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THE GUT-BRAIN AXIS: EFFECTS OF THE PROBIOTIC *LACTOBACILLUS FERMENTUM* INTRODUCED IN THE DIET OF ETHANOL-FED MICE

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List of abbreviations

ADH	Alcohol Dehydrogenase
ALDH2	Acetaldehyde Dehydrogenase 2
ANS	Autonomic Nervous System
BBB	Blood–Brain Barrier
BEVs	Bacterial Extracellular Vesicles
BGA	Brain-Gut Axis
BGMA	Brain-Gut-Microbiota Axis
CNS	Central Nervous System
CYP2E1	Cytochrome P450 2E1
EGCs	Enteric Glial Cells
ENS	Enteric Nervous System
EtOH	Ethanol
EVs	Extracellular Vesicles
GABA	γ-Aminobutyric Acid
GI	Gastrointestinal
GIT	Gastrointestinal Tract
GFAP	Glial Fibrillary Acidic Protein
GL	Granular Layer
GM	Gut Microbiota
HPA	Hypothalamic-Pituitary-Adrenal
HSFs	Heat Shock Transcription Factors
HSPs	Heat Shock Proteins
H_2O_2	Hydrogen Peroxide
H&E	Haematoxylin and Eosin
IBS	Irritable Bowel Syndrome
IECs	Intestinal Epithelial Cells
IκB-α	NF kappa B inhibitor-α
IL-1β	Interleukin 1β
IMA	Inferior Mesenteric Artery
IMV	Inferior Mesenteric Vein
LAB	Lactic Acid Bacteria
LPS	Lipopolysaccharide
MALT	Mucosa-Associated Lymphatic Tissue
MEOS	Microsomal Ethanol-Oxidizing System
ML	Molecular Layer
MP	Myenteric Plexus
MMP-9	Matrix Metalloproteinase 9
NAD+	Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NADPH	Dihydronicotinamide Adenine Dinucleotide Phosphate
NF-κB	Pro-inflammatory Nuclear Factor kappa B
OMVs	Outer Membrane Vesicles
pAKT	Phosphorylated AKT
PCs	Purkinje cells
PGN	Peptidoglycan
PKR	Protein Kinase R
pNF-κB	Phosphorylated NF-κB
ROS	Reactive Oxygen Species
S100β	Calcium-Binding Protein

SCFAs	Short-Chain Fatty Acids
SMA	Superior Mesenteric Artery
SMV	Superior Mesenteric Vein
SMP	Submucosal Plexus
TJs	Tight Junctions
TNF-α	Tumor Necrosis Factor alpha
WHO	World Health Organization
WM	Subcerebellar White Matter
ZO-1	Zonula Occludens-1

Abstract

Chronic alcohol consumption alters the composition of the intestinal microbiota favouring intestinal permeability. Endotoxin and non-metabolized alcohol reach the systemic circulation and other organs, including the central nervous system (CNS), leading to neurodegeneration. Probiotics could play a key role in protection from alcohol damage, modulating the gut microbiota.

The aim of this thesis was the study of the protective effects of the probiotic *Lactobacillus fermentum* (*L. fermentum*) against oxidative stress damage and inflammation induced by chronic ethanol (EtOH) consumption, using an animal model of alcohol abuse. In fact, female 12-month-old mice (BALB/cAnNHsd) were orally given EtOH (96%) alone (n=5) or in combination with *L. fermentum* (10^9 CFU) (n=5), every day for twelve weeks (12 EtOH and 12 EtOH-P, respectively). Both groups were compared with mice (n=5) fed with the standard diet (Ctrl). At the end of the treatment, the mice were sacrificed and their brains and intestine were dissected, fixed and embedded in paraffin for histological analysis.

Hematoxylin-eosin staining showed a *leaky gut* in the mice treated with EtOH, characterized by altered barrier structure and loss of the normal morphology of Purkinje cells in the cerebellum. Since glial cells participate in the regulation of inflammatory mechanisms, immunomorphological analyses were conducted to identify Glial Fibrillary Acid Protein (GFAP), S100β-Calcium-Binding Protein (S100β) and Fatty Acid Binding Protein-7 (FABP7) in small intestine and cerebellum slices. The glial markers (GFAP, and S100β) were increased after EtOH consumption, probably as a consequence of dysbiosis. While, GFAP, S100β, and FABP7 were also increased in cerebellum slices, probably as a consequence of astrogliosis. The 12 EtOH-P group showed a decreased amount of GFAP, S100β, and FABP7, suggesting a protective role of *L. fermentum* against inflammation. In addition, immunomorphological and quantitative real-time PCR analyses were used to evaluate the effects of *L. fermentum* on the proinflammatory nuclear factor (NF- κB) signalling pathway, and on the levels of cytokines, as interlukin-6 (IL-6) and Tumor Necrosis Factor alpha (TNF-α), and molecular chaperones (HSPs), such as HSP60 and HSP90.

The levels of phosphorylated NF-κB (pNF-κB) were elevated in the 12 EtOH group but were lower in the 12 EtOH-P. *L. fermentum* induced downregulation of TNF-α production and increased expression of the NF-κB inhibitor-α (IκB-α). Moreover, immunomorphological and immunofluorescence analyses showed an increase of HSPs levels in both tissues. In particular, HSP60 and HSP90 were induced by probiotic, inhibiting IκB-kinase (IKK) and preventing NF-κB activation. These data indicate that *L. fermentum* had anti-inflammatory and cytoprotective effects, suggesting to its possible application to treat intestinal inflammatory disorders.

Introduction

1.1 The gastrointestinal tract

The human digestive system consists mainly of a series of interconnected hollow organs, including the gastrointestinal tract (GIT) (or alimentary canal), that, together with the glands, enable the process of digestion (**figure 1a**). Digestion occurs by mechanical and chemical processes. Specifically, food and liquids pass through them to be transformed into nutrients needed by the body, that pass through the small intestine wall into blood and lymphatic vessels and are transported to the liver for storage or further processing; while unnecessary components are eliminated through the faeces (Dworken HJ., et al., 2020).

The GIT is composed of the oral cavity, pharynx, oesophagus, stomach, small intestine (duodenum, jejunum, and ileum), large intestine (cecum, colon, and rectum), anal canal, and anus. The four distinct concentric layers of the gut wall are the mucosa, the submucosa, the muscularis externa, and either the adventitia or the serosa (Reed KK., and Wickham R., 2009) (**figure 1b**). The innermost layer of the gut wall is represented by the mucosa and consists of epithelium, lamina propria, and muscularis mucosa. The mucosal epithelium is differentiated along the GIT, correlating with the regional function of the tract. In these regions, the cells secrete mucous, enzymes, and other biochemicals that either protect the mucosa or aid in digestion. The lamina propria and muscularis mucosa lie outside the mucosal epithelium. The lamina propria consists primarily of connective tissue, as well as blood and lymphatic vessels that deliver nutrients to the mucosal epithelium, transport hormones secreted by the endocrine epithelium, and absorb end products of digestion from the lumen of the GIT. In addition, the lamina propria is infiltrated with lymphocytes and lymph nodules, collectively known as the GALT that protect the GIT wall from resident bacterial flora and ingested pathogens. Lastly, the muscularis mucosa, is a thin layer of smooth muscle that is the boundary between the mucosa and the submucosa. The submucosa

is a connective tissue layer that lies outside of and supports the mucosa, containing blood vessels, lymphatic vessels, submucosal glands, and Meissner's (submucosal) plexus (a nerve network that influences the smooth muscle of the muscularis). The next outer layer of the gut wall is the muscularis externa having has two smooth muscle layers: an inner circular layer and an outer longitudinal layer. Auerbach's (myenteric) plexus lies between the two layers, and this nerve network coordinates contractions of the layers resulting in rhythmic peristalsis. Finally, the outermost layer of the gut wall that lies farthest from the lumen is either the adventitia or the serosa. If the outermost layer is attached to surrounding tissue, it is adventitia, connective tissue that supports the organ it surrounds. When the outermost layer lies adjacent to the peritoneal cavity, it is called serosa. Serosa is connective tissue that has a surface of mesothelium, which is composed of simple squamous epithelial cells (King D., et al., 2008; Reed KK., and Wickham R., 2009).

