AWARD NUMBER: W81XWH-20-1-0940

TITLE: Potential Role of the Microbiome-Endocannabinoidome Connection in the Gut-Brain Axis After Traumatic Brain Injury and Its Association with Alzheimer's Disease

PRINCIPAL INVESTIGATOR: FABIANA PISCITELLI

CONTRACTING ORGANIZATION: Istituto di Chimica Biomolecolare CNR
Pozzuoli (Na) ITALY

REPORT DATE: October 2023

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2023	Annual	30Sep2022-29Sep2023

4. TITLE AND SUBTITLE

Potential Role of the Microbiome-Endocannabinoidome Connection in the Gut-Brain Axis After Traumatic Brain Injury and Its Association with Alzheimer's Disease

5a. CONTRACT NUMBER W81XWH-20-1-0940

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Fabiana Piscitelli

E-Mail: fpiscitelli@icb.cnr.it

5d. PROJECT NUMBER AZ190044

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

CONSIGLIO NAZIONALE DELLE RICERCHE -
CNR
ICB ISTITUTO DI CHIMICA BIOMOLECOLARE
Via C. Flegrei 34, 80078 Pozzuoli
(Na) ITALY

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Based on published preclinical, clinical, and epidemiological data, we hypotesised that the gut-brain axis and the connection with the endocannabinoidome may peripherally influence the physiopathology of TBI and the subsequent risk of latent neurodegenerative diseases. Therefore, the objective of this research is to investigate the effects of a mild TBI on the subsequent development of Alzheimer's disease-related neuropathology and cognitive impairments in an APP/PS1 mice, the role of inflammation, the potential perturbation of the gut microbiome and how the potential alteration in gut microbiota composition may determine the severity of these disorders by regulating the activity of endocannabinoid and related mediators using a multidisciplinary approach. To date, our data in control mice confirm previous studies showing that the mTBI induces a induces a characteristic dual behavioral phenotype (aggressive/depressive) in mice In addition, we demonstrate that mTBI causes significant impairments in the discriminative and spatial memory tasks.

15. SUBJECT TERMS Microbiome, TBI, Alzheimer, endocannabinoids, gut-brain					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRDC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified	Unclassified	42	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

	Page
1. Introduction	5
2. Keywords	6
3. Accomplishments	6
4. Impact	33
5. Changes/Problems	34
6. Products	36
7. Participants & Other Collaborating Organizations	38
8. Special Reporting Requirements	42
9. Appendices	42

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Background: Traumatic brain injury (TBI) is the leading cause of death under the age 45 in the Western World and is followed by secondary brain damage leading to long-term consequences, such as increased prevalence of dementia, and Alzheimer's disease (AD). Recent evidence suggested that both TBI and AD have an alteration in the brain-gut microbiota axis that may significantly contribute to their pathogenesis and could be the missed link to understand their association. Furthermore, accumulating evidences in literature have showed that the endocannabinoid (eCB) system with the accompanying "endocannabinoidome" (eCBome), have a key role in numerous physiological and pathological conditions, including neuroprotection. The endocannabinoidome is increasingly emerging as a system of lipid mediators of the health-disease continuum and its strong connection with the gut microbiome has been so far suggested only in the context of inflammatory metabolic and intestinal disorders and has never been investigated in other disorders. **Hypothesis**: Based on published preclinical, clinical, and epidemiological data, we propose a theoretical framework that highlights the potential mechanisms by which the gut-brain axis and the connection with the eCBome may peripherally influence the physiopathology of TBI and the subsequent risk of latent neurodegenerative diseases. **Specific aims**: Therefore, the objective of this research is to investigate the effects of a mild TBI on the subsequent development of AD-related neuropathology and cognitive impairments in an APP/PS1 mice, the role of inflammation, the potential perturbation of the gut microbiome and how the potential alteration in gut microbiota composition may determine the severity of these disorders by regulating the activity of endocannabinoid and related mediators. **Research strategy:** We plan to analyse the microbiome of amyloid precursor protein APP-SWE mice after mTBI and their healthy controls. Feces and intestinal tissues from these animals will be used to compare the taxonomic composition, genome, transcriptome, proteome and metabolome of the gut microbiome. The endocannabinoidome will be profiled in the gut and in other target tissues, with particular emphasis on brain. Microbiome analyses will be related to the biochemical characterization of the endocannabinoidome in key target tissues. To accomplish this aim we will take advantage to be part of the Joint International Research Unit (UMI) that is a bilateral research unit between the Italian National Research Council (CNR) and the Université Laval of Quebec that has among its proposed ambitious goals the development of research projects, and the innovation, education and knowledge transfer in the emerging field of the biomolecular study of the intestinal microbiome. **Innovation and impact:**

This represents an unique opportunity to carry out this pilot study that could open new perspectives for the development of novel microbiome-based interventions for neurodegenerative diseases and to prevent long-term consequences of TBI.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*
mTBI, behavior, cognition, Alzheimer, endocannabinoids, microbiome, gut-brain axis

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

SOW Task	Timeline for completion	Progress
Major Task 1 "Characterization of behavioral impairments mTBI-induced in mice and identification of novel biomarkers.**"** Subtask 1: Institutional and ACURO approvals. Italian Ministry of Health approval	**Y1Q1Q2**	Completed
Subtask 2: Inducing mTBI in wild type mice and behavioral characterization	Y1Q3	Completed
Subtask 3: Specific markers analysis, brain immunohistochemistry and molecular biology analyses	Y1Q4	Completed
Subtask 2: Specific markers analysis, brain immunohistochemistry and molecular biology analyses	**Y2Q4**	**Completed**
Major Task 2: to investigate if the GBM-eCBome is altered in a murine model of mTBI, using a novel integrative approach, the endocannabinoidomics	**Y2Q1Q2**	
Subtask 1: Gut microbiome and eCBome analyses	Y2Q1-Q4	**Completed**
Major Task 3: to characterize the mTBI- mediated behavioral and biochemical dysfunctions in a	**Y3Q1**	**Completed**

transgenic AD mouse model. <u>Subtask 1:</u> Inducing mTBI in APP mice and behavioral characterization (N=50) <u>Subtask 2</u>: Specific markers analysis, brain immunohistochemistry and molecular biology		
Major Task 4: to investigate if the GBM-eCBome is altered in in a transgenic AD mouse model post-TBI, using a novel integrative approach, the endocannabinoidomics <u>Subtask 1</u>: Gut microbiome and biomarkers analyses <u>Subtask 2</u>: Measure the levels and expression of eCBome mediators, metabolic enzymes and molecular targets by lipidomics and metabolomics approachesresults at end of study.	**Y3Q1-Q4**	**Completed**

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

During the third year we have performed ex vivo experiments as indicated in the subtask 2 (Y2). In mild traumatic brain jury (m-TBI) in APP and related controls sham mice we performed immunohistochemical analysis.

Morphological activation and phenotype characterization of microglia cells in traumatized mice and relative controls at day 60 post injury. In particular, the number of the total and activated microglia cells (Iba-1 positive) have been assessed at hippocampal and cortical levels by immunofluorescence. Moreover, the expression of specific markers, such as milk fat globule EGF factor 8 protein (MFG-E8), Receptor Expressed on Myeloid cells (TREM2) and CD33, a sialic acid receptor, have been also evaluated in microglia cells.

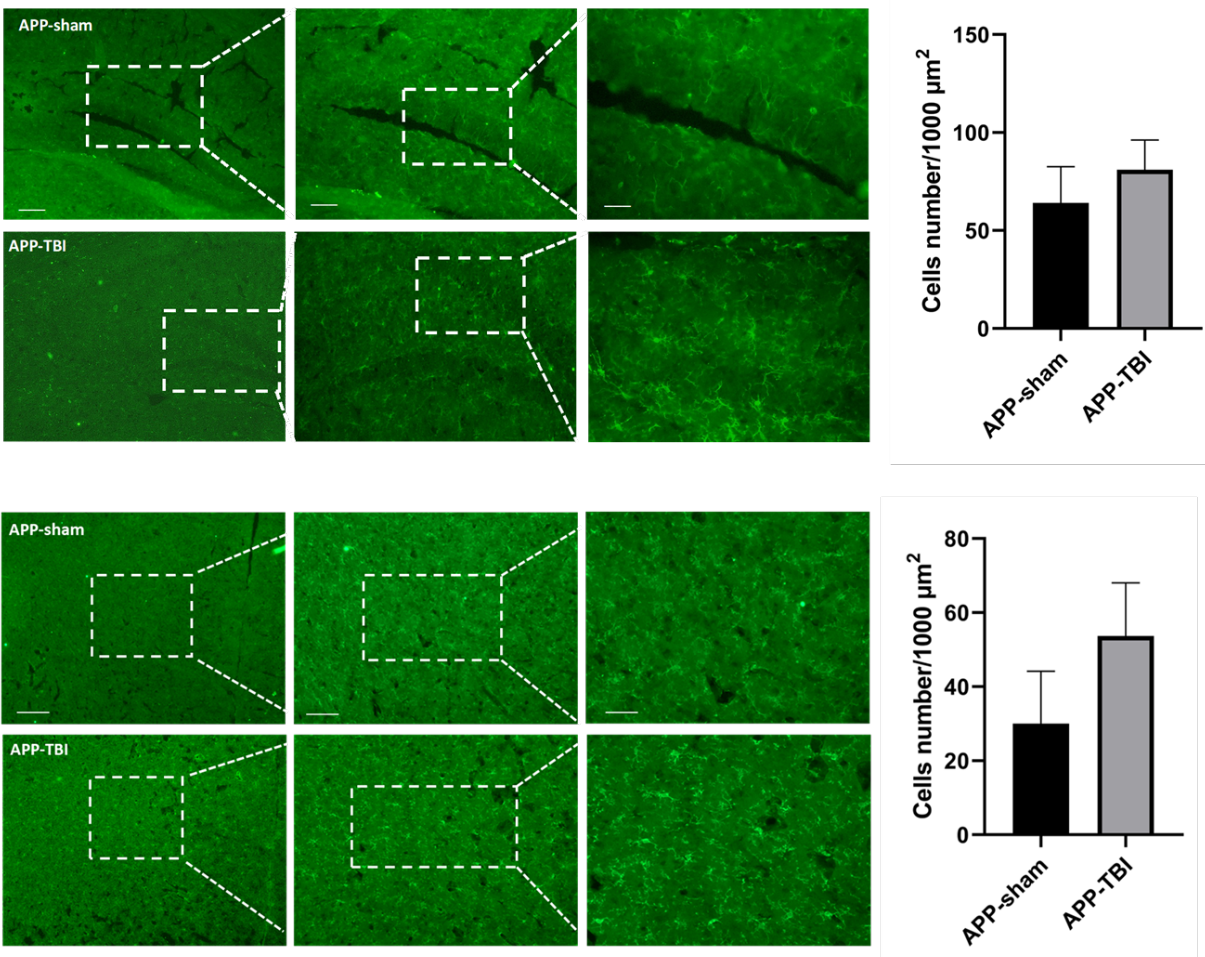


Figure 1. Representative images and quantitative analysis of Iba-1 positive profiles in the hippocampus (upper panel) and cortex (lower panel) of APP sham and mTBI mice 60 days after injury. Data are expressed as the number of the cells/area (n = 2 mice per group), scale bars 200, 100 and 50 µm in the low, medium and higher magnification, respectively.

In 60 days TBI mice, we did not detect any significant change in the number of total microglia, labeled Iba-1-positive cells in the hippocampus (dentate gyrus), as well as, in cortex.

Hippocampus: SHAM: Total cell number: 64 ± 10.69 and mTBI: Total cell number: 81 ± 6.197

Cortex: SHAM: Total cell number: 30 ± 7.083 and mTBI: Total cell number: 53.67 ± 8.293

No positive cells for MFG-E8, TREM2 and CD33, a sialic acid receptor, were detected.

We have analyzed the gut microbiota of WT and APP-SWE mice (sham and mTBI), nut no difference in the relative abundance of the microbiota composition was found.

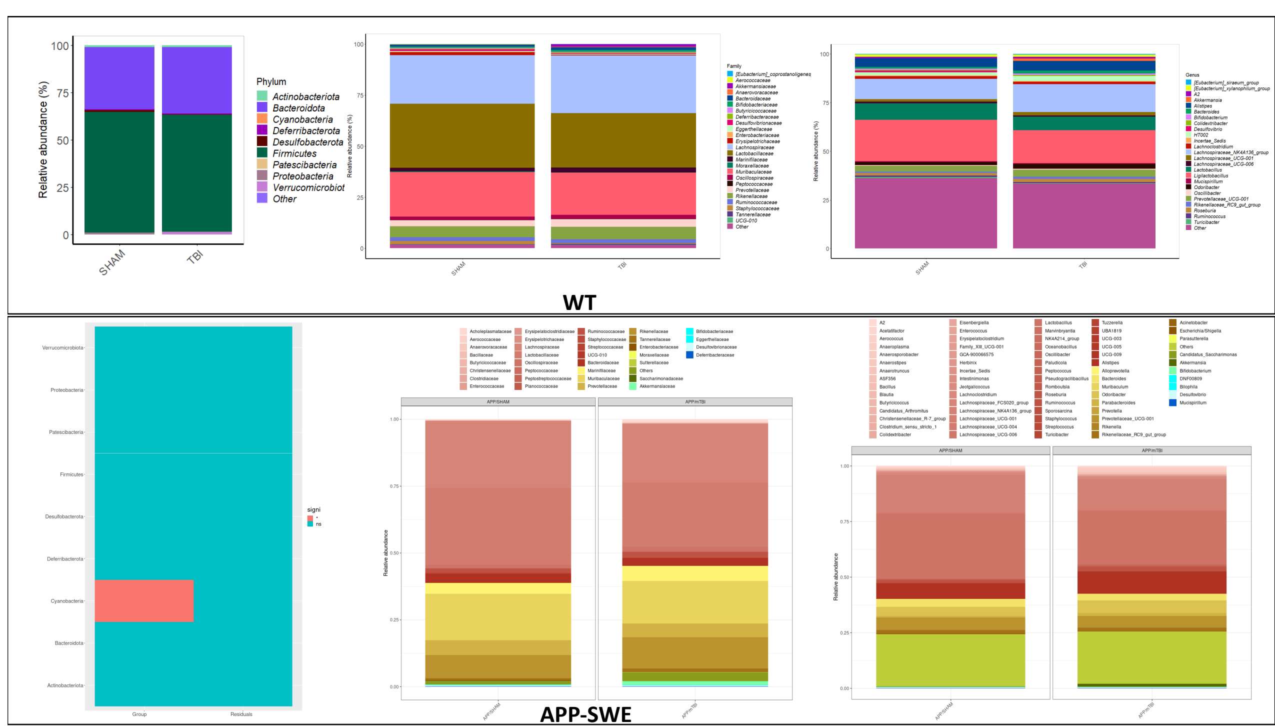


Figure 2: Relative abundance of the gut microbiota in WT and APP-SWE mice in terms of (from left to right) phylum, family and genus.

