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DETERMINING SOME MOLECULAR BIOLOGY ASPECTS AND MICROBIOME RELEVANCE IN THE ETIOPATHOPHYSIOLOGY OF PARKINSON'S DISEASE

PHD THESIS SUMMARY

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Contents

List of abbreviations and keywords

PD - Parkinson’s disease

AD - Alzheimer’s disease

SN - substantia nigra

DA - dopamine

LB - Lewy body

αS - alpha-synuclein

DIP - drug-induced parkinsonism

GM - gastrointestinal microflora

HMP - Human Microbiome Project

HGP - Human Genome Project

iHMP - Integrative Human Microbiome Project

IBS - irritable bowel syndrome

GI - gastrointestinal tract

GBA - gut-brain axis

IESC - intestinal epithelial stem cell

GC - Goblet cell

EEC - enteroendocrine cell

IEC - intestinal epithelial cell

GALT - gut-associated lymphoid tissue

MUC2 - mucin 2

AMP - antimicrobial peptide

TFF3 - trefoil factor 3

RELMβ - resistin-like molecule β

LPS - lipopolysaccharide

CD - cluster of differentiation

BBB - blood-brain barrier

HPA - hypothalamic-pituitary-adrenal axis

PPR - pattern recognition receptor

TLR - toll-like receptor

NOD - nucleotide-binding oligomerization domain-like receptor

CLR - C-type lectin receptor

RLR - retinoic acid-inducible gene-I-like receptor

ALR - absent in melanoma 2-like receptor

cGAS - cyclic GMP-AMP synthase

RAGE - receptor for advanced glycation endproducts

NF-κB - nuclear factor kappa B

IRF3 - interferon regulatory factor 3

PAMP - pathogen associated molecular pattern

DAMP - damage-associated molecular pattern

DC - dendritic cell

APC - antigen-presenting cell

MC - microfold cell

Th - T-helper

IgA - immunoglobulin A

SCFA - short chain fatty acid

GPCR - G protein-coupled receptor

NA - noradrenaline

5-HT - 5-hydroxytryptamine

EC - enterochromaffin cell

ASF - altered Schaedler flora

ACh - acetylcholine

VN - vagus nerve

ENS - enteric nervous system

OS - oxidative stress

DNV - dorsal nucleus of the vagus

LF - lipofuscin

SNC - central nervous system

DMT1 - divalent metal transporter-1

FPN1 - Ferroportin 1

NM - neuromelanin

MPTP - 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

CT - computed tomography

SC - stercoral colitis

DRE - digital rectal exam

ACPO - Acute colonic pseudo-obstruction

BMS - burning mouth syndrome

QOL - quality of life

VF - videofluoroscopy

HRM - high resolution manometry

LES - lower esophageal sphincter

DGE - delayed gastric emptying

GET - gastric emptying time

WMC - wireless motility capsule

SBTT - small bowel transit time

CTT - colonic transit time

WGTT - whole gut transit time

GES - gastric electrical stimulation

SIBO - small intestinal bacterial overgrowth

CFU - colony forming unit

HBT - glucose breath test

ACG - American College of Gastroenterology

HRAM - high-resolution anorectal manometry

MR - magnetic resonance

FAO - Food and Agriculture Organization

WHO - World Health Organization

FMT - fecal microbiota transplantation

CDI - *Clostridium difficile* infection

IBD - Inflammatory bowel disease

MTT - microbial transfer therapy

LBP - live biotherapeutic product

FIFRA - Federal Insecticide, Fungicide, and Rodenticide Act

ATP - adenosine triphosphate

ROS - reactive oxygen species

ETC - mitochondrial electron transport chain

WT - wild type

UE - European Union

ARRIVE - Animal Research: Reporting of *In Vivo* Experiments

SOD - superoxide dismutase

GPx - glutathione peroxidase

MDA - malondialdehyde

ARN - ribonucleic acid

ELISA - enzyme-linked immunosorbent assay

HE - hematoxylin-eosin

IHC - immunohistochemistry

GFAP - glial fibrillary acidic protein

S100b - S100 calcium-binding protein B

PCNA - proliferating cell nuclear antigen

p53 - tumor antigen p53

cox4i1 - cytochrome c oxidase subunit 4 isoform 1, mitochondrial

PBS - phosphate-buffered saline

IgG - immunoglobulin G

DAB - 3,3′-diaminobenzidine

ANOVA - analysis of variance

SEM - standard error of the mean

NSC - neural stem cell

RGC - radial glial cell

ip - intraperitoneal injection

PFC - prefrontal cortex

SVZ - subventricular zone

Parkinson’s disease, zebrafish, *Danio rerio*, rotenone, valproic acid, levodopa, carbidopa, probiotics, *Bifidobacterium longum* BB536, *Lactobacillus rhamnosus* HN001, *Lactobacillus casei* W56, *Lactobacillus acidophilus* W22, *Lactobacillus paracasei* W20, *Lactobacillus salivarius* W24, *Lactobacillus lactis* W19, *Lactobacillus plantarum* W62, *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52, *Bifidobacterium bifidum* W23, sociability, antisocial, aggressivity, locomotion, T-maze, three-dimensional conformations, time spent in arms, active status, distance swum, velocity, inactivity episodes, counter clockwise rotation, oxidative stress, SOD, GPx, MDA, Bradford, spectrophotometry, gene expression, RT-PCR, LRRK2, PINK1, PARKIN, alfa-SNCA, ACTIN, ELISA, dopamine, immunohistochemistry, GFAP, S100b, PCNA, p53, cox4i1

THEORETICAL PART

Introduction

PD affects between 8.7 - 9.3 million individuals globally, currently occupying the second position in frequency after AD. It is even predicted that the number of cases will increase or double (Dorsey et al., 2007) by 2050 (Poewe et al., 2017). Cumulatively, AD and PD exert a colossal financial pressure on the resources of public health systems, costs that will amount to €357 billion over the next three decades (Maresova et al., 2016).

Contrary to expectations, there is a relatively limited number of studies focusing on the prevalence and mortality of PD. The latest published statistics indicated a global prevalence of 6.1 million cases and a mortality of 3.2 million in 2016 (Dorsey et al., 2018). At the level of Europe, the estimated prevalence oscillates around the value of 65.6 - 12.500, and the incidence between 5 and 346 per 100.000 inhabitants (von Campenhausen et al., 2005), figures that are correlated with the age, geographical location and sex of the individuals, especially in men in their fifties (Pringsheim et al., 2014).

According to the classification scale of the evolution of the disease defined by the five stages of severity introduced by (Hoehn and Yahr, 1967), in parallel with the age of onset as the first endogenous risk factor, PD can have a juvenile form (< 20 years), early-onset (20 - 50 years) and late-onset (> 50 years) (Tysnes and Storstein, 2017).

This pathology is characterized by an irreversible degeneration of the dopaminergic neurons in the SN, a unidirectional process restricted in the prodromal stages only to the ventrolateral segment without affecting the mesencephalic dopaminergic neurons. Consequently, it causes an inhibition of DA biosynthesis and leads to an aggregation of αS-positive LBs. Thus, a predominance of motor symptoms was observed in the diagnosed patients, but also the predisposition towards non-motor dysfunctions. Clinical signs for a favorable differential diagnosis brought together under the umbrella of parkinsonism include essential tremor, rigidity, and bradykinesia. Impairment of postural balance occurs gradually over the course of the patient's life (Poewe et al., 2017).

Despite the best efforts, the etiology of PD remains obscure. Recent studies have revealed that PD possesses a multifactorial substrate, reflected either by abnormal gene expression (Blauwendraat et al., 2020), endogenous and/or exogenous stressors, but also lifestyle (Ascherio and Schwarzschild, 2016). Due to clinical heterogeneity, the nomenclature has been constantly re-evaluated to be able to distinguish PD of idiopathic

cause, overlap of dementia in PD, dementia with LB, but also other types (Marras et al., 2016).

Non-motor symptomatology is complex, with important ramifications because it encompasses neuropsychiatric disorders commonly seen in the healthy population. An underexplored but crucial role in PD is played by anxiety (Chen and Marsh, 2014) and depression (Marsh, 2013) as promoters and/or indicators of PD. Analogous for healthy patients exposed to prolonged stress as a risk group (Mah et al., 2016; Trifu et al., 2020). Moreover, this palette incorporates sleep, autonomic, sensory, but also gastrointestinal impairments (Chaudhuri and Schapira, 2009).

PD is a neurological disease that benefits from symptomatic (Charvin et al., 2018), surgical or alternative treatment as appropriate (Sharma et al., 2020) in advanced disease stages compared to AD. On the other hand, particular circumstances can arise in which patients under drug treatment with antipsychotics can manifest side effects, a syndrome known under the pseudonym of DIP (Brigo et al., 2014). Pharmaco-kinetics/dynamics is mainly shaped by GM which proved to be the missing link as a transport vector, but also a modulator, especially of internal environment parameters, independent of age and sex (Scheperjans, 2016).

Purpose, hypotheses and objectives of the thesis

The topic addressed orbits around the non-invasive potential of using probiotic lactic strains as an alternative management strategy, simultaneously with issuing some hypotheses aimed at testing an anticonvulsant, but also the main agents administered in Parkinson's disease as a last defensive barrier.

In this way, it was followed how chronic exposure to different concentrations of rotenone administered alone or in combination with valproic acid, levodopa and carbidopa or with probiotics changes internal parameters in zebrafish (*Danio rerio*) as a study model of Parkinson's disease.

As it is a relatively new field of activity, the idea of manipulating the gastrointestinal microflora for clinical purposes is promising and unfortunately still insufficiently explored. Thus, in the framework of this work, it was pursued:

- Evaluation of the spatial conformations of social, anti-social, aggressive and locomotor behavior following chronic exposure of zebrafish to distinct concentrations of

rotenone, personalized of valproic acid, levodopa and carbidopa, respectively preset concentrations of probiotic lactic strains;

- Evaluation of the status of oxidative biomarkers by spectrophotometry (SOD, GPx, MDA);
- Evaluation of gene expression by RT-PCR of a panel of specific PD genes (*LRRK2*, *PINK1*, *PARKIN*, *alpha-SNCA*, *ACTIN*);
- Dopamine level evaluation by ELISA;
- Evaluation of histological changes by means of immunohistochemistry protocols (GFAP, S100B, PCNA, p53 and cox4i1).

Chapter 1. The current status of knowledge

1.1. Introductory aspects

The HMP marked by the "Jumpstart" phase was launched only at the end of 2007 and was officially declared completed in 2013. It was the most promising research project after the HGP which had as its main objectives: (I) the exploration of the symbiotic relationship, (II) development of new working algorithms and (III) computational analysis of microbiome variability (Group et al., 2009).

In order to deepen this research direction, the iHMP was born a year later, being completed in 2016. In contrast to its predecessor, the iHMP was mainly oriented towards the complete characterization of the human microbiome, in the case of the impact exerted by the transient stages on the structure and GM composition: (I) pregnancy, type of delivery, and preterm birth, (II) pathogenesis of IBS, and (III) influence of stressors on patients with prediabetes (Consortium, 2019).

The total number of commensal, symbiotic, and pathogenic microorganisms exceeds that of eukaryotic cells by a factor of ten. These communities are grouped into four major ecosystems: skin (Byrd et al., 2018), oral cavity (Kilian et al., 2016), urogenital tract (Jones-Freeman et al., 2021), and digestive tract ($10^{\sim 14}$) in the following segments: stomach, small intestine (duodenum, jejunum, ileum), colon and appendix (Kastl et al., 2020). Relative to the human genome, GM possesses more than 150.000 times more bacterial genes (Turnbaugh et al., 2007), whose biomass production is greater than the weight of the human brain (Macfarlane and Macfarlane, 2003).

GI hosts one thousand and fifty-seven species in different ratios (8, 92, 957) that have already been cultivated and phylogenetically analyzed, among which are *Archaea*, *Eukarya* and *Bacteria* (Rajilić-Stojanović and de Vos, 2014), with the mention that the most abundant communities belong to the phyla *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia* (Hollister et al., 2014). In summary, *Ruminococcus*, *Bacteroides* and *Prevotella* are the constitutive enterotypes of GM (Arumugam et al., 2011; Bäckhed et al., 2005).

1.2. Functionality of the gastrointestinal microflora

GM homeostasis is ensured by GBA through two mechanisms: (1) segregation and (2) mediation. Segregation implies the delimitation by means of a physical and biochemical barrier of the microflora from the host immune cells, while mediation requires the emission of signals between the microflora and the host immune cells. The intestinal epithelium together with commensal microorganisms form the gastrointestinal barrier, the main guard that prevents pathogens from adhering to the intestinal wall (Okumura and Takeda, 2017).

Systematically, the intestinal epithelium is organized in a monolayer of Lieberkühn's crypts (intestinal glands) located in the lamina propria and intestinal villi in the lumen, a coating that is constantly renewed by pluripotent IESCs that are found at the base of the crypts. Key functions such as proliferation, differentiation and functional potential are regulated by the stem cell niche in proximity (Crosnier et al., 2006; van der Flier and Clevers, 2009).

The intrinsic barrier brings together secretory IESCs including GC, Paneth and EEC, the latter representing the link between the enteric neuroendocrine system and the central neuroendocrine system that ensures digestive function and prevents penetration of the intestinal epithelium. IECs are protected by immune structures such as Peyer's patches and crypts, but also lymphoid follicles, structures brought together in the GALT (Hill and Artis, 2010; Kamada et al., 2013). GCs of the small intestine secrete MUC2 (Johansson et al., 2008), coupled to AMP and GC by-products, TFF3 and RELMβ that strengthen the extrinsic barrier against the antagonistic action of pathogenic LPSs (Bevins and Salzman, 2011).

Essentially, the instability created is defined by low-grade inflammation, cell death, and an imbalance of cellular energy (Noble et al., 2017). Abnormal expression of CD1,

CD4 and CD17 (Kamada et al., 2013) inhibits peripheral immune cell responses (Berer and Krishnamoorthy, 2012) resulting in disruption of BBB integrity.

Lymphocytes and macrophages are mainly responsible for controlling the immune cascade. Prolonged exposure stimulates intestinal permeability as a result of denaturation of tight junctions due to the action of pathogenic endotoxins. Consequently, they induce the so-called "leaky gut" characterized by a dysbiosis/dysbacteriosis (Hakansson and Molin, 2011) that affects the functionality and integrity of the HPA (Rea et al., 2016).

Enterocytes are directly involved in host defense because they display evolutionarily conserved, germline-encoded receptors called PPRs. These sensors bring together receptor families including TLR (Abreu, 2010; Kawai and Akira, 2011), NOD (Motta et al., 2015), CLR (Brown et al., 2018), RLR (Kell and Gale, 2015), ALR (Lugrin and Martinon, 2018), cGAS (Sun et al., 2013) and RAGE (Palanissami and Paul, 2018) which mark the activation of the transcription factors NF-κB and IRF3.

NOD performs a similar function to that of enterocytes by targeting microbial templates known as PAMPs. Additionally, PAMPs activate NF-κB, NOD, and DAMPs (Williams et al., 2010). Sequestered pathogens are presented to DCs as APCs (Williams et al., 2010), and together with MCs, they take up APCs and present them further to naive CD4 cells, a transient process of Th cell differentiation and IgA synthesis (Hooper and Macpherson, 2010).

Anaerobic commensal bacteria stimulate the fermentation of non-digestible carbohydrates, on the one hand resulting in SCFA (acetate, butyrate and propionate), but also secondary products (Wang et al., 2019). SCFA in turn bind to GPCRs, GPR41, GPR43 and GPR109a with roles in regulating energy balance and inflammatory responses (Levy et al., 2017; Silva et al., 2020). While butyrate is involved in blocking the accumulation of toxic byproducts and down-modulating inflammatory reactions (Canani et al., 2011), acetate and propionate cross the enteric blood barrier participating as substrates in the process of gluconeogenesis and lipogenesis (Krishnan et al., 2015; Silva et al., 2020).

GM is also involved in the synthesis of the B vitamin complex (Yoshii et al., 2019), pantothenic acid (B5) and cyanocobalamin (B12), but also of some neurotransmitters, in particular NA and DA (Strandwitz, 2018), but also 5-HT. ECs are responsible in this context for the synthesis, storage and release of 5-HT in order to regulate intestinal motility. The involvement of *Bacteroides* spp., ASF and spore-forming *Clostridium* was previously described during intestinal colonization (Ma et al., 2018). B12 is a modulator of

ACh and cortisol production in the adrenal gland. A suboptimal level of these vitamins has been reported in a multitude of gastrointestinal, psychiatric and hematological disorders (Chi et al., 2017).

1.3. Braak's hypothesis regarding the pathogenesis of Parkinson's disease

Researchers in the 1980s hypothesized regarding the pathodynamics of Lewy structures that the origin of PD resides at the GI level (Edwards et al., 1992). Studies have provided further evidence for the chronological evolution behind PD (Braak et al., 1996), associated with specific neuropathological changes in olfactory neurons and VN, suggesting that PD may be triggered at a peripheral site before targets the brain, αS aggregates being observed in the myenteric (Auerbach) plexus of the ENS. Defined by six stages, the progression of PD is described by the system proposed by Braak; rhombencephalon pathology in stage I and II, midbrain in stage III and IV, the point at which motor symptoms become visible, and cognitive symptoms in stage V and VI when the neocortex is completely affected (Braak et al., 2003).

Nineteen years ago, Braak and his collaborators put forward a new "gut-brain" hypothesis according to which PD could be caused by a neurotropic pathogen that penetrates the mucosal barrier (Hawkes et al., 2007) and induces OS by triggering an immune inflammatory response by which defective folding of αS is gradually produced. The aggregates produced migrate to the ENS by anterograde and retrograde axonal transport (Ulusoy et al., 2017, 2013) through the VN to the DNV and implicitly into the brain (Braak et al., 2003). αS-immunoreactive gastric inclusions have been observed in Auerbach's plexuses and submucosal Meissner via postganglionic neurons that can trigger the formation of αS and fibrils (Braak et al., 2006). Thus, this aberrant protein could act as a template for the formation of other structures that spread from neuron to neuron in a prion-like manner. In this context, GM could be the intermediary because a dysregulation of host eubiosis leads to an immune reactivity that disrupts the BBB, causing systemic inflammation and neurodegeneration (Jan et al., 2021).