Whitin all organs of the GIT, the gut (small and large intestine) is a complex system where many events (nutrient absorption, immune modulation, antigen sampling) take place (Funk MC., et al., 2020). In particular, most absorption of nutrients and water happen in the small intestine lying between the stomach and large intestine, and by receiving bile and pancreatic juice, it aids digestion. It also plays a role in the immune system, acting as a barrier to a multitude of flora that inhabits the gut and to make sure no harmful bacteria enter the body. Due to the destruction of the small intestine, there can be a decrease in the absorption of essential vitamins, minerals, and other nutrients to the body which can cause a myriad of problems that can interfere with other systems in the body (Collins JT., et al., 2021).

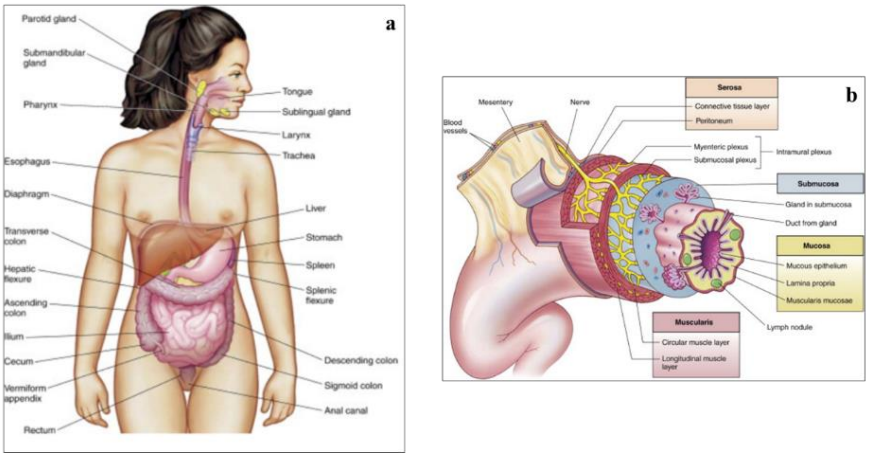

Figure 1. The human digestive system: **a**) Anatomy of the human GIT and associated glands; **b**) The wall of the GIT is made up of four layers. Notice that the serosa is continuous with a fold of serous membrane called a mesentery. Notice also those digestive glands may empty their products into the lumen of the GIT by way of ducts (Reed KK., and Wickham R., 2009).

1.1.1 Gross Anatomy

The small intestine is a tubular organ extending from the pyloric sphincter to the ileocecal valve, where it empties into the large intestine. It is the longest part of the digestive system, with an average length of 3 to 5 meters. It is referred to as the "small" intestine because its lumen is smaller in diameter (at about 2,5 centimeters) than the large intestine.

Anatomically, the small intestine is divided into the duodenum, jejunum and ileum.

The duodenum is the shortest segment of the small intestine, measuring only 20-25 cm. Its proximal end in connected to the antrum of the stomach, separated by the pylorus, a valve that opens and closes, allowing food to pass from stomach into the small intestine. Distally, the duodenum curves around the pancreas and ends in the area of the upper left quadrant of the abdomen, where it blends into the beginning of the jejunum. Therefore, the duodenum receives chyme from the stomach, and pancreatic enzymes and bile from the liver, containing enzymes that help break down ingested food.

The jejunum and ileum are the distal two parts of the small intestine. In contrast to the duodenum, they are intraperitoneal. The jejunum is the upper portion of the small intestine which connects to the duodenum at the duodenojejunal flexure and to the

ileum at the other end. The jejunum is roughly 2,5 m in length comprising approximately 40% of the small bowel in humans. It contains plicae circulares and villi to absorb the product of digestion. The ileum is the final portion of the small intestine, with a length of about 3 meters, constituting approximately 60% of the small intestine in humans. It opens into the large intestine, at the level of the ileocecal valve, and has the function of absorbing all the final nutrients, including vitamin B12 (Caballero B., 2005; Federle MP., et al., 2017).

The mesenteric circulation consists of three major arteries (celiac axis, superior mesenteric artery [SMA], and inferior mesenteric artery [IMA]) and two major veins (superior [SMV] and inferior mesenteric [IMV] veins), connected by arterioles, capillaries, and venules. This cascade, referred to as the splanchnic circulation, courses through the mesentery, providing blood to and draining it from the digestive organs. The small intestine is highly vascularized. In particular, the SMA gives rise to the inferior pancreaticoduodenal artery, the middle colic artery, right colic artery, and many jejunal and ileal branches. The jejunal and ileal branches supply the jejunum and ileum, respectively. Instead, The IMV joins the splenic vein, and the SMV and splenic vein anastomose to form the portal vein.

The branches of these vessels form anastomotic systems that provide a rich blood supply to the adjoining organs. In many areas the systems overlap while in other regions linkages are limited. Interrelations and weak points are of significant clinical importance. As well as this, there is a great individual variability in the anatomy of the gastrointestinal vasculature (Geboes K., et al., 2001).

The GIT is innervated by intrinsic neurons of the enteric nervous system (ENS) and by the axons of extrinsic sympathetic, parasympathetic, and visceral afferent neurons. 1) The myenteric plexus (MP), the outer of the two major ENS plexuses, is the network of neurons situated between the muscle layers of the GIT, and is primarily involved in the initiation and control of smooth muscle motor patterns such as peristalsis; 2) The submucosal plexus (SMP), the second of the two major ENS plexuses, is the network of neurons located in the submucosa between the smooth muscle layers and mucosa. The SMP coordinates reflexes such as secretion and absorption, as well as motor control of smooth muscle. Enteric glia is prominently located within the ganglia of both the MP and SMP where they interdigitate with neurons, within the villi of the

mucosa where they make close contacts with the epithelial cell layer. Classically, enteric glia was thought to act only as structural support for neurons, but recently the list of their candidate functions has expanded to include metabolic, trophic, and protective support of neurons. Additionally, it is clear that glia plays an important role in the health of the enteric innervation of the GIT.

In addition, the gut's extrinsic innervation is supplied by neurons located in prevertebral ganglia, the brainstem, and peripheral afferent ganglia. The noradrenergic fibers within the wall of the GIT originate from cell bodies located within the prevertebral sympathetic ganglia. The celiac-mesenteric ganglia provide fibers to the stomach, small intestine and, to some extent, the proximal large intestine. The inferior mesenteric ganglia provide fibers to the large intestine, and the remaining noradrenergic fibers to the rectum originate from the pelvic ganglia. The noradrenergic innervation from these prevertebral ganglia supplies an extensive network of fibers to the smooth muscle wall, ganglia of the MP and SMP, and arteries of the GIT.

The vagus nerve supplies both sensory (afferent fibers) and motor (efferent fibers) functions. The neurites of vagal afferents form extensive networks of endings in the smooth muscle, MP, and mucosa. Vagal sensory innervation of the mucosa consists of terminal arborizations mainly between the crypts and the villous lamina propria, with terminal branches reaching the basal lamina but not penetrating it so as to make direct contact with epithelial cells. Vagal motor innervation of the GIT is restricted to the MP where individual efferent fibers collateralize extensively throughout the plexus, with terminal swellings (i.e., presumptive synaptic contacts) in close approximation with individual myenteric neurons. Primary afferent sensory fibers originating from cell bodies in dorsal root ganglia project along the mesenteric arteries to enter the GIT and provide a perivascular network of fibers to the arterial system, around neurons in the ganglia of the MP and SMP, and within the villi of the mucosa. The role of dorsal root afferents in the control of gastrointestinal (GI) function echoes this pattern of innervation, and includes regulation of blood flow, neuronal excitability, motility, and secretion (Phillips RJ., et al., 2007).

1.1.2 Microanatomy

The histological structure of the small intestine is similar to that of the other organs of the digestive tract. There are four main layers (**figure 2**): 1) the mucosa, which is the innermost layer, contains the epithelium, lamina propria and muscularis mucosae. The epithelium is made up of several components. The enterocytes, tall columnar cells, form a completely continuous barrier so that molecules present in the intestinal lumen cannot immediately reach the extracellular spaces. This barrier is created by the presence of tight junctions, including Zonula occludens-1 (ZO-1), in the apical zone of the enterocytes. However, in the basal zone there are adherent junctions, such as desmosomes, which allow the passage of molecules. They contain brush border enzymes on the surface which have an important digestive function. Interspersed between them, there are the globet cells, exocrine gland secreting mucin, and Lieberkuhn's crypts containing numerous cells such as stem cells that produce new cells to replace cells lost through abrasion and enteroendocrine cells that synthesise and secrete hormones. Furthermore, to protect against pathogens, there are Paneth cells that secrete protective agents (such as defensins and lysozymes) and Peyer's plaques (only in the ileum), containing mucosa-associated lymphatic tissue (MALT) that harbours white blood cells and lymphocytes. These cells can produce antibodies to further protect the small intestine from infection. 2) The submucosa, a layer of connective tissue that contains the blood and lymph vessels and the submucosal plexus; 3) The muscularis consists of two layers of smooth muscle. The outermost layer is the thin, longitudinal muscle that contracts, relaxes, shortens, and lengthens the gut allowing food to move in one direction. The innermost layer is a thicker, circular muscle. This layer enables the gut to contract and break apart larger food particles. It also stops food from moving in the wrong direction by blocking the more proximal end. The two muscle layers work together to propagate food from the proximal end to the distal end. 4) Finally, most of the small intestine is covered by serosa, the outermost layer covered by mesothelium (Volk N., et al., 2017; Willie R., et al., 2021).