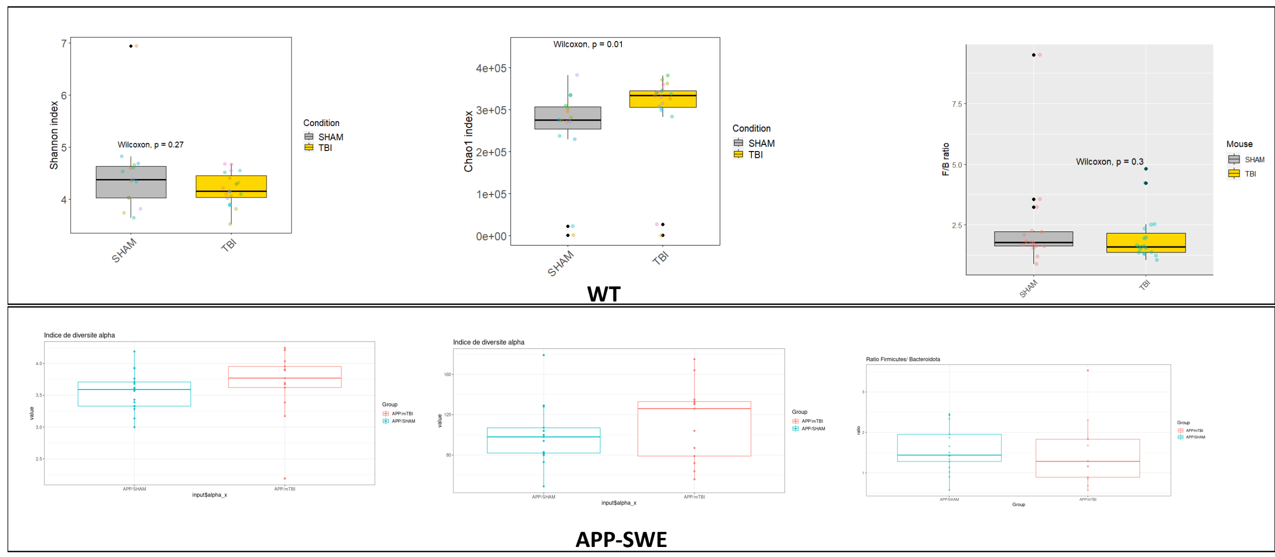
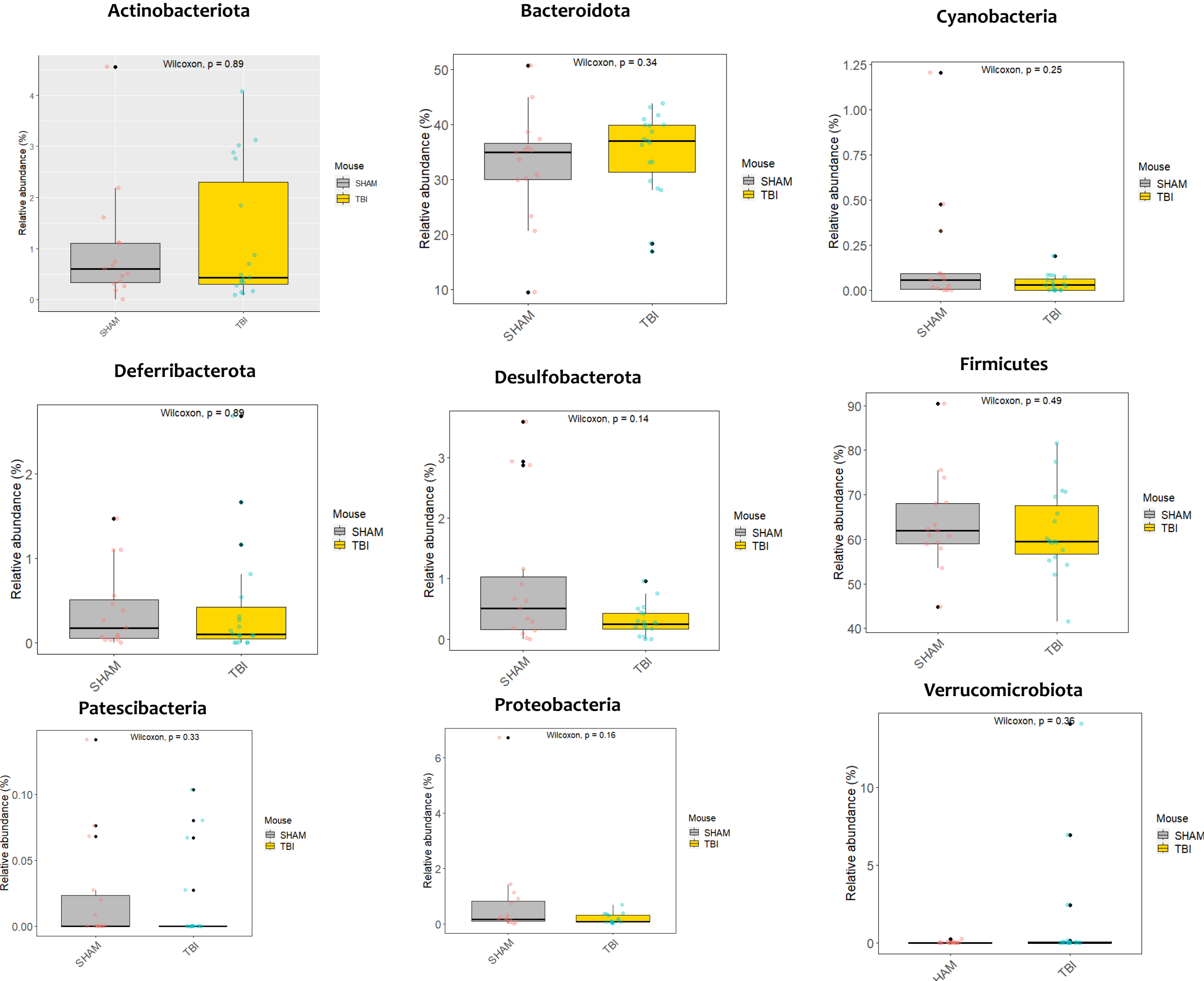
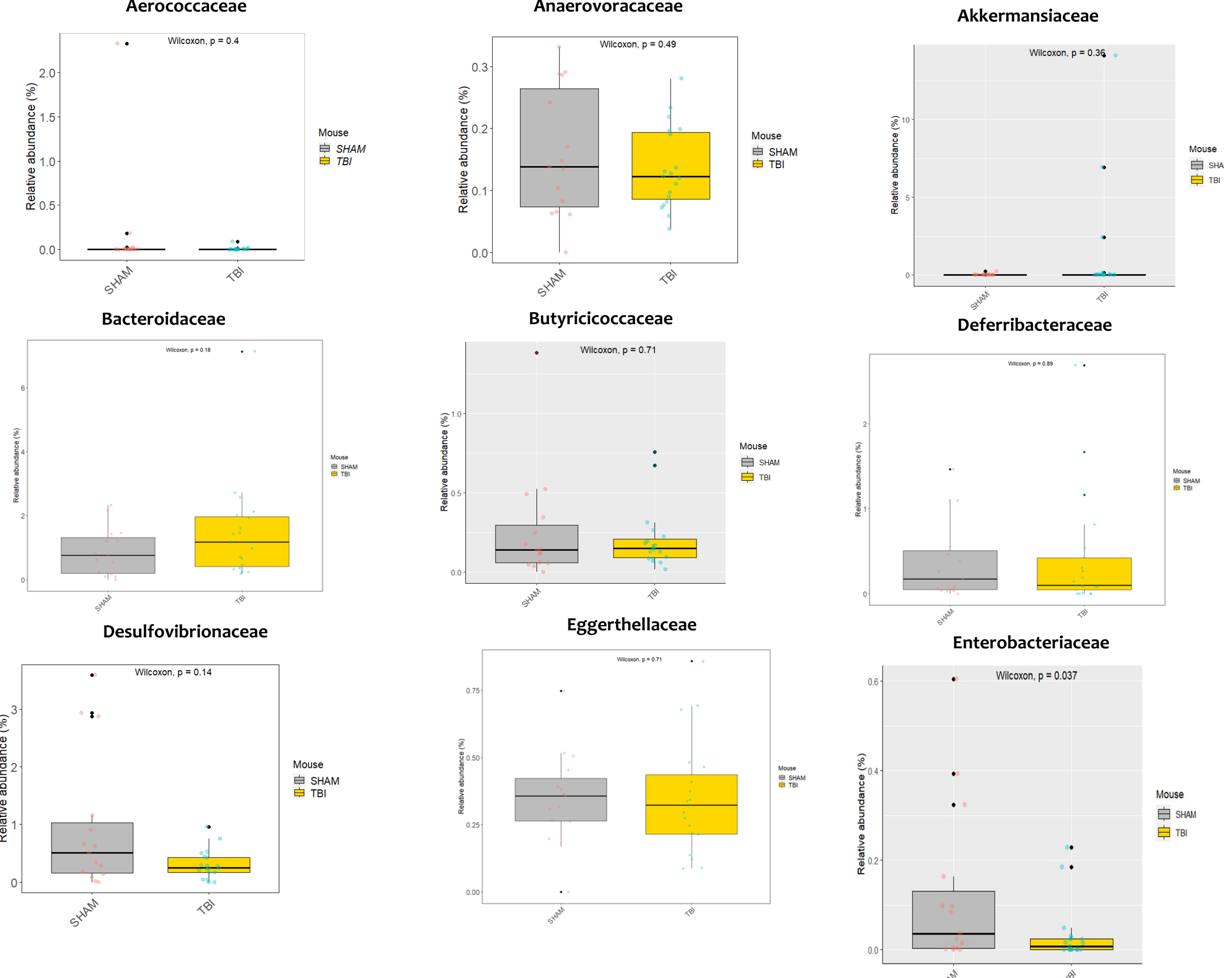


Figure 3: Shannon alpha diversity, Chao1 index and Firmicutes/Bacteroidetes (F/B) ratio in WT and APP-SWE mice (sham and mTBI).

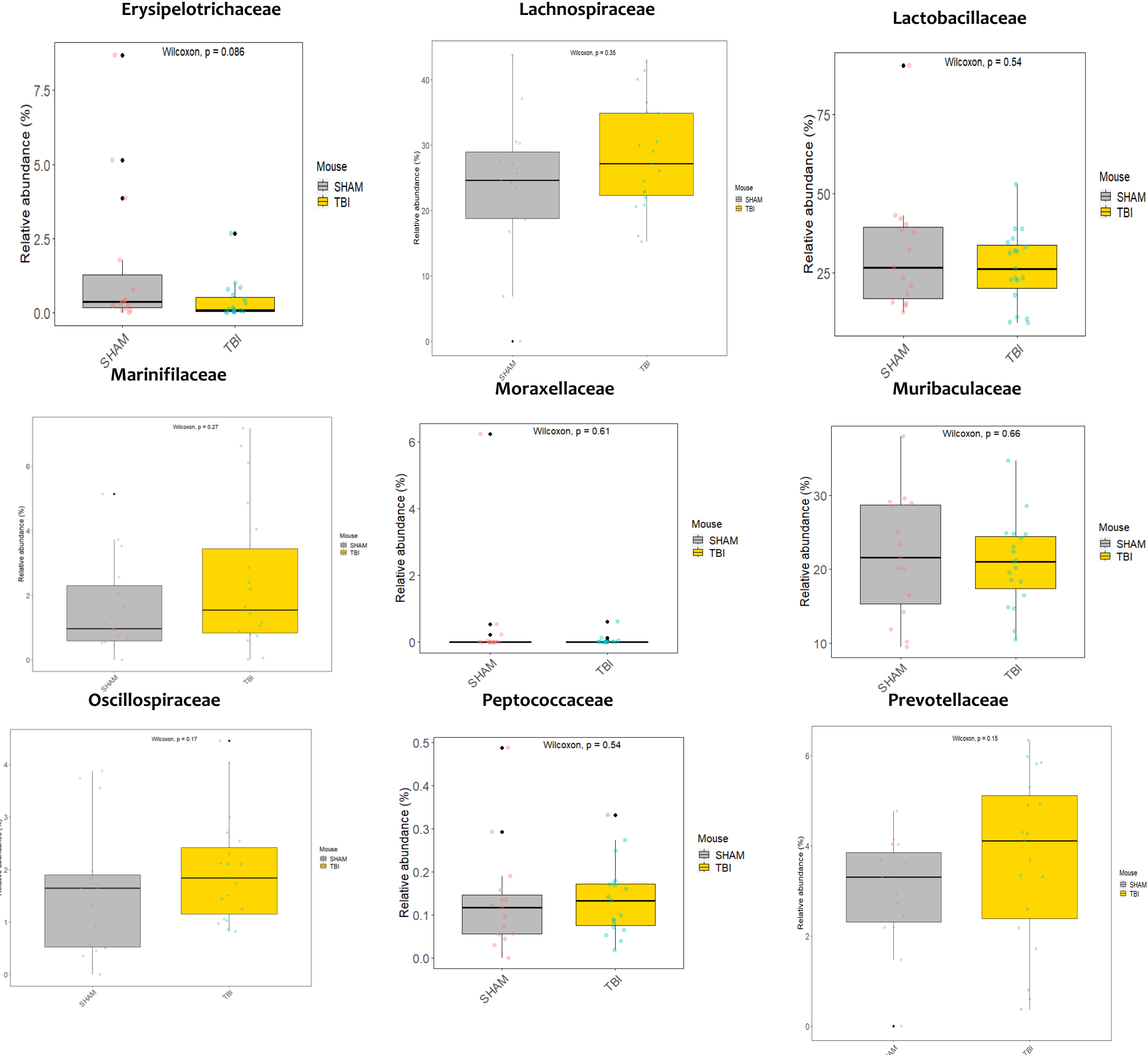
However, the statistical analysis of single species revealed some significant change.