1.4. Lipofuscin aggregates and Parkinson's disease

Aging, as an inherent attribute of the finite cycle of life, induces a progressive disruption of homeostasis, especially molecular abnormalities (Riga et al., 2015), which is why the rise of biomedicine was an imperative stage (Jung et al., 2007). Most studies in the current literature have primarily aimed to delineate the origin of LF versus ceroid (Porta,

2002), the main markers of brain vulnerability, OS and senescence/senility-related pathologies (Riga and Riga, 1995).

Even though they influence a similar spectrum of diseases, a clear differentiation was needed (Seehafer and Pearce, 2006). The characteristic profiles of neuropathological aging are influenced by the concentration of lipopigment which generates a cascade of negative events at the subcellular level. These specific and associated negative consequences of lipopigment accumulation have multiple deleterious effects on neuron and glia homeostasis, from neuronal function to CNS physiology (Riga and Riga, 1995).

Thus, granular aggregates of LF, in parallel with an overexpression of αS seem to be directly involved in the selective degeneration of dopaminergic neurons (Lv et al., 2011). Additionally, overexpression of DMT1 and inhibition of FPN1 is reflected in CNS iron level, further suggesting involvement in selective degeneration of dopaminergic neurons (Lv et al., 2011).

Only those neuronal types containing LF or NM have been shown to show increased susceptibility to pathological changes, including cytoskeletal changes and LB deposits with αS (Braak et al., 2001). αS appears to be a constituent of LF deposits present in SN neurons of PD patients and in nigral neurons of MPTP-treated mice, suggesting that LF deposits may play a role in the selective degeneration of nigral neurons (Braak et al., 2001; Meredith et al., 2002).

In non-dopaminergic regions such as medullary nuclei that are affected in the early phases of PD (Braak et al., 2001) LB and LF granules accumulate (Wellings et al., 2017), also identified in LF stores of medullary neurons (Braak et al., 2003).

Chapter 2. Gastrointestinal symptoms

2.1. Acute manifestations

2.1.1. Acute dysphagia

It is a prevalent symptom in PD with gradual onset and acute or chronic evolution in particular cases. The impact of the food bolus or a foreign body should always be considered, especially if secretory functions are affected, hence the prioritization of performing an endoscopy if acute dysphagia is associated with acute onset of odynophagia (Suttrup and Warnecke, 2016).

2.1.2. Colonic volvulus

It can occur in either the sigmoid colon or the cecum, but rarely causes obstruction of the large intestine and, untreated, progresses to intestinal gangrene. Cecal volvulus is common in young patients, correlated with increased cecal mobility, while sigmoid volvulus is common in septuagenarian men with neuropsychiatric disorders, long-term constipation or colonic dysmotility. Even though the presentation may be (sub)acute, associated with a progressive worsening of abdominal pain, in more than half of the patients an abdominal X-ray or abdominal CT is used to rule out the suspicion of obstruction. Management of colonic volvulus consists of endoscopic reduction if intestinal necrosis is not suspected, although there is a risk of recurrence. In such situations, elective resection after initial endoscopic reduction may be necessary to avoid the risk of recurrence. Otherwise an emergency sigmoid resection is performed if the colon is not viable or perforated (Bauman and Evans, 2018; Gingold and Murrell, 2012).

2.1.3. Fecal impact

One of the many consequences of prolonged constipation is the increased risk of fecal impaction, sometimes SC. A DRE is recommended for all patients. Repercussions of fecal impaction reverberate in the region of the proximal rectum and sigmoid colon, which is why abdominal radiography should be performed if the DRE is negative. Current management strategies include colonic disimpaction if manual or enemas fail (Fernando and Sarma, 2021; Obokhare, 2012).

2.1.4. Acute colonic pseudo-obstruction

Also known as Ogilvie syndrome, ACPO brings together symptoms of colonic obstruction, but with the absence of any mechanical blockage. A number of conditions associated with this condition have been described, including electrolyte disturbance, infections, medication, surgery, and neurological disease. In practice, the diagnosis is established by performing abdominal radiography/abdominal CT to rule out mechanical obstruction. Conservative therapy should be started in the early phase by digestive rest and correction of (hydro)electrolyte imbalances and monitoring for potential intestinal ischemia and perforation. Intravenous neostigmine or the opioid agonist methylnaltrexone is used after failure of supportive treatment, i.e. colonoscopic, surgical or percutaneous decompression if there is no improvement in the patient's condition and in emergency situations (Harnsberger, 2019; Jain and Vargas, 2012).

2.2. Chronic manifestations

2.2.1. Oropharynx and esophagus

2.2.1.1. Sialorrhea

Hypersalivation begins late in PD, probably secondary to a loss of the swallowing reflex and motor impairment of the pharyngeal muscles. Even if they do not promote comorbidities, motor symptoms in PD, hypomimia and camptocormia could exacerbate the secretion. An effective conventional method has been shown to be chewing gum. Side effects on the oral cavity of anticholinergic drugs, glycopyrrolate and atropine should be considered before starting treatment with them. Ipratropium bromide is an alternative because it has low systemic toxicity, but it did not objectively but only subjectively improve sialorrhea, and injections of the parotid or submandibular glands with botulinum toxin are administered only in severe situations (Lakraj et al., 2013; Miller et al., 2019).

Patients with PD also experience oral and dental conditions. They pay minimal attention to oral hygiene in contrast to healthy patients due to decreased dexterity of effective brushing and difficulty opening the jaw. As a result, periodontal and temporomandibular disorders, bruxism, mandibular dislocation, but also BMS are frequently present in PD patients. Sialorrhea could induce changes in pH, and xerostomia influences the self-cleaning mechanisms of the oral cavity (Zlotnik et al., 2015).

2.2.1.2. Dysphagia

Characterized by difficulty in effective initiation of swallowing due to motor symptoms of bradykinesia and motor control of the tongue, dysphagia negatively affects QOL (Keage et al., 2015; Suttrup and Warnecke, 2016). In this context, patients are referred for an oropharyngeal and/or esophageal evaluation guided by history and clinical status because it can potentially cause malnutrition, weight loss, and aspiration pneumonia (Suttrup and Warnecke, 2016). VF is considered to be the gold standard in the assessment of oropharyngeal dysfunction (Martin-Harris and Jones, 2008). LBs have been detected in multiple sites including the esophageal myenteric plexus using HRM systems, barium radiography, but also scintigraphy indicating abnormalities of the esophageal body and LES, but also in the glossopharyngeal nerve and superior internal laryngeal nerve (Bushmann et al., 1989; Potulska et al., 2003; Su et al., 2017; Tanei et al., 2021).

2.2.2. Stomach

2.2.2.1. Gastroparesis

DGE is characterized by a reduction in gastric motility in the absence of mechanical obstruction, symptomatically indicating gastroparesis, with nausea, bloating, vomiting, weight loss, reduced appetite, and early satiety among the most common symptoms. Scintigraphic methods are currently used to measure gastric emptying, of which solid mass

scintigraphy is commonly used to estimate GET, and WMC has been shown to be a reliable alternative that accurately measures GET, SBTT, CTT and WGTT. Treatment is a real challenge because levodopa has been documented as a precursor agent of DGE in a dose-dependent manner, while metoclopramide, domperidone, erythromycin, 5-HT agonists, nizatidine, relamorelin are agents that do not cross the BBB, have not received approval or have been withdrawn, studies are limited or are in the experimental phase. Pyloric sphincter botulinum injection could have a beneficial effect in reducing gastroparesis, as could GES implantation for refractory cases (Camilleri et al., 2018; Parkman et al., 2004; Usai-Satta et al., 2020).

2.2.2.2. Helycobacter pylori infection

Independent of the involvement of *Helycobacter pylori* as a risk factor in gastric cancer and peptic ulcer, chronic systemic inflammation could be a causative factor in PD. It is certain that the presence of this bacterium interferes with current management strategies. Eradication of *Helycobacter pylori* improves not only levodopa absorption but also motor symptoms. This topic is underexplored, which is why two theoretical mechanisms have been proposed to explain the pathophysiological relationship between *Helycobacter pylori* infection and PD. Specifically, it causes a neurotoxic effect by increasing the level of cholesterol glycosides that cause the destruction of dopaminergic neurons (Schulz et al., 2006) or *Helycobacter pylori* crossing the BBB by initiating dopaminergic neuron apoptosis (Dobbs et al., 2008; Kountouras et al., 2012).

2.2.3. The small intestine

2.2.3.1. Bacterial overpopulation of the small intestine

Although small intestinal microflora is an integrative component of GM, bacterial overpopulation has been confirmed in PD patients. This phenomenon is known as SIBO, most likely caused by impaired intestinal motility. SIBO is characterized by a concentration greater than 10^5 CFU/mL and/or the presence of colonic bacteria in the small intestine (Gasbarrini et al., 2007; Losurdo et al., 2020). Prolonged exposure increases intestinal permeability, inducing overstimulation of the innate immune system, but also systemic inflammation. Both mechanisms are involved in the initiation of αS deposits (de Vos and de Vos, 2012; Klingelhoefer and Reichmann, 2015; Visanji et al., 2013). SIBO is diagnosed by jejunal aspirate cultures, but the technique is invasive and reliability is variable (Quigley and Quera, 2006) or HBT (20 - 22%) and glucose (0 - 12.5%) to measure hydrogen or methane concentration (sensitivity; 60 - 70%, specificity; 40 - 80%) (Bohm et al., 2013) produced by *Enterococci*, *Serratia*, *Pseudomonas*, *Streptococcus viridans* or

Staphylococcus aureus (Fasano et al., 2013). Eradication of SIBO by antibiotic administration has been shown to promote an improvement in motor fluctuations, in parallel with an increase in levodopa absorption (Dănău et al., 2021; Losurdo et al., 2020).

2.2.4. Colon

2.2.4.1. Constipation

It is the only recognized and documented symptom that precedes the motor features of PD by more than twenty years before the actual onset (Savica et al., 2009; Schrag et al., 2015), correlated with bowel movements (Gao et al., 2011; Kamada et al., 2013; Savica et al., 2009), but also the severity (Edwards et al., 1993; Lin et al., 2014), the elderly representing the risk category (Mozaffari et al., 2020; Yu et al., 2018). In the absence of a clear diagnostic criterion, the ACG provided a definition in 2005 of constipation (Longstreth et al., 2006). The number of bowel movements (< 3/7 days) is the most used frequency criterion (50%), followed by straining (33% - 83%) (Knudsen et al., 2017), 64% vs. 49% in case of akinetic-rigid and tremor-dominant phenotype (Khedr et al., 2013) in advanced stages or 45% vs. 21% *de novo* (Pont-Sunyer et al., 2015). The risk of PD is three to eleven times higher in individuals with chronic constipation (Yu et al., 2018). Middle-aged men with infrequent bowel movements are four times more likely to develop PD over the next 20 to 25 years, a condition that can cause complications such as volvulus, intestinal perforation, megacolon, and fecal impaction (Bharucha and Lacy, 2020).

2.2.5. The anorect

2.2.5.1. Dyssynergic defecation

Another reason for constipation in PD is dyssynergic defecation. Anorectal function is based on a series of coordinated movements that include relaxation of the internal and external sphincters along with the puborectal muscle. Often, anorectal dysfunction in PD is caused by paradoxical contraction of the puborectal muscle or failure of anal relaxation during exertion. In such conditions, an HRAM must be carried out. An inability to relax the anal sphincters is correlated with constipation and anism, while a decrease in anal tone at rest with faecal incontinence. An MR defecography may be indicated in the case of a rectocele or internal rectal prolapse. Biofeedback is the most recommended treatment strategy in cases of dyssynergic defecation, of no value in patients with colonic inertia, but as an alternative, botulinum injections in the puborectal muscles or in the anal sphincter can be used (Amieva-Balmori and Remes-Troche, 2020; Rao and Patcharatrakul, 2016).

2.2.5.2. Fecal incontinence

Treatment for constipation often leads to faecal incontinence in PD, with low resting anal tone observed in some patients, which explains the increased predisposition following laxative use. HRAM can be applied in this context to diagnose anorectal dysfunction that may precede fecal incontinence. Management strategies include appropriate titration of bulking agents and laxatives, incontinence pads may be needed as a precaution at first titration for chronic constipation, but also biofeedback if the condition is due to decreased sphincter tone or tenesmus (Saldana Ruiz and Kaiser, 2017; Wang and Abbas, 2013).

Chapter 3. Techniques dedicated to microflora reconstruction

3.1. Pro-, pre- and synbiotics

More than two decades have passed since a scientific definition of probiotics was provided with guidelines to ensure the appropriate use of terminology. According to the FAO, in parallel with the directives established by the WHO, this formula is cataloged as "live microorganisms that, administered in adequate doses, enhance the general state of health of the host", from which they derived two other terms - prebiotics and synbiotics. Prebiotics, on the other hand, are supplements that have been defined as "a non-viable food component that confers host health benefits associated with microbiota modulation". Synbiotics are a mixture of the two previously mentioned categories, usually administered in order to increase the lifespan of those already existing in the digestive tract (Sanders et al., 2019).

In the last half decade, a limited number of studies have been conducted with the aim of exploring the impact of GM in the prodromal and early stages of PD (Mertsalmi et al., 2017; Scheperjans, 2018). Fortunately, the most potent vehicle used in current clinical practice to alleviate PD-related symptomatology and associated causal or non-causal comorbidities are probiotics (Tan et al., 2021a).

3.2. Faecal microbiota transplantation and microbial transfer therapy

FMT is another technique dedicated to GM reconstruction. It is a relatively simple procedure that involves the transfer of stool samples from a healthy donor to a patient for cases of CDI, IBD and IBS (Khoruts and Sadowsky, 2016). MTT is a similar protocol with substantial utility and potential in clinical practice. There have been controversies

surrounding this topic because the inter-individual microbial composition is similar in small percentages, hence the need to implement personalized strategies (Khoruts and Sadowsky, 2016).

There are also LBPs that, unlike FMT, are less diverse and contain a reduced number of bacterial species, but superior to the use of conventional probiotics that gather only a few microorganisms (Paquet et al., 2021). LBPs are defined as "biological products containing living organisms, used to prevent, treat and cure a disease or condition in human patients".

Chapter 4. Experimental induction of Parkinson's disease

4.1. Overview

The zebrafish (*Danio rerio*) is a tropical species that was identified in the Kosi River in Bengal and first described in 1822 by Francis Buchanan Hamilton (1762 - 1829) (Arunachalam et al., 2013; McCluskey and Postlethwait, 2015). Etiologically, *Danio* originates from the Bengali "dhani" which can be translated as "those of the rice field" (Rainboth, 1994), ensuring continuity as a food source for species of *Channa*, *Notopterus notopterus*, *Ardeola grayii*, *Alcedo atthis* (Spence et al., 2008).

Physiological homology with that of human individuals, short replication time and low cost of reproduction in contrast to other experimental models, and feasibility in pharmaceutical studies are just a few advantages for using zebrafish (Vaz et al., 2018).

4.2. Rotenone, dopaminergic agonist?

This tasteless, odorless and crystalline isoflavone isolated by Emmanuel Geoffroy (1862 - 1894), is one of the earliest natural compounds identified in plants; *Robinia nicou*, currently *Lonchocarpus nicou* (nicoline) (Turner, 1932) and used in several products marketed for crops as an insecticide, pesticide or piscicide (Robea et al., 2020). It can be extracted from the leaves, seeds, and stem of the Mexican turnip (*Pachyrhizus erosus*), known as the Jicama vine, and from the roots of the *Fabaceae* family - the genera *Derris*, *Lonchocarpus*, *Tephrosia*, and *Mundulea* (Gupta, 2012; Lawana and Cannon, 2020). In addition to *Lonchocarpus utilis* and *Nolina lindheimeriana*, native to South and North America, *Lonchocarpus nicou* and *Derris elliptica* are candidate species for rotenone

extraction (Gupta, 2012; Lawana and Cannon, 2020), with FIFRA recording rotenone in 1947 (Gupta, 2007).

Although the implications in small amounts are minimal, in larger doses it can become toxic. Compared to incomplete GI absorption in humans and rodents, it is complete in fish due to the absence of degrading enzymes (Gupta, 2012). Thus, it was possible to establish a causal relationship between rotenone administration and PD in zebrafish (Razali et al., 2021), rodents (Innos and Hickey, 2021) and humans (Tanner et al., 2011).

Rotenone has been shown to be a dopaminergic agonist that directly crosses the BBB and enters the CNS by continuous accumulation in cell organelles, predominantly in mitochondria due to its lipophilic structure (Bové et al., 2005; Robea et al., 2020; Sherer et al., 2003). Rotenone induces neuronal toxicity (Bastías-Candia et al., 2019), leading to a decrease in ATP generation and exacerbation of ROS generation by inhibiting complex I of the ETC (Yurtsever et al., 2020). Thus, rotenone causes microglia activation, reflected by neuroinflammation (Gao et al., 2013) and αS aggregation known for its involvement in LB pathology (Hijaz and Volpicelli-Daley, 2020).

EXPERIMENTAL PART

Chapter 5. Research materials and methods

5.1. Experimental model

The experimental studies within the present research concerned zebrafish (*Danio rerio* - Hamilton, 1822), adults (6 - 8 months), WT, genetic line AB, purchased from a local authorized breeder in Iași county.

The individuals were kept for 14 days in a 90L aquarium equipped with a thermometer and water recirculation system, fed with dechlorinated system water, stage preceding the acclimatization phase.

The zebrafish were transferred at the end of the acclimatization period to new 10L aquaria for another 7 days in order to adapt to the new experimental conditions. They were housed in the same aquaria for the entire behavioral analysis period.

The temperature in the laboratory was kept constant at 26 ± 2°C, pH 7.5 and a 14h light/10h dark cycle (Reed and Jennings, 2011). The regimen consisted of TetraMin Flakes twice a day, and the water in each experimental tank was changed daily.

The animals were maintained and treated in accordance with the Recommendation of the EU Commission (2007), Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010, normative act relating to guidelines for the housing, care and protection of animals used in experimental purposes (Commission Recommendation, 2007; Directive 63, 2010). The experiments were carried out in accordance with the Helsinki Declaration, but also with Romanian and EU legislation regarding the use of animals in biomedical research. All procedures were performed by limiting to as few individuals as possible according to the ARRIVE guidelines (Kilkenny et al., 2010).

5.2. Behavioral typologies

Although zebrafish behavior has become an increasingly studied attribute in biomedical research (Klee et al., 2011; Stewart et al., 2011; Suriyampola et al., 2015), there is very little data on the natural behavior of WT.

