Candida rugosa lipase administration alters Alzheimer's disease pathology

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Introduction

The gut microbiome consists of distinct symbionts including bacteria, yeast, virus, archaea and fungi that have pivotal functions in and beyond the gut. Diet, stress, chemical exposure and age can significantly alter the microbiome's composition, impacting metabolite secretion and the organism's resiliency. Several inflammatory disease such as diabetes, inflammatory bowel disease and cancer have been shown to be directly affected by the gut microbial composition[1,2]. Although the precise mechanism of gut to brain bidirectional pathway(s) remain unknown, four pathways have been proposed as communication routes from the gut to the brain and vice versa: endocrine, metabolic, neuronal and immunological. All pathways allow transmission of microbial induced cell signaling or microbial-derived metabolites to the brain[3]. Since microbial-derived metabolites have been shown to signal directly or indirectly to the brain, neurodegenerative disease such as Alzheimer's disease (AD) might benefit from their release by alleviating neuroinflammation and consequently neurodegeneration[4].

To test this hypothesis, we investigated the impact of Candida rugosa lipase (CRL), a commercially available enzyme supplement, on the gut microbial composition and AD pathology in a transgenic mouse model of AD (APPPS1: Swedish mutation K670N/M671L on the amyloid precursor protein (APP) and deletion of exon 9 on the presenilin 1 gene (PSEN1dE9)) as compared to its wildtype (C57BL6/J) littermates. Four groups of mice were used in this study: (1) APP/PS1 (n=9) and (2) C57BLg/J mice (n=9) receiving 5000 FiP/kg CRL via drinking water for two months; (3) APP/PS1 (n=9) and (4) WT mice (n=9) receiving regular drinking water. To determine changes in the gut microbial environment, we analyzed the gut microbial composition by 16S rRNA gene sequencing and the cecal metabolome by gas-chromatography mass-spectrometry (GCMS). In addition, brain pathology was analyzed by immuno-histochemistry staining of GFAP, a marker for activated astrocytes determining severity of astrocytosis in the brain. Finally, we performed behavioral testing in the Barnes maze to determine spatial memory performance.

Our investigation will contribute to the understanding of the gut-brain axis and the link between the gut microbiome and the brain in AD. The additional comparison with wildtype mice will allow the identification of treatment-driven effects independent of genotype, which contribute to alleviate disease pathology in mice with AD-like pathology, but could also translate into humans.

Hypothesis

Administration of Candida rugosa lipase, an enzyme supplement, in mice with AD-like pathology and control mice improves gut and brain health by modulation the gut microbial composition.

Methods

Animals

Transgenic male and female APPPS1 (n=9/group) were bred in-house and compared to C57BL6/J litter mates (n=9/group). All mice in this study were 8 month of age, co-housed, maintained in a controlled environment (regulated 12 h day/12 h night cycle) and received a standard diet. Dependent on the treatment group, water was supplemented with 5000 FiP/kg CRL or water for two month. Food and water were supplied ad libitum. All animal experiments were approved by the Roskamp Institute's Institutional Animal Care and Use Committee and were conducted in accordance with the Office of Laboratory Animal Welfare and the Association for the Assessment and Accreditation of Laboratory Animal Care. In accordance with the approved IACUC protocol, animals were humanely euthanized, brain, plasma and the intestinal tract were collected and stored at -80°C.

16S rRNA gene sequencing

Cecum (30 mg) samples were extracted using the Fast DNA stool mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer protocol. Volumes were adjusted to account for lower sample weight. Cecal DNA extracts were analysed by V3-V4 region 16S rRNA gene amplicon sequencing using the Illumina MiSeq sequencing platform (Prof. Klatt, University of Miami) following the Earth Microbiome Project protocols (http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/). The following modifications have been made: Platinum™ II Hot-Start PCR Master Mix (2X) with the 515FB-806RB primer pair was used for DNA amplification of the V4 variable region in the 16S rRNA gene resulting in approximately 400 bp amplicons. The reaction was performed in triplicates, quantified with Qubit dsDNA High Sensitivity Assay Kit (ThermoFisher Scientific, Waltham, MA) and pooled. The LabChip GX (PerkinElmer, MA) were used for visualization of PCR results. The library pools were quantified using the KAPA Library Quantification Kit (KAPA Biosystems, MA), combined (0.4 ng per sample) into a single sample and purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). 300-cycle Illumina MiSeq Kit was used to analyze 8 pM of pooled library with 30% PhiX Control v3 Library as control following the EMP protocol.