In Figure 4 we have reported the statistical analysis on single species at the family and genus level in WT mice and the only two taxa that decrease significantly after TBI is the *Enterobacteriaceae* (p=0.037, Figure 8) and *Desulfovibrio* (p=0.0099, Figure 5).
Enterobacteriaceae are often found in the lungs of patients with severe Traumatic Brain Injury (sTBI) causing respiratory tract infections and a very recent study have shown that these gut bacteria translocated to the lungs after sTBI (Yang et al., Microorganisms. 2022). *Desulfovibrio* species are Gram negative species characterized by the ability to reduce sulphate to hydrogen sulfide in anaerobic respiration of organic matter (Gibson et al., J Appl Bacteriol. 1988). *Desulfovibrio* is frequently found in the human intestine but limited knowledge is available regarding the relationship between this taxa and host health (Chen et al., PeerJ. 2021). Very interestingly, a recent paper has reported the potential role of this taxa in the development of Parkinson's disease (Murros et al., Front. Cell. Infect. Microbiol. 2021). In particular, they have shown that all 20 PD patients harbored *Desulfovibrio* bacteria in their gut microbiota and these bacteria were present at higher levels in PD patients than in healthy controls. Additionally, the concentration of *Desulfovibrio* species correlated with the severity of PD. Moreover, the ability of *Desulfovibrio* to produce hydrogen sulfide (H_2S) is interesting because H_2S has been observed to release mitochondrial cytochrome c into the cytosol, where the cytochrome is able to form α-synuclein (α-Syn) radicals and thereby initiate α-Syn oligomerization (Guo et al., Mol. Med. Rep. 2015; Kumar et al., Mol. Neurodegener. 2016). Further, H_2S can interfere with iron metabolism by increasing iron levels in the cytosol (Cassanelli and Moulis, Biochim. Biophys. Acta 2001; Hälldin and Land, Biometals 2008), an event potentially inducing α-Syn aggregate formations (Joppe et al., Front. Neurosci. 2019). α-Syn is also involved in the pathophysiology of AD through a pathological feedback loop: Aβ could increase serine/threonine kinase glycogen synthase kinase 3-β (GSK3β) activity which in turn can induce tau phosphorylation and αSyn production (and heterotrimeric complexes), all of which (Aβ, tau, and αSyn) can seed the aggregation of one another, leading to potentially more Aβ production, GSK3β activation (via more Aβ and αSyn), tau phosphorylation, cellular dysfunction and apoptosis (Twohig and Nielsen, Molecular Neurodegeneration 2019).
In APP mice we found an increase in Cyanobacteria in TBI group. Cyanobacteria were formerly called blue-green algae and classified as eukaryotes, but they are now classified as prokaryotes and the presence of cyanobacterial DNA in the human microbiome may not be a result of short-term exposure to Cyanobacteria, but it may result from cumulative exposure to Cyanobacteria toxins since birth through the food chain (Cox et al., Proc. Natl. Acad. Sci. 2003*)*
This taxa produces different complex lipid secondary metabolites (Shah et al., Marine Drugs, 2017) and possess strong biological properties and application value, such as antibacterial, antifungal, anticancer, antituberculosis, immunosuppressive, and anti-inflammatory properties.
In AD, cyanobacteria have shown an increasing therapeutic potential and a feasible source of novel active drugs. Its neuroprotective effect may come from the production of neuroactive substances such as anatoxin-a(s), microcystins, and nodularin, which exert a competitive advance in grazing defense, by reducing palatability and avoid predation. Particularly regarding the cholinergic system, AChE inhibitors in cyanobacteria seems to be involved in inhibiting the colonization of colonies and filaments by other organisms, since AChE inhibitors were found to inhibit invertebrate larval settlement (Castaneda et al., Mar Drugs. 2021). Therefore, the increase in the gut microbiome of APP-mTBI mice might be an endogenous mechanism of neuroprotection.


Actinobacteriota
Wilcoxon, p = 0.89
Bacteroidota
Wilcoxon, p = 0.34
Cyanobacteria
Wilcoxon, p = 0.25
Deferribacterota
Wilcoxon, p = 0.89
Desulfobacterota
Wilcoxon, p = 0.14
Firmicutes
Wilcoxon, p = 0.49
Patescibacteria
Wilcoxon, p = 0.33
Proteobacteria
Wilcoxon, p = 0.16
Verrucomicrobiota
Wilcoxon, p = 0.36
Relative abundance (%)
Mouse
SHAM
TBI



Aerococcaceae
Wilcoxon, p = 0.4
Anaerovoracaceae
Wilcoxon, p = 0.49
Akkermansiaceae
Wilcoxon, p = 0.36
Bacteroidaceae
Wilcoxon, p = 0.18
Butyricicoccaceae
Wilcoxon, p = 0.71
Deferribacteraceae
Wilcoxon, p = 0.89
Desulfovibrionaceae
Wilcoxon, p = 0.14
Eggerthellaceae
Wilcoxon, p = 0.71
Enterobacteriaceae
Wilcoxon, p = 0.037
Relative abundance (%)
Mouse
SHAM
TBI



Erysipelotrichaceae
Wilcoxon, p = 0.086
Relative abundance (%)
Mouse
SHAM
TBI
Lachnospiraceae
Lactobacillaceae
Wilcoxon, p = 0.54
Relative abundance (%)
Mouse
SHAM
TBI
Marinifilaceae
Moraxellaceae
Wilcoxon, p = 0.61
Relative abundance (%)
Mouse
SHAM
TBI
Muribaculaceae
Wilcoxon, p = 0.66
Relative abundance (%)
Mouse
SHAM
TBI
Oscillospiraceae
Peptococcaceae
Wilcoxon, p = 0.54
Relative abundance (%)
Mouse
SHAM
TBI
Prevotellaceae

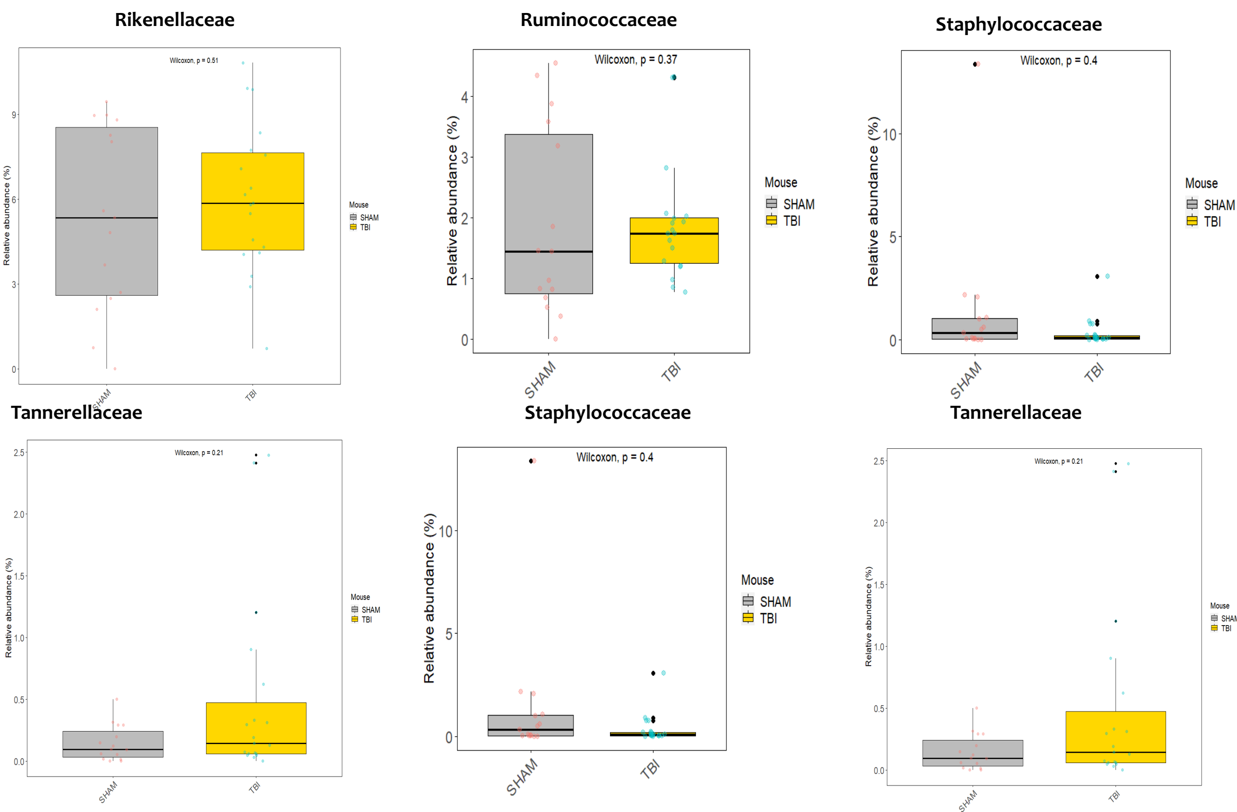
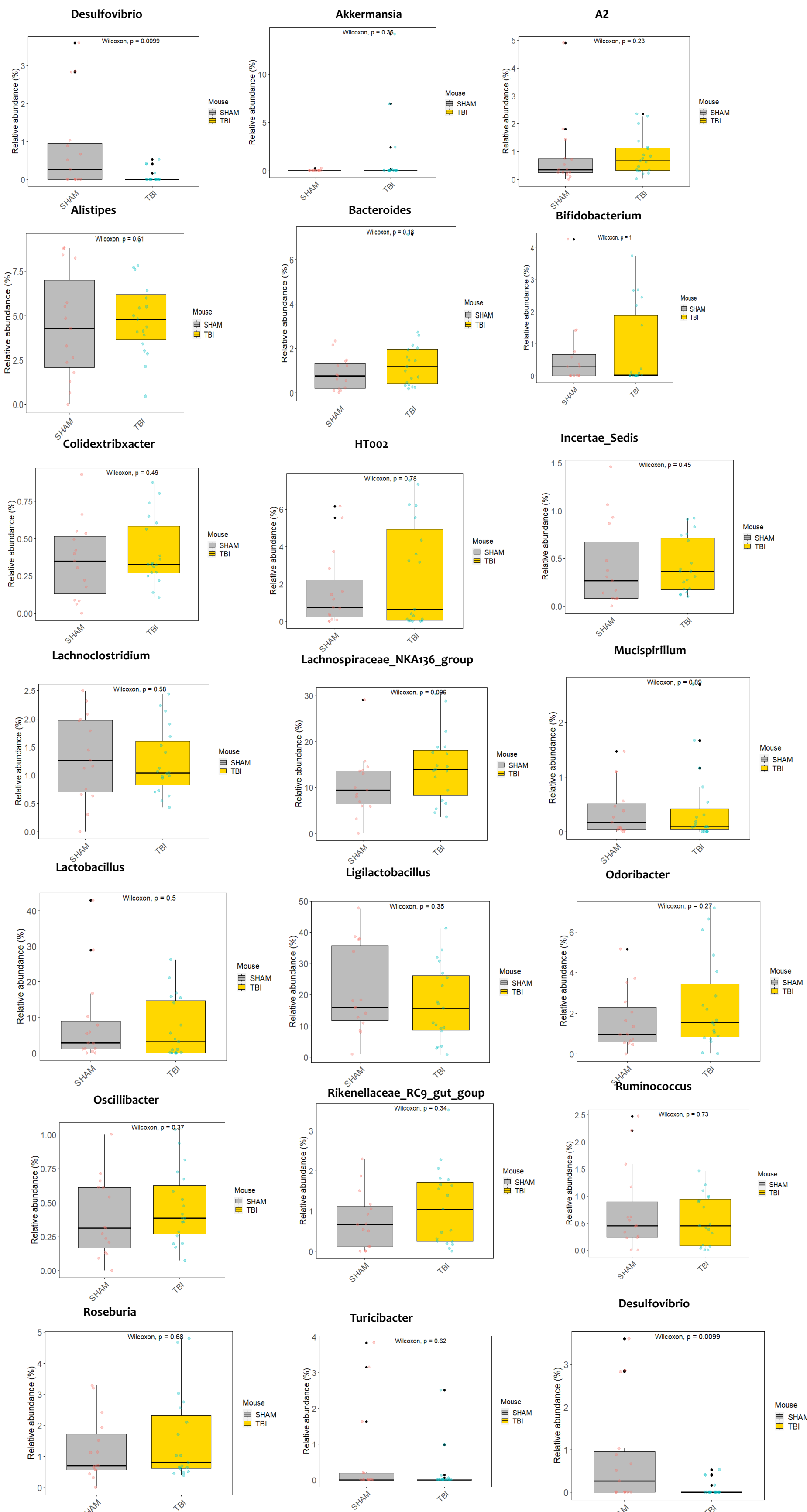


Figure 4. Statistical analysis on single species at the family level in WT mice (Sham and mTBI)



Desulfovibrio
Wilcoxon, p = 0.0099
Akkermansia
Wilcoxon, p = 0.38
A2
Wilcoxon, p = 0.23
Alistipes
Wilcoxon, p = 0.61
Bacteroides
Wilcoxon, p = 0.18
Bifidobacterium
Wilcoxon, p = 1
Colidextribxacter
Wilcoxon, p = 0.49
HT002
Wilcoxon, p = 0.78
Incertae_Sedis
Wilcoxon, p = 0.45
Lachnoclostridium
Wilcoxon, p = 0.58
Lachnospiraceae_NKA136_group
Wilcoxon, p = 0.096
Mucispirillum
Wilcoxon, p = 0.89
Lactobacillus
Wilcoxon, p = 0.5
Ligilactobacillus
Wilcoxon, p = 0.35
Odoribacter
Wilcoxon, p = 0.27
Oscillibacter
Wilcoxon, p = 0.37
Rikenellaceae_RC9_gut_goup
Wilcoxon, p = 0.34
Ruminococcus
Wilcoxon, p = 0.73
Roseburia
Wilcoxon, p = 0.68
Turicibacter
Wilcoxon, p = 0.62
Desulfovibrio
Wilcoxon, p = 0.0099
Relative abundance (%)
Mouse
SHAM
TBI

Figure 5. Statistical analysis on single species at the genus level in WT mice (Sham and mTBI)

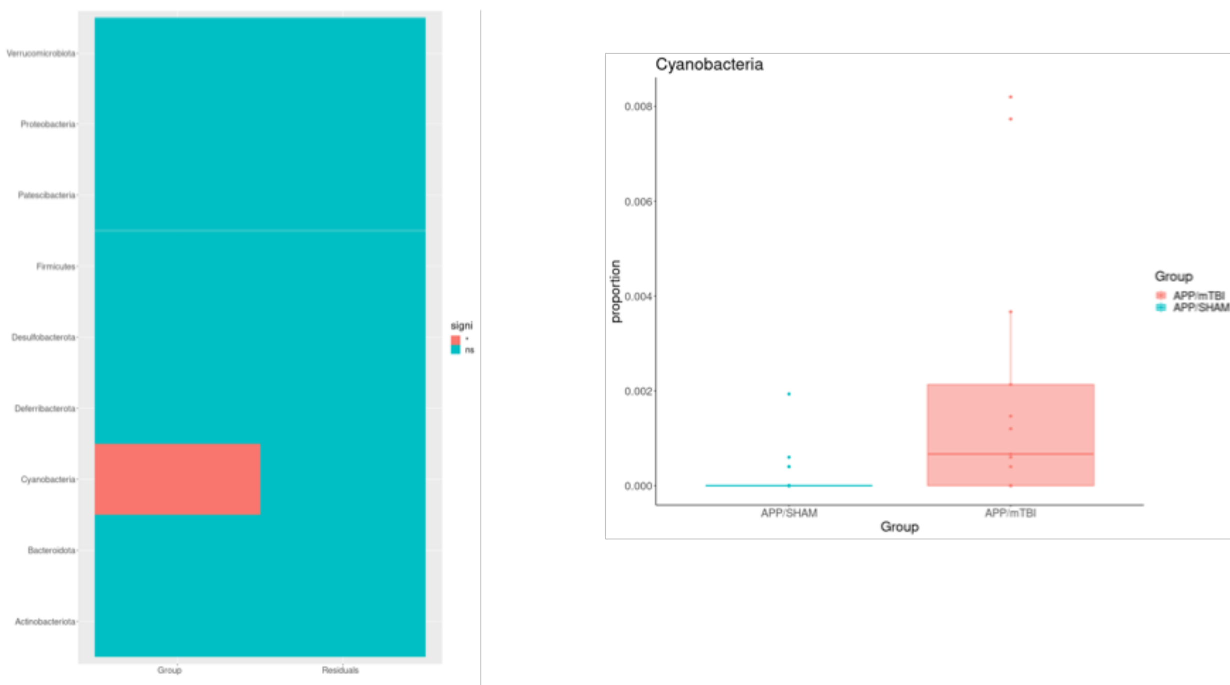


Figure 6. Individual taxa at the phylum level showing significant increase in APP-mTBI vs. APP-sham.