To optimize digestive and absorptive operations, the mucosa forms multitudes of projections protruding into the lumen, called villi. These villi are covered with

absorptive epithelial cells that take up nutrients from the lumen and transport into the blood. Furthermore, the luminal plasma membrane of absorptive epithelial cells is studded with densely-packed microvilli.

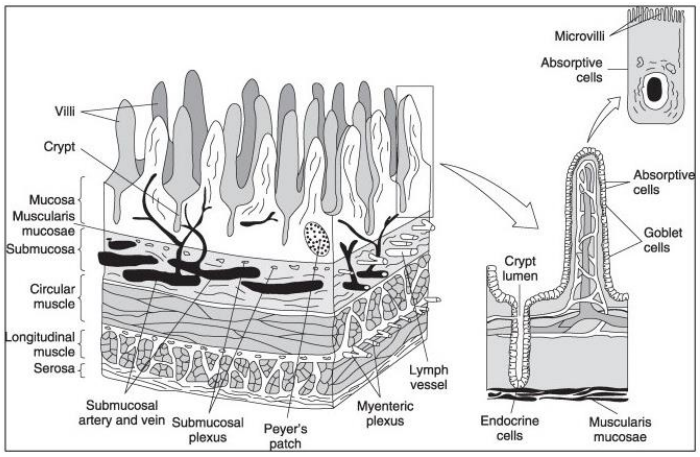

Figure 2. Wall of the small intestine. The intestinal surface area is enhanced by finger-like villi (Caballero B., 2005).

In addition, Cappello F., et al., have suggested that the innermost lining of the intestinal lumen consist of the mucus produced by the epithelial cells of the mucosa and contains about 100 billion microbial cells, called the gut microbiota (GM). For this reason, Cappello F. et al., have proposed to name this layer “MuMi layer” on account of its two major components, namely the mucous and the microbiota (Cappello F., et al., 2019). Although this layer is not visible on routine histological examination, it may have a loose (as compared with the other intestinal layers) and changeable structure mostly provided by the biofilms formed by bacteria, archaea, and micro-eukaryotes that constitute the microbiota (Cappello F., et al., 2019).

The nervous system of the small intestine is made up of the parasympathetic and sympathetic divisions of the autonomic nervous system. While parasympathetic fibres from the vagus nerve control secretions and motility; sympathetic fibres control blood vessels, intestinal secretions, motility and pain stimuli (Caballero B., 2005).

1.2. The human cerebellum

Originating from the metencephalon, the human cerebellum is an important part of the vertebrate central nervous system (CNS). It is the largest part of the hindbrain, containing more than half the total number of neurons (Roostaei, T., et al., 2014). It is enshrined in posterior cranial fossa beneath the tentorium cerebelli behind the pons and medulla oblongata (**figure 3**). Cerebellum is separated from the pons and medulla by the cavity of fourth ventricle. When looking at the cerebellum from behind, it has two hemispheres which are joined together by a longitudinal depression running down the midline of both hemispheres called the vermis. The cerebellar cortical surface is convoluted into numerous folia. The midsagittal view of these folia resembles a branching tree, called the *arbor vitae* (Roostaei, T., et al., 2014).

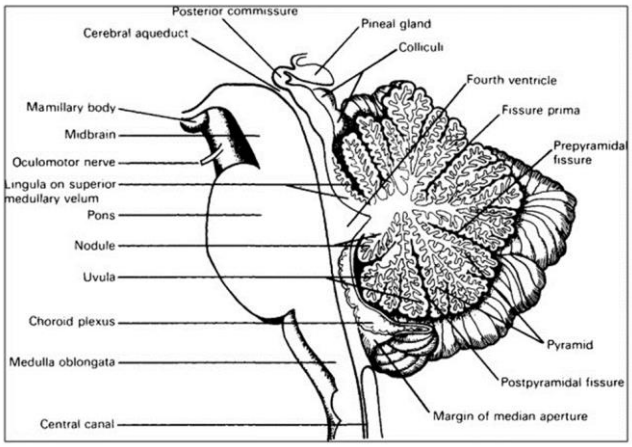

Figure 3. The cerebellum: Median sagittal section of the cerebellum and brainstem (Wilkinson, JL. 1992).

Although the cerebellum has traditionally been regarded as a motor control center, several recent neuroimaging studies have revealed anatomical and functional connections between the cerebral cortex, indicating that the cerebellum is not only involved in motor function (Tanabe HC., et al., 2018). These cerebro-cerebellar connections enable the cerebellum to contribute to higher cognitive functions,

including language, working memory and executive functions (Buckner, RL., et al., 2011; Marvel, CL., & Desmond, JE. 2010; Ito M., 2008). In fact, its activity is made possible by the large amount of information it receives from the spinal cord, vestibule and cerebral cortex, and the outgoing connections with the brainstem and cerebral cortex. In addition, in the area of cognitive aspects, such as memory and learning, have recently been demonstrated connections with the limbic lobe and basal ganglia (Barbatelli, G., et al. 2018).

The microscopic structure of the cortex is uniform, whit a geometric arrangement such that its cellular constituents are precisely oriented. Externally, the cerebellum has a superficial, convoluted cortex of grey matter that constitutes the cerebellar cortex, folded to form narrow leaf like bands called folia and containing most of the cerebellar neurons. Each folium covers a dense central part consisting of white matter arranged in the form of the branching tree so called *arbor vitae* cerebelli.

Three layers comprise the cerebellar cortex as opposed to the six layers of the cerebral cortex. These are the granular layer, Purkinje cell layer, and the molecular layer (**figure 4**) (Voogd, J., et al., 1998: Britannica, T. 2020). 1) The molecular layer consists of unmyelinated nerve fibres derived from axons of granule, stellate and basket cells, dendrites of Purkinje and Golgi cells. The molecular layer consists of GABAergic neuronal groups and nerve fibres. 2) Purkinje cell layer consists of single layer of flask shaped Purkinje cells. Dendrites of these cells travel upwards into the molecular layer in which these cells undergo profuse branching. Axons of Purkinje cells (PCs) travel through granular layer into white matter where they form synaptic connections with intracerebellar nuclei and exert inhibitory influence on these nuclei. Most PCs use the neurotransmitter γ-aminobutyric acid (GABA) and therefore exert strong inhibitory influences upon the cells that receive their terminals. 3) The inner granular layer composed of numerous granule cells and few Golgi cells. Axons of these cells' courses into molecular layer where these bifurcates and branches pass parallel to the long axis of cerebellar folium. These fibres are known as parallel fibres and synapse with the dendrites of Purkinje cells. Golgi cells are prominent but scanty, and their dendrites ramify in molecular layer.

Human cerebellum contains about 30–50 billion granule cells, 30 million PCs and 100 million stellate and basket cells. Purkinje cells, granule cells, stellate cells, basket cells

and Golgi cells constitute intrinsic neurons of cerebellar cortex. All intrinsic neurons except granule cells are inhibitory and such collection of inhibitory neurons is not found anywhere in the central nervous system except cerebellum.

As a result, all sensory input into the cerebellum results in inhibitory impulses' being exerted upon the deep cerebellar nuclei and parts of the vestibular nucleus. Cells of all deep cerebellar nuclei, on the other hand, are excitatory (secreting the neurotransmitter glutamate) and project upon parts of the thalamus, red nucleus, vestibular nuclei, and reticular formation.

Injuries or disease affecting the cerebellum usually produce neuromuscular disturbances, in particular ataxia, or disruptions of coordinated limb movements. The loss of integrated muscular control may cause tremors and difficulty in standing.