Sequqencing data analysis

The 16S rRNA gene sequencing data was analyzed with the QIIME 2 software packages. Paired end reads were demultiplexed, trimmed, corrected for chimera, merged and filtered with the demux and dada2 plugin. Alpha-diversity (Shannon index and Simpson index) and beta-diversity (Bray-Curtis dissimilarity) were determined and significance tested with Kruskal Wallis H test (alpha) and Permutational multivariate analysis of variance (PERMANOVA, beta). Sequence classification was performed with the Naïve Bayes classifier trained on Greengenes 13_8 and subsequently binned into amplicon sequence variant (ASVs) at 99% sequence similarity. The Greengenes 13_8 was then used than to classify ASVs and to determine relative abundance at various taxonomic levels allowing visualization and statistical analyses with analysis of composition of microbiomes (ANCOM). Plots of taxonomies were acquired in Qiime 2 and GraphPad Prism.

Metabolomics

Cecal metabolites were investigated through GCMS analysis. 50 mg of cecum were mixed with 50 uL internal standard mix (energy metabolites). Samples were crashed with 80% methanol and subsequently methoximated. Finally, samples were trimethylsilylated to allow metabolite transition in gas phase. Samples were measured on a Agilent 7890A GCMS instrument and separated on a Rxi-5ms fused silica column (30m x 0.25mm, 0.25 um, Restek). 100 nL sample were injected. Quality control samples, which were composed of 5 uL of each sample, were injected after each batch run. Samples were identified with the NIST database in AMDIS and deconvoluted with XCMS and MSDial. Data and statistical analysis was performed in MetaboAnalyst.

Immunohistochemistry

The collected brain hemisphere was fixed in 4% para-formaldehyde solution for 24h and subsequently dehydrated and embedded in paraffin. 9 um brain sections were deparaffinized followed by rehydration through histoclear, an ethanol gradient and hydrogen peroxide treatment to reduce background staining. Sections were washed in Phosphate Buffer Saline (PBS) and incubated in blocking serum before an overnight incubation at 4°C in a 1:8000 dilution of Glial Fibrillary Acidic Protein (GFAP) antibody (Polyclonal Rabbit Anti-GFAP, DAKO, Z0334) solution. Next, the sections were washed in PBS and placed in secondary antibody Vectastain Elite ABC reagent for 30 minutes and DAB incubation for 1-3 minutes. Afterwards tissue sections were counterstained with Hematoxylin, dehydrated through an ethanol gradient and histoclear and mounted in toluene. Once the slides were dry, images of 10x magnification of the hippocampus and cortex were taken using an Olympus BX63 Intelligent microscope and quantified by ImageJ.

Barnes Maze

The Barnes maze had a flat, circular platform with 18 equally spaced holes around the outer perimeter of the maze. The target hole had a box positioned directly beneath it that allowed the mice to exit the maze. Distinct visual cues were positioned on each of the four walls. Mice were trained for 4 days (4 min/trial/day) to use the cues to locate the target hole and escape the maze, which was achieved when the mouse entered the target box positioned under the target hole. On day 5, the target box was removed in order to test for learning and spatial memory in a 90-second probe trial. Each trial was tracked and recorded using EthoVision XT 14 software. Statistical analysis was performed with 3-way ANOVA testing for acquisition and one- or two-way ANOVA dependent on the respective parameter with post-hoc multiple comparisons.

Literature

(1) The Gut Microbiota in the Pathogenesis and Therapeutics of Inflammatory Bowel Disease, Zuo et al. 2018
(2) The influence of the gut microbiome on cancer, immunity, and cancer immunotherapy, Gopalakrishnan et al., 2019
(3) The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems, Carabotti et al., 2015
(4) Neuroinflammation and the Gut Microbiota: Possible Alternative Therapeutic Targets to Counteract Alzheimer's Disease?, Cerovic et al., 2019
(5) Ethanolamine utilization in bacterial pathogens: roles and regulation, Garsin, 2010
(6) Neuroprotective potential of aromatic alcohols against oxidative cell death, Moosmann et al., 1998