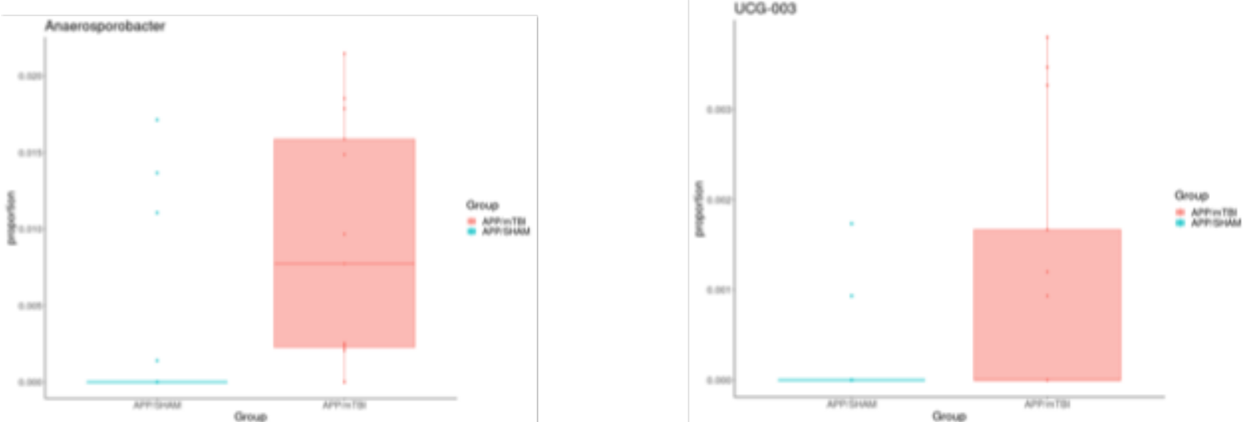


Figure 7. Individual taxa at the genus level showing significant increase in APP-mTBI vs. APP-sham.

To pinpoint and evaluate the fecal metabolic alterations specifically induced by traumatic brain injury in APP experimental mice, we performed OPLS-DA to fecal samples collected from the treated and untreated control group (**sham-WT**/**TBI-WT**) and the APP animals (**sham-APP**/**TBI-APP**). We obtained a statistical model with 3 predictive and 2 orthogonal components and parameters R2= 61% and Q2=40%. CV-ANOVA test *p* value = CV-ANOVA= 1.6 x10-6 (significant if p<0,05). The parallel or predictive component t[1] accounts for the main differences between the treated (**TBI-WT**) and all other classes, in particular the APP animals . On the other

hand, the second component t[2] is able to distinguish the **sham-WT** class from the not-separated treated or untreated APP profiles, which form an unique cluster placed at t[2] negative coordinates.

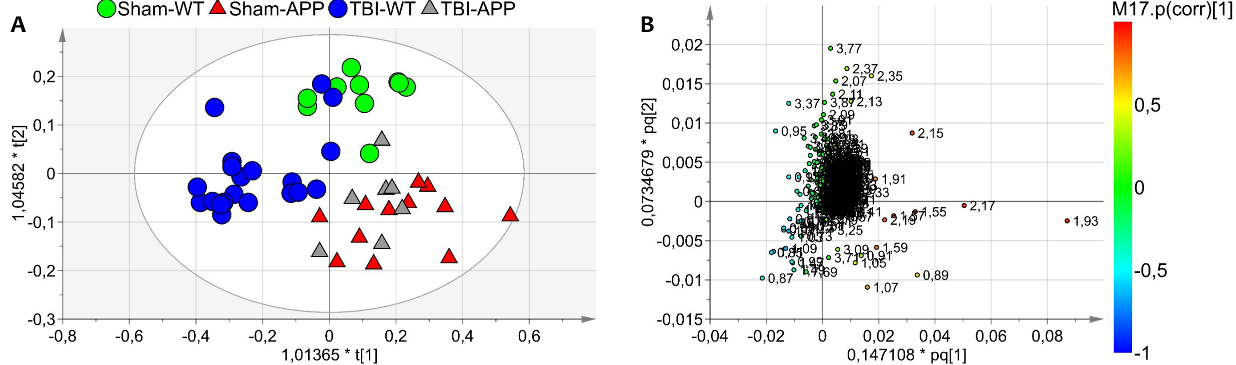


Figure 8. OPLS-DA of NMR metabolic profiles of mice feces. (A) Scores plot. (B) Loadings plot.

The third component t[3] of the model is useful to display a minor data variation, which is able to better characterize and separate **TBI-APP** (t[3] positive values) from **Sham-APP** profiles (t[3] negative values).

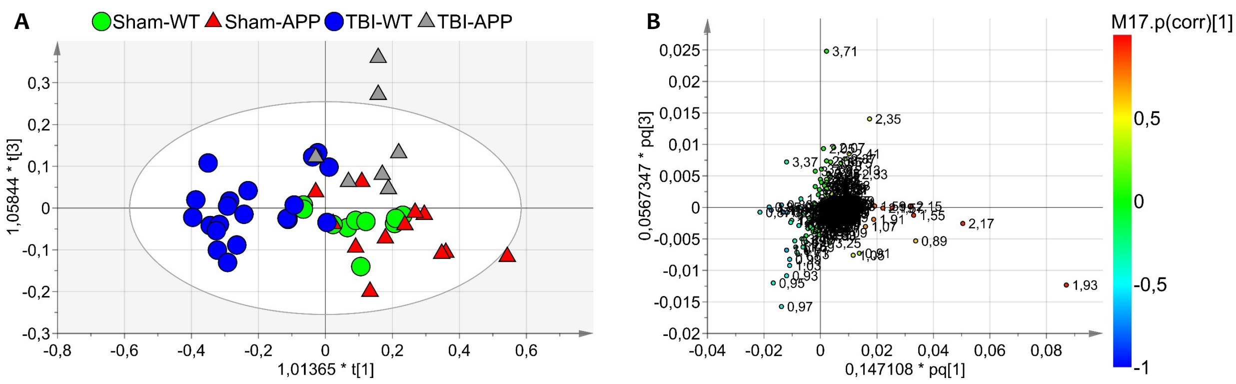


Figure 9. OPLS-DA of NMR metabolic profiles of mice feces. (A) Scores plot. (B) Loadings plot.

To identify and to highlight the subset of most responsible metabolites in TBI feces metabolic profiles characterizing the discrimination found for control/TBI classes in all experimental groups, NMR variables were selected using a combination of VIP (Variable Influence in Projection) value >1 and correlation loadings values |p(corr)|> 0.5 in the OPLS-DA model classification (Figure 13).

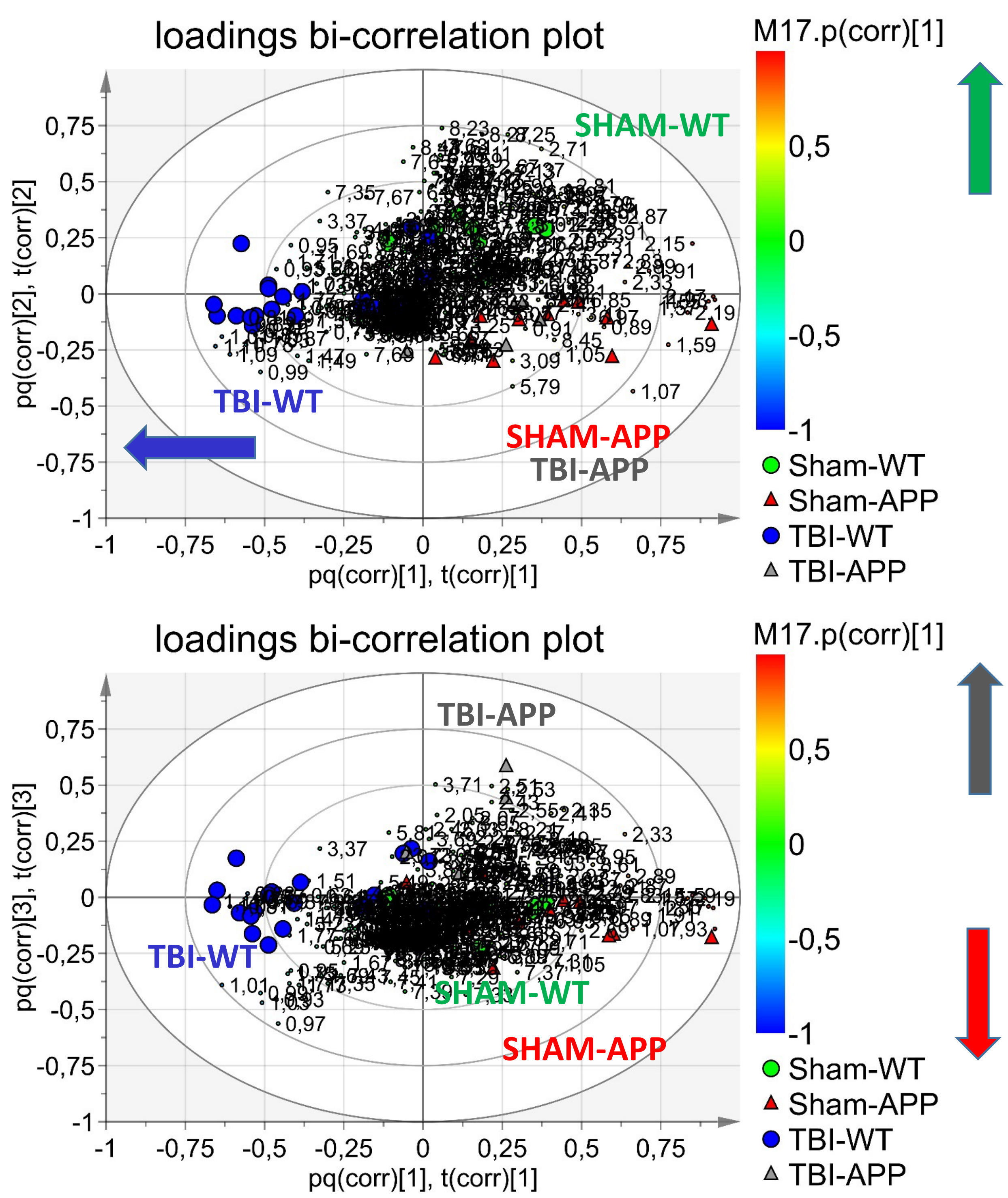


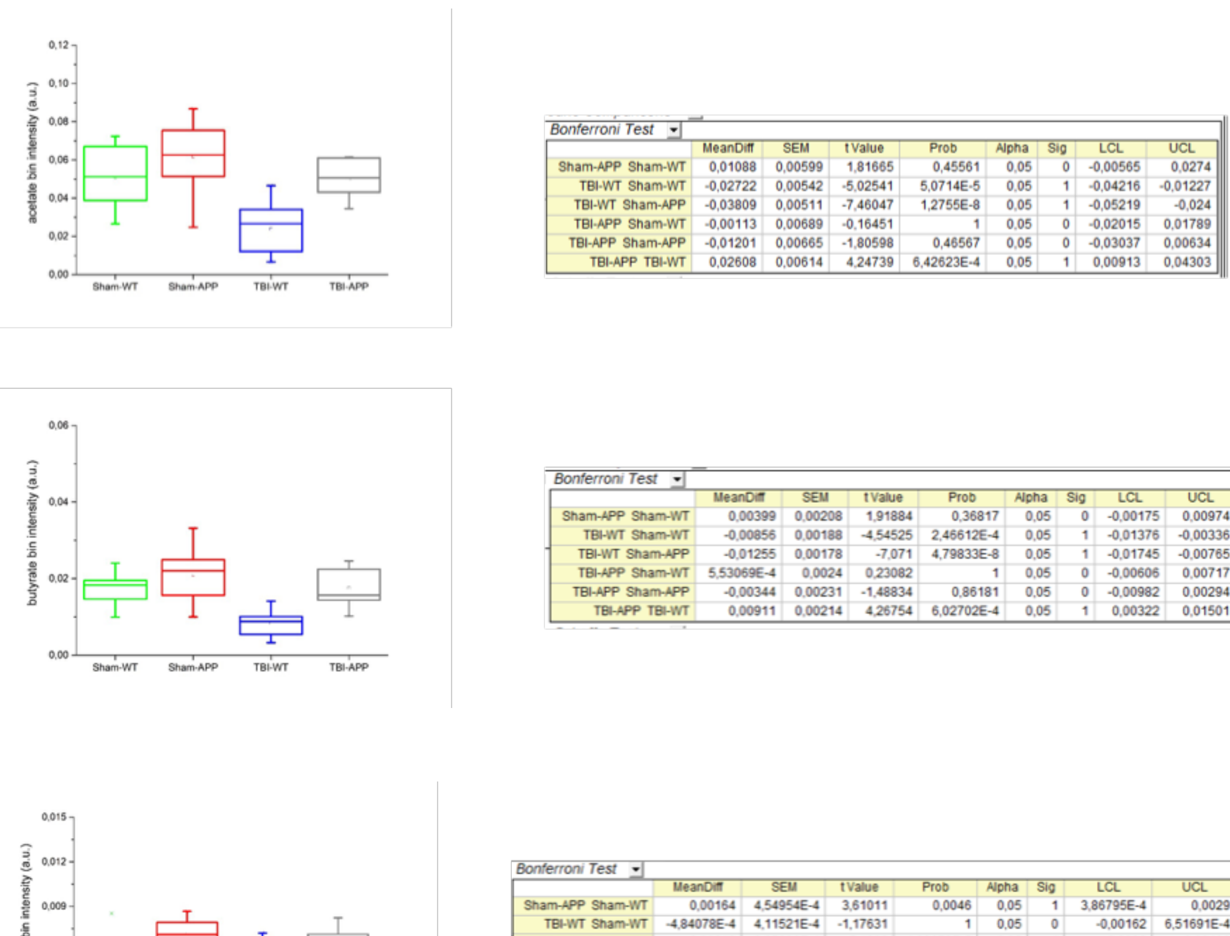
Figure 10. Loadings bi-correlation plot omp2 vs comp2 (top) and comp3 vs comp1 (down).