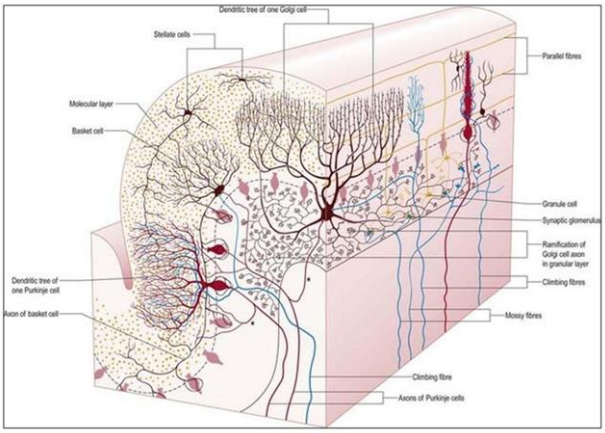

Figure 4. General organization of the cerebellar cortex (Gray' S Clinical Neuroanatomy).

1.3 The Brain-Gut-Microbiome Axis

For decades, out knowledge of the digestive system has been based on a mechanistic model. According to this, food is firstly processed in the mouth, process which leads to the formation of the bolus. Afterwards, the stomach breaks it up before eliminating it in the small intestine, where calories and nutrients are absorbed. The remaining, undigested part is sent to the large intestine (Boland M., 2016). So, the intestine

represents the part of the digestive tract deputed to the enzymatic digestion of the ingested material, to the absorption of nutritive molecules, vitamins, ions and water. Of course, this is an extremely simplistic model. In fact, the mechanisms governing our digestive system are much more complicated, complex and powerful than we could have imagined in the past.

It is now known that the digestive system and the brain are intimately connected to each other. The intestine has long been referred as the "*second brain*" as it has its own nervous system, known in the scientific literature as ENS, composed of about 100 million neurons and is a part of the CNS separating during development; however, it maintains a two-way communication pathway with the CNS (Furness JB., 2012; Lake JI., et al., 2013). The enteric neurons are connected to the central and peripheral nervous system to regulate digestive functions. Specifically, type I enteric neurons innervate GI smooth muscle and, behaving like motor neurons, regulate ganglionic nicotinic synaptic transmission; type II neurons receive mechanical, chemical and thermal stimuli and are in a hyperpolarised state after a stimulus rendering them unresponsive; and type III enteric neurons generate excitatory postsynaptic potentials following stimulation (Furness JB., 2000 and 2012). Moreover, the gut is controlled by the autonomic nervous system (ANS) composed of the parasympathetic and sympathetic systems, and also by the local enteric nervous system composed of the myenteric (Auerbach's plexus) and Meissner's (submucosal plexus). Parasympathetic control of the CNS occurs through the vagal nerve with efferent cholinergics acting on the myenteric plexus (motor movements) and Meissner's plexus (secretions of submucosal glands). Sympathetic control is through the splanchnic nerves which decrease the motility of the intestine and the blood supply to the splanchnic circulation (Anadure, et al. 2019). So, the GIT exerts an influence on brain function, and vice versa.

Furthermore, the intestinal mucosa contains endocrine cells specialized in containing up to twenty different types of hormones that can be released into the bloodstream (Gribble FM., et al., 2016), and it is also the body's largest deposit of serotonin (O'Mahony SM., et al., 2015). These intestinal functions are under the control of the hypothalamic-pituitary-adrenal (HPA) axis, acting as a coordinator of neuroendocrine

responses to stress and playing a crucial role in vital functions such as sensitivity to pain and sleep (Sudo N., 2014).

This creates a two-way communication between the CNS and ENS, knowns as the brain-gut axis (BGA), which links the emotional and cognitive centres of the brain with peripheral gut functions. This places BGA as an attractive target for the development of new therapies against an ever-growing list of disorders related to mental health and cognitive function (Cryan JF, et al., 2019).

However, while the bidirectional BGA is a well-known mechanism for regulating gut function in both healthy and diseased states, the fundamental role of the enteric flora in these interactions has only been recognised in recent years, forming the so-called brain-gut-microbiota axis (BGMA). (Grenham S., et al., 2011; Rhee SH, et al., 2009). Indeed, from birth to death, we are colonized by a vast, complex and dynamic consortium of microorganisms which, due to the presence of nutrients, mainly occupy the intestine (Hooper LV., et al., 2001; Sender R., et al., 2016). These commensal microorganisms are vital for maintaining body homeostasis and may also be involved in the aetiology of several metabolic, immune and mental disorders (Grenham S., et al., 2011; Martin CR., et al., 2018). The bidirectional interaction between microbiota and brain takes place through a complex neural, endocrine, immune and humoral network (**figure 5**).

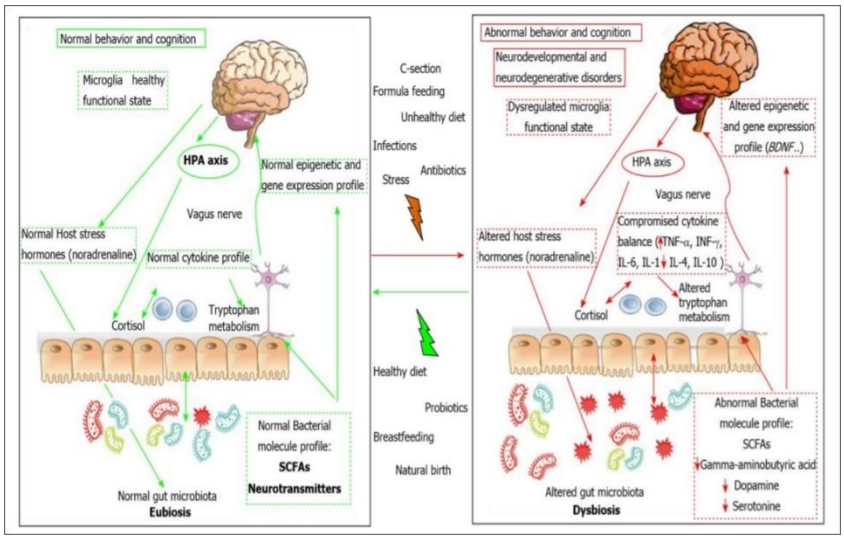

Figure 5. Schematic representation of mechanisms involved in the relationship between the microbiota and brain development and function: cytokine balance and microglia activation

(immune pathway), cortisol (endocrine pathway) and vagus and enteric nervous system (neural pathway). An altered composition of the gut microbiota increases the risk of neurodegenerative disorders, probably resulting from microbiota-derived products such as short-chain fatty acids (SCFAs) and neurotransmitters (Cenit MC., et al., 2017).

The role of the GM in neurodegenerative disorders is explained through the HPA axis, intervening under stressful conditions (Sudo N., 2014). Animal studies confirm that stress induces the brain to influence the microbial composition of the intestine trough the release of hormones and neurotransmitters that affect intestinal physiology and modify the habitat of bacteria, leading to the preferential growth of specific communities (Takada M., et al., 2016). On the other hand, it is now known that some bacteria influence phenomena at neuronal level though the production of neurotransmitters and neuromodulators: e.g., Lactobacillus spp. and Bifidobacterium spp. produce GABAand acetylcholine; Bacillus spp. secrete noradrenaline; Escherichia spp., Streptococus spp. and Enterococcus spp. release serotonin (Chen Y., et al., 2021; Jameson KG., et al., 2018; Strandwitz P., 2018). When neurotransmitter is secreted, it is released into the intestinal lumen where it stimulates epithelial cells to release molecules to modulate the nerve signal in the ENS and, thus, control brain function. Thus, under stressful conditions, the HPA axis is activated, inducing an increase in cortisone in the blood, which stimulates bowel movement and changes in the microbiota. The latter, however, to relive the state of anxiety can act on the HPA axis to inhibit the increase in cortisone produced and restore the physiological condition. This is the fundamental role played by the HPA axis in the bidirectional regulation of the BGMA. In this way, the brain can influence the microbiota indirectly, via changes in gastrointestinal motility and secretion, and intestinal permeability; or directly, via signalling molecules released into the gut lumen from cells in the lamina propria, such as the enterochromaffin cells that are important bidirectional transducers regulating communication between the gut lumen and the nervous system (Rhee SH, et al., 2009). On the other hand, the communication from enteric microbiota to the host can occur via multiple mechanisms, including epithelial-cell, receptor-mediated signalling and, when intestinal permeability is increased, through direct stimulation of host cells in the lamina propria (Rhee SH, et al., 2009).