Because animal behavior is complex and provides responses to internal (physiological and/or biochemical) and external (social or environmental) reactions, behavioral objectives are more sensitive and rapid compared to classical toxicological methods that assess mortality, reproduction and growth (Melvin and Wilson, 2013). In this way, behavioral changes as effects of contaminants can be quantified shortly after exposure even if the concentration is low (Huang et al., 2014).

5.2.1. Locomotion

Motor activities are largely engaged in the spinal cord and hindbrain, segmented structures that possess a relatively small number of identifiable sets of neurons in the embryonic stage. Many types of neurons, but also the two types of muscle cells have been classified according to their morphology, molecular signals for cell differentiation have recently been identified and mutations that affect cell development have been isolated (Bae et al., 2009).

Motor behaviors in the embryonic stage occur sequentially and involve an early phase characterized by transient, spontaneous contractions. Subsequently, twitching responses to touch are observed, which are crystallized by the development of swimming ability. Spiral contractions are generated by an electrically coupled network of a set of

spinal neurons while a glutamatergic and glycinergic synaptic unit constitutes the backbone underlying touch and swimming responses. Sustained swimming in the larval stage occurs with the development of the neuromodulatory serotonergic system (Drapeau et al., 2002).

5.2.2. Aggression

Aggressiveness is an important component on which the degree of adaptability is based, including hierarchy, feeding and reproduction. As expected, zebrafish engage in territorial battles based on habitat structure, preferring rich vegetation that reduces visual space and thus provides them with protection and shelter from predators (Spence et al., 2008).

Aggressive behavior is frequently encountered in particular circumstances such as the spawning period, disruption of the nyctemeral cycle, but compensated when the fish form peaceful schools (Spence and Smith, 2005). Territorial aggression is a normal feature of aggressive behavior during the spawning period where biting can often be observed which can cause scale loss (Ruhl et al., 2009).

5.2.3. Sociability

Another argument in favor of the role of zebrafish in biomedical research is due to the wide repertoire it benefits from, especially behavioral patterns (Kalueff et al., 2013; Vaz et al., 2018). They are intrinsically gregarious species that live in shoals, which is why they display a varied palette of social behaviors.

5.3. The phenotype associated with Parkinson's disease

In order to deepen the spectrum of knowledge through animal models, PD-specific symptoms were artificially induced by chronic rotenone administration based on predetermined doses and exposure intervals.

5.3.1. The influence of probiotic lactic strains on sociability and locomotion

In the first study, we wanted to establish the possible beneficial role of the probiotic lactic strains *Bifidobacterium longum* BB536 - 4 x 10^9 CFU (150 mg) and *Lactobacillus rhamnosus* HN001 - 1 x 10^9 CFU (25 mg) and vitamin B6 - 1.4 mg on social behavior and locomotor activity in the T-maze. Performances were recorded daily using the 2D camera. Subjects were chronically exposed to 2 µg/L rotenone for 21 days.

5.3.1.1. The experimental plan

A total of 60 individuals divided into four equal experimental groups (n = 15) were used.

5.3.2. The influence of valproic acid, the combination of levodopa and carbidopa and probiotic lactic strains on sociability and aggression states

In the second study we evaluated how valproic acid (0.5 mg/mL), levodopa + carbidopa (250 mg + 25 mg) and probiotics (3 g - *Lactobacillus casei* W56, *Lactobacillus acidophilus* W22, *Lactobacillus paracasei* W20, *Lactobacillus salivarius* W24, *Lactobacillus lactis* W19, *Lactobacillus plantarum* W62, *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52 and *Bifidobacterium bifidum* W23) could exacerbate/decrease the neurotoxicological effect of rotenone after chronic exposure for 32 days at 2.5 µg/L. The primary objectives were to evaluate changes in social behavior and/or aggression states in the T-maze using counter clockwise rotation as a neurological indicator. Performances were recorded at 4-day intervals to avoid chronic stress. We used the 2D camera for the evaluation of (anti)social behavior, respectively the 3D for counter clockwise rotation.

5.3.2.1. The experimental plan

A total of 40 individuals divided into eight equal experimental groups (n = 5) were used.

5.3.3. The influence of valproic acid, the combination of levodopa and carbidopa and probiotic lactic strains on locomotion

In the last study we looked at how valproic acid (0.5 mg/mL), levodopa + carbidopa (250 mg + 25 mg) and probiotics (3 g - *Lactobacillus casei* W56, *Lactobacillus acidophilus* W22, *Lactobacillus paracasei* W20, *Lactobacillus salivarius* W24, *Lactobacillus lactis* W19, *Lactobacillus plantarum* W62, *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52 and *Bifidobacterium bifidum* W23) could act as GBA axis modulators by assessing locomotor activity in 3D after chronic exposure to 2.5 µg/L rotenone for 32 days. Performances were recorded at 4-day intervals to avoid chronic stress.

5.3.3.1. The experimental plan

A total of 120 individuals divided into eight equal experimental groups (n = 15) were used.

5.4. Ant(agonists) used

5.4.1. Rotenone administration

Rotenone (5 g) was purchased from Toronto Research Chemicals, North York, Canada, (Cat # R700580) as a white powder.

5.4.2. Valproic acid administration

Valproic acid (100 g) was purchased from Sigma-Aldrich, Saint Louis, Missouri, USA, (#SLBC9758V), having the appearance of a white powder.

5.4.3. Levodopa and carbidopa administration

Isicom - ((250 mg levodopa + 25 mg carbidopa - 10 pills/blister x 3) - Desitin Arzneimittel GmbH, Hamburg, Germany) was purchased from a local pharmacy in tablet form.

5.4.4. Probiotics administration

Zircombi (ALFASIGMAS.p.A.) is a dietary supplement having the appearance of a white powder that was purchased from a local pharmacy. It contains two strains - *Bifidobacterium longum* BB536 - 4 x 10^9 CFU (150 mg) and *Lactobacillus rhamnosus* HN001 - 1 x 10^9 CFU (25 mg) and vitamin B6 - 1.4 mg.

OMNi BIOTIC STRESS Repair- Institut Allergosan Pharmazeutische Produkte Forschungs- und Vertriebs GmbH, Graz, Austria (3 g) was purchased from a local pharmacy. It contains the following strains: *Lactobacillus casei* W56, *Lactobacillus acidophilus* W22, *Lactobacillus paracasei* W20, *Lactobacillus salivarius* W24, *Lactobacillus lactis* W19, *Lactobacillus plantarum* W62, *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52 and *Bifidobacterium bifidum* W23.

5.5. Justification of administered concentrations

5.5.1. Rotenone

We performed a series of preliminary tests before the actual implementation of the protocol in which up to 5 subjects per tank were exposed to three different doses (from 2 µg/L, 2.5 µg/L to 5 µg/L) for 24h, up to 72h and we concluded that 2.5 µg/L might be the optimal dose because 5 µg/L causes excessive mortality (data not shown) (Ilie et al., 2022a, 2022b).

5.5.2. Valproic acid

A similar approach was applied to valproic acid where we tested four doses (0.5 mg/mL, 2 mg/mL, 5 mg/mL and 10 mg/mL). Concentrations of 5 mg/mL and 10 mg/mL valproic acid resulted in excessive mortality, while at 2 mg/mL zebrafish showed episodes of immobility to touch (data not shown) during the first 6h - 12h after administration. Based on these considerations, we managed to keep the survival rate constant among the

subjects throughout the analyzed period by administering 0.5 mg/mL (Ilie et al., 2022a, 2022b).

5.5.3. Levodopa and carbidopa

Regarding the associated dose of levodopa and carbidopa, we used the study by (Idalencio et al., 2021) as computer support.

5.5.4. Lactic probiotic strains

There are no restrictions on the bacterial dose and ratio, these being customized according to the study concept, the strains pooled in the product used if it is a commercially available one, and CFU rather than gender and species.

5.6. Behavioral testing

5.6.1. The (anti)social interaction test, aggression and locomotion

Behavioral readings were performed in a multipurpose cross-maze enclosed by a clear Plexiglas slot and converted to a T-maze filled with dechlorinated system water (5 cm).

The duration of each test was 4 min per individual with a 30 sec acclimatization period in a 10L aquarium recreating the usual conditions, but filled to half of the total volume (5L). Images were recorded with a professional infrared camera placed above the experimental chamber connected to a computer and analyzed using EthoVision XT 11.5 software (Noldus Information Technology, Wageningen, The Netherlands) previously calibrated for this type of tests.

5.6.2. Locomotor activity test

Analysis of locomotor activity was also performed in 3D using a 10L aquarium recreating the usual conditions that was filled with 6L of system dechlorinated water.

The duration of each trial was 4 min per individual with a 30 sec habituation period, images that were recorded with a professional infrared camera placed above and to the side of the experimental chamber connected to a computer and analyzed using the Track3D module of the tracking software video EthoVisionXT 14 (Noldus Information Technology, Wageningen, The Netherlands).

5.7. Euthanasia, sample collection and distribution

Animals were euthanized according to standard procedures by immersion in ice water at 2 - 4°C for 10 min until the disappearance of opercular movements.

Five samples were aliquoted for each subsequent analysis in 1.5 mL Eppendorf tubes and stored at -20°C.

5.8. Determination of oxidative stress biomarkers

5.8.1. Determination of superoxide dismutase activity

The SOD determination kit (19160-1KT-F) was purchased from Sigma-Aldrich, Saint Louis, Missouri, USA and used following the manufacturer's instructions. The wavelength at which the readings were taken was 450 nm.

5.8.2. Determination of glutathione peroxidase activity

The GPx determination kit (CGP1-1KT) was purchased from Sigma-Aldrich, Saint Louis, Missouri, USA and used following the manufacturer's instructions. The wavelength at which readings were taken was 340 nm.

5.8.3. Determination of malondialdehyde level

The level of MDA was determined according to the method described by (Ciobica et al., 2012). The wavelength at which the readings were taken was 532 nm.

5.8.4. Bradford method for quantification of soluble proteins

Analogous to the technique for obtaining the homogenate, this protocol is based on the dosage of the protein content according to the method described by (Bradford, 1976) and adapted by (Artenie, 2008).

5.9. Real-time polymerase chain reaction

Total brain RNA was extracted using peqGOLD TriFast (Peqlab Biotechnologie, Erlangen, Germany) and reverse transcription in one step with GoTaq 1-Step RT-qPCR (Madison, Wisconsin, USA) following the manufacturer's instructions.

Reactions were performed on the AriaMx Real-Time PCR System (Santa Clara, California, USA).

The sequences used (IDTDNA, San Diego, California, USA) are as follows: *PINK 1* (NM_001008628.1) (2088 bp) forward: 5'-GGCAATGAAGATGATGTGGAAC-3', *PINK 1* reverse: 5'-TTGTGGGCATGAAGGAACTAAC-3', *PARKIN* (NM_001017635.1) (1465 bp) forward: 5'-GAGGAGTTTCACGAGGGTCC-3', *PARKIN* reverse: 5'-TGAGTGGTTTTGGTGATGGTC-3', *LRRK2* (NM_001201456.2) (9170 bp) forward: 5'-ACTCGGATTAAGTTCCCACCAGA-3', *LRRK2* reverse: 5'-CAGTGAGGGTTGATGGTCTGTA-3', *alpha-SNCA* (NM_001017567.2) (1294 bp) forward: 5'-ATGCACTGAAGAAGGGATTCTC-3', *alpha-SNCA* reverse: 5'-

AGATTTGCCTGGTCAGTTGTTT-3' and *ACTIN* (NM_181601.5) (1843 bp) forward: 5'-GGCATCACACCTTCTACAATGA-3', *ACTIN* reverse: 5'-TACGACCAGAAGCGTACAGAGA-3' serving as reference gene.

We also included a melting curve to increase specificity in the event of primer dimers. The amplification protocol was as follows: RT: 20:00 min at 38°C (1 cycle), hot start: 10:00 min at 95°C (1 cycle), amplification: 00:10 sec at 95°C , 00:30 sec at x°C (gene dependent), 00:30 sec at 72°C (40 cycles), melting curve: 00:30 sec at x°C (gene dependent) x 2 and 00:30 sec at 95°C (1 cycle); *ACTIN*, *PINK1* and *alpha-SNCA* at 55°C, *LRRK2* at 57.5°C, and *PARKIN* at 58.5°C.

Relative expression was determined using the formula $2^{-\Delta\Delta Ct}$, with final results normalized to *ACTIN*.

5.10. Determination of dopamine levels

DA in brain samples was measured by ELISA using the Fish Dopamine kit (antibodies, Aachen, Germany) according to the manufacturer's instructions. Readings were performed using a Tecan Sunrise (Tecan, Crailsheim, Germany) at a wavelength of 450 nm.

Calculations using standards were performed by applying a polynomial regression with the formula: y = a + bx + c/x^2.

5.11. Histological and immunohistological analysis

Organ samples were fixed in Bouin's solution for 24h, and sections approximately 0.5 cm thick were dehydrated in a solution of decreasing concentration of ethanol, then cleared in xylene and embedded in paraffin. From the microtome sections, ten microscope slides were selected from each paraffin block, specifically stained and read under an Olympus CX41 microscope (Olympus Europa SE & Co, Wendenstraße, Hamburg, Germany).

These were initially stained with HE then IHC using 5 antibodies; GFAP, S100B, PCNA, p53 and cox4i1, were used to perform IHC staining. After sections were deparaffinized in xylene, hydrated in ethanol, and microwaved for 10 min at 95°C in 10 mmol citric acid buffer pH 6, they were cooled for 20 min, then washed twice in PBS for 5 min. Sections were treated with 3% hydrogen peroxide and rinsed with PBS, after which they were incubated overnight at 4°C in a humid atmosphere with primary antibodies at 1:1000 dilutions GFAP and S100b; 1:250 for PCNA, p53 and cox4i1. Slides were washed

3 times in PBS for 5 min, being incubated with secondary antibodies. Secondary goat anti-rabbit IgG was used for GFAP, S100b, p53 and PCNA. Microscope slides were developed in DAB and finally counterstained with hematoxylin.

5.12. Statistical analysis

Microsoft Excel 2010 spreadsheet software (Microsoft Corporation, Redmond, Washington, USA) was used to edit, sort and code the raw data which were subsequently tested for normality and distribution by the Shapiro-Wilk test using either the Graph software Pad Prism (v 9.1.0.221, San Diego, California, USA) or OriginPro (v 9.3-2016, OriginLab Corporation, Northampton, Massachusetts, USA). Comparisons between groups or between test days were performed by one-way ANOVA followed by post-hoc parametric tests such as Tukey HSD or Dunnett. Data are expressed as mean ± SEM. The p value < 0.05 was considered statistically significant.

Chapter 6. Results

6.1. The social interaction test

6.1.1. Exposure to 2 µg/L rotenone

No significant changes were observed for the CONTROL and ROT group ($p > 0.05$ ANOVA). Single administration in the ROT and PROBIO groups had no effect on zebrafish sociability after 21 days of chronic exposure to 2 µg/L rotenone as can be seen in Figure 6.1. Moreover, a similar effect was observed in the ROT + PROBIO group, except for D_19 compared to the pretreatment period (182.5 ± 16.5 sec vs. 56.2 ± 19.8 sec, $p = 0.01$ Tukey, ANOVA).

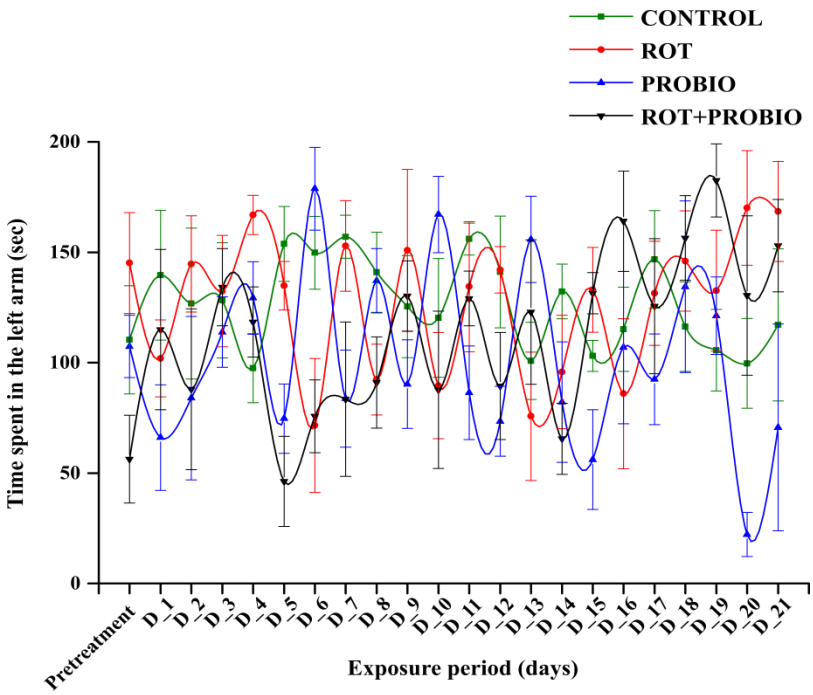


Figure 6.1. Time spent in the left arm in the social interaction test (n = 15). The mean of the groups was compared with the mean of pretreatment days and the results were represented as mean ± SEM

6.2. Anti-social behavior test

6.2.1. Exposure to 2.5 µg/L rotenone

Statistically significant differences were observed on separate days after centralization and analyzing the data for time spent in the right and center arms. Thus, group (b) supplemented only with VPA showed a preference for the right arm in D_1 - p = 0.006 and D_8 - p = 0.022, while group (c) given LEV/CARB only in D_12 - p = 0.008. Regarding group (e) ROT and group (f) ROT + VPA, zebrafish show antisocial behavior in D_1 - p = 0.002 and D_4 - p = 0.011. Exploratory capacity was somewhat influenced as the behavior corresponded to an anxiety state in D_4 - p = 0.004, D_20 - p = 0.002, D_28 - p = 0.003, D_32 - p = 0.048 in (b) VPA, and in D_4 - p = 0.028 , D_24 - p = 0.021, D_28 and D_32 $p < 0.001$ in group (c) LEV/CARB. Groups (f) ROT + VPA and (g) ROT + LEV/CARB were the only ones compared to (e) ROT and (h) ROT + PROBIO in which there were noticeable changes; D_12 - p = 0.041, D_16 - p = 0.005 in (e) (ROT) and D_8 - p = 0.008, D_24 - p = 0.033 in (f) ROT + VPA. Interesting is the lack of efficacy of the lactic strains administered in groups (d) PROBIO and (h) ROT + PROBIO ($p > 0.05$), but also in group (g) ROT + LEV/CARB. The (a) CONTROL group maintained a linear trend throughout the experiment (Figure 6.2).

