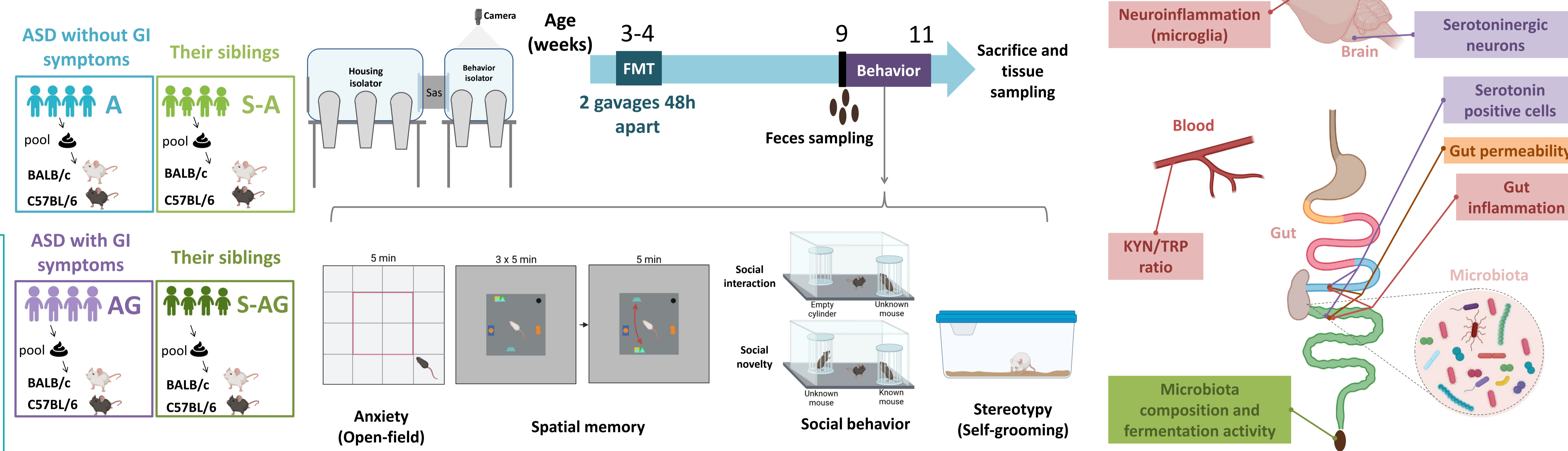


Fig 4: A) Total number of TPH2 positive neurons in the raphe nuclei of whole left hemisphere after immunostaining and transparization using the iDisco method. Fluorescence was measured on a light sheet microscope and analysed using the Imaris software (spot analysis algorithm) B) Total number of serotonin positive cells in sections of a swiss roll of ileum. Number of 5-HT positive cells were counted by a blinded experimenter in eight consecutive vili in 5 different sections for each animal using BZ-II analyser C) Representative pictures of an analysed zone of ileum.

Results: A decrease of serotonergic neurons in the raphe nuclei and of 5-HT positive cells in the ileum was observed in both A and AG groups compared to their respective sibling group. (Ileum : S-A vs A $p=0.0016$; S-AG vs AG $p=0.0033$; Raphe nuclei S-A vs A $p=0.0077$; S-AG vs AG $p=0.0206$)

Study design



First results on C57BL/6 mice:

5 objects spatial recognition test

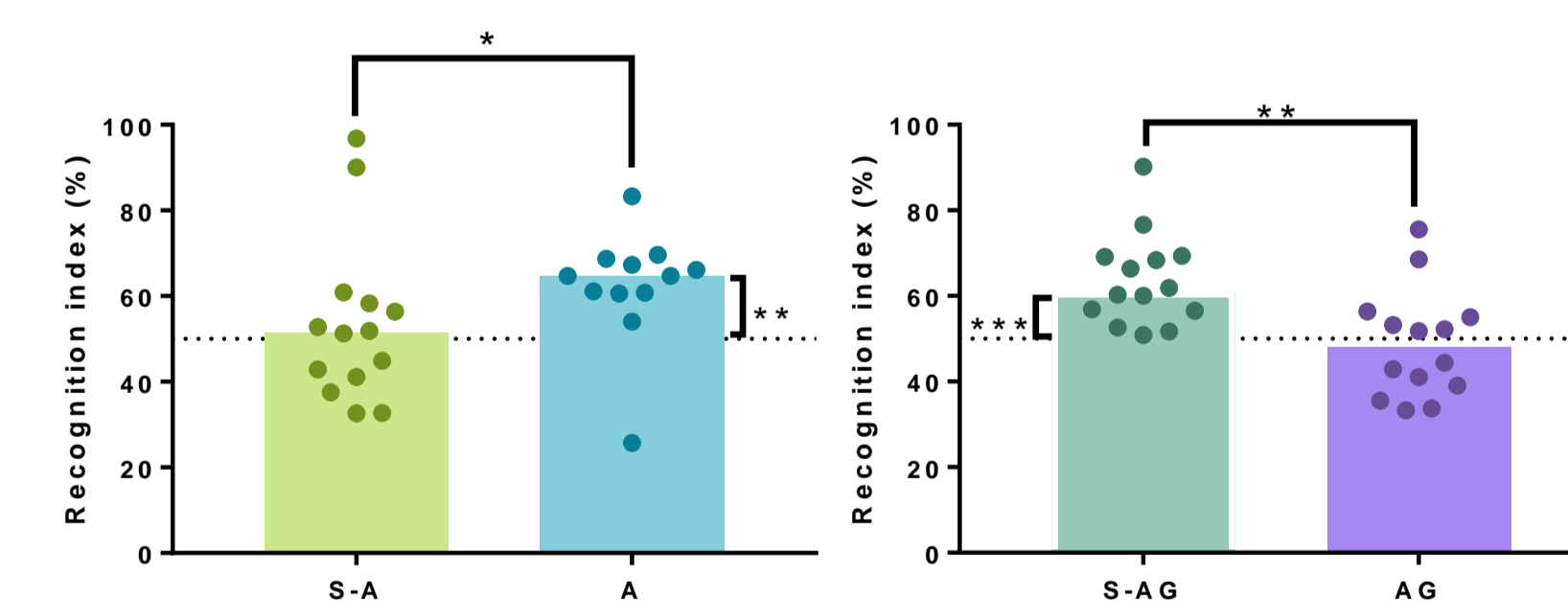
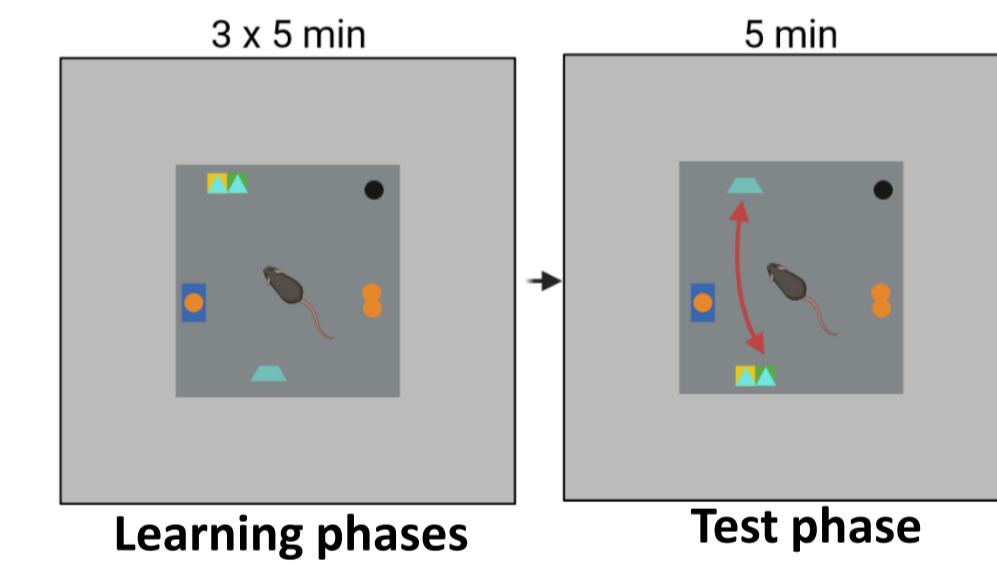


Fig 5: Recognition index in the test phase of the object recognition test. Tests were filmed and analysed using ANY-maze (Stoelting.co)

Results: Recognition index (RI) in mice from the AG group is significantly lower than the one of the S-AG group ($p=0.0030$), indicating a decreased short term spatial memory in this group. In the A group however, RI was significantly higher than the one of the S-A group ($p=0.261$). In addition, only the A and S-AG groups showed a RI significantly different from the theoretical value of 50%, indicating a preference for the displaced objects (A: $p=0.009$; S-AG $p=0.0004$). These results indicate that the microbiota from children with ASD and GI symptoms negatively impacts spatial memory when implanted in originally GF C57BL/6 mice whereas microbiota from children with ASD and no GI symptoms seems to positively impact it.

Conclusion

The microbiota that was transferred to the BALB/c mice has a distinct diversity, composition and fermentation activity in our four groups, which is consistent with literature for ASD patients. For C57BL/6 mice, the analysis is still ongoing.

The effect of the FMT on behavior was limited. In BALB/c mice we did not observe any difference between our four groups. In C57BL/6 mice, we saw an effect of the microbiota on short term spatial memory, however, it is hard to interpret this result as the effects are opposite between A and AG groups. It may be noted that two previous papers (Sharon et al. *Cell*, 2019; and Xiao et al. *ASM*, 2021) with similar study designs both found an impact of microbiota from patients with ASD on anxiety, social and repetitive behaviors in GF mice.

However, the different microbiota did have an impact of biochemical factors, as the number of serotonergic or serotonin positive neurons is decreased in the brain and the gut in both "ASD" groups in BALB/c mice. This is interesting as the serotonin pathway has been shown to be dysregulated, both in ASD patients and various animal models of ASD (Muller et al. *Neuroscience*, 2016). Those analyses are still ongoing for C57BL/6 mice, and further analyses on gut inflammation and permeability are still ongoing for both strains, which will hopefully bring more information on the effects of the FMT at a biochemical level.

Overall, our results show that the impact of the gut microbiota on behavioral manifestations of ASD is still unclear, and more research is needed to further this hypothesis.

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