In addition, the BGMA is also supported by the vagus nerve, the tenth pair of nerves that runs from the medulla oblongata of the brainstem to the chest and abdomen. In fact, the vagal parasympathetic nerve fibres control the smooth muscles of the stomach and intestines, playing a fundamental role in the control of intestinal peristalsis. Thus, the vagus nerve plays an extrinsic role as the primary connection between the brain and the proximal intestinal tract. In particular, the humoral connection involves the HPA axis, which coordinates the stress response and stimulates intestinal endocrine cells to produce neuropeptides that act locally and, through vagal innervation, also at a cerebral level (Anglin R., et al., 2015).

Several recent studies have also revealed the potential existence of another bacterial-host communication mechanism: extracellular vesicles (EVs). EVs are nanosized, heterogenous membrane structures released from members of all three kingdoms of life and provide a highly conserved, efficient pathway for long-distance intercellular and interkingdom communication. Like exosomes, bacterial EVs (BEVs), including the Gram-negative outer membrane vesicles (OMVs), can cross tissue barriers, diffuse across long distances, and deliver RNA, DNA, and protein cargo to target cells to affect gene expression and function (Pirolli NH., et al., 2021). In this way, microbiome-released BEVs are capable of entering the blood circulation, crossing the blood–brain barrier (BBB), and are involved in host colonization and invasion, microbial interactions, antimicrobial resistance, stress and inflammatory response, endothelial cell adhesion, and systemic inflammatory and metabolic response, which all play key roles in the pathogenesis of diverse infectious and inflammatory diseases (Zhao L., et al., 2021).

Given its importance, disruption of the bidirectional interactions between the enteric microbiota and the nervous system may be involved in the pathophysiology of acute and chronic gastrointestinal disease states. In clinical practice, evidence of microbiota and brain interactions comes from the association of gut dysbiosis with central nervous disorders (e.g., Parkinson’s disease, anxious-depressive behaviour) and functional GI disorders, as irritable bowel syndrome (IBS) (Anadure, et al. 2019; Hooper LV., et al., 2001; Lach G., et al., 2018; Martin CR., et al., 2018).

1.3.1 The gut microbiota

The term “microbiota” refers to the entire population of microorganisms that colonizes a particular location. In fact, the mammals have complex microbial communities associated with them, composed primarily of bacteria, but also including other microbes such as fungi, archaea and viruses (Anadure, et al. 2019; Sekirov I., et al., 2010). They amount for more than 1 kg of weight, and outnumber human eukaryotic cells at least ten times (about 10^{14}). These microorganisms essentially coat all the skin and mucosal surface of the host, but the majority of them reside within the gastrointestinal tract, constituting the so-called GM (Rajilić-Stojanović M., et al., 2014).

Since a large part of microorganisms is anaerobes, it is not cultivable through conventional microbiology techniques, so our understanding of GM is substantially limited. However, the development of culture-independent tools, based on metagenomics technologies, has led us to take a step forward in the assessment of GM composition. The healthy GM is predominantly composed of four main microbial phyla accounting for over 90% of the bacterial component of the GM: *Firmicutes*, *Bacteroides*, *Proteobacteria* and *Actinobacteria* (Dominguez-Bello M. G., et al., 2010; Lozupone CA., et a., 2012). Moreover, our GM includes viruses, especially phages, Eukarya, as Fungi, Amoebozoa, and Archaea (Columpsi P., et al., 2016). Even though this general profile remains constant, GM exhibits both temporal and spatial differences in distribution at the genus level and beyond (**figure 4**). So, the intestinal microbiota is not homogenous throughout the gut. The number of bacterial cells follows a gradient starting at approximately 10^1 to 10^3 cells per gram in the stomach and duodenum contents, and increasing up to 10^{11} to 10^{12} cells per gram in the caecum and colon (Sekirov I., et al., 2010).

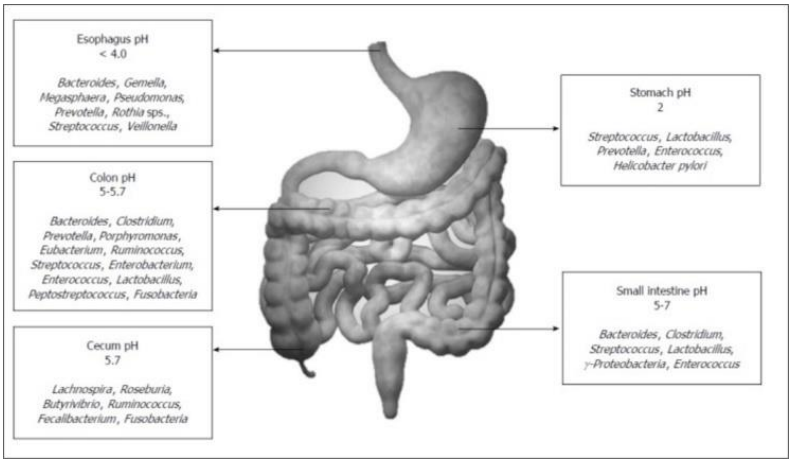

Figure 6. Distribution of the normal human gut flora.

Another gradient is present from the lumen to the epithelium of the gut, with large numbers of bacteria in the lumen, but few adhering to the mucus or epithelium of the colon (Sekirov I., et al., 2010). Most species reside in the lumen, while a few well-adapted species of bacteria can adhere to, and reside within, the mucus layer close to the tissue (Swidsinski A, et al., 2005).

Furthermore, the microbes present in the intestine depend on many factors, such as the maternal microbiota, nutrition and psychophysical state (Dominguez-Bello MG., et al., 2010; Lozupone CA., et al., 2012). In health, GM plays a relevant role within our body, mainly being involved in the development and growth of immunity and in the regulation of several fundamental metabolic pathways. Quantitative and/or qualitative alterations of GM bring to the impairment of this homeostasis, leading to the development of several GM-related diseases, such as functional GI diseases, inflammatory bowel disease, obesity, metabolic syndrome, autism and others (D'Aversa F., et al., 2013; Li Q., et al., 2016; Manichanh C., et al., 2012; Viggiano D., et al., 2015).

As mentioned above, the microbiota communicates with the host in different ways, such as humoral signalling molecules and hormonal components, through ENS and HPA. Essentially, the BGMA provides a network for signals from the brain to influence motor, sensory and secretory functions of the gut, allowing signals and metabolites from the GM to influence brain development, biochemistry, function and behaviour (Ganci M., et al., 2019). Moreover, the collaboration between the host and

the bacteria seems to play a role in the development of the immune system, as the intestinal mucosa is the main interface between the immune system and the external environment. It was shown that mice germ-free have a poorly developed lymphatic system, with few Peyer's plaques and intestinal dendritic cells (Haverson K., et al., 2007). Normally, the intestinal mucosa signals a threat to the innate immune system through Toll-like receptors, which recognize and bind specific microbial macromolecules, including lipopolysaccharide (LPS). This event initiates the nuclear factor kappa B (NF-κB) pathway, activating a cascade of signals and leading to the production and release of protective peptides, cytokines and chemokines. Thus, commensal bacteria in the GIT play an active role in the development and homeostasis of the immune system (Mazmanian SK., et al., 2005).

Diet is arguably one of the most important environmental factors in shaping the composition and metabolic activities of microbiota. The GM has a broad metabolic capacity as they possess many enzymes that enable them to use ingested nutrients to produce a wide range of microbial metabolites that can influence the metabolism and health of the host (Ramos S., et al., 2021). For example, it has been shown that intestinal microbes metabolize dietary fiber into short-chain fatty acids (SCFAs) that can reach various organs in the bloodstream and serve as activators of lipogenesis and/or 24lcohol24g24nesis, exerting different effects on host metabolism (Ramos S., et al., 2021). In addition, SCFAs also play an important role in determining the intestinal environment, influencing pH, intestinal transit, nutrient absorption and microbial balance in the large intestine. Thus, SCFAs not only provide energy sources for host tissues, but also exert anti-inflammatory and anti-apoptotic effects that may be important for the prevention of various GI diseases (Flint HJ., et al., 2015).

This indicates that diet plays a dominant role in shaping the GM, also causing dysbiosis, an alteration of the microbial community with consequent biological changes in the host. Due of the intricate balance that exists within the microbiota, alterations in one group or species may not only directly affect the host, but may also disrupt the entire microbial community, inducing profound effects on the host's inflammatory and metabolic responses (Brown K., et al., 2012). Therefore, dysbiosis can also alter the metabolic activity of other members of the GM promoting the growth

of microbes that could have detrimental effects on their host while other dietary factors could promote beneficial microbes (Brown K., et al., 2012).