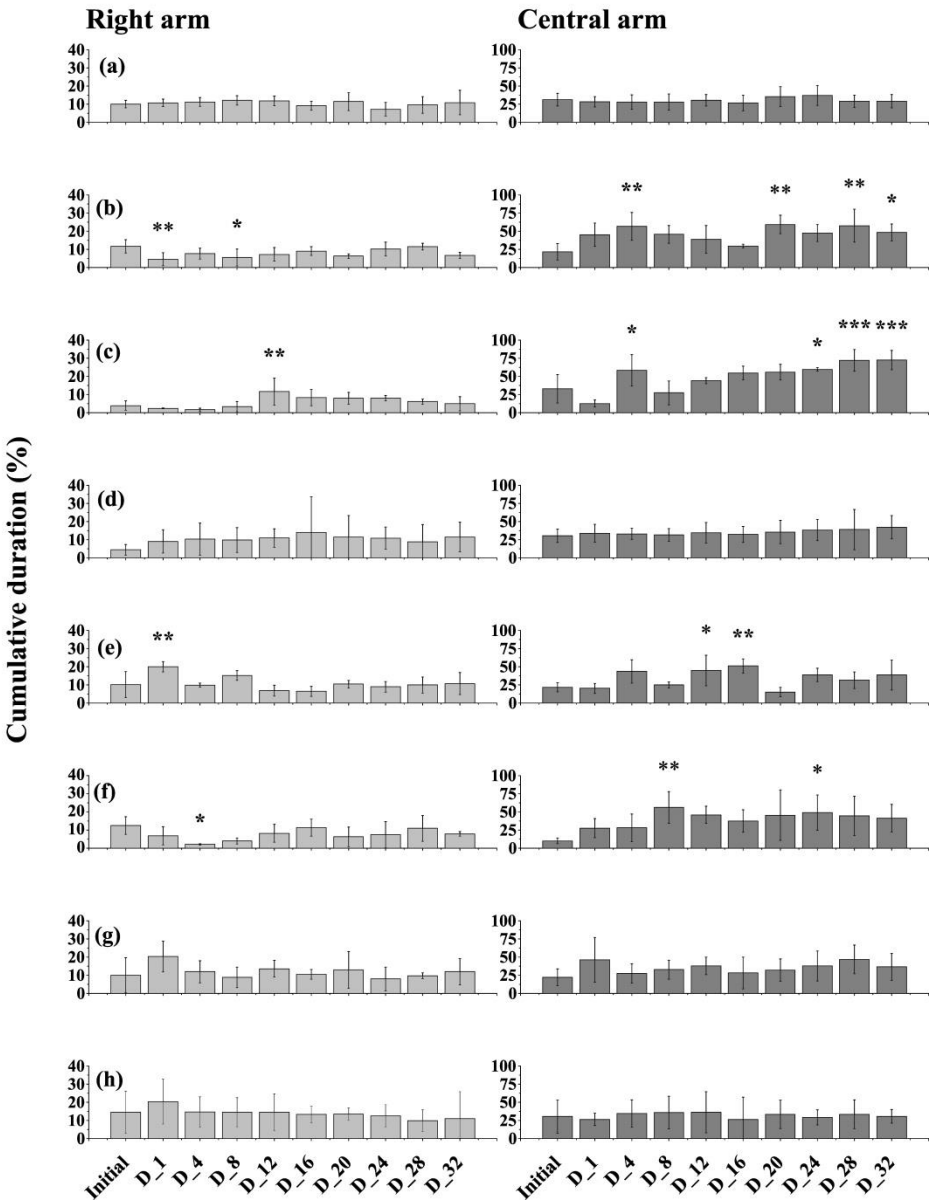


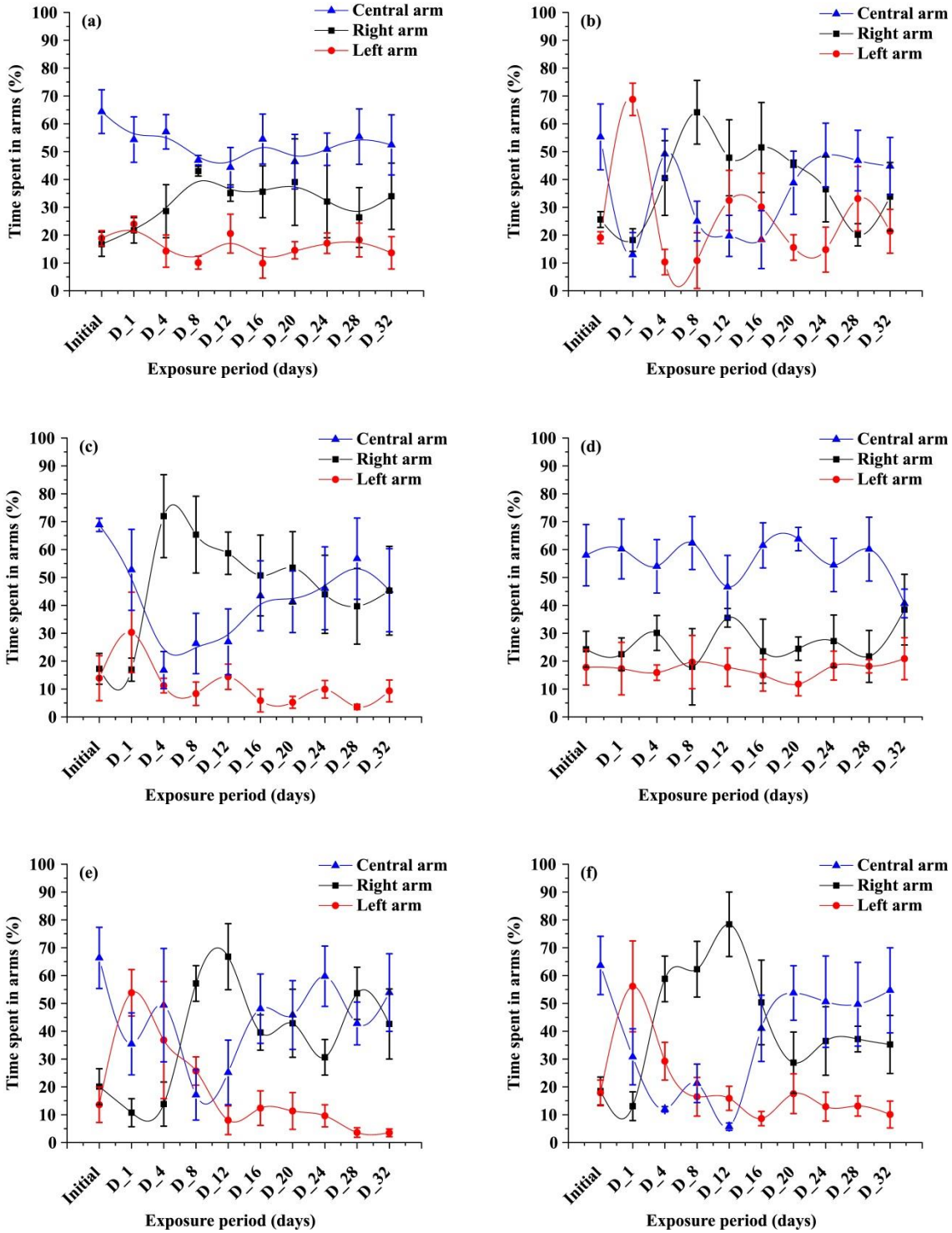
Figure 6.2. Antisocial pattern in *Danio rerio* (n = 5) and their trends towards both arms (values expressed as mean with SEM followed by Dunnett's test; * - $p < 0.05$, ** - $p < 0.005$, * - $p < 0.0005$)**

6.3. The mirror test

6.3.1. Exposure to 2.5 µg/L rotenone

Compared to the pre-treatment period, even in the (a) CONTROL group a deducible phenotype was observed based on the test performed. There was no statistically significant difference ($p > 0.05$) between baseline behavior and time spent in the left arm. Analogous for the right and central arm to (a) CONTROL, but also to those in (b) VPA, (c) LEV/CARB, (d) PROBIO or in combination with ROT (e - h), specific patterns of aggression being recorded ($p < 0.05$, 0.005 and 0.001) either relative to baseline or between different days. It should be noted, however, the lack of significance in the (d)

PROBIO group ($p > 0.05$) regarding the time spent in the left arm, but also by comparison with the pretreatment ($p > 0.05$) in the other two arms. It can be concluded that the administered probiotics really had a beneficial effect, an argument that is not valid in the case of group (h) ROT + PROBIO (Figure 6.3).



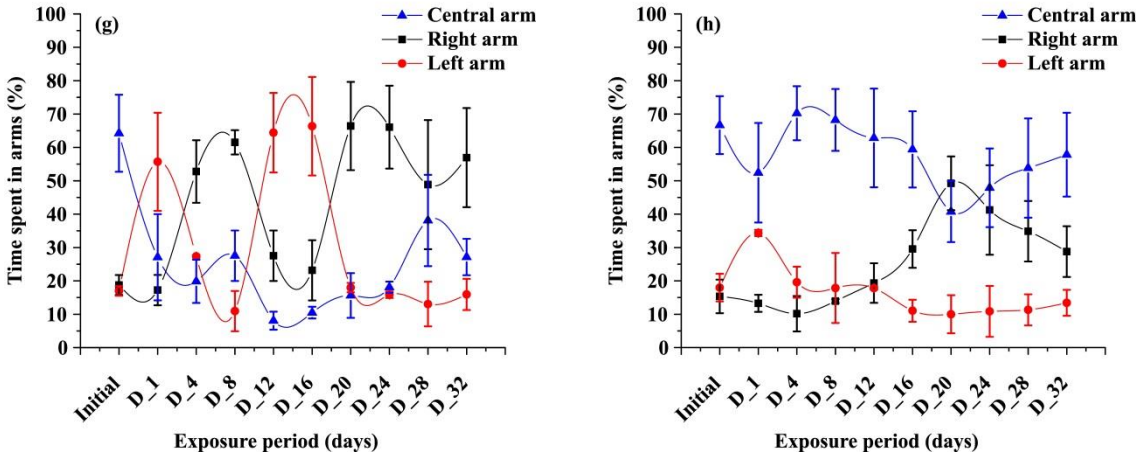


Figure 6.3. Aggressive type patterns in *Danio rerio* (n = 5) and their trends towards all three arms (values expressed as mean with SEM followed by Dunnett's test)

6.4. Assessment of locomotor activity

6.4.1. Exposure to 2 µg/L rotenone

6.4.1.1. Total distance swum

Contrary to our expectations and contrary to existing literature, no significant changes were observed following rotenone or probiotic administration after 21 days according to our parameters of interest; total distance swum (cm), average speed (cm/sec) and active state (sec).

More precisely, in the CONTROL group per total distance swum (cm) there were particular situations where zebrafish reached maximum points during the experimental period in contrast to the pretreatment days: D_10 (1240.03 ± 169.9 cm, p = 0 .01 Tukey, ANOVA), D_13 (1209.58 ± 71.7 cm, p = 0.02 Tukey, ANOVA) and D_15 (1220.13 ± 104.2 cm, p = 0.02 Tukey, ANOVA) vs. 620.7 ± 127.8 cm. The ROT group was not significantly influenced, statistically significant differences being noted in D_6 (1369.2 ± 255.8 cm, p = 0.008 Tukey, ANOVA) and D_14 (1642.9 ± 165.1 cm, p = 0.002 Tukey, ANOVA). Moreover, in the PROBIO group an increase in swimming distance was observed in the first days of treatment with the highest value recorded in D_5 (1626.4 ± 138.2 cm, p = 0.006 Tukey, ANOVA). Regarding the ROT + PROBIO group, the values obtained for this parameter were not significant (p > 0.05 ANOVA) (Figure 6.4).

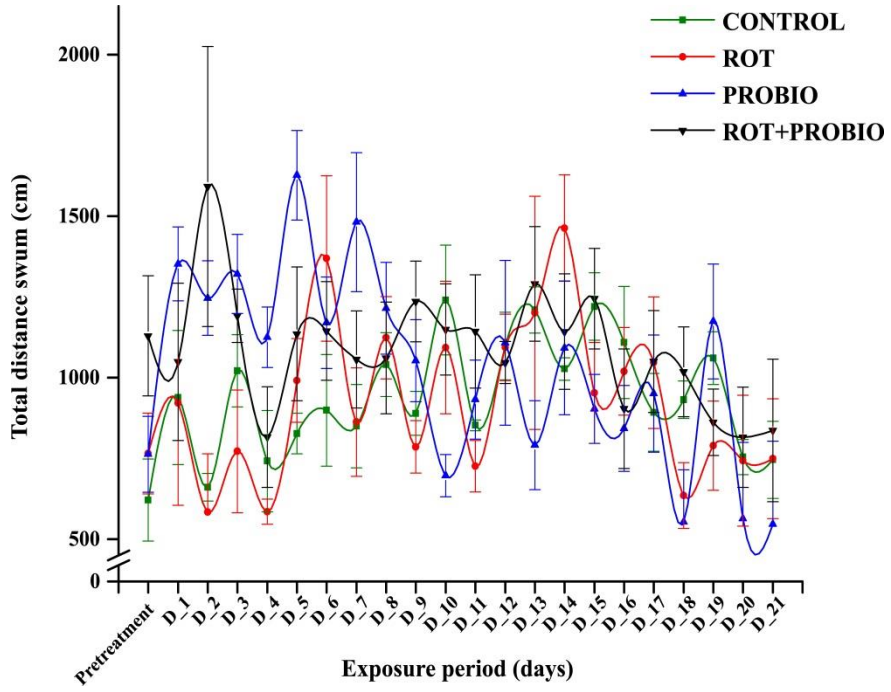


Figure 6.4. Total distance swum during the locomotor activity test (n = 15). The mean of the groups was compared with the mean of pretreatment days and the results were represented as mean ± SEM

6.4.1.2. Velocity

Analogous to the above parameter, no significant changes were observed for the CONTROL, ROT and ROT + PROBIO group regarding the speed parameter (cm/sec) (p > 0.05 ANOVA). The trend was also similar for the PROBIO group, except for a pattern of hyperlocomotion in D_5 (6.77 ± 0.57 cm/sec, p = 0.005 Tukey, ANOVA) compared to pretreatment: 3.18 ± 0.42 cm/sec (Figure 6.5).

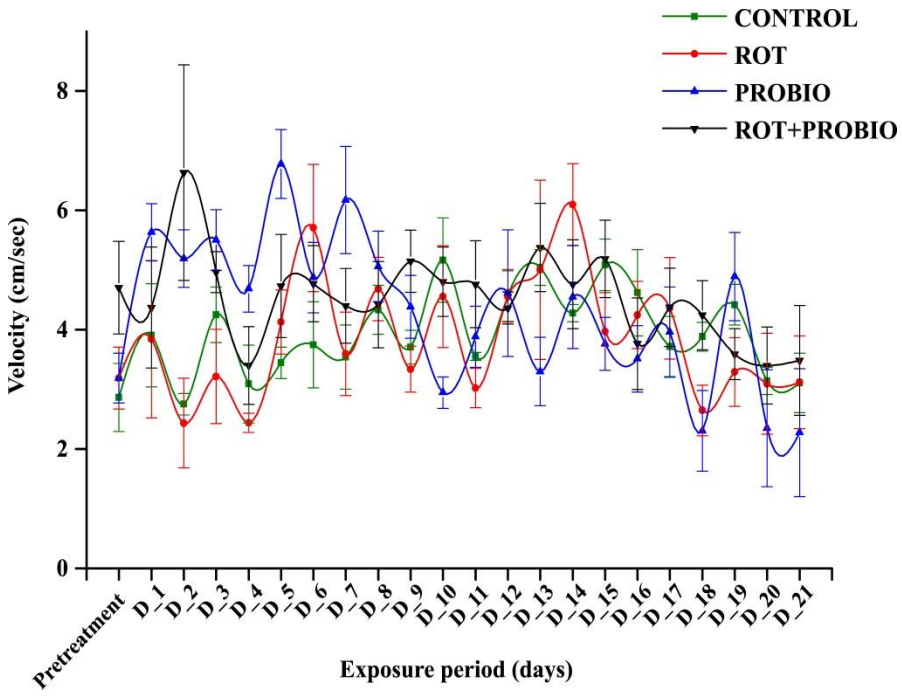


Figure 6.5. Velocity during locomotor activity test (n = 15). The mean of the groups was compared with the mean of pretreatment days and the results were represented as mean ± SEM

6.4.1.3. Active status

We also determined active status (sec) which measures the time spent by active fish during the session. On the pretreatment days the fish in the CONTROL group showed a decrease in movement time, but the activity throughout the experimental period followed a constant trend. The ROT group recorded increases in time spent moving as D_6 (224.8 ± 10.8 sec, p = 0.03 Tukey, ANOVA), D_8 (228.8 ± 5.22 sec, p = 0.02 Tukey, ANOVA), D_12 (227.2 ± 3.52 sec, p = 0.02 Tukey, ANOVA), D_14 (236.3 ± 1.50 sec, p = 0.007 Tukey, ANOVA), D_16 (232.1 ± 3 .78 sec, p = 0.01 Tukey, ANOVA) and D_17 (223.5 ± 4.55 sec, p = 0.04 Tukey, ANOVA) compared to 139.8 ± 12.7 sec at pretreatment. Regarding the activity for the PROBIO group, the activity was similar to those observed for the parameters mentioned above; increase in the first part of the administration, and then it is fluctuating. No significant changes were observed in the ROT + PROBIO group (p > 0.05 ANOVA) (Figure 6.6).