Up-regulated in TBI-WT ↑	down-regulated in TBI-WT ↓
valine	butyrate
isoleucine	nicotinate
	acetate
	aspartate
	propionate
	3-phenylpropionate (ns)

Up-regulated in sham-WT ↑	down-regulated in sham-WT ↓
thymidine	uracil
deoxyuridine	propionate
methionine	
glutamate	
o-cresol	
nicotinate	

Up-regulated in TBI-APP ↑	down-regulated in TBI-APP ↓
glutamate	leucine
succinate	valine
guanidine succinate	phenylalanine (ns)
glucose (ns)	isoleucine
α-Ketoglutarate (ns)	3-phenylpropionate (ns)

Figure 11. Summary of the main metabolites that are up or down-regulated in the different groups-ns=not significantly



Bonferroni Test

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
Sham-APP Sham-WT	0,01088	0,00599	1,81665	0,45561	0,05	0	-0,00565	0,0274
TBI-WT Sham-WT	-0,02722	0,00542	-5,02541	5,0714E-5	0,05	1	-0,04216	-0,01227
TBI-WT Sham-APP	-0,03809	0,00511	-7,46047	1,2755E-8	0,05	1	-0,05219	-0,024
TBI-APP Sham-WT	-0,00113	0,00689	-0,16451	1	0,05	0	-0,02015	0,01789
TBI-APP Sham-APP	-0,01201	0,00665	-1,80598	0,46567	0,05	0	-0,03037	0,00634
TBI-APP TBI-WT	0,02608	0,00614	4,24739	6,42623E-4	0,05	1	0,00913	0,04303

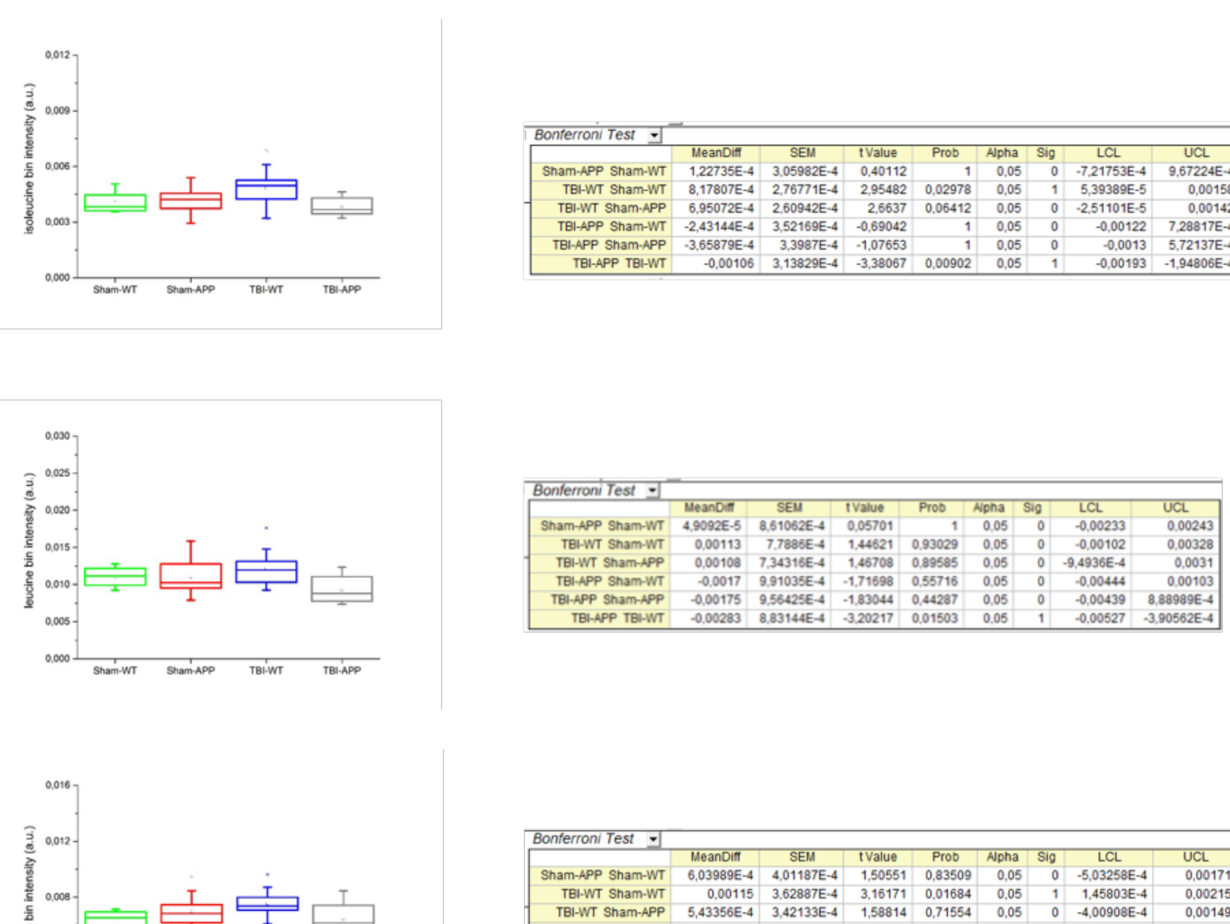
Bonferroni Test

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
Sham-APP Sham-WT	0,00399	0,00208	1,91884	0,36817	0,05	0	-0,00175	0,00974
TBI-WT Sham-WT	-0,00856	0,00188	-4,54525	2,46612E-4	0,05	1	-0,01376	-0,00336
TBI-WT Sham-APP	-0,01255	0,00178	-7,071	4,79833E-8	0,05	1	-0,01745	-0,00765
TBI-APP Sham-WT	5,53069E-4	0,0024	0,23082	1	0,05	0	-0,00606	0,00717
TBI-APP Sham-APP	-0,00344	0,00231	-1,48834	0,86181	0,05	0	-0,00982	0,00294
TBI-APP TBI-WT	0,00911	0,00214	4,26754	6,02702E-4	0,05	1	0,00322	0,01501

Bonferroni Test

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
Sham-APP Sham-WT	0,00164	4,54954E-4	3,61011	0,0046	0,05	1	3,86795E-4	0,0029
TBI-WT Sham-WT	-4,84078E-4	4,11521E-4	-1,17631	1	0,05	0	-0,00162	6,51691E-4
TBI-WT Sham-APP	-0,00213	3,87986E-4	-5,4809	1,09673E-5	0,05	1	-0,0032	-0,00106
TBI-APP Sham-WT	8,0077E-4	5,23627E-4	1,52927	0,79918	0,05	0	-6,44404E-4	0,00225
TBI-APP Sham-APP	-8,41666E-4	5,0534E-4	-1,66554	0,61651	0,05	0	-0,00224	5,53037E-4
TBI-APP TBI-WT	0,00128	4,66621E-4	2,75351	0,05084	0,05	0	-2,99379E-6	0,00257

Figure 12. Short-chain fatty acids (SCFAs) butyrate (up), acetate (middle) and propionate (down) analysis though NMR-based metabolomics



Bonferroni Test

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
Sham-APP Sham-WT	1,22735E-4	3,05982E-4	0,40112	1	0,05	0	-7,21753E-4	9,67224E-4
TBI-WT Sham-WT	8,17807E-4	2,76771E-4	2,95482	0,02978	0,05	1	5,39389E-5	0,00158
TBI-WT Sham-APP	6,95072E-4	2,60942E-4	2,6637	0,06412	0,05	0	-2,51101E-5	0,00142
TBI-APP Sham-WT	-2,43144E-4	3,52169E-4	-0,69042	1	0,05	0	-0,00122	7,28817E-4
TBI-APP Sham-APP	-3,65879E-4	3,3987E-4	-1,07653	1	0,05	0	-0,0013	5,72137E-4
TBI-APP TBI-WT	-0,00106	3,13829E-4	-3,38067	0,00902	0,05	1	-0,00193	-1,94806E-4

Bonferroni Test

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
Sham-APP Sham-WT	4,9092E-5	8,61062E-4	0,05701	1	0,05	0	-0,00233	0,00243
TBI-WT Sham-WT	0,00113	7,7886E-4	1,44621	0,93029	0,05	0	-0,00102	0,00328
TBI-WT Sham-APP	0,00108	7,34316E-4	1,46708	0,89585	0,05	0	-9,4936E-4	0,0031
TBI-APP Sham-WT	-0,0017	9,91035E-4	-1,71698	0,55716	0,05	0	-0,00444	0,00103
TBI-APP Sham-APP	-0,00175	9,56425E-4	-1,83044	0,44287	0,05	0	-0,00439	8,88989E-4
TBI-APP TBI-WT	-0,00283	8,83144E-4	-3,20217	0,01503	0,05	1	-0,00527	-3,90562E-4

Bonferroni Test

	MeanDiff	SEM	t Value	Prob	Alpha	Sig	LCL	UCL
Sham-APP Sham-WT	6,03989E-4	4,01187E-4	1,50551	0,83509	0,05	0	-5,03258E-4	0,00171
TBI-WT Sham-WT	0,00115	3,62887E-4	3,16171	0,01684	0,05	1	1,45803E-4	0,00215
TBI-WT Sham-APP	5,43356E-4	3,42133E-4	1,58814	0,71554	0,05	0	-4,00908E-4	0,00149
TBI-APP Sham-WT	4,44539E-5	4,61744E-4	0,09627	1	0,05	0	-0,00123	0,00132
TBI-APP Sham-APP	-5,59535E-4	4,45618E-4	-1,25564	1	0,05	0	-0,00179	6,70339E-4
TBI-APP TBI-WT	-0,0011	4,11475E-4	-2,68033	0,06144	0,05	0	-0,00224	3,27513E-5

Figure 13. Branched-chain amino acids (BCAAs) leucine (up), isoleucine (middle) and valine (down) analysis though NMR-based metabolomics

Very interestingly, in WT mice butyrate and acetate were reduced in TBI group accordingly to previous findings in which a different model of TBI led to a reduction of SCFAs, especially acetate, in stool samples (Opeyemi et al., J Neurotrauma 2021). On the other hand, in APP mice if we compare WT-TBI vs APP-TBI we observed an increase of these metabolites (Figure 14). However, even not significantly, these metabolites tend to decrease if we compare APP-sham vs APP-TBI, as well as in WT mice. Regarding BCAAs, while in WT-TBI tend to increase, in APP-TBI tend to decrease, as shown in Figure 16. Early studies of the cerebrospinal fluid amino acid composition have demonstrated a significant reduction (by about 35%) in the concentration of valine in AD patients compared to healthy controls (Basun et al., J Neural Transm Park Dis Dement Sect.1990). Two more recent studies in APP/PS1 mice showed that AD is a systemic disorder characterized by impaired glucose metabolism, mitochondrial dysfunction, and abnormal metabolism of BCAAs (González-Domínguez et al., Mol Biosyst. 2015) and serious disturbed polyamines and BCAA metabolism (Pan et al., Neurobiol Aging. 2016).

Moreover, the targeted lipidomics analysis of the eCBome in feces and in the intestine (ileum, cecum, jejunum and duodenum) of WT and APP mice reported significant alterations (Figures 16-20).

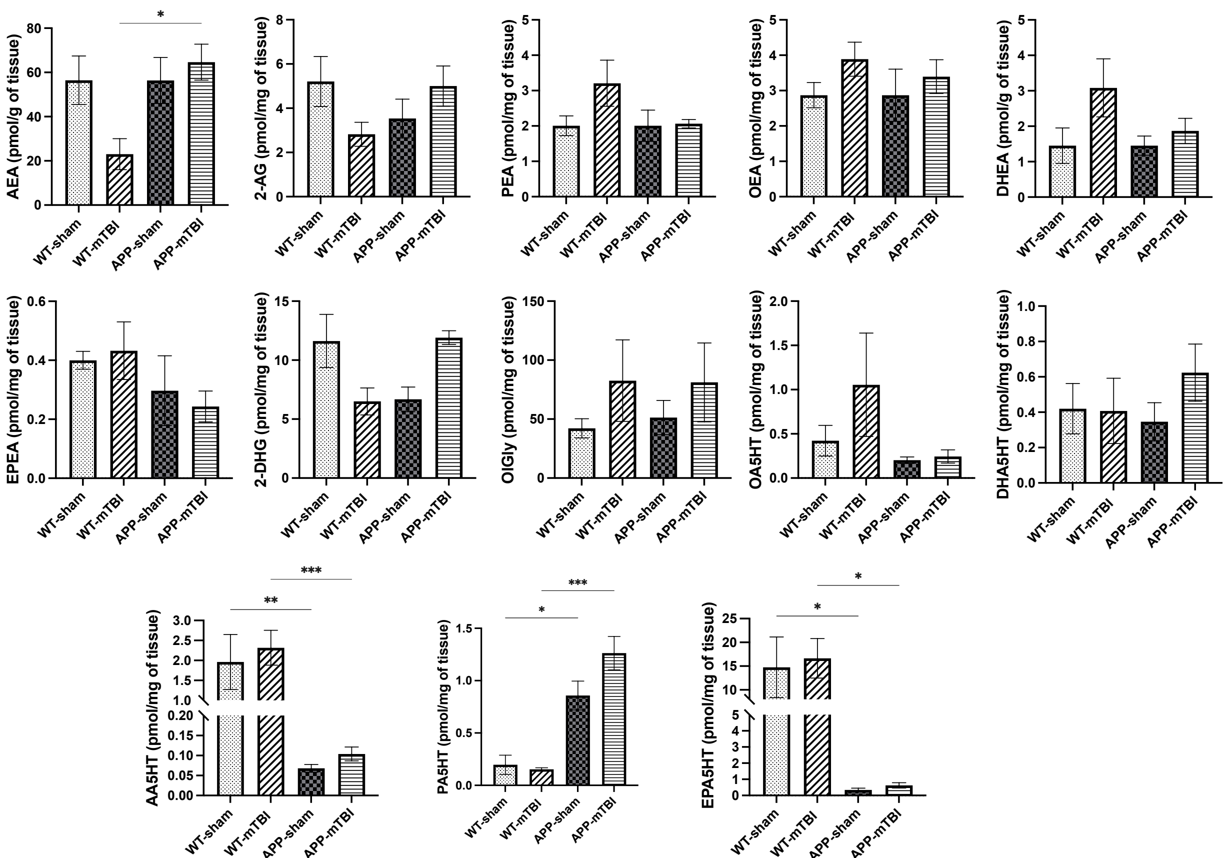


Figure 14. Levels of targeted eCBome mediators in feces of WT and APP (sham and m-TBI) mice at the end of in vivo tests.