Under physiological conditions, the intestinal epithelium acts as an effective and continuous barrier preventing bacterial translocation. However, various exogenous and/or endogenous situations are associated with an alteration of the protective function, causing a defect in the epithelial barrier (*leaky gut*) with changes in the distribution and localization of its tight junction proteins, Z0-1 and 25lcohol25g in the intestinal tissue. This increased leakiness facilitates the translocation of bacteria, such as Escherichia coli, and their products, such as LPS, promoting metabolic endotoxaemia and leading to a proinflammatory environment in the gut (Martin C. R., et al., 2018). Among many factors, recent scientific studies have suggested that alcohol consumption may lead to dysbiosis and increased intestinal permeability. (Le Brun ES., et al., 2020; Lowe PP., et al., 2017; Vetreno RP., et al., 2021).

1.4 Alcohol Metabolism

Chronic alcohol consumption represents one of the most common causes of mortality worldwide (Rehm J., et al., 2013). According to the World Health Organization (WHO), 2.3 billion people consume alcohol in the world and about 75 million are classified as having alcohol disorders (World Health Organization, 2018). Alcohol misuse induces damages to multiple organs, predominantly the liver, the gut, and the brain, thus triggering multi-systemic injuries (Meroni M., et al., 2019).

Chemically and physically, alcohol (CH_3CH_2OH) is a small, water-soluble molecule that is relatively slowly absorbed from the stomach, more rapidly absorbed from the small intestine, and freely distributed throughout the body (Paton A., 2005). The effects of alcohol on the body depending on the individual: their sex, body composition, the amount of alcohol consumed, the presence of food, and the ability of the liver to produce the enzyme alcohol dehydrogenase (ADH). For example, women have lower levels of ADH in the stomach than men, so that less alcohol is metabolized before absorption. This may be important in the increased sensitivity to alcohol and

the higher blood 26lcoholl concentrations in women than in men after an equivalent oral dose of EtOH (Cederbaum AI., 2012).

Regarding its metabolism (**figure 7**), the liver is the primary organ responsible for the detoxification of alcohol. Liver cells produce the enzyme ADH which metabolizes alcohol producing acetaldehyde, a highly reactive and toxic byproduct that may contribute to tissue damage. This oxidation process involves an intermediate carrier of electrons, nicotinamide adenine dinucleotide (NAD+), which is reduced by two electrons to form reduced dinucleotide (NADH). As a result, alcohol oxidation generates a highly reduced cytosolic environment in the hepatocytes, leaving them in a state particularly vulnerable. In addition, cytochrome P450 isozymes, including Cytochrome P450 2E1 (CYP2E1), located mainly in the endoplasmic reticulum, and catalase, present in peroxisomes, also contribute to alcohol oxidation. In particular, CYP2E1 plays an important role in metabolizing ethanol to acetaldehyde at high concentrations of ethanol, acting in other tissues, such as the brain, where ADH activity is low. Conversely, although considered a minor pathway, catalase is able to oxidize ethanol in vitro in the presence of a system that generates hydrogen peroxide (H_2O_2), such as the enzyme complex dihydronicotinamide-adenine dinucleotide phosphate (NADPH) oxidase or the enzyme xanthine oxidase (Handler JA., et al., 1990; Zakhari S., 2006).

Acetaldehyde, produced by alcohol oxidation through any of the mechanisms outlined above, is rapidly metabolized in the mitochondria to acetate and NADH, mainly by acetaldehyde dehydrogenase 2 (ALDH2). While NADH then is oxidized by the mitochondrial electron transport chain, or respiratory chain, most of the acetate resulting from EtOH metabolism escapes the liver into the blood. In particular, the acetate is metabolized to carbon dioxide (CO_2) in heart, skeletal muscle, and brain cells. Furthermore, the acetate is metabolized to acetyl-coenzyme A (acetyl-CoA), involving in lipid and cholesterol biosynthesis in the mitochondria of peripheral and brain tissues (Cederbaum AI., 2012). Moreover, in mammalian, the acetyl-CoA then is used to acetylate histones, resulting in gene activation (Zakhari S., 2006 and 2013).

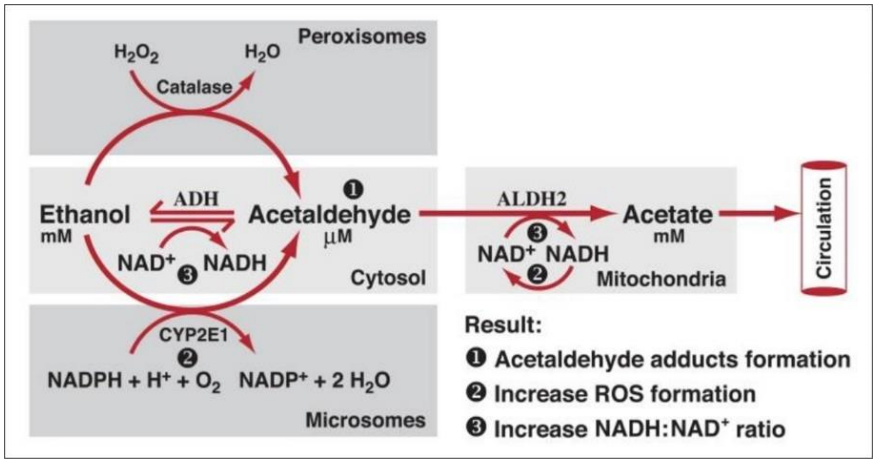

Figure 7. Oxidative pathways of alcohol metabolism. The enzymes ADH, CYP2E1, and catalase all contribute to oxidative metabolism of alcohol. ADH, present in the fluid of the cell (i.e., cytosol), converts alcohol (i.e., EtOH) to acetaldehyde. This reaction involves an intermediate carrier of electrons, +NAD, which is reduced by two electrons to form NADH. Catalase, located in peroxisomes, requires H_2O_2 to oxidize alcohol. CYP2E1, present predominantly in the cell's microsomes, assumes an important role in metabolizing ethanol to acetaldehyde at elevated ethanol concentrations. Acetaldehyde is metabolized mainly by ALDH2 in the mitochondria to form acetate and NADH (Zakhari S., 2006 and 2013).

Another alcohol metabolism pathway is the microsomal ethanol-oxidizing system (MEOS) that acts when the body need to process larger amounts of alcohol, leading to the production of reactive oxygen species (ROS), which can cause cellular damage (Bishehsari F., et al., 2017; Cederbaum AI., 2012). In addition, nonoxidative alcohol metabolism occurs in the intestines via reactions with membrane phospholipids and/or free fatty acids. This alternative pathway may become particularly relevant when intestinal injuries occur after chronic alcohol consumption (Bishehsari, F., et al., 2017; Elamin EE., et al., 2013).

Moreover, to being ingested orally, EtOH can be produced endogenously by bacterial fermentation of carbohydrates. The endogenously produced ethanol is absorbed and transferred via the portal vein to the liver, where it is metabolized. Endogenous and exogenous EtOH can also be further metabolized by the GM. For instance, some bacteria and yeasts, have been found to possess ADH activity, others have also been shown to oxidize ethanol aerobically into acetaldehyde by an ADH-dependent reaction. This shows that changes in the GM due to EtOH abuse may be suggested as a key factor in the development of alcohol-associated diseases such as, alcoholic liver disease (ALD) (Llopis M., et al., 2016; Meroni M., et al., 2019; Yan AW., et al., 2011).

1.4.1 Ethanol Effects on Intestinal Permeability and Integrity

Alcohol consumption is associated with dysfunction of various organs and systems, including the intestine, liver and CNS. These effects in different tissues are mediated by alcohol and its metabolites as the result of its oxidative and nonoxidative metabolism, such as acetaldehyde and free radicals (Handler JA., et al., 1990; Zakhari S., 2006 and 2013). In particular, several studies show that alcohol induces intestinal inflammation through various pathways, including changes in intestinal microbiota composition and function, increased permeability of the intestinal mucosa and disruptions of the immune system of the intestinal mucosa (Bishehsari F., et al., 2017; Engen PA., et al., 2015; Mutlu EA., et al., 2012). Therefore, an intact intestinal barrier formed mainly by the epithelium is essential for normal physiological function and disease prevention. The space between adjacent epithelial cells is mainly sealed by tight junctions (TJs), which control the barrier function of an intact intestinal epithelium (**figure 8**). So, several mechanisms underlying the ethanol-induced barrier dysfunction have been proposed, including direct damage to epithelial cells, loss of integrity of TJs through the destruction of ZO-1 and 28ccluding. In particular, EtOH has been shown to induce activation of NF-κB, resulting in instability of the F-actin cytoskeleton and, consequently, dysfunction of the intestinal barrier (Banan A., et al., 2007; Elamin EE., et al., 2013). In addition, in Caco-2 monolayers, EtOH downregulated the target gene ZO-1 of miRNA-212 and, consequently, decreased the mRNA and protein levels (Tang Y., et al., 2008). Finally, EtOH is also able to induce post-translational modifications in intestinal cell proteins, inducing microtubule hyperacetylation, resulting in redistribution of ZO-1 and, consequently, loss of TJs integrity (Elamin E., et al., 2012 and 2013).