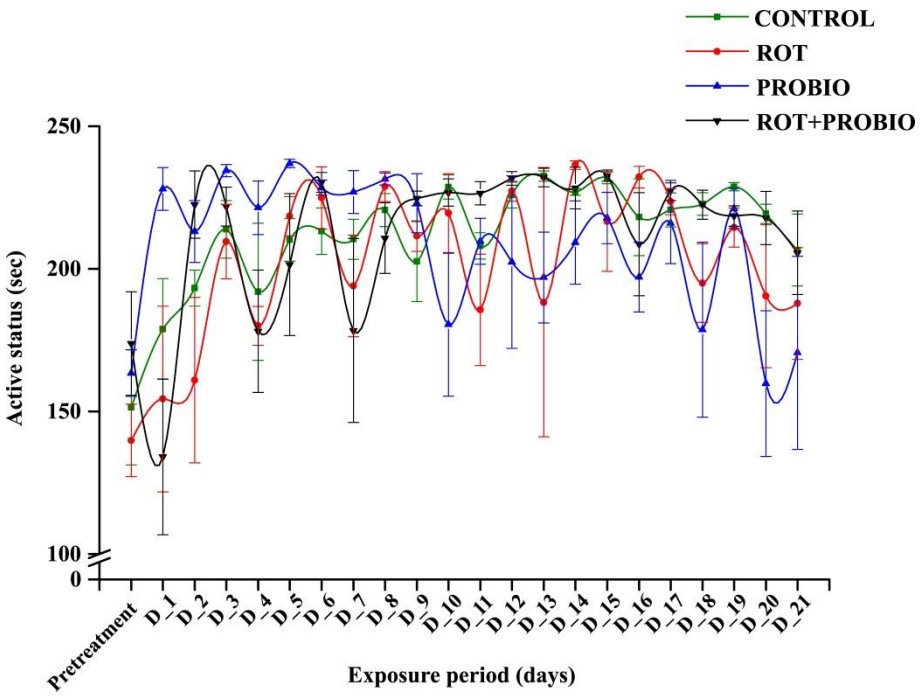


Figure 6.6. Active status during the locomotor activity test (n = 15). The mean of the groups was compared with the mean of pretreatment days and the results were represented as mean ± SEM

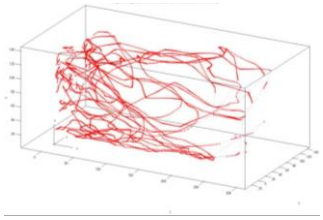
6.4.2. Exposure to 2.5 µg/L rotenone

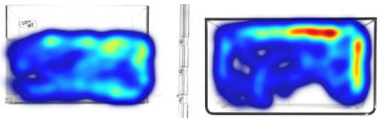
In order to evaluate the neurotoxicological effect of rotenone after 32 days of chronic administration and the possible neuroactive effect of valproic acid, the combination of levodopa and carbidopa and probiotics, we performed the 3D locomotor activity test. Spatial conformations can be observed following the reconstruction of 3D

swimming paths (Figure 6.7). The analyzed parameters of interest such as swimming distance (mm), speed (mm/sec) and freezing episodes (sec) are detailed in Figure 6.8 - Figure 6.10.

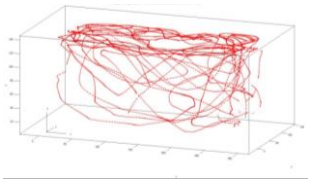
As expected, in the four groups not exposed to rotenone (a - d), zebrafish maintain their exploratory behavior throughout the analyzed period, with slight phenotypic changes in (b) VPA and (c) LEV/CARB defined by “touching the surface of the water” indicating the possible neuroactive potential of these two agents, while in group (d) PROBIO a similar behavioral pattern to (a) CONTROL can be observed. On the other hand, groups exposed to rotenone (e - h) show an affinity for the bottom of the aquarium, as seen in (e) ROT and (g) ROT + LEV/CARB. Moreover, the swimming patterns in (f) ROT + VPA and (h) ROT + PROBIO are antithetical in contrast to (e) ROT and (g) ROT + LEV/CARB.

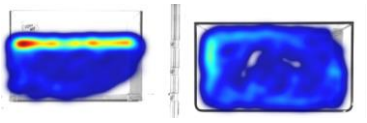
(a) CONTROL



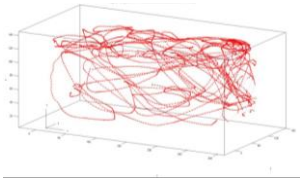


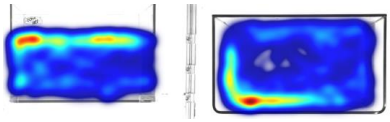
(b) VPA



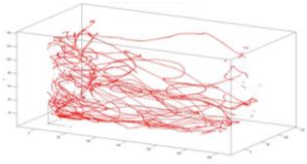


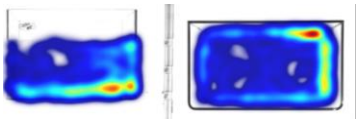
(c) LEV/CARB



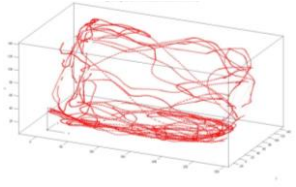


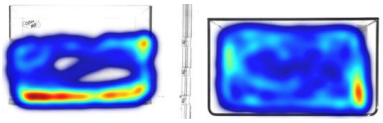
(d) PROBIO





(e) ROT





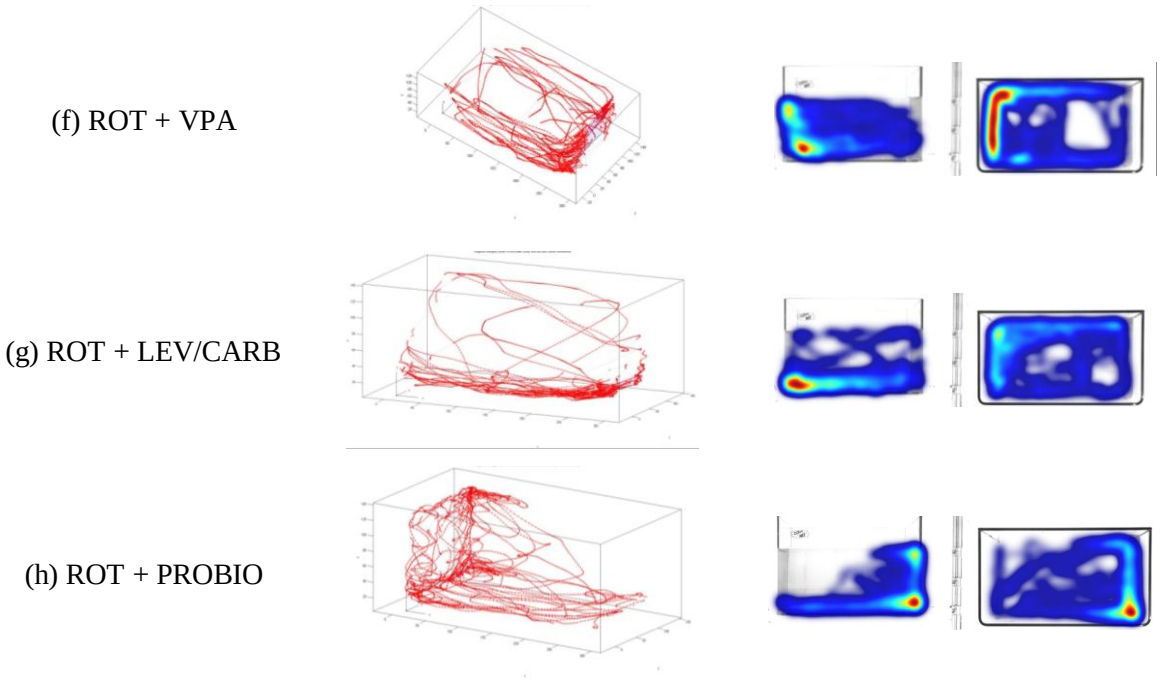


Figure 6.7. Results of 3D swimming tracks for *Danio rerio* (n = 5) in (a) CONTROL and groups treated with (b) VPA - 0.5 mg/mL, (c) LEV/CARB - 250 mg + 25 mg, (d) PROBIO - 3 g, (e) ROT - 2.5 µg/L, (f) ROT + VPA - 2.5 µg/L + 0.5 mg/mL, (g) ROT + LEV/CARB - 2, 5 µg/L + 250 mg LEV + 25 mg CARB and (h) ROT + PROBIO - 2.5 µg/L + 3 g. An automatic integration using Track3D software results in 3D swimming tracks reflected in red, right panel showing the vertical (X, Y) and lateral (Y, Z) image in the 240 sec test

6.4.2.1. Distance swum

Regarding the swimming distance parameter (mm) recorded, significant differences appear between (a) CONTROL and several experimental groups: compared to (e) ROT, $p = 0.002/p < 0.0001$, and (h) ROT + PROBIO, $p = 0.004/p = 0.000$, according to both cameras, while in (d) PROBIO, $p = 0.002$; (f) ROT + VPA, $p = 0.004$; and (g) ROT + LEV/CARB, $p = 0.000$ based on recordings made by the lateral camera only. Other circumstances where we observed differences were in (b) VPA compared to (e) ROT, $p = 0.009/p = 0.038$, also based on both cameras, and (h) ROT + PROBIO, $p = 0.014$, by the vertical camera. In addition, the (c) LEV/CARB group showed differences from (e) ROT, $p = 0.001$; (g) ROT + LEV/CARB, $p = 0.022$; and (h) ROT + PROBIO, $p = 0.025$ (Figure 6.8).

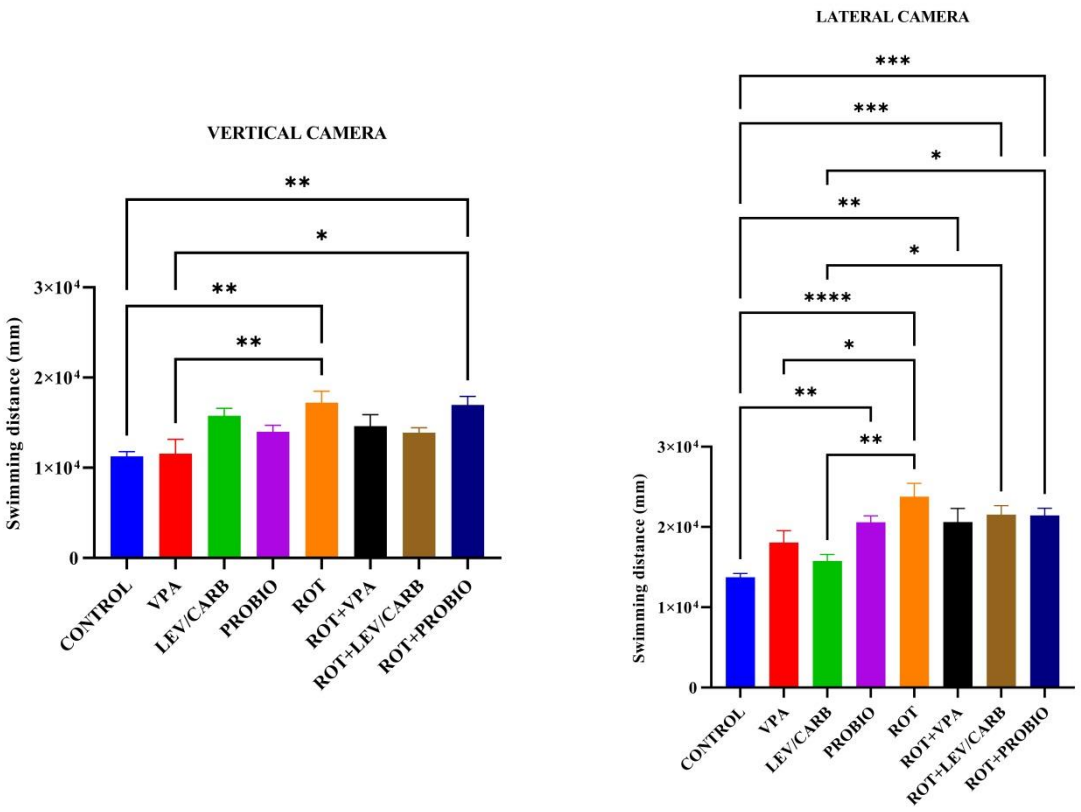


Figure 6.8. Swimming distance patterns (mm) in studied *Danio rerio* groups (n = 5) (* - $p < 0.05$; ** - $p < 0.005$; * - $p < 0.0005$; **** - $p < 0.0001$; values expressed as mean with SEM followed by Tukey HSD test)**

6.4.2.2. Velocity

Interesting behavioral patterns were further observed following centralization of the data assigned to the velocity parameter (mm/sec) in (a) CONTROL by comparison with the experimental groups. More specifically, the only group in which we did not observe a significant difference was in (b) VPA ($p > 0.05$) and with a particular case in (c) LEV/CARB, $p = 0.024$ according to the side camera recording. Continuing with this concept, we noted major differences between healthy zebrafish and (d) PROBIO, $p = 0.004/p = 0.004$; (e) ROT, $p = 0.000/p = 0.000$; (f) ROT + VPA, $p = 0.008/p = 0.007$; (g) ROT + LEV/CARB, $p = 0.019/p = 0.005$; and (h) ROT + PROBIO, $p = 0.003/p = 0.000$, based on recordings from both cameras (Figure 6.9).

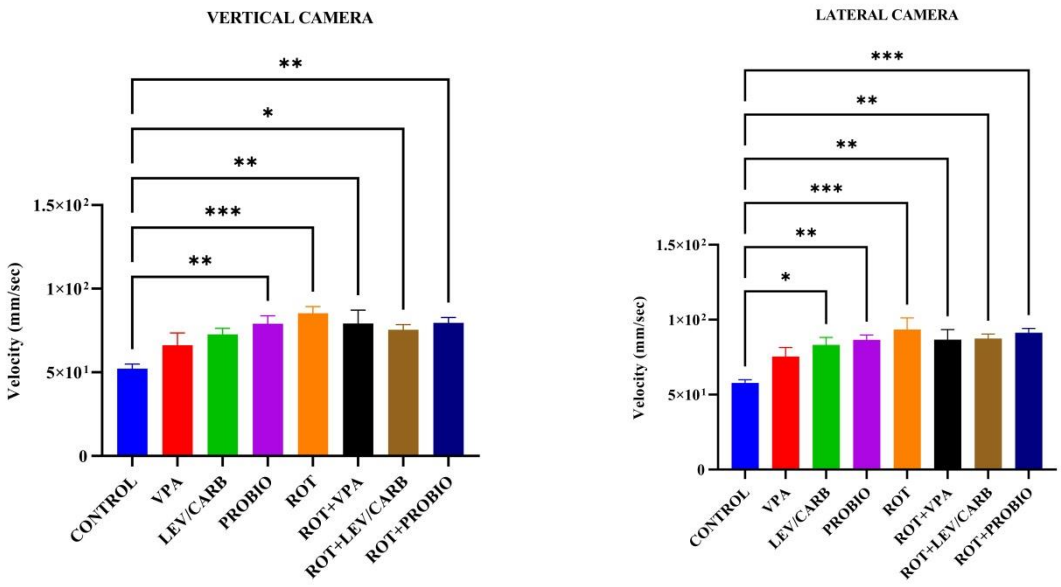


Figure 6.9. Velocity patterns (mm/sec) in *Danio rerio* groups (n = 5) studied (* - $p < 0.05$; ** - $p < 0.005$; * - $p < 0.0005$; values expressed as mean with SEM followed by Tukey HSD test)**

6.4.2.3. Freezing episodes

In addition to the observations made in light of the above two parameters, more episodes of inactivity were observed in (a) CONTROL in (e) ROT relative to (c) LEV/CARB, $p = 0.000$/$p = 0.010$ and (d) PROBIO, $p = 0.031$/$p = 0.042$ by vertical and lateral camera and between (e) ROT, $p = 0.007$ and (g) ROT + LEV/CARB, $p = 0.036$ by lateral camera. Two unique cases where we observed differences were in (b) VPA versus (c) LEV/CARB, $p = 0.013$ according to vertical camera recording, and (e) ROT versus (f) ROT + VPA, $p = 0.045$ by lateral camera (Figure 6.10).

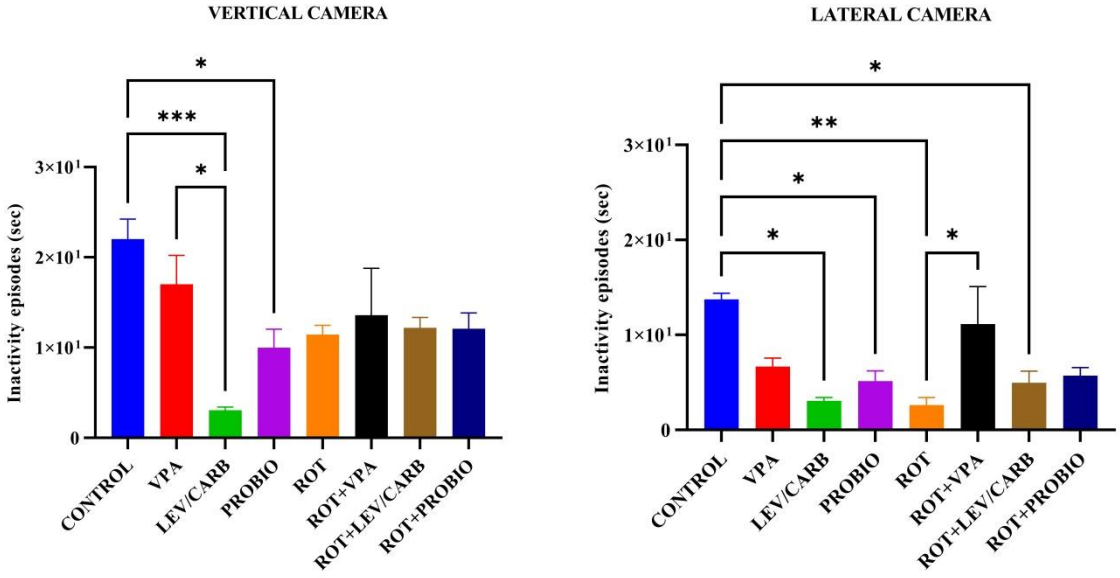
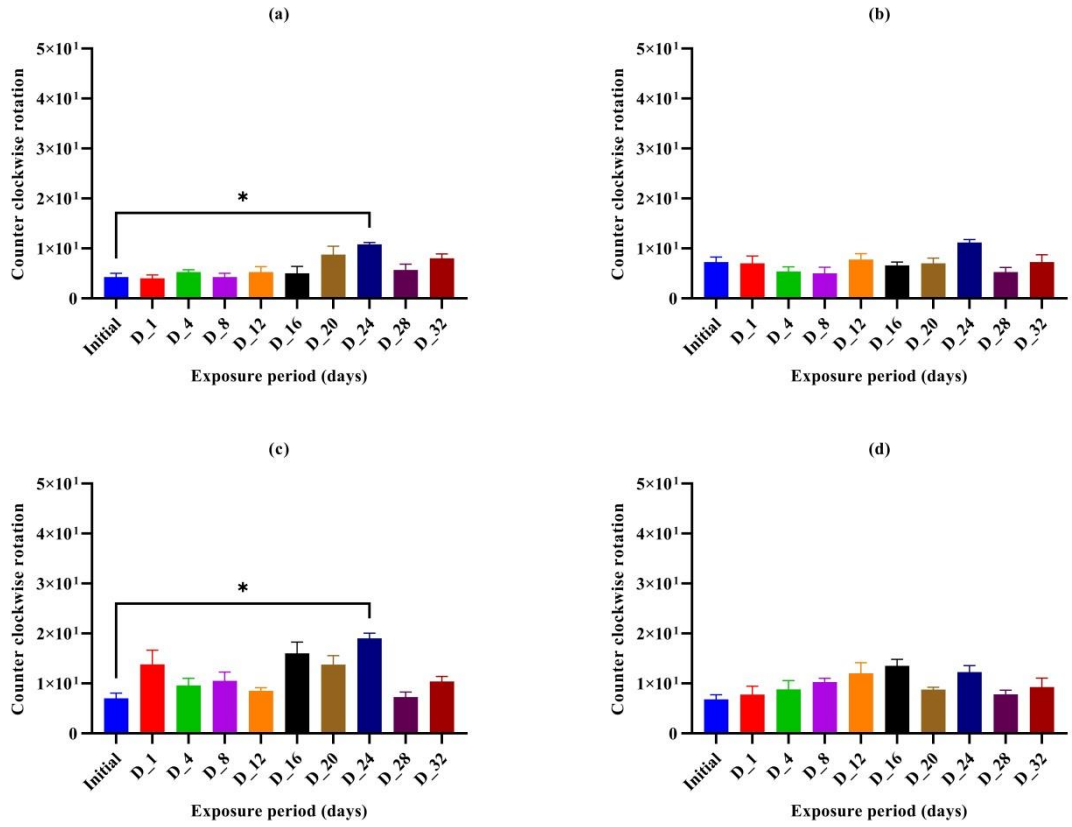