Gut microbiome

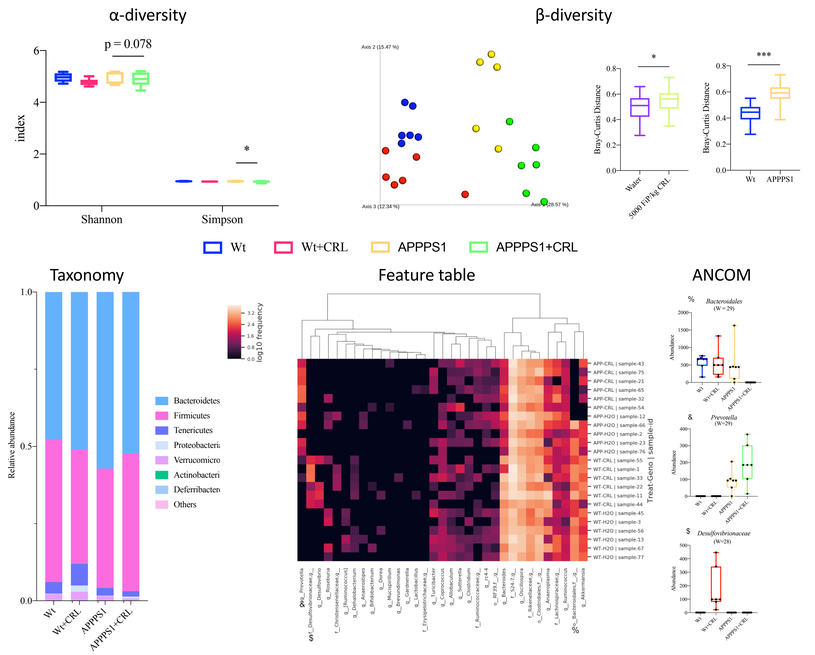
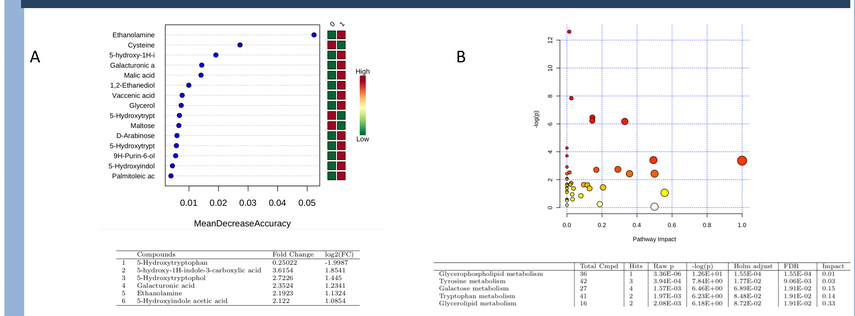


Figure 1: Cecal microbiome analysis. α-diversity. The shannon and simpson index were determined to investigate differences in α-diversity, which remained mostly unchanged. β-diversity. Bray-Curtis dissimilarity showed a significant separation between genotypes and treatment indicating compositional alterations due to treatment. The taxonomy showed *Proteobacteria* expanded due to CRL administration in both treatment groups. While *Verrucomicrobia* and *Tenericutes* only expanded in wildtype mice. ANCOM. The differential abundance analysis identified three bacterial genera. Significance: *p<0.05, **p<0.01, ***p<0.001.

Gut metabolomics



Compounds	Fold Change	log2(FC)
1 5-Hydroxytryptophan	0.25022	-1.9987
2 5-hydroxy-1H-indole-3-carboxylic acid	3.6154	1.8541
3 5-Hydroxytryptophol	2.7226	1.445
4 Galacturonic acid	2.3524	1.2341
5 Ethanolamine	2.1923	1.1324
6 5-Hydroxyindole acetic acid	2.122	1.0854

	Total Cmpd	Hits	Raw p	-log(p)	Holm adjust	FDR	Impact
Glycerophospholipid metabolism	36	1	3.36E-06	1.26E+01	1.55E-04	1.55E-04	0.01
Tyrosine metabolism	42	3	3.94E-04	7.84E+00	1.77E-02	9.06E-03	0.03
Galactose metabolism	27	4	1.57E-03	6.46E+00	6.89E-02	1.91E-02	0.15
Tryptophan metabolism	41	2	1.97E-03	6.23E+00	8.48E-02	1.91E-02	0.14
Glycerolipid metabolism	16	2	2.08E-03	6.18E+00	8.72E-02	1.91E-02	0.33