One of the consequences associated with the loss of the epithelial barrier and increased intestinal permeability is the translocation of endotoxins, including LPS, a major component of the cell wall of Gram-negative bacteria, which can cause injury to the liver and other organs (Purohit V., et al., 2008). As discussed previously, EtOH has been shown to alter the composition of the intestinal microbiota, inducing an

overgrowth of gram-negative anaerobic bacteria in the jejunum of alcoholics, predisposing them to increased levels of luminal LPS (Schnabl B., et al., 2014).

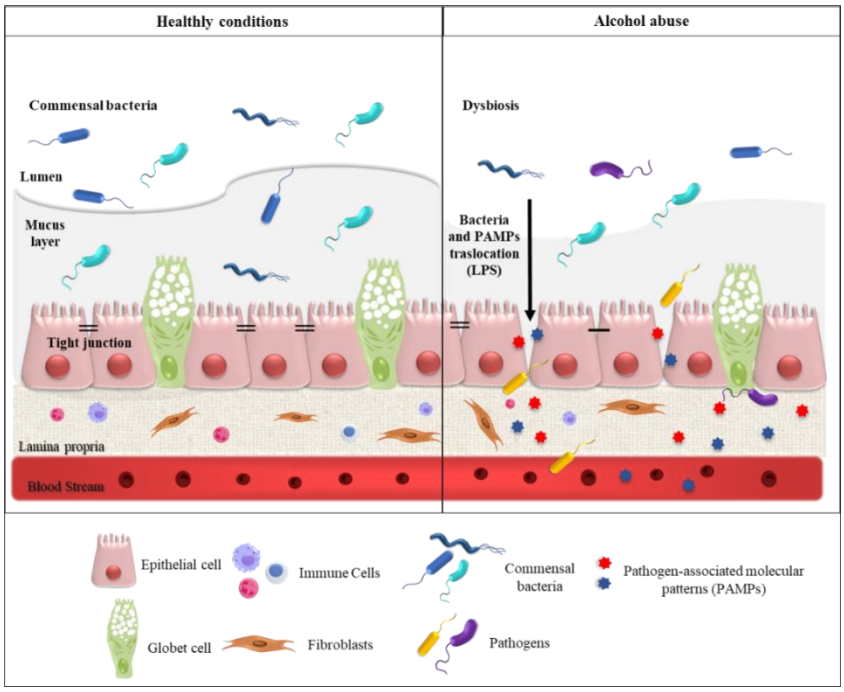

Figure 8. Alcohol-induced gut barrier dysfunction. Schematic diagrams of the gut barrier under healthy and alcohol-intoxicated conditions.

When these pathogenic antigens reach the liver via the portal vein, they are enabled to induce the activation of the inflammatory processes, stimulating the innate immunity response. As a consequence, pro-inflammatory mediators are secreted, such as chemokines and cytokines, such as TNF-α and Interleukin 1β (IL-1β), corroborating the systemic inflammation (Leclercq S., et al., 2014; Meroni M., et al.,2019).

Moreover, the *leaky gut* may alter CNS functionality, influencing psychological and cognitive function (Meroni M., et al.,2019). Although the mechanisms by which the gut–brain axis conveys the effects of alcohol on the CNS are not well established, several studies suggest that systemic inflammation, like that caused by alcohol-provoked *leaky gut*, can influence the nervous system in several ways. For example, alcohol-induced gut inflammation can result in a systemic inflammation that subsequently affects neuronal function and may drive some symptoms of alcohol

withdrawal, including autonomic disturbances and anxiety (Retson TA., et al., 2015). In addition, elevated cytokines caused by the inflammatory response may be able to enter the brain and disrupt the BBB and the passage of activated peripheral immune cells, thus enhancing the EtOH-induced neuroinflammation mainly in microglia and astrocytes (Banks WA., et al., 2015). Finally, alcohol-induced dysbiosis may have its own effect on the CNS via vagal afferent nerve fibers, which influence areas of the brain, including the thalamus, hippocampus, amygdala, and prefrontal cortex. Specifically, accumulating evidence suggests that alcohol-induced dysbiosis and gut microbiome may contribute to modifications in the vagal response and neuroinflammation in the CNS linked with alcohol-associated behaviours (Bishehsari F., et al., 2017; Gorky J., et al., 2016).

1.5 Probiotics

As previously mentioned, chronic and acute alcohol consumption lead to bacterial overgrowth and dysbiosis in both the small and large intestine in experimental animals (Li F., et al., 2016). Intestinal dysbiosis is defined as an imbalance of the various microbial entities in the intestine with a disruption of symbiosis (Fuenzalida C., et al., 2021). Specifically, using metagenomics-based techniques, a decline in the abundance of the phyla Bacteroidetes and Firmicutes was observed in alcohol-fed mice, with a proportional increase in the phyla Gram-negative Proteobacteria and Gram-positive Actinobacteria (Li F., et al., 2016; Yan AW., et al., 2011).

Since SCFAs are products derived from bacterial fermentation, changes in intestinal microbial composition might be related to differences in intestinal metabolism responsible for decreased SCFA levels observed after alcohol intake (Hartmann P., et al., 2015). SCFAs provide energy to enterocytes and exert a protective effect on the gut barrier function by promoting an anti-inflammatory environment, thus mediated by regulatory mechanisms of immune response activation (Litwinowicz K., et al., 2020). Additionally, the dysbiosis is associated with increased intestinal permeability leading direct damage to the CNS as microbial products, including LPS, can cross the

BBB, causing oxidative stress that contributes to neuroinflammation (Fuenzalida C., et al., 2021).

In this scenario, it is not difficult to imagine that, by an indirect effect of probiotics on microbiota modulation and the reduction of systemic inflammation, they could be a good therapeutic alternative against damage caused by alcohol abuse.

Generally, probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (Mack DR., 2005). Probiotics' beneficial effects have been widely studied in different pathologies, such as gastrointestinal diseases, and to treat various central disorders by restoring microbiota properties and the capability to modulate systemic and CNS inflammation (Fuenzalida C., et al., 2021). Currently, the most often used probiotics are Bifidobacteria and lactic acid bacteria (LAB). Lactobacilli, major contributors to the LAB group, are frequently used probiotics. Various species and strains of Lactobacilli have been used in the practice in animals and humans, including *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Lactobacillus helveticus*. Most of these species belong to the phylum Firmicutes. *Bifidobacterium*, producing lactic acid, is another commonly used probiotic genus and belongs to the Actinobacteria phylum (Li F., et al., 2016; Mutlu EA, et al., 2009). To date, a large number of probiotics have been reported to be suitable for the treatment of a variety of diseases, and this number is still growing. Clearly, due to the critical role of microbiota in GBA, restoration of GM contributes to the beneficial effects of probiotics in systemic inflammation. Despite many proof-of-effectiveness of probiotics have been widely investigated in multiple animal models and clinical studies, the mechanisms by which probiotics function are still poorly understood. To date, several important mechanisms including the modification of GM, improvement of the intestinal epithelial barrier function, regulation of the immune system and inflammation, and alteration of hepatic lipid homeostasis have been proposed. For example, several studies show that probiotics administration increased the expression of TJ proteins, such as claudin-1, ZO-1, and occludin at both protein and mRNA levels and normalized barrier function, restoring also the expression of mucus-related genes, which were decreased by alcohol ingestion in mice (Wang Y., et al., 2012). Furthermore, considering that bacterial metabolism produces endogenous ethanol,

which might also have deleterious effects on the gut barrier, probiotics could contribute to intestinal barrier function by modulating certain gut bacteria leading to reduced metabolism of alcohol and ROS production in the intestine (Forsyth CB., et al., 2009; Llopis M., et al., 2016; Meroni M., et al., 2019; Spinucci G., et al., 2006; Yan AW., et al., 2011). In this way, probiotic administration decreased alcohol-induced systemic and intestinal TNF-α and IL-1β levels, which might contribute to the beneficial effects of probiotics on gut barrier integrity (Chang B., et al., 2013; Hong M., et al., 2015; Segawa S., et al., 2008).