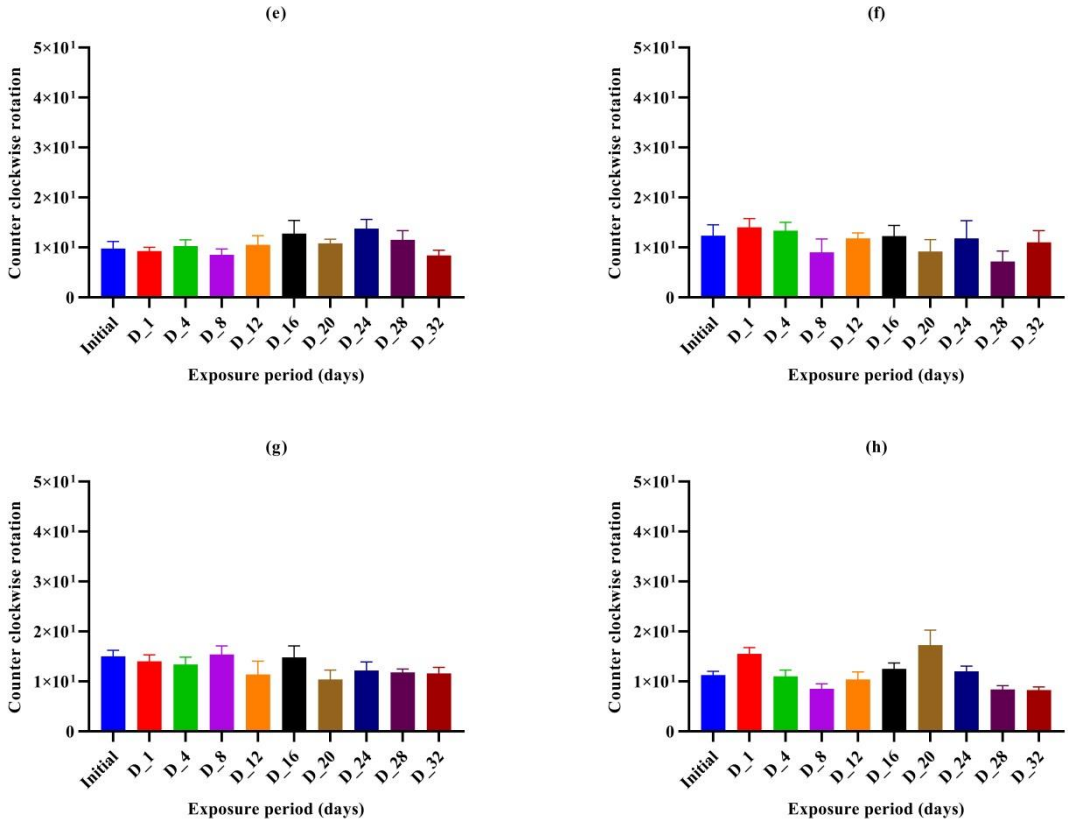
Figure 6.10. Inactivity episodes (sec) in *Danio rerio* groups (n = 5) studied (* - $p < 0.05$; ** - $p < 0.005$; * - $p < 0.0005$; values expressed as mean with SEM followed by Tukey HSD test)**

6.5. Counter clockwise rotation

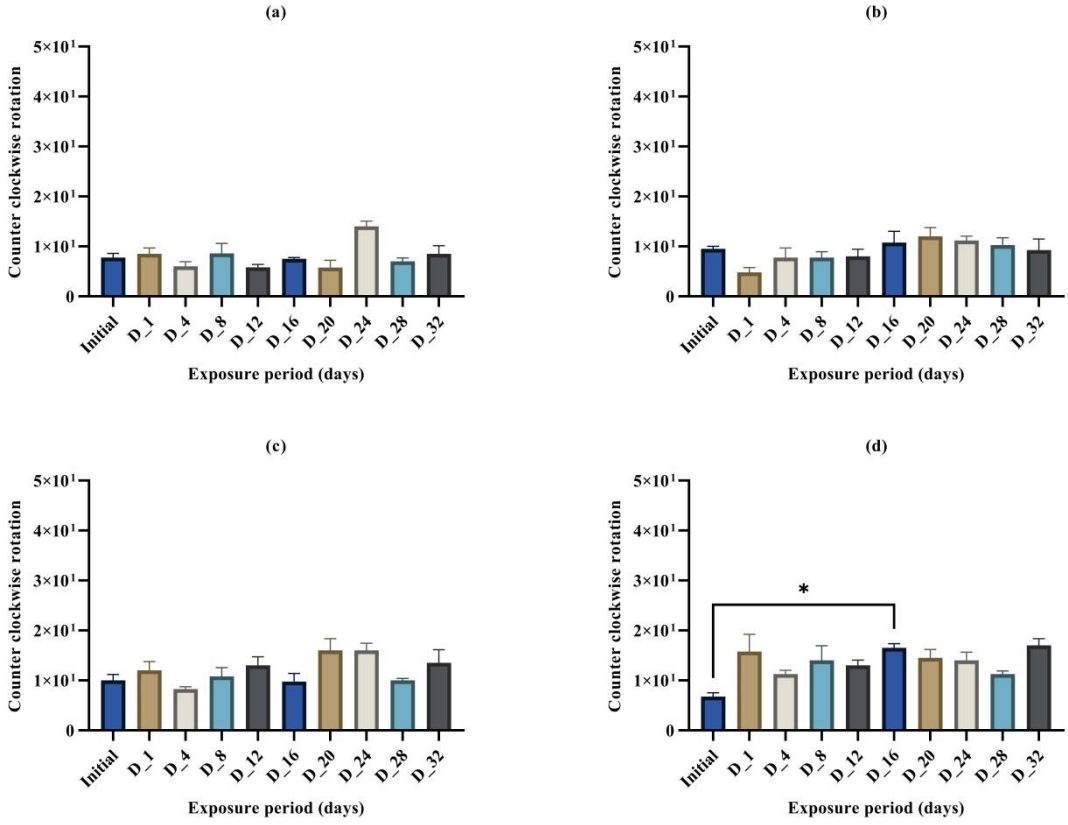
6.5.1. Exposure to 2.5 µg/L rotenone

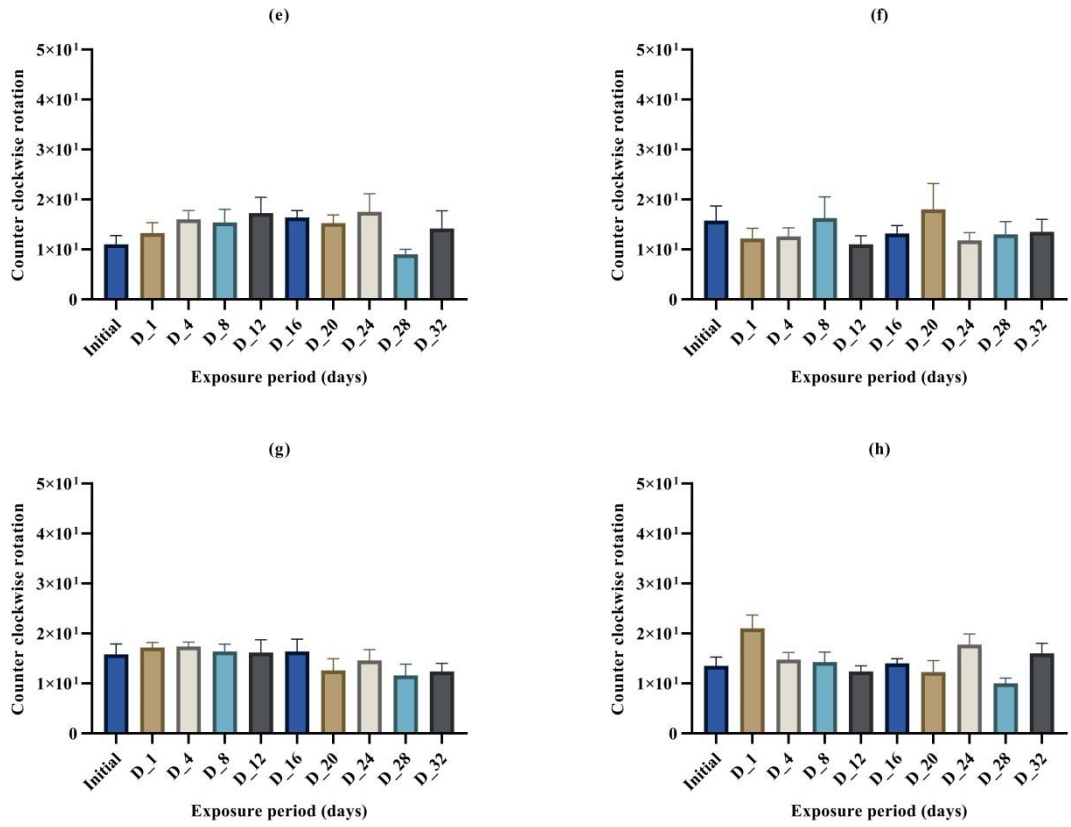
Although fluctuations in behavioral patterns are observable in all eight groups, only in three did we observe a statistically significant difference over 32 days of analysis. We observed an abnormal pattern reflected by their turning tendencies in (a) CONTROL group in D_24 - p = 0.026 and (c) LEV/CARB group in D_24 - p = 0.013 on the same day according to the vertical camera recordings. Moreover, a significant difference was observed in the (d) PROBIO group in D_16 - p = 0.022 based on the route filmed by the side camera. Abnormal behavioral patterns in the other five groups were no longer noted (p > 0.05). However, in groups that were not exposed to rotenone (a - d) a continuously increasing rotation pattern can be observed. In the other four groups (e - h) that received rotenone in combination with other agents, this behavior was amplified, but not statistically significant (Figure 6.11).





VERTICAL CAMERA





LATERAL CAMERA

Figure 6.11. Counter clockwise rotation parameter in *Danio rerio* groups (n = 5) studied (values expressed as mean with SEM followed by Dunnett's test; * - $p < 0.05$)

6.6. Assessment of oxidative biomarkers

6.6.1. Exposure to 2 µg/L rotenone

Analyzing OS data after 21 days of chronic rotenone administration in individual zebrafish, we observed several changes in oxidative biomarkers (Figure 6.12). For SOD there was no significant difference between the CONTROL group and the other three ($p > 0.05$), but rather when we compared the ROT group with PROBIO ($p = 0.014$) and ROT + PROBIO ($p = 0.011$). Enzyme activity decreased in the ROT group compared to the CONTROL group, but not statistically significant. In contrast, the PROBIO group showed an increase in the enzymatic activity of SOD, a result that confirms the data from the specialized literature according to which probiotics can enhance the expression of SOD in living cells (Kong et al., 2020) and the differential response of the investigated organ (Ünal et al., 2019). Meanwhile, the last ROT + PROBIO group also had an increase in SOD activity, but without any considerable change compared to the CONTROL group.

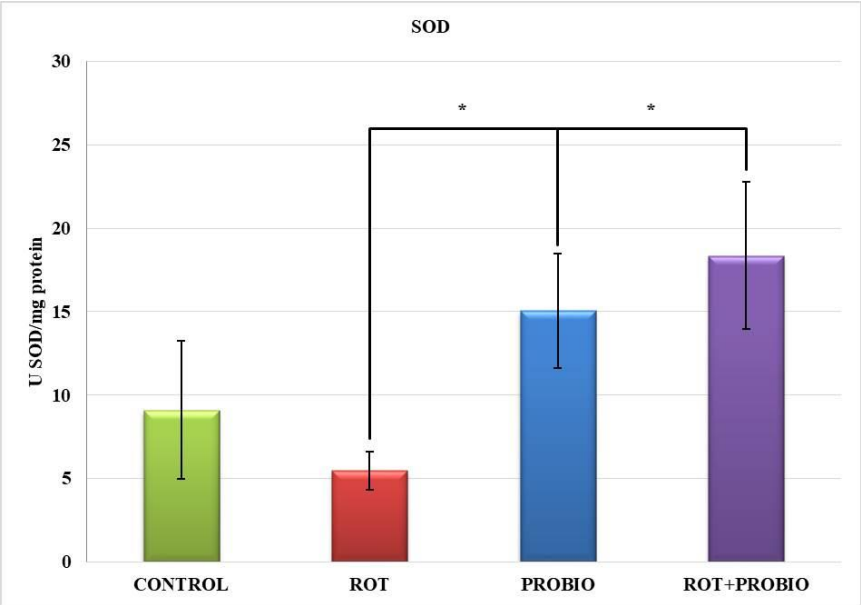


Figure 6.12. Enzymatic activity of SOD. Data are expressed as mean ± SEM (* - $p < 0.05$)

In the case of GPx, similar results were obtained because there were no statistically significant differences between the CONTROL group and the rest of the investigated groups ($p > 0.05$). On the other hand, there was a significant change in GPx expression compared to ROT and PROBIO ($p = 0.001$) and ROT + PROBIO ($p = 0.004$) (Figure 6.13).

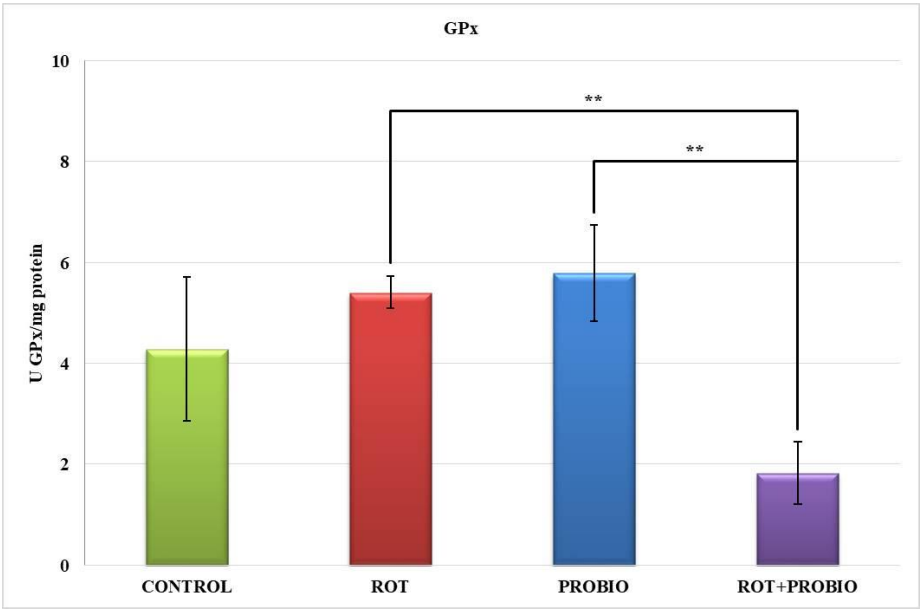


Figure 6.13. Enzymatic activity of GPx. Data are expressed as mean ± SEM (- $p < 0.005$)**

Moreover, the main marker of lipid peroxidation showed a high level ($p < 0.05$) in the CONTROL group compared to PROBIO ($p = 0.033$) and in contrast to ROT + PROBIO ($p = 0.032$). Also, there were significant changes in the level of MDA between

the ROT and PROBIO group (p = 0.009), respectively ROT + PROBIO (p = 0.01) (Figure 6.14).