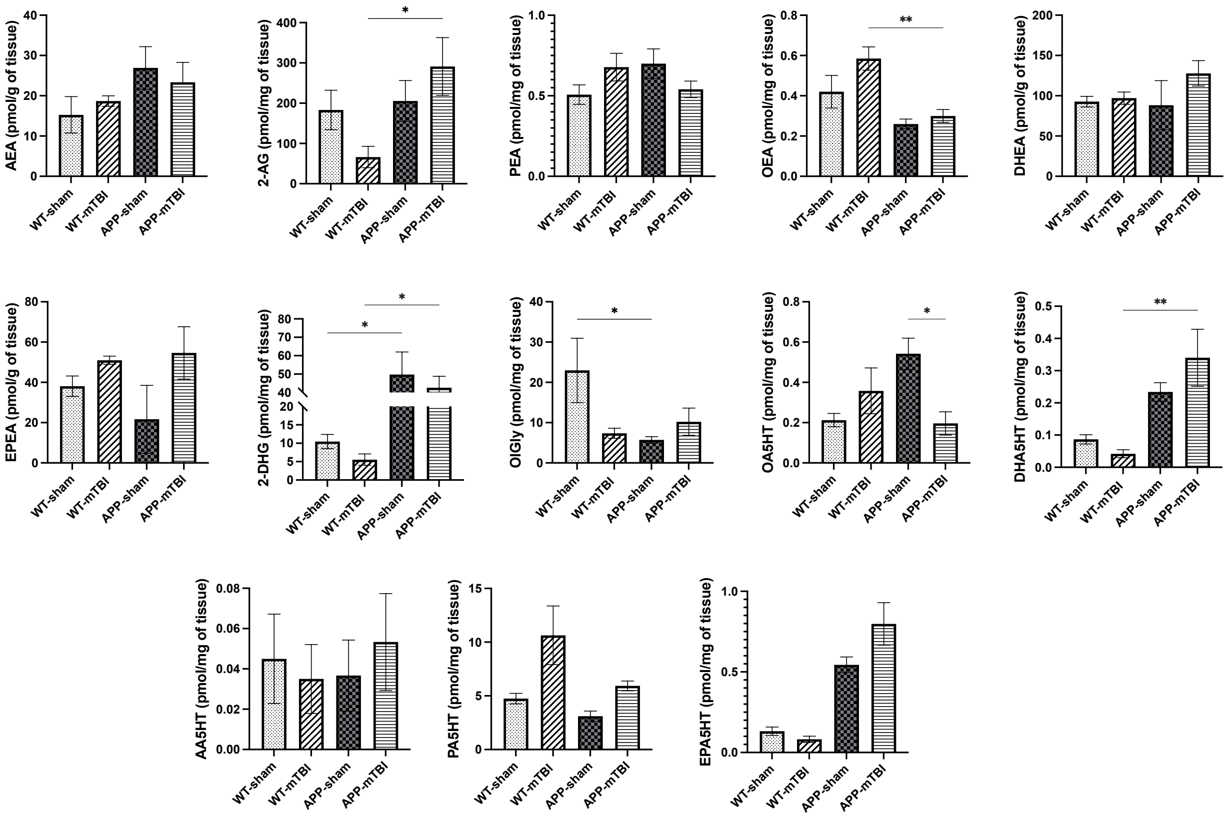


Figure 15. Levels of targeted eCBome mediators in ileum of WT and APP (sham and m-TBI) mice at the end of in vivo tests.

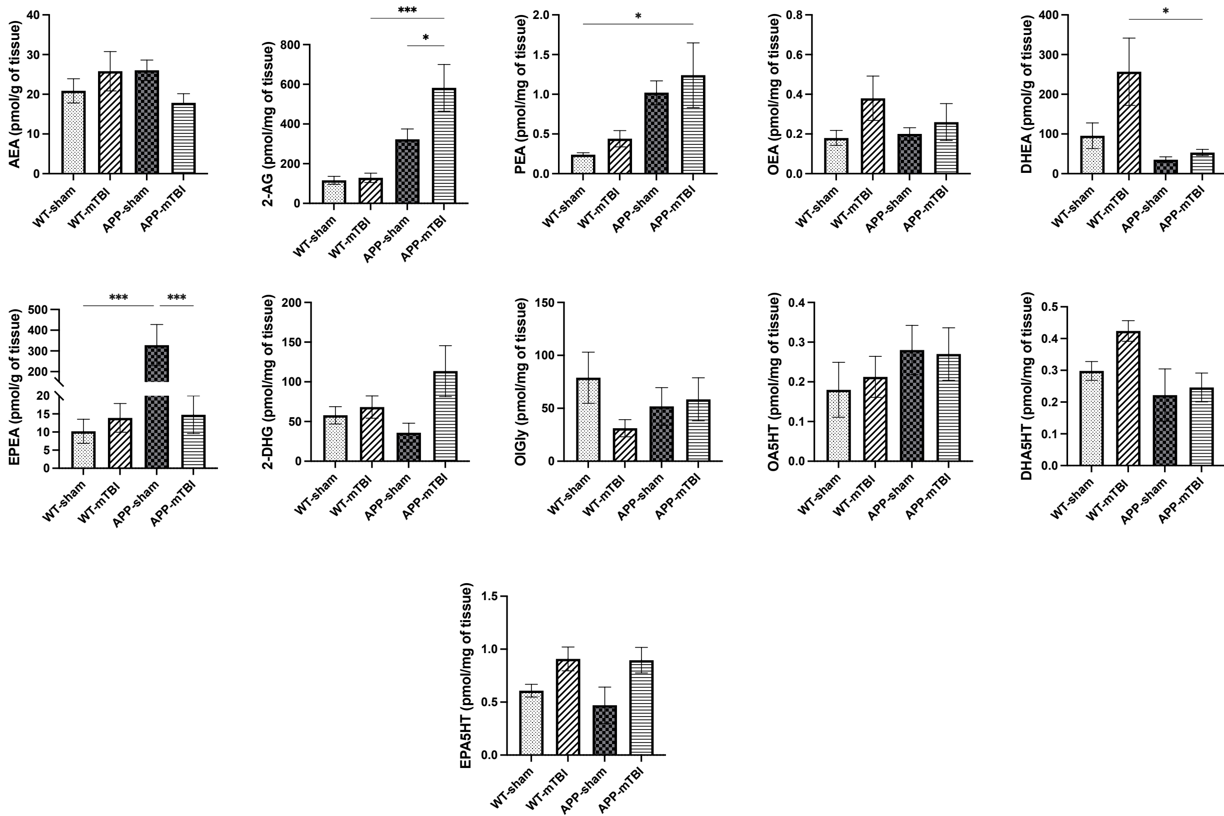


Figure 16. Levels of targeted eCBome mediators in duodenum of WT and APP (sham and m-TBI) mice at the end of in vivo tests.

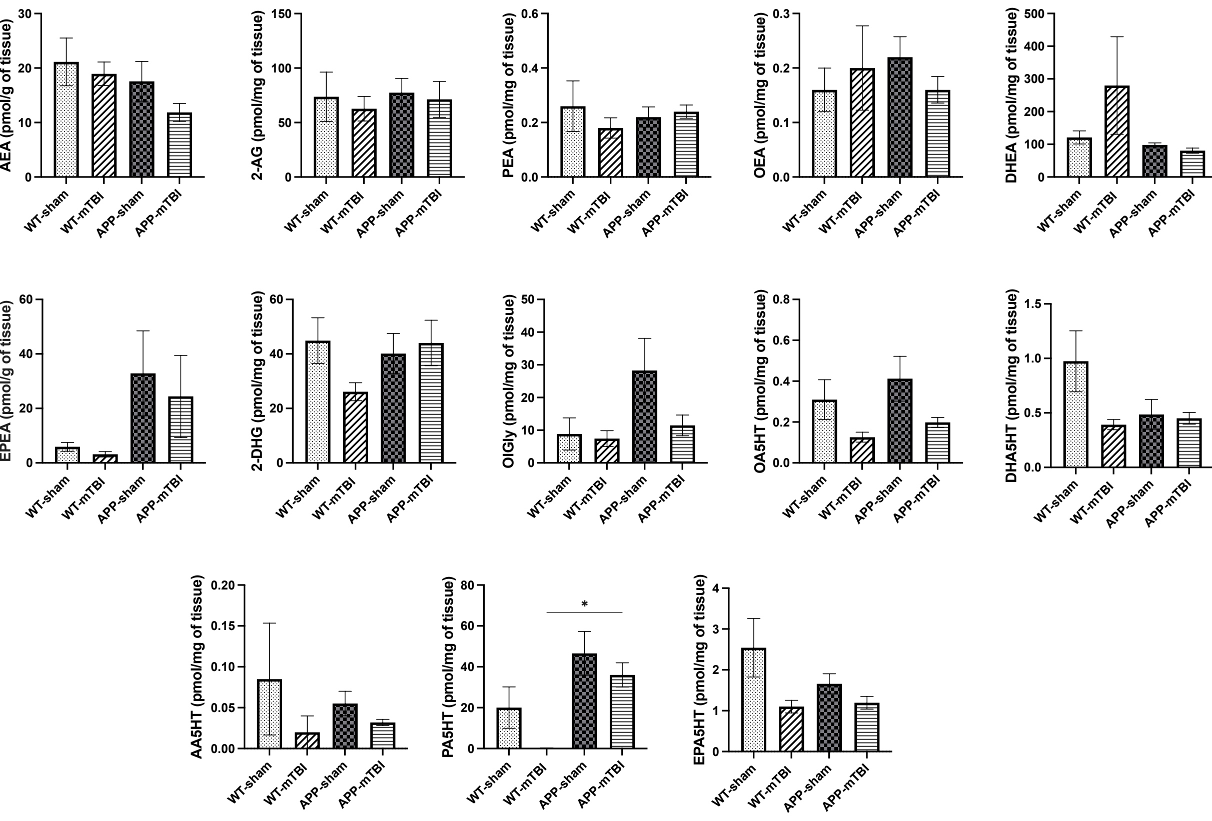


Figure 17. Levels of targeted eCBome mediators in jejunum of WT and APP (sham and m-TBI) mice at the end of in vivo tests.

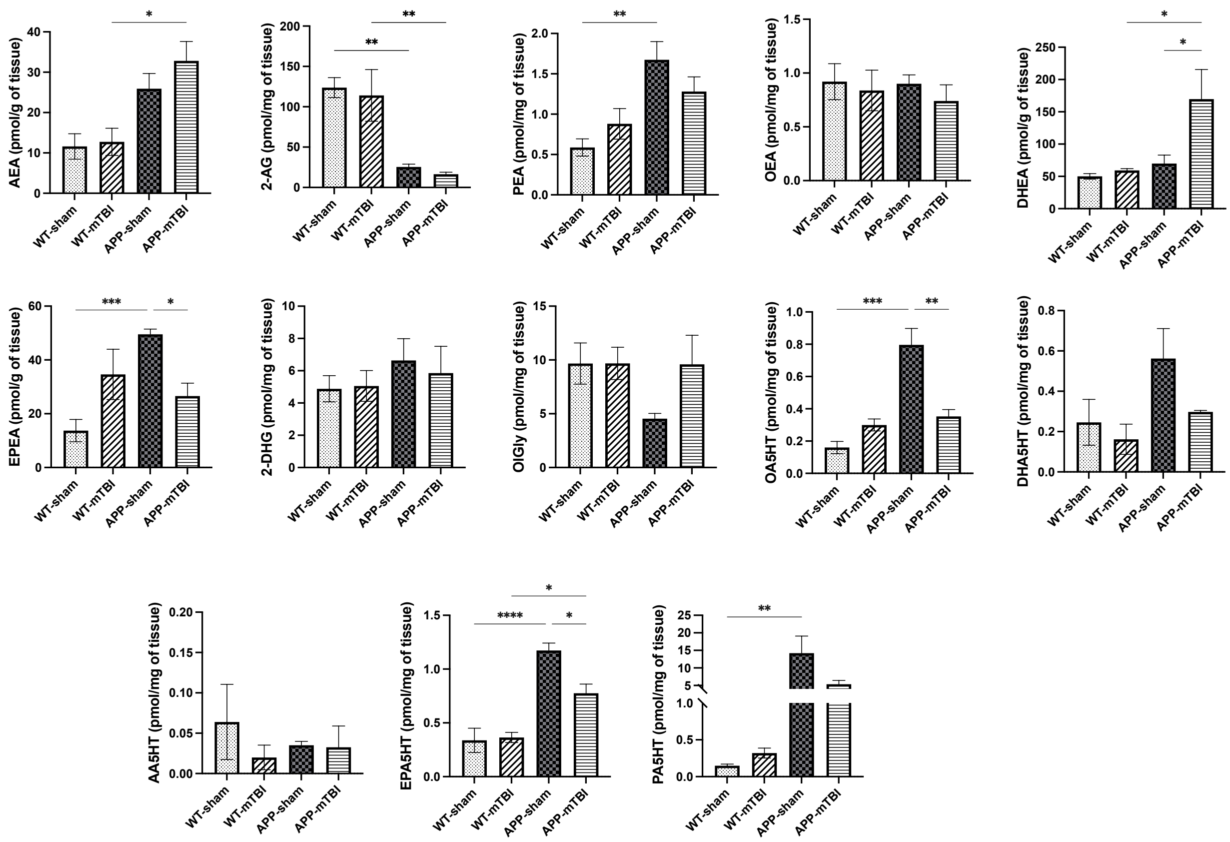
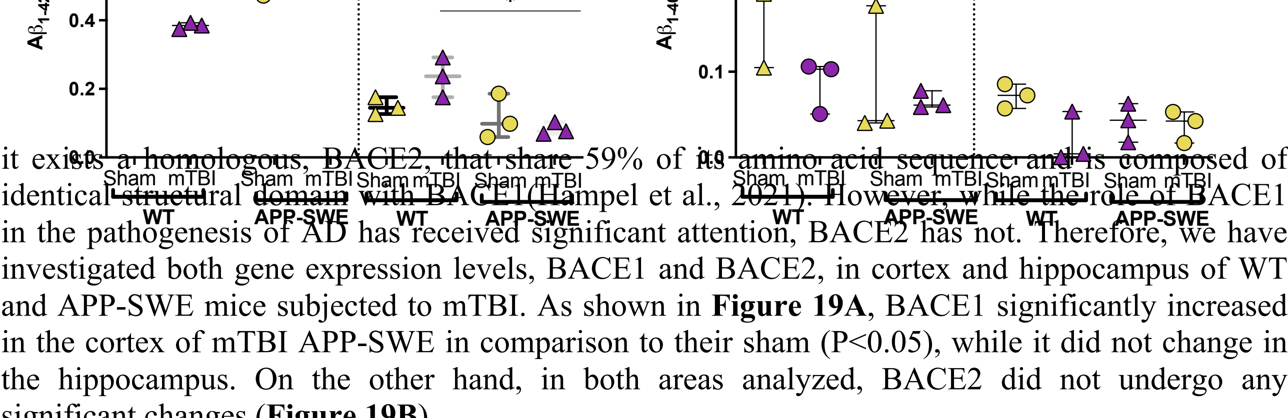


Figure 18. Levels of targeted eCBome mediators in cecum of WT and APP (sham and m-TBI) mice at the end of in vivo tests.