Figure 2: Cecal metabolome in CRL treated mice compared to control. A. Random forest analysis of combined treatment and control groups, highlighting three metabolite groups: ethanolamine, galacturonic acid and hydroxy indoles. B. Pathway analysis of identified metabolites identified five significant all connected by the six

Astrocytosis

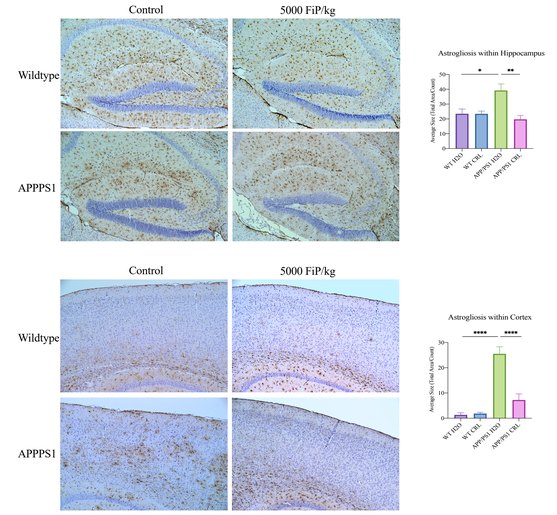


Figure 3: Brain sections were stained for astrocytosis marker GFAP. Astrogliosis is significantly reduced in APPPS1 mice after 2 month of CRL treatment in both cortex and hippocampus compared to non. Both treatment and genotype effect were detected. Significance: *p<0.05,**p<0.01, ***p<0.001, ****p<0.0001.

Learning and spatial memory

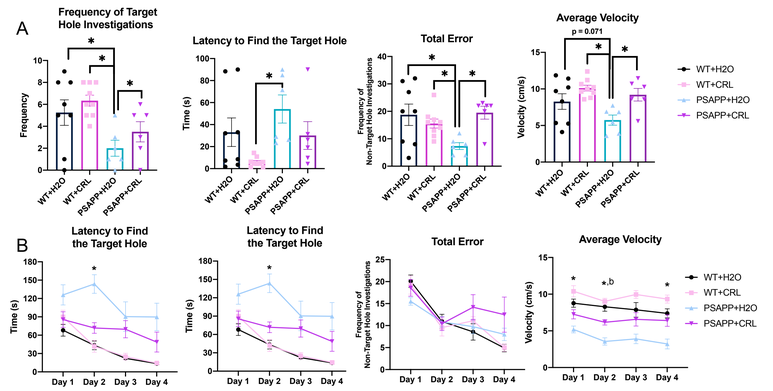


Figure 4: Barnes Maze performance of CRL treated mice. A. The CRL treatment in APPPS1 was significantly improved after CRL treatment, enhancing parameters comparable to wildtype mice. CRL treatment had a trend to improve Wt memory treatment-dependent. B. APPPS1 animals move generally slower independent of treatment, while only Wt significantly improved over time in the Barnes maze. Significance probe: *p<0.05. Significance acquisition: *p<0.05 between WT and PSAPP receiving water, [b]p<0.05 between WT and APPPS1 receiving CRL.

Conclusions

Our results demonstrated a significant difference in β-diversity when comparing treatment and genotype effects, which suggests, as expected, a stronger genotype than treatment dependent effect on the gut microbial composition, however, both cause significant dissimilarities in the microbial composition. To determine the functional effect of these differences, we performed a metabolomics analysis in the cecal environment. Our focus on the treatment dependent metabolites resulted in identification of ethanolamine, which can promote growth of pathogenic bacteria[5]. Although this might be in correlation with the treatment-dependent increase of *Proteobacteria* and detected genera, it needs to be further investigated to determine the pathway and effect of this metabolite in the host environment. The identified hydroxy indole group, however, which comprises metabolites of the tryptophan metabolism, are directly synthesized by the gut microbiota, show neuroprotective effects in the brain against oxidative cell death and could be connected to the determined brain pathological effects[6]. We determined that astrocytosis was reduced in both cortex and hippocampus due to CRL treatment and similarly, that learning and spatial memory in the Barnes maze improved due to CRL treatment. Based on these results, we have shown that the microbiome is significantly impacted by CRL treatment not only a mouse model with AD-like pathology but also in wildtype mice. Although, we couldn't determine the bacterial species evoking these drastic changes in the brain pathology, we could determine microbial-derived metabolites, which could be causing these effects. This contributes to the understanding of the gut-brain axis, but this needs to be further investigated in future studies.

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