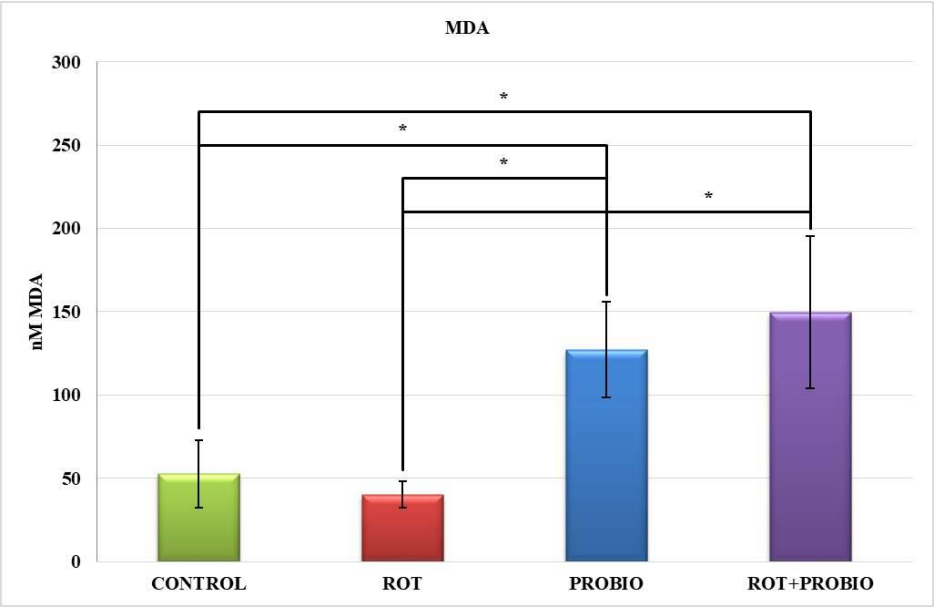


Figure 6.14. MDA level. Data are expressed as mean ± SEM (* - p < 0.05)

6.6.2. Exposure to 2.5 µg/L rotenone

Assessment of oxidative biomarkers revealed a statistically significant difference in MDA level between (b) VPA and (c) LEV/CARB (p = 0.023) and compared to (d) PROBIO (p = 0.009). There are also changes in SOD enzyme activity when comparing the same groups (p = 0.025). SOD enzyme activity is lower in (e) ROT compared to (c) LEV/CARB (p = 0.034), relative to MDA level in (e) ROT group. Contrary to our expectations, there is no difference between the (a) CONTROL group and the experimental groups exposed to rotenone. Even though SOD enzyme activity in (f) ROT + VPA is similar to that of (e) ROT, there is no notable difference when comparing the results between groups exposed to rotenone (p > 0.05). Slight changes in MDA level are observed in (f) ROT + VPA, (g) ROT + LEV/CARB and (h) ROT + PROBIO analogous to (a) CONTROL and (h) ROT + PROBIO in SOD (Figure 6.15).

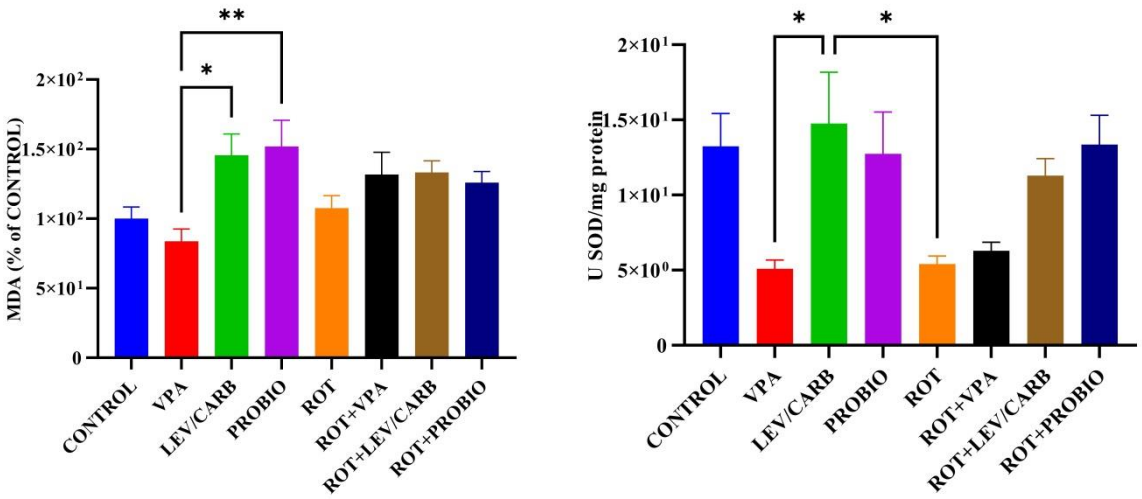


Figure 6.15. SOD enzyme activity and MDA level in studied *Danio rerio* groups (n = 5) (* - $p < 0.05$; ** - $p < 0.005$; values expressed as mean with SEM followed by Tukey HSD test)

6.7. Determination of changes in gene expression

6.7.1. Exposure to 2.5 µg/L rotenone

Analyzing the results of four PD-related genes in zebrafish exposed to rotenone, it was found that there was no statistically significant difference in *LRRK2* and *alpha-SNCA* ($p > 0.05$). There are statistically significant differences in *PARKIN* reflected by an overexpression in (g) ROT + LEV/CARB compared to each experimental group. Therefore, we observed in (a) CONTROL vs. (g) ROT + LEV/CARB, $p = 0.000$; (b) VPA vs. (g) ROT + LEV/CARB, $p = 0.000$; (c) LEV/CARB vs. (g) ROT + LEV/CARB, $p = 0.000$; (d) PROBIO vs. (g) ROT + LEV/CARB, $p = 0.000$; (e) ROT vs. (g) ROT + LEV/CARB, $p = 0.001$; (f) ROT + VPA vs. (g) ROT + LEV/CARB, $p = 0.002$; and (g) ROT + LEV/CARB vs. (h) ROT + PROBIO, $p = 0.007$. Finally, another exacerbated expression of *PINK1* in (e) ROT promoted three situations where we observed a statistically significant difference: (d) PROBIO vs. (e) ROT, $p = 0.007$; (e) ROT vs. (g) ROT + LEV/CARB, $p = 0.010$; and (e) ROT vs. (h) ROT + PROBIO, $p = 0.008$ (Figure 6.16).

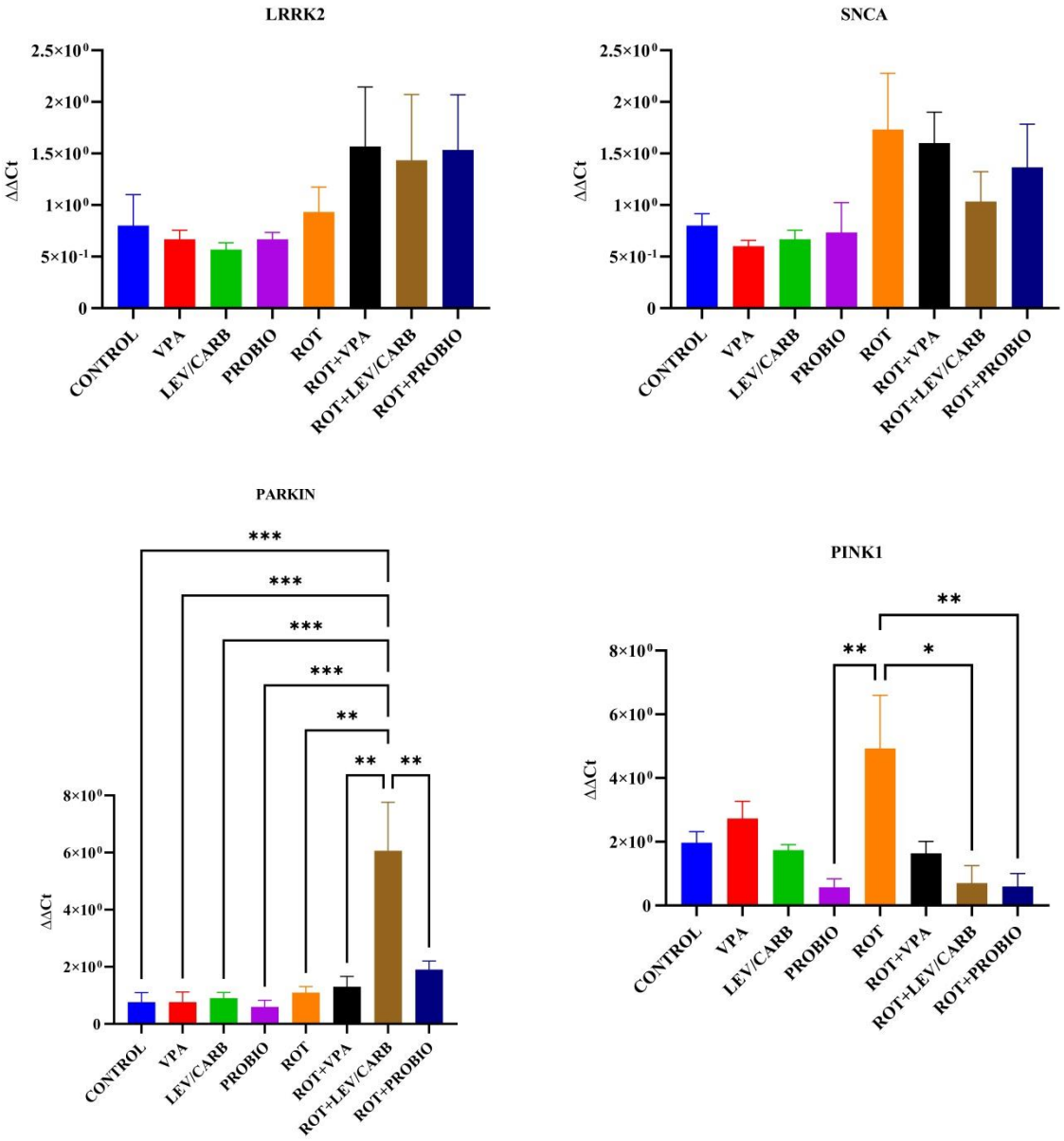


Figure 6.16. Relative expression values for *LRRK2, alpha-SNCA, PARKIN* and *PINK1* in the brain of *Danio rerio* groups (n = 3) studied (*- $p < 0.05$; ** - $p < 0.005$; * - $p < 0.0005$; values expressed as mean with SEM followed by Tukey HSD test)**

6.8. Establishing dopamine levels

6.8.1. Exposure to 2.5 µg/L rotenone

The DA level peaked in the (a) CONTROL group, exceeding all experimental groups except (b) VPA where there is no significance ($p > 0.05$). There are statistical differences in the following cases: (c) LEV/CARB, $p = 0.000$; (d) PROBIO, $p = 0.000$; (e) ROT, $p = 0.001$; (f) ROT + VPA, $p = 0.002$; (g) ROT + LEV/CARB, $p < 0.0001$; and (h) ROT + PROBIO, $p = 0.000$. Also, in group (b) VPA there were three situations where DA

level is increased compared to (c) LEV/CARB, p = 0.024; (g) ROT + LEV/CARB, p = 0.009; and (h) ROT + PROBIO, p = 0.048 (Figure 6.17).

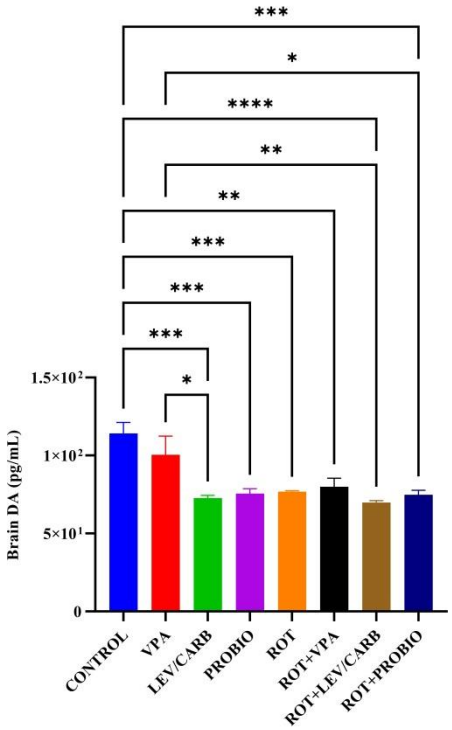


Figure 6.17. Brain DA level in *Danio rerio* groups (n = 5) studied (*- p < 0.05; ** - p < 0.005; * - p < 0.0005; **** - p < 0.0001; values expressed as mean with SEM followed by the Tukey HSD test)**

6.9. Highlighting histological alterations by immunohistochemistry

6.9.1. Exposure to 2.5 µg/L rotenone

Moderate labeling was observed in the optic tectum of the CONTROL group (a) based on the IHC markers used where PCNA labels two small areas of NSCs and neuroblasts, respectively.

In (b) VPA, areas of neurogenesis in the midbrain and angiogenesis in the periventricular gray area can be seen. PCNA, S100b, GFAP and cox4i1 markers show intense expression in torus longitudinalis, torus semicircularis (gray periventricular zone) and basal tegmentum. S100b protein immunoreactivity was detected particularly in the mesencephalic optic tectum, labeling nerve fibers, especially profiles rather than cells. In fact, immunoreactivity was concentrated in fiber profiles crossing the optic tectum perpendicular to the outer side as a whole. Furthermore, S100b protein immunoreactivity was further localized to the medial and lateral areas of the cerebral valve. The inner area of the optic tectum located in the tectal ventricle was also lined with cells positive for S100b and GFAP proteins showing morphological characteristics of ependymal cells and

subependymal cells. Ependymal cells are large and round in shape, while subependymal cells and glial cells show long radial processes that pass through the optic tectum and reach the pial surface. The dorsal and lateral parts of the longitudinal torus were covered by ependymal cells that have S100b protein, and the nerve fibers that form the commissure in the ventral part of the longitudinal torus were also positive for S100b protein.

Group (c) LEV/CARB showed islands of PCNA cells compared to the other groups, newly formed capillaries in diencephalon and midbrain. S100b and GFAP markers were positively labeled in a higher number of cells, while cox4i1 and p53 showed moderate labeling.

In group (d) PROBIO, positive labeling was observed, especially for PCNA, S100b and GFAP and moderately for p53 and cox4i1.

On the other hand, in (e) ROT a reduction to absence was observed for PCNA, GFAP, S100b and a moderate labeling for p53 and cox4i1.

In (f) ROT + VPA, (g) ROT + LEV/CARB, and (h) ROT + PROBIO there was an increase in all IHC markers, and in (h) ROT + PROBIO, PCNA labeled a small number of cells.

Rotenone caused a reduction in PCNA labeling, which may suggest a decrease in neurogenesis and, by implication, an increase in neuronal dysfunction by reducing GFAP and S100b labeling. This could be explained by the expression of p53 and cox4i1, which further indicate apoptosis and mitochondrial dysfunction.

However, in the (b) VPA, (c) LEV/CARB and (d) PROBIO groups there is positive labeling for neurogenesis (PCNA), apoptosis (p53) and the presence of mature, active radial neurons and glial cells (GFAP and S100b). Cox4i1 is positivity in these clusters, denoting intense mitochondrial activity.

In the cerebellum, GFAP, S100b, p53, and cox4i1-labeled RGCs and gray matter neurons in the molecular, Purkinje, and granular layers were highlighted with close intensity to the optic tectum in each experiment. The distribution pattern of S100b protein in the cerebellum differed from that of other CNS segments because it was mainly localized in neurons, more frequently than in glial cells. In the cerebellum, S100b protein labeled small neurons in the superficial molecular layer. In addition, neurons that make up the cerebellar body and deep nuclei were also immunoreactive for S100b. Purkinje neurons located in the basal zone showed a strong reaction to S100b protein in both the perikaryon and the dendritic tree (Figure 6.18).

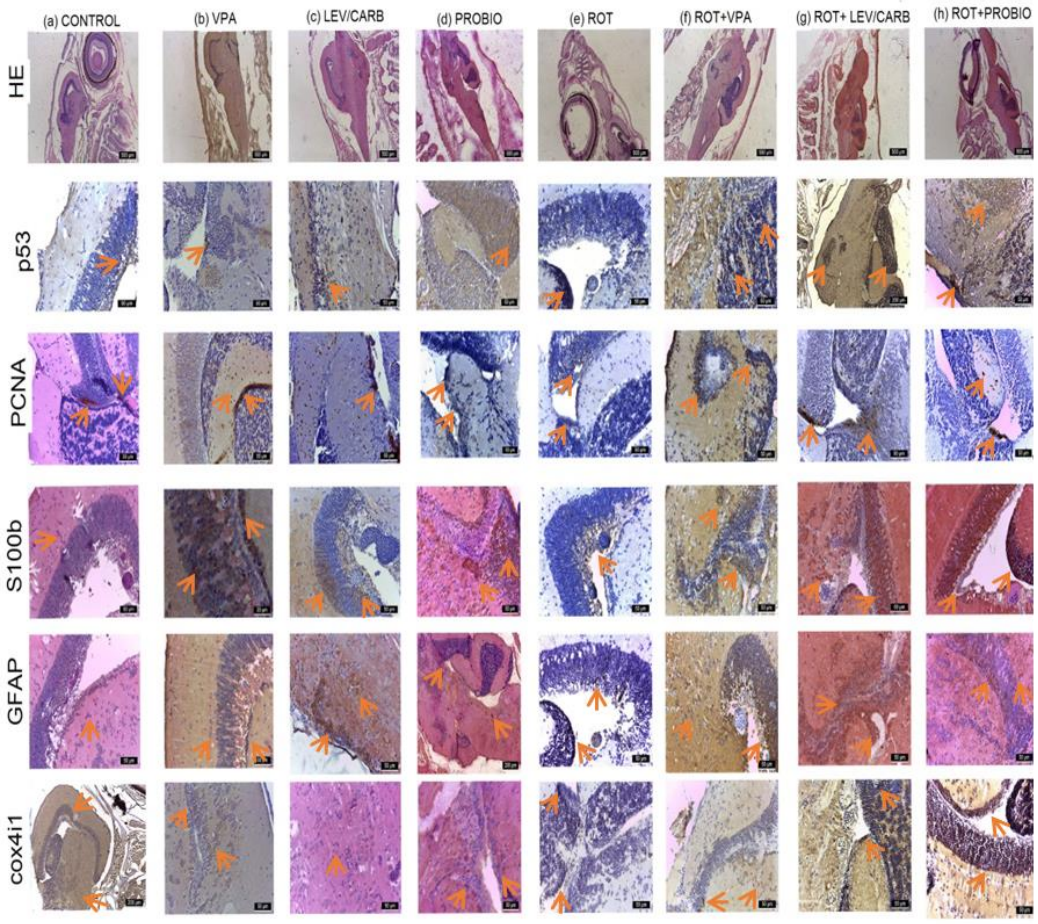


Figure 6.18. Nervous system reactivity in studied *Danio rerio* groups (n = 5) to HE, p53, PCNA, S100b, GFAP and cox4i1

Chapter 7. Discussions

7.1. Impact of 2 µg/L rotenone on sociability and locomotion

We observed that the administration of 2 µg/L rotenone changed the values of only one of all locomotion-specific parameters. Thus, the results associated with the total distance swum are congruent with another existing study in the literature describing the induction of a non- and mild motor phenotype after administration of the same concentration for 28 days (Wang et al., 2017).

Interestingly, active status was different for the second and fourth groups that received rotenone and rotenone and probiotics on the first day of treatment, respectively. Rotenone administration led to abnormal activity of zebrafish throughout the period compared to the group exposed to rotenone and probiotics whose activity was adjusted after only one week of probiotic treatment. When rotenone and probiotics were

administered together this parameter was not significantly influenced. This could be an effect triggered by the presence of probiotics in the environment, a fact suggested by the results obtained for the third group exposed only to probiotics, in which case their activity was increased in the first week of treatment compared to the phenotype recorded in pretreatment. Unfortunately the spectrum of knowledge is limited only to the present study in terms of affecting and evaluating the social component in parallel with locomotor activity in zebrafish.