In particular, AEA levels increased significantly in feces of APP-mTBI mice in comparison to WT-mTBI (Figure 14). The others lipid mediators analyzed did not undergo any significant change. Moreover, AA5HT and EPA5HT decreased significantly in APP mice compared to WT mice (WT-sham vs APP-sham and WT-mTBI vs APP-mTBI). On the other hand, the opposite trend is observed for PA5HT.

In the intestinal tissues we were able also to identify and quantify other *N*-acylserotonin species, as PA5HT and EPA5HT. In ileum and duodenum 2-AG levels increased in APP mice vs WT mice, as well as the congener 2-DHG, even though the increase of the latter in the duodenum is not significant (Figures 15-16). Probably this increase might be a mechanism to lock the intestinal barrier and reduce intestinal inflammation. On the other hand, in the cecum 2-AG levels decreased significantly in APP mice (Figure 18). Further statistical analysis need to be performed in order to correlate all the data obtained.

In order to better investigate the possibility that mTBI induced an accelerated AD phenotype, we analyzed gene expression levels of beta-secretase 1, also known as beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) which is the enzyme responsible for amyloid precursor protein (APP) cleavage and subsequent formation of Aβ peptides, including $A\beta_{1-42}$, which aggregates into bioactive conformational species and likely initiates toxicity in AD[11]. Furthermore,



it exists a homologous, BACE2, that share 59% of its amino acid sequence and is composed of identical structural domain with BACE1(Hampel et al., 2021). However, while the role of BACE1 in the pathogenesis of AD has received significant attention, BACE2 has not. Therefore, we have investigated both gene expression levels, BACE1 and BACE2, in cortex and hippocampus of WT and APP-SWE mice subjected to mTBI. As shown in **Figure 19A**, BACE1 significantly increased in the cortex of mTBI APP-SWE in comparison to their sham (P<0.05), while it did not change in the hippocampus. On the other hand, in both areas analyzed, BACE2 did not undergo any significant changes (**Figure 19B**).

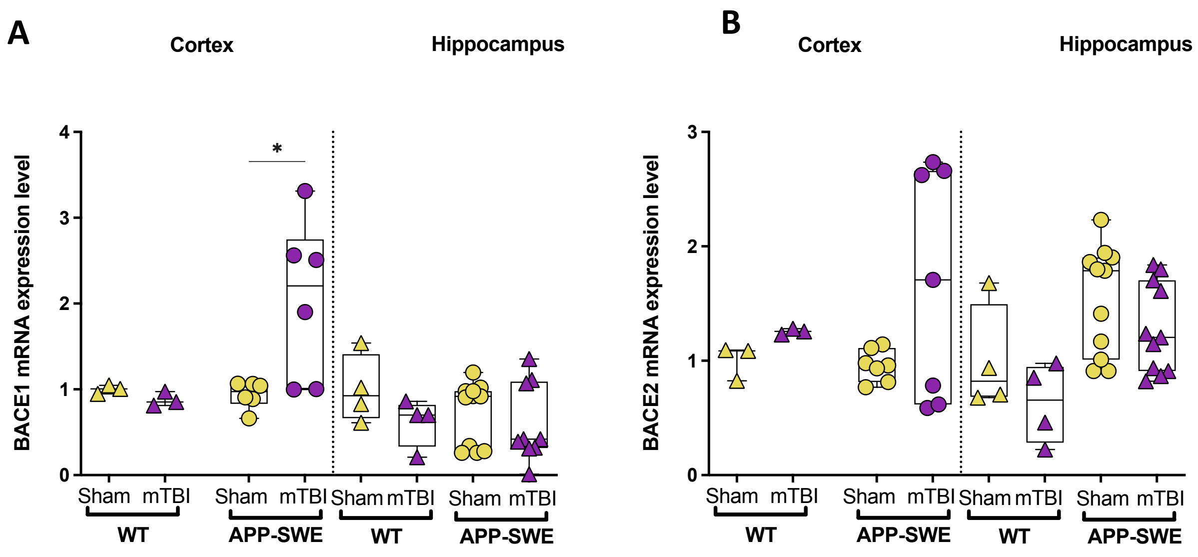


Figure 19. Key Alzheimer's hallmarks in WT and APP mice. Gene expression analysis of BACE 1 (A) and 2 (B) in cortex and hippocampus.

Moreover, we completed the eCBome analysis in brain tissues (cortex and hippocampus) in terms of gene expression analysis. As shown in Figure 20A,C, we have analyzed genes involved in the biosynthesis (*Napepld, Gde1, Daglalpha*) and degradation (*Faah, Magl*) of endocannabinoids and endocannabinoid-like molecules. Instead in Figure 20B,D we have reported gene expression analysis of receptors (*Cb1, Pparalpha and Ppargamma*).

As reported in previous reports, 2-AG levels were upregulated in both cortex and hippocampus of APP mice accordingly to gene expression analysis that showed an increase in *Daglalpha* (2-AG biosynthetic enzyme) and a decrease in Magl (2-AG degradative enzyme). Moreover, *Cb1* increased in cortex and 2-AG is one of its ligand. In both cortex and hippocampus, *Ppargamma* increased significantly in APP mice and some of its ligands too (OEA and OlGly), suggesting an involvement also of this receptor. Interestingly, increased levels of 2-AG might act as a neuroprotective mechanism to inhibit BACE1 and Aβ production, as well as PPARgamma activation, as previously reported (Zhang et al., J Neurosci 2014). On the opposite hand, the overactivation of Cb1 and 2-AG

tone might also contribute to the progression of the disease. However, further studies are needed to better investigate this mechanism.

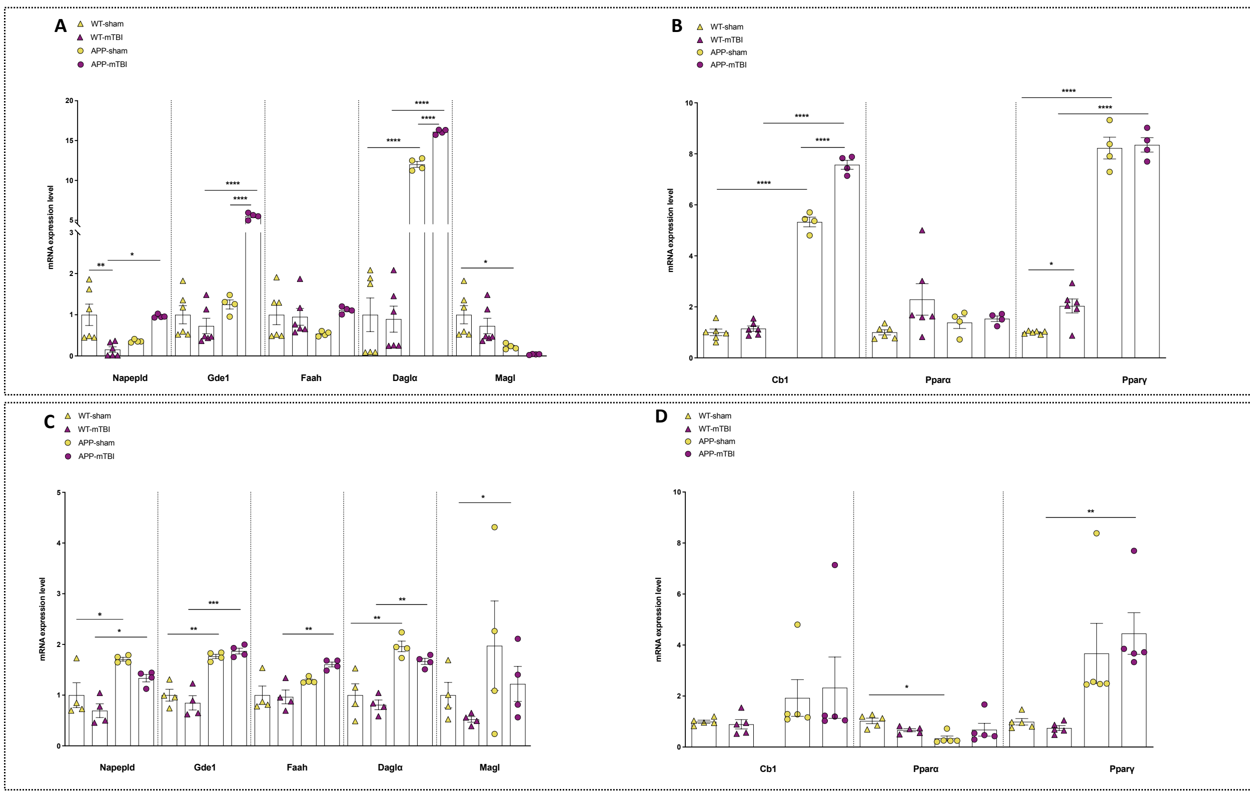
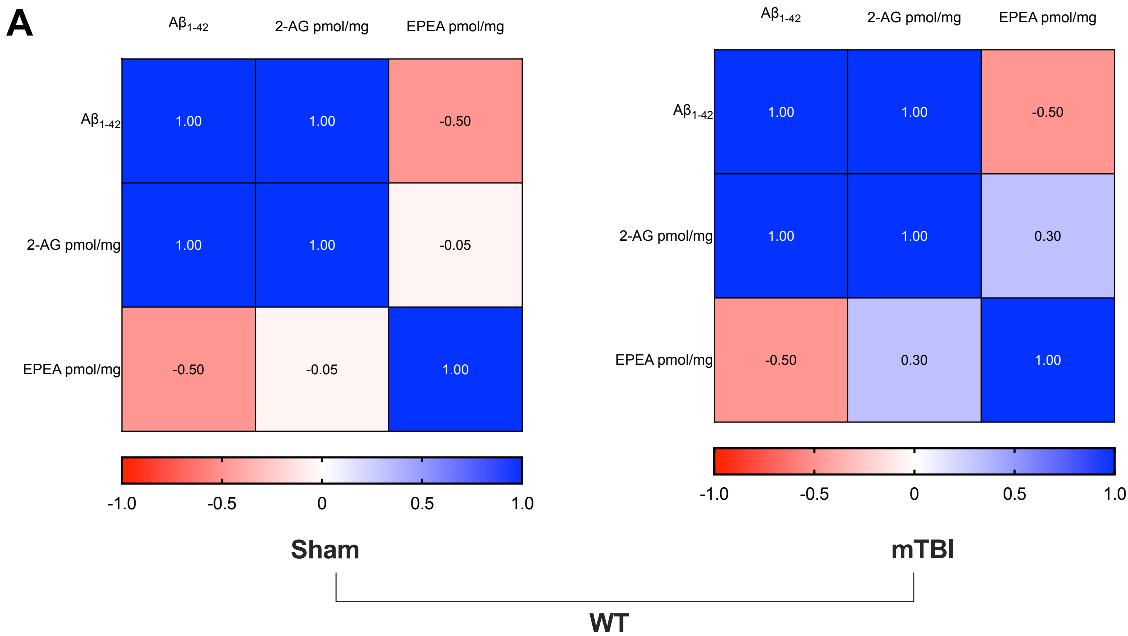


Figure 20. eCBome gene expression analysis. Gene expression analysis of biosynthetic and degradative enzymes (A, C) and receptors (B, D) in cortex (A, B) and hippocampus (C, D).

To better understand a possible correlation between eCBome mediators that underwent significant alterations, and the possible predisposing effect of mTBI and APP genotype, we carried out a Spearman correlation analysis (Figure 21). Very interestingly, in the cortex of WT mice, $A\beta_{1-42}$ strongly correlated with 2-AG levels (R^2=1), in both sham and mTBI groups. Conversely, in APP-SWE mice, while in the sham group only EPEA positively correlated with $A\beta_{1-42}$ (R^2=1), in mTBI mice 2-AG directly correlated whereas EPEA showed a negative correlation (R^2=-1)



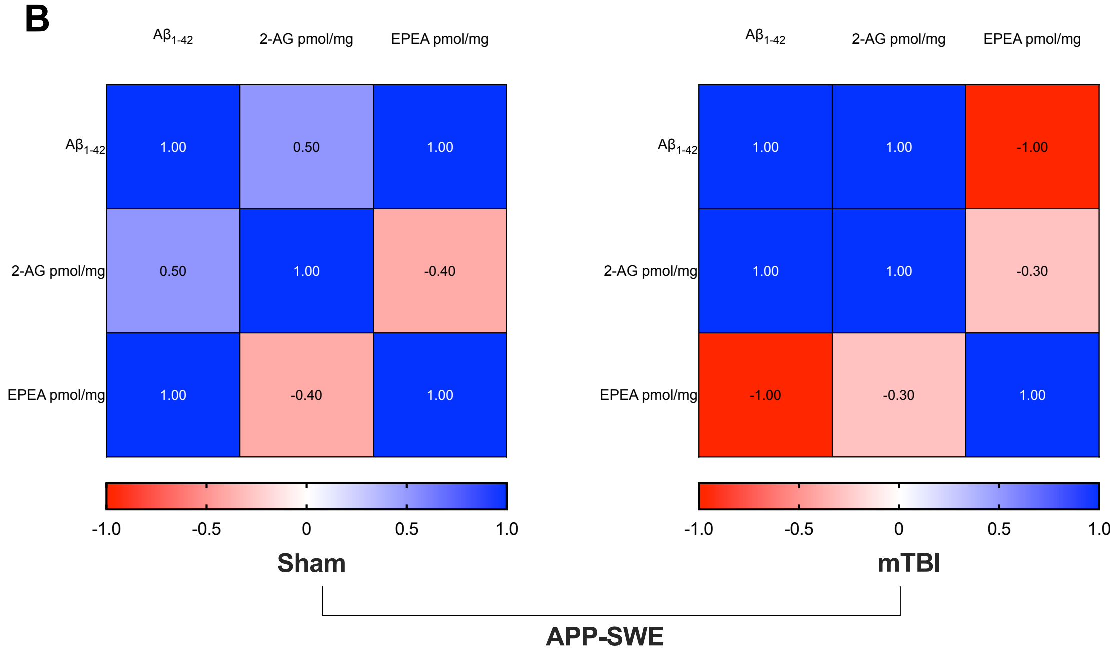


Figure 21. Spearman correlation analysis in cortex in WT (A) and APP-SWE (B) mice.