1.5.1 Lactobacillus

Lactobacilli are ubiquitous gram-positive rod-like bacteria, acid-resistant, functioning as a barrier of many bacteria. As acidophiles, lactobacilli are the first kind of micro-organisms that can start growing in the proximal small bowel and therefore they are the first micro-organisms encountered in the GIT. In contrast to most other intestinal bacteria, most lactobacilli are limited in depolymerizing polysaccharides, as starch in food, and/or hydrolysing sugars from glycoproteins, e.g., the human intestinal mucins. In addition, they prefer lactose, sucrose, and glucose as energy sources. As facultative anaerobes, these bacteria resist intestinal traces of molecular oxygen, and gain only a small part of the energy content of the sugars, i.e., the energy released by enzymatic substrate phosphorylation in sugar fermentation. Due to the absence of adequate enzymes and cytochromes in these bacteria, most energy present in lactic acid and ethanol is inaccessible. Thus, lactobacilli ferment suitable sugars to either lactic acid, e.g., the homolactic *L. acidophilus*, or to lactic acid, EtOH and CO_2, e.g., the heterolactic *L. fermentum* (Bongaerts G., et al., 2001).

Amongst probiotics, *Lactobacillus* is one of the most commonly used, having beneficial outcomes, for example by reducing gastrointestinal and upper respiratory tract infections (Azagra-Boronat I., et al., 2020; Maldonado J., et al., 2012). In addition, several of these strains have been demonstrated to have immunomodulatory properties, as have an impact on both innate and acquired immunity, inducing the

production of a number of cytokines and chemokines, such as TNF-α, IL-1β (Pérez-Cano FJ., et al., 2010).

Regarding their role against oxidative stress damage, several studies have shown that *L. fermentum* has a protective effect in the liver, preventing endotoxemia and improving inflammation by modulating the expression of NF-κB and TNF-α (Frick JS., et al., 20017; Osman N., et al., 2006; Barone R., et al., 2016). Moreover, several studies support the notion that Lactobacillus spp. can improve gut barrier function in a wide range of pathological conditions. For example, *Lactobacillus GG* treatment was recently shown to protect against gastric hyperpermeability and injury caused by acute alcohol exposure in a rat model (Lambert D., et al. 2007).

Finally, *Lactobacillus* influences neurotransmitter and neurotrophin expression in the brain and intestine, implying that probiotic supplementation can enhance brain development, especially memory and brain plasticity (Ranuh R., et al., 2019). Thus, in vitro and in vivo animal models have shown that probiotics and especially Lcatobacillus sp. can preserve barrier function in response to alcohol and diverse other injurious stimuli (Forsyth CB., et al., 2009).

1.6 Heat Shock Proteins

As described previously, alcohol abuse causes a change in cellular homeostasis due to a strong induction of factors promoting oxidative stress both locally (gut, liver) and systemically (brain and other organs).

Among the mediators of the cellular response to stress are Heat shock proteins (HSPs), an important class of molecular chaperones with various functions (Rappa F., et al., 2012). Molecular chaperones are a family of proteins forming an accurate and sophisticated system responsible for the quality control and homeostasis of the proteome. These proteins help the other cellular proteins to achieve their native state (correct folding or functional conformation), reach their final destination (e.g., the endoplasmic reticulum or the mitochondria), resist denaturing by stressors, and regain the native state after partial denaturation (Macario AJ., 1995).

In eukaryotic cells, there are many structurally and functionally distinct classes of chaperones that vary in size, complexity, target proteins and mechanisms of action. However, these different classes of molecular chaperones work together to form elaborate, cooperative networks and ensure cellular proteostasis (Strauch A., et al., 2016). A specific class of chaperones are the HSPs, which comprise five major families classified according to their molecular weight (HSP40, HSP60, HSP70, HSP90, HSP100 and the so-called small HSPs) (Kampinga HH., et al., 2009). Some HSPs are constitutively expressed and are up-regulated in response to stress, while others are induced only upon exposure of cells to environmental and physiological stresses to counteract the increased concentration of misfolded/unfolded proteins that occurs.

Following heat shock or other stresses, HSPs are produced after transactivation of the genes by a family of DNA-binding proteins called heat shock transcription factors (HSFs). In response to stress, HSF is released and translocated into the nucleus, where activates constitutive HSPs that, together with the induced HSPs, affect cytoprotection (Santoro MG., 2000).

Given their important role, it is not surprising that the main effectors of the HSPs are evolutionarily conserved proteins in all three domains of life, existing in a variety of species, originally characterized on the basis of their strong induction by heat shock (Richter K., et al., 2010).

The HSPs occupy the stage's center whenever and wherever there is cellular and tissue injury caused by local or systemic stressors via protein damage. It follows that chaperone failure will have an impact, possibly serious, on one or more cellular function, which may lead to disease (Macario AJ., 1995).

Among other roles, HSPs are also induced by a number of chemical agents including ethanol, modulating the effects of inflammatory cascades leading to endogenous generation of ROS and intrinsic apoptosis through the inhibition of pro-inflammatory factors. In addition, a number of studies suggest a close reciprocal relationship between the activities of HSF and NF-κB. In these studies, the inhibitors of NF-κB activation that effectively prevented NF kappa B inhibitor-α (IκB-α) degradation were potent activators of HSF transcription (Malago JJ., et al., 2002).

Thus, HSPs have a major role in protecting, eukaryotic and prokaryotic cells from harmful stimuli, playing a cytoprotective role (Ikwegbue PC., et al., 2017).
With specific reference to the GIT, HSPs have a pivotal role in protecting intestinal epithelial cells (IECs) from transport and barrier changes associated with gut inflammation (Ciancio MJ., et al., 2008; Otaka M., et al., 2006; Petrof EO., et al., 2004). So, the inducible HSPs, produced in response to stressors such as hypoxia, oxidative radicals, hyper- and hypothermia, and malnutrition act to prevent protein denaturation and maintain actin organization during times of stress (Arnal ME., et al., 2016; Ciancio MJ., et al., 2008; Petrof EO., et al., 2004). *In vitro* studies have demonstrated that inducible HSP70 protect critical cellular proteins, maintaining IEC's tight junctional integrity, to reduce permeability (Liu TS., et al., 2003; Musch MW., et al., 1999). Furthermore, given their protective and beneficial effects on IECs function, some studies have suggested that one of the mechanisms of probiotic action may involve the induction of cytoprotective HSPs (Arnal ME., et al., 2016; De Angelis M., et al., 2004; Liu H., et al., 2014; Liu HY., et al., 2015; Tao Y., et al., 2006).

1.6.1 HSP60

HSP60 is a chaperone belonging to the Chaperonins of Group I and typically functions inside mitochondria in which, together with the co-chaperonin HSP10, maintains protein homeostasis. In addition to this canonical role, outside and inside the mitochondria, HSP60 plays many others functions including participation in inflammation, autoimmunity, carcinogenesis, cell replication, and other cellular events in health and disease (Cappello F., et al., 2014; Caruso Bavisotto C., et al., 2020).
Although modern chaperonology has taught us that HSP-chaperones, including HSP60 and HSP10, play a critical role in several diseases (Cappello F., et al., 2005, 2011 and 2018; Rappa F., et al., 2016; Rodolico V., et al., 2010; Tomasello G., et al., 2011), many studies have focused on the interaction between exogenous HSPs and the host. In fact, of all the molecular chaperones, HSP60 is highly conserved among all species and is called the *'common antigen'*. For instance, exogenous HSP60 either derived

from bacteria or human cells, may protect cells, including IECs, from apoptosis by affecting cell survival (Takada M., et al., 2010; Zhang L., et al., 2004).

It is pertinent to note that probiotics have the ability to inhibit NF-κB of the inflammatory pathway by inducing the expression of HSP60 (Moura CS., et al., 2018; Petrof EO., et al., 2004; Tao Y., et al., 2006).

Furthermore, in studies conducted by Barone R., et al., it is noted that the introduction of Pleurotus eryngii as a dietary supplement leads to a significant decrease in HSP60 protein levels in C26 colon cancer (Barone R., et al., 2021).

These results indicate that the chaperone function of HSP60 may protect small IECs from cytotoxic agents in both apoptotic and necrotic conditions. Furthermore, these data suggest that the protective role of probiotics may