7.2. Impact of 2.5 µg/L rotenone on antisocial character and aggression

We observe a specific phenotype in group (a) CONTROL and (c) LEV/CARB ($p < 0.05$), but also in group (d) PROBIO compared to baseline ($p < 0.05$). Intriguingly, there were no significant additional behavioral changes in the counter clockwise rotation parameter in groups exposed to valproic acid and rotenone alone or in combination. No significant abnormal oscillations in behavior were observed in (a) CONTROL, (d) PROBIO, (g) ROT + LEV/CARB, and (h) ROT + PROBIO. In (b) VPA, (c) LEV/CARB, (e) ROT and (f) ROT + VPA the most pronounced atypical behavioral patterns were observed with the most time spent in both arms ($p < 0.05$, 0.005 , 0.001). Groups (a) CONTROL and (d) PROBIO showed a reduced degree of aggressiveness, comparable to the fluctuations shown in (b) VPA, (c) LEV/CARB, (e) ROT, (f) ROT + VPA, (g) ROT + LEV/CARB and (h) ROT + PROBIO.

7.3. Impact of 2.5 µg/L on locomotor activity

It was shown that 2.5 µg/L rotenone did not significantly influence any of the evaluated parameters. Animals that received this dose showed above average normal behavior compared to the rest of the experimental groups as they recorded the longest swimming distance with the highest speed and the fewest freezing episodes. Our results are congruent with those of our previous study (Ilie et al., 2021) and the study of (Wang et al., 2017) following administration of 2 µg/L rotenone for 21 - 28 days.

However, three hypothetical case scenarios can be derived from this point that could explain this situation: the zebrafish starts to metabolize the administered rotenone, the route of administration does not ensure adequate ingestion of the compound, or the exposure period is not sufficient at the selected concentration. Furthermore, there are other variables related to stress-related behavioral analyses. We hypothesized that daily testing might accelerate metabolic rate, which may further stimulate rotenone's effect, reflected by

abnormal free radical generation. On the other hand, testing at specific intervals may not exert a strong impact on metabolic rate. Unfortunately, most of the current data on locomotor dysfunction have been reported by week (Khotimah et al., 2015a, 2015b) or are not specified (Cansız et al., 2021; Ünal et al., 2020) and have not concerned information regarding the renewal of the substances used.

7.4. Impact of 2 µg/L rotenone on oxidative biomarkers

In our study we observed several changes in the enzymatic activity of the main antioxidants, but also in the level of the specific marker of lipid peroxidation.

Treatment with 2 µg/L rotenone for 21 days leads to a decrease in SOD enzyme activity that was in agreement with that of (Khan and Ali, 2018) in PD patients. On the other hand, the activity of SOD and GPx was enhanced by probiotics as demonstrated by administration of *Lactobacillus fermentum* to pigs (Wang et al., 2009).

7.5. Impact of 2.5 µg/L rotenone on oxidative biomarkers

SOD enzyme activity and MDA level in the CONTROL group were non-significantly higher than those of the ROT group ($p > 0.05$) and lower than those receiving *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001 alone or in combination with rotenone in the case MDA ($p < 0.05$) (Ilie et al., 2021).

Although there were slight differences in the level of MDA in contrast to the enzymatic activity of SOD, these levels peaked in the (c) LEV/CARB and (d) PROBIO groups. Interestingly, there were no significant differences between (a) CONTROL and experimental groups, especially in zebrafish exposed to rotenone.

Although the level of MDA is lower in contrast to the four groups exposed to rotenone, there are no significant changes, similar to the enzymatic activity of SOD which is objectively higher for the same groups, but not significant ($p > 0.05$). These data are not in agreement with our previous study (Ilie et al., 2021), but this is the second study to assess the status of oxidative markers at low concentrations using the whole individual, not only brain or gut (Cansız et al., 2021; Ünal et al., 2020). While SOD enzyme activity is higher in healthy adult zebrafish, rotenone-treated zebrafish had elevated brain and gut MDA compared to healthy individuals under some circumstances.

A possible explanation that should be tested for the lack of significance is that valproic acid, levodopa and carbidopa and probiotics combined with rotenone exert an antagonistic phenomenon, canceling each other’s effects.

7.6. Impact of 2.5 µg/L rotenone on gene expression

Our results further strengthen the observations made by (Ünal et al., 2020) and contradict those of (Wang et al., 2017). While rotenone in the rotenone-exposed groups (e - h) increases the expression of *LRRK2* compared to the other four groups that were not exposed to rotenone (a - d), it does not inhibit the expression of *alpha-SNCA*, a trend similar to that of *LRRK2* . Furthermore, rotenone upregulates the expression of *PINK1* and *PARKIN*, possibly attributable to the neuroactive potential of levodopa and carbidopa. The results of (Ünal et al., 2020) and (Wang et al., 2017) are confirmed to some extent as they claim that rotenone decreases the expression of *PINK1* and *PARKIN*, but in particular cases.

7.7. Impact of 2.5 µg/L rotenone on dopamine levels

The DA level in the brain in the (a) CONTROL group was higher in six distinct situations, supported by a statistically significant difference, in parallel with the three situations in the (b) VPA group. *Centella asiatica* (Khotimah et al., 2015a) and mitoquinone (Ünal et al., 2020) increase DA levels in rotenone-depleted zebrafish (Khotimah et al., 2015b). One of the most eloquent examples of the effect of rotenone on the DA level is provided by the studies of (Alam and Schmidt, 2002) and (Biehlmaier et al., 2007). Ip with 1.5 mg/kg, 2 mg/kg/day, and 2.5 mg/kg between 10 days and 2 months induces degeneration of dopaminergic neurons (Landau et al., 2021) in the posterior striatum, PFC, and SN.

7.8. Histological changes associated with administration of 2.5 µg/L

Most NSCs under homeostatic conditions are considered resting type I RGCs, a notable pattern in the (a) CONTROL group. When the telencephalon is damaged and subjected to aggression, more NSCs are activated, enter the cell cycle and begin to express proliferation markers (März et al., 2010), a situation observed in every experimental group except (e) ROT. In addition, intense expression of ventricular GFAP and S100b of the telencephalon and midbrain, which are densely populated by RGC cell bodies, was observed in all experimental groups except (e) ROT.

Valproic acid attenuates tissue and neuronal damage, improves functional restoration, and stimulates neurogenesis and functional integration. This stimulation of neurogenesis was observed in (b) VPA and (f) ROT + VPA. In this experiment, we suggested that the levodopa effects observed in (g) ROT + LEV/CARB were due to its

uptake into the neuronal or glial system in the cell cytoplasm. Glial cells of the SVZ play an essential role in the neurogenesis of adult zebrafish (Wasel and Freeman, 2020), which explains the presence of GFAP and S100b expression in the SVZ in fish in all experimental groups except (e) ROT. Interestingly, angiogenesis was observed in this experiment three times: in the group (b) VPA, (c) LEV/CARB, and (g) ROT + LEV/CARB.

General conclusions

Judging by the prism of the expected results, obtained and highlighted in the present doctoral thesis, a series of conclusions are derived.

1. The zebrafish (*Danio rerio*) can be seen as an ideal model organism in biomedical research based on the vast repertoire it benefits from, mainly the behavioral typologies that ensure the decoding of the mechanisms behind neurodegenerative disorders including the phenotype associated with Parkinson's disease.

2. Rotenone is a viable agent with a broad spectrum of applicability in neuroscience, whose neurotoxicological profile allows the induction of certain behavioral features in a manner dependent on the duration of exposure and the administered dose that specifically mimics the symptomatic picture of Parkinson's disease.

3. Although the literature argues for the successful use of 5 µg/L, it proved to be lethal, which is why we considered a preliminary evaluation appropriate, concluding that 2 µg/L, respectively 2.5 µg/L for 21 - 32 can be administered successfully to maintain a constant survival rate.

4. The concentration of 2 µg/L rotenone was insufficient to significantly alter the behavior of individuals in the social interaction test administered in parallel with or without *Bifidobacterium longum* BB536 (150 mg) and *Lactobacillus rhamnosus* HN001 (25 mg) at the end of 21 days of testing in the T-maze (2D).

5. Contrary to our expectations, individuals exposed only to 2 µg/L rotenone, respectively *Bifidobacterium longum* BB536 (150 mg) and *Lactobacillus rhamnosus* HN001 (25 mg) in a mixed dose displayed behavioral patterns diametrically opposed to those typical of chronic exposure per locomotor parameters analyzed in the T-maze (2D) as indicated by total distance swum, velocity and active status at the end of the analysis period at 21 days.

6. Only four of the eight experimental groups showed preference for the right and center arm during the T-maze (2D) session, further suggesting the neuroactive potential of valproic acid (0.5 mg/mL) administered alone or in combination with rotenone (2.5 µg/L) and that of levodopa and carbidopa (250 mg + 25 mg) based on the preset interval of chronic exposure for 32 days.

7. *Lactobacillus casei* W56, *Lactobacillus acidophilus* W22, *Lactobacillus paracasei* W20, *Lactobacillus salivarius* W24, *Lactobacillus lactis* W19, *Lactobacillus*

plantarum W62, *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52 and *Bifidobacterium bifidum* W23 (3 g) modulate the aggressive behavior of zebrafish in the mirror test performed in the T-maze (2D), an observation not valid for the other seven groups, including the one given rotenone (2.5 µg/L) in parallel for 32 days.

8. The values associated with 3D counter clockwise rotation as a reference parameter for a possible neurological disorder were constant in all eight experimental groups during the entire period of behavioral analysis, including when valproic acid (0.5 mg/ mL), levodopa and carbidopa (250 mg + 25 mg) and *Lactobacillus casei* W56, *Lactobacillus acidophilus* W22, *Lactobacillus paracasei* W20, *Lactobacillus salivarius* W24, *Lactobacillus lactis* W19, *Lactobacillus plantarum* W62, *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52 and *Bifidobacterium bifidum* W23 (3 g) were administered to zebrafish alone or concurrently with rotenone (2.5 µg/L) for 32 days.

9. Similar to observations following exposure to 2 µg/L rotenone for 21 days, healthy fish not exposed to any additional exogenous chemical traveled the least distance at the slowest swimming speed coupled with the most episodes of immobility in contrast to the other experimental groups, especially by reference to those exposed to 2.5 µg/L rotenone for 32 days in 3D.

10. The concentration of 2 µg/L rotenone did not significantly influence the status of oxidative biomarkers, but caused an inter-individual difference, more specifically in zebrafish exposed to rotenone and *Bifidobacterium longum* BB536 (150 mg) and *Lactobacillus rhamnosus* HN001 (25 mg) where there was observed an increase in SOD activity and MDA level compared to GPx where the ratio changed between groups exposed to rotenone alone or in mixed dose with *Bifidobacterium longum* BB536 (150 mg) and *Lactobacillus rhamnosus* HN001 (25 mg).

11. The concentration of 2.5 µg/L rotenone did not significantly influence the status of oxidative biomarkers, but an exacerbation of the enzymatic activity of SOD and the level of MDA was found in the group that received levodopa and carbidopa (250 mg + 25 mg), similarly for the group supplemented with *Lactobacillus casei* W56, *Lactobacillus acidophilus* W22, *Lactobacillus paracasei* W20, *Lactobacillus salivarius* W24, *Lactobacillus lactis* W19, *Lactobacillus plantarum* W62, *Bifidobacterium lactis* W51, Bifidobacterium lactis W52 and *Bifidobacterium bifidum* W23 (3 g).

12. Although objectively an amplification of expression in *LRRK2* and *alpha-SNCA* was observed in groups exposed to 2.5 µg/L rotenone, these were not significant,

but it promoted an exacerbation of *PARKIN* gene expression or in mixed dose with levodopa and carbidopa (250 mg + 25 mg) in *PINK1*.

13. The concentration of 2.5 µg/L rotenone did not cause a depletion of dopamine levels in the brain in the four experimental groups exposed, including the groups exposed to *Lactobacillus casei* W56, *Lactobacillus acidophilus* W22, *Lactobacillus paracasei* W20, *Lactobacillus salivarius* W24, *Lactobacillus lactis* W19, *Lactobacillus plantarum* W62, *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52 and *Bifidobacterium bifidum* W23 (3 g) and levodopa and carbidopa (250 mg + 25 mg).

14. Valproic acid (0.5 mg/mL), levodopa and carbidopa (250 mg + 25 mg) and *Lactobacillus casei* W56, *Lactobacillus acidophilus* W22, *Lactobacillus paracasei* W20, *Lactobacillus salivarius* W24, *Lactobacillus lactis* W19, *Lactobacillus plantarum* W62, *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52 and *Bifidobacterium bifidum* W23 (3 g) exert a neuroprotective effect by supporting angiogenesis and neurogenesis against the antagonistic action of 2.5 µg/L rotenone where the engagement of the apoptosis process was noted.

15. Although the protective neuroactive effect exerted by agents hypothesized to be rotenone inhibitors is certified, in the present research no protein quantification by ELISA or Western blot or inflammatory cytokines was performed to confirm neuroinflammation.

16. Cumulatively, we want to extrapolate by creating and applying working protocols aimed at analyzing the gastrointestinal microflora in zebrafish to establish both the ratio and the microbial load as a consequence of the administration of rotenone, valproic acid, levodopa and carbidopa and probiotics.

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Published articles

ISI articles with IF

Synthesis of the literature

Ilie, O.-D., Ciobica, A., McKenna, J., Doroftei, B., Mavroudis, I., 2020. Minireview on the Relations between Gut Microflora and Parkinson's Disease: Further Biochemical (Oxidative Stress), Inflammatory, and Neurological Particularities. Oxidative Medicine and Cellular Longevity. 4518023, https://doi.org/10.1155/2020/4518023 (IF 5,076).

Original data

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Complementary articles to the doctoral thesis

Synthesis of the literature

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Participation in international conferences in the poster section

PROBIOTICS DID NOT IMPROVE SOCIAL INTERACTION IN A ZEBRAFISH (DANIO RERIO)-MODEL OF PARKINSON'S DISEASE #2649 - WORLD PSYCHIATRIC ASSOCIATION 2022

IS ZEBRAFISH SUITABLE FOR MODELING GASTROINTESTINAL DISEASES? #2660 - WORLD PSYCHIATRIC ASSOCIATION 2022

LACTIC ACID BACTERIA STRAINS IN MEDIATING PARKINSON'S DISEASE-RELATED CONSTIPATION #2661 - WORLD PSYCHIATRIC ASSOCIATION 2022

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Keywords: oxidative stress; functional gastrointestinal disorder; animal behavior

EPV0768

Microbiome-dependent antioxidant, gastrointestinal and neurological modulation of irritable bowel syndrome symptomatology

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Introduction: Irritable bowel syndrome (IBS) is a multifactorial, multigenic and environmental-dependent disorder exhibiting a wide range of functional gastrointestinal symptoms. IBS pathophysiology includes the immune system activation, disturbance of intestinal function accompanied by inflammatory process and dysbiosis which lead to brain-gut axis impairments. The bidirectional brain-gut communication contribution is suggested by comorbidity between gastrointestinal and psychiatric illnesses.
Objectives: Given that the microbiome was recently described as a key modulator in mood and brain development, neurodegeneration, ageing, inflammatory processes and oxidative stress, our main goal was to review the existing data that addresses this topic of high interest, the relationship between microbiome and antioxidant, gastrointestinal and neuropsychiatric modulation in IBS.
Methods: The literature search was conducted using the keywords "irritable bowel syndrome", "microbiome", "gut-brain axis" "stress", "depression", "behavior"," antioxidants" in Science Direct, Oxford Journals, Medline and Google Scholar databases. Only English publications have been taken into consideration. This inquiry was conducted by three separate researchers. Any differences of opinions were solutioned by common consent.
Results: Mood disorders, also modulated by the microbiome, affect more than half of IBS patients, antidepressants being commonly administered to IBS patients for both gastrointestinal and neuropsychiatric symptoms. However, it was observed that the changes in gut microbial species could lead to several gastrointestinal and neuropsychiatric symptoms. Moreover, the microbiota impairments could lead to colonic cells and systemic inflammatory processes and oxidative stress.
Conclusions: The discussed modulatory potential of microbiome in gastrointestinal tract, nervous system and molecular pathways suggested that the microbiome –gut–brain axis could be the key component in an IBS future treatment.
Disclosure: PN-III-P1-1.1-TE2016-1210, named "Complex study on oxidative stress status, inflammatory processes and neurological manifestations correlations in irritable bowel syndrome pathophysiology (animal models and human patients)"

Keywords: Microbiome; oxidative stress; neuropsychiatric comorbidies; Irritable bowel syndrome

EPV0770

Preventing the progression of cognitive impairments in epilepsy

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Introduction: Varying different degrees of cognitive impairments have a considerable effect on the functioning of patients, their socialization, and the level of disability. Cognitive deficits deteriorate the quality of patients life.
Objectives: The aims of research were detection of versatile cognitive impairments in epilepsy and studying the results of cognitive training.
Methods: We studied the features of Clinical and psychopathological manifestations in patients suffering from epilepsy. The study covered 100 patients (35 men and 65 women) who were in inpatient care. The following psychodiagnostic techniques were used: the Toronto Cognitive Assessment TorCA, the test of 10 words of Luria, the MOCA test, the Münsterberg test, the quality of life scale, the Hamilton scale of depression and anxiety.
Results: The following results of the study were observed: decreased memory in 88 % patients, mild dementia in 48%, moderate dementia in 24% and severe dementia in 16%. We used non-pharmacological rehabilitation methods for correction of cognitive impairment with patients who have mild and moderate memory decreas.
Conclusions: The results of the conducted research indicate the need for further study of the features of cognitive disorders in epilepsy and implementation of training aimed at improving cognitive function and preventing the progression of cognitive impairment.

Conflict of interest: No
Keywords: Cognitive disorders; Epilepsy

EPV0771

Comparison of visual p300 amplitude and latency in people with schizophrenia and bipolar disorder

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Introduction: Reduction in the amplitude of P300, is found more frequently in the auditory mode but has also been reported using visual stimulus in people with schizophrenia. Previous research may imply that visual P300 alterations are specific markers of schizophrenia, but they have small sample sizes, and few make comparisons with other psychiatric disorders.

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