Methods

DNA extraction and 16S rRNA gene sequencing. DNA was extracted from fecal samples using the QIAmp PowerFecalDNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentrations of the extracts were measured fluorometrically with the Quant-iT PicoGreen dsDNA Kit (ThermoFisher Scientific, MA, USA), and the DNAs were stored at -20°Cuntil16S rDNA library preparation. Briefly, 1 ng of DNA was used as a tem-plate, and the V3-V4 region of the 16S rRNA gene was amplified bypolymerase chain reaction (PCR) using the QIAseq 16S RegionPanel protocol in conjunction with the QIAseq 16S/ITS 384-Index I (Sets A, B, C, D) kit (Qiagen, Hilden, Germany). The 16S metagenomicl ibraries were eluted in 30ul of nuclease-free water, and 1ul was qualified with a Bioanalyser DNA 1000 Chip (Agilent, CA, USA) to verify the amplicon size (expected size~600 bp) and quantified with a Qubit (Thermo Fisher Scientific, MA, USA). Libraries were then normalized and pooled to 2 nM, denatured, and diluted to a final concentration of 6 pM. Sequencing (2×300 bp paired-end) was performed using the MiSeq Reagent Kit V3 (600 cycles) on an Illumina MiSeqSystem. Sequencing reads were generated in less than 65 h. Image analysis and base calling were carried out directly on the MiSeq. Datawere processed using the DADA2 pipeline (Callahanet al,2016), and taxonomic assignation was performed against the SILVA 132 rRNAreference database (Quastet al,2013). Relative microbiota abun-dances were obtained by Cumulative Sum Scaling (CSS, Metagen-omeSeq R package) (Paulsonet al,2013), and microbiota composition was assessed by calculatinga and bdiversity indexes and intra- and inter-individual variations in microbial composition using PERMA-NOVA (vegan R package).

NMR-metabolomics analysis

To extract the metabolites of interest (e.g., lipids, carbohydrates, amino acids and other small metabolites), leaving other compounds (e.g. DNA, RNA, proteins) in the pellet, the feces were mechanically broken down. The extraction of polar metabolites was performed using the methanol / chloroform protocol suggested by the Standard Metabolic Reporting Structures working group (Lindon et al., 2005). Homogenization of 30 mg of frozen stool samples was performed in 8 ml / g of wet tissue of methanol and 1.70 ml / g of wet tissue of water (all solvents were cold) with UltraTurrax for 2 minutes on ice . At this point, 4 ml / g of wet chloroform tissue was added and the homogenate was gently shaken and mixed, on ice (the solution must be monophasic). Subsequently, an additional 4 ml / g of chloroform wet tissue and 4 ml / g of water wet tissue were added and the final mixture was shaken well and centrifuged at 12,000 g for 15 minutes at 4 ° C. Through this procedure, three phases are separated: water / methanol in the upper part (aqueous phase, with the polar metabolites), denatured proteins and cellular debris in the middle and chloroform in the lower part (lipid phase, with lipophilic compounds). The upper and lower layers were transferred into glass vials and the solvents were removed under a stream of dry nitrogen and stored at -80 ° C. For each extract, two samples are obtained for NMR measurements. The first sample was resuspended in 700 µl of phosphate buffer saline (PBS, pH 7.4) and transferred to an NMR tube. The second sample was used for two-dimensional (2D) analyzes and resuspended in 700 µl of D2O. This procedure avoided possible differences in chemical displacement due to separate extractions. The mono (1D-NMR) and two-dimensional (2D-NMR) spectra were obtained at a temperature of 27 ° C (300K) on a high resolution Bruker Avance spectrometer operating at the frequency of 600.13 MHz and equipped with TCI CryoProbe ™ technology. For one-dimensional proton spectra (1H), the

water peak signal was suppressed by using the excitationsculpting sequence. To improve the resolution of the peaks in the one-dimensional spectra and to facilitate their assignment, homonuclear 1H-1H (TOCSY = clean Total Correlation SpectroscopY) and hetero nuclear 1H-13C (HSQC = Heteronuclear Single Quantum Coherence) NMR experiments were acquired. The TOCSY spectra were recorded using the standard pulse sequence that incorporates the suppression of the water signal (excitation-sculpting). After transformation, each spectrum was calibrated on the peak of the TSP standard set at δ = 0.00 ppm. The HSQC spectra were recorded on the same Avance-600 spectrometer operating at 150.90 MHz for the 13C core. After the appropriate transformation, each spectrum was calibrated on the lactate signal (βCH3) consisting of a resonant doublet at δ = 1.33 ppm for 1H and δ = 20.76 ppm for 13C. The values of the chemical shifts (positions) of the peaks identified in the spectra were then compared with the data present in the dedicated on-line databases.

Lipid Extraction and eCBome analysis.

Feces and intestinal tissues were frozen in liquid nitrogen immediately after dissection, which took place within 5 min from sacrifice. Frozen tissues were then homogenized and extracted with chloroform/methanol/Tris-HCl 50 mM pH 7.5 (2:1:1, v/v) containing internal deuterated standards for AEA, 2-AG, PEA, OEA, DHEA, EPEA, OlGly and *N*-acylserotonins quantification by isotope dilution (5 pmol for $[^2H]_8$AEA; 50 pmol for $[^2H]_5$2-AG, $[^2H]_4$PEA, and $[^2H]_2$OEA; 10 pmol for $[^2H]_4$DHEA, $[^2H]_4$EPEA, $[^2H]_2$OlGly, $[^2H]_{17}$OA5HT). Then the lipid extract was purified using open bed chromatography with silica gel. Fractions enriched in eCBs, *N*-acylethanolamines, *N*-acylglycines and *N*-acylserotonins (9:1, CHCl3/CH3OH, v/v) were analyzed by liquid chromatography-atmospheric pressure chemical ionization-single quadrupole mass spectrometry, as previously described (Guida et al, BBI 2018; Piscitelli et al., ACS Chem Neurosci 2020). Endogenous levels of eCBome mediators were calculated on the basis of their area ratio with the internal deuterated standard signal areas, all *N*- acylserotonins were calculated on the basis of their area ratio with the OA5HT deuterated standard signal areas.

Immunoistochemistry.

Under pentobarbital anesthesia (50 mg/kg, i.p.), animals were transcardially perfused with saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were excised, post fixed for 3 h in the perfusion fixative, cryoprotected for 72 h in 30% sucrose in 0.1 M phosphate buffer, and frozen in Optimal cutting temperature-embedding compound. Prefrontal cortex and hippocampus were analyzed. Transverse sections (20 µm) were cut using a cryostat and thaw-mounted onto glass slides. Slides were incubated overnight with primary antibody solutions for the microglial cell marker Iba-1 (rabbit antiionized calcium binding adapter molecule-1; 1:1000; Wako Chemicals, Germany). Possible non-specific labeling of rabbit secondary antibody was detected by using secondary antibody alone. Following incubation, sections were washed and incubated for 2 h with secondary antibody solution (donkey anti-rabbit Alexa FluorTM 488; 1:1000; Molecular Probes, USA). Slides were washed, coverslipped with Vectashield mounting medium (Vector Laboratories, USA), and visualized under a Leica fluorescence microscope. Quantitative analysis was performed by counting in areas measuring 1.7×10^4 µm2.

RNA Extraction and quantitative PCR (qPCR)
Total RNA was isolated from hippocampus and cortex by use of the TRIzol Reagent (Cat# 15596026; ThermoFisher, Italy), reacted with DNase-I (Cat# 180680151U/µl; ThermoFisher, Italy) for 15 min at room temperature, followed by spectrophotometric quantification. The final preparation of RNA was considered DNA- and protein-free if the ratio between readings at 260/280 nm was ≥ 1.7. Isolated mRNA was reverse-transcribed by the use of iScript™ Reverse Transcription Supermix (Cat# 1708840; Biorad, Italy). Quantitative PCR (qPCR) was carried out in a real-time PCR system CFX384 (Bio-Rad) using the SYBR Green PCR Kit (Cat# 1725274, Biorad; Italy) Each sample was amplified simultaneously in quadruplicate in a one-assay run with a nontemplate control blank for each primer pair to control for contamination or primer-dimer formation, and the cycle threshold (Ct) value for each experimental group was determined. The housekeeping gene ribosomal protein S16 was used to normalize the Ct values, using the $2^{\wedge -\Delta Ct}$ formula. Differences in mRNAs content between groups were expressed as $2^{\wedge -\Delta\Delta Ct}$, as previously described (Iannotti et al. 2018).

Statistical analysis
Data analysis was performed by Prism Software 9.0. Data are represented as mean ± SEM of 8-10 mice per group, (∗) P< 0.05, (∗∗) P 0.01, and (∗∗∗) P <0.001 versus Sham group. Unpaired T test in the Resident intruder and Sociability. Two way ANOVA followed by post hoc Sidak or Tukey for all other tests.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Conferences
Oral and poster presentation at the Gordon Conference "Cannabinoid Function in the CNS", 16-21 July 2023, Castelldefels Barcelona (Spain)
Oral presentation: **Exploiting the Endocannabionoidome in Traumatic Brain Injury and Alzheimer's Disease**
Poster presentation: **Potential role of the microbiome-endocannabinoidome connection in the gut-brain axis after traumatic brain injury and its association with Alzheimer's disease**

Monthly meetings with co-PIs and main collaborators

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Dissemination activities
Preparation of the first draft of the paper to be submitted in few weeks

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We asked an integration for the animal study (ACURO approval AZ190044.e001 08/31/2023) to obtain more lipidomic and metabolomic analysis as the project needs further investigation in this direction.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The results provided so far confirmed that mild brain trauma might induce and/or exacerbate some biochemical hallmarks of AD in genetically predisposed animals and the eCBome played a key role.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:
- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:
- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to report.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

No use of human subjects

Significant changes in use or care of vertebrate animals

We asked an integration for the animal study (ACURO approval AZ190044.e001 08/31/2023) to obtain more lipidomic and metabolomic data. The approval of the Italian Ministry of Health is submitted.

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to Report

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year*

(international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Oral and poster presentation at the Gordon Conference "Cannabinoid Function in the CNS", 16-21 July 2023, Castelldefels Barcelona (Spain)
Oral presentation: **Exploiting the Endocannabionoidome in Traumatic Brain Injury and Alzheimer's Disease**
Poster presentation: **Potential role of the microbiome-endocannabinoidome connection in the gut-brain axis after traumatic brain injury and its association with Alzheimer's disease**

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  Nothing to Report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name: *Fabiana Piscitelli*
Project Role: *PD*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *4*
Contribution to Project: *Dr Piscitelli has prepared all the documentation needed to start the project, order mice and has supervised all the experimentation.*

Name: *Francesca guida*
Project Role: *co-PI*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *4*
Contribution to Project: *Dr. Guida has prepared the documentation for the Italian Ministry of Health approval. Dr. Guida has participated in the in vivo experimentation and supervised the behavioral tests.*

Name: *Serena Boccella*
Project Role: *Other professional*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *2*
Contribution to Project: *Dr. Boccella has done in vivo experimentation.*

Name: *Sabatino Maione*
Project Role: *Other professional*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1*
Contribution to Project: *Prof. Maione conducted animal surgery and behavioral analysis*

Name: *Livio Luongo*
Project Role: *Other professional*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *2*
Contribution to Project: *Prof. Luongo is conducting experiments for the avaluation of neuroinflammation*

Name: *Roberta Verde*
Project Role: *Other professional*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *2*
Contribution to Project: *Dr. Verde has performed targeted lipidomics analysis.*

Name: *Adele Cutignano*
Project Role: *Other professional*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1*
Contribution to Project: *Dr. Cutignano has performed targeted lipidomics analysis.*

Name: *Debora Paris*
Project Role: *Other professional*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1*
Contribution to Project: *Dr. Cutignano has performed untargeted metabolomics analysis.*

Name: *Monica Iannotta*
Project Role: *Other professional*
Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: full-time
Contribution to Project: Dr. Iannotta started her post-doc position to work full-time in the project.

Name: Anna Lauritano
Project Role: Other professional
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: full-time
Contribution to Project: Dr. Lauritano started her post-doc position to work full-time in the project.

Name: Fabio Arturo Iannotti
Project Role: Other professional
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2
Contribution to Project: Dr. Iannotti has performed gene expression analysis.

Name: Vincenzo Di Marzo
Project Role: Co-PI
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Dr. Di Marzo is working on gut microbiome analysis.

Name: Pal Pacher
Project Role: Co-PI
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1
Contribution to Project: Prof Pacher is working on inflammatory markers analysis.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name: ***Università della Campania "L. Vanvitelli"***
Location of Organization: (if foreign location list country) ***Via Costantinopoli 16, 80138 Napoli, Italy***
Partner's contribution to the project (identify one or more)
- *Facilities;*
- *Collaboration;*
- *Personnel exchanges*

Organization Name: ***Université Laval***
Location of Organization: (if foreign location list country) **1050, avenue de la Médecine Quebec City, Quebec Canada**

Partner's contribution to the project (identify one or more)
- *Facilities;*
- *Collaboration;*

Organization Name: ***NIH***

Location of Organization: (if foreign location list country) **5625 Fishers Lane Rockville, MD 20852 USA**
Partner's contribution to the project (identify one or more)

- *Facilities;*
- *Collaboration;*

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil/Pages/Resources.aspx) should be updated and submitted with attachments.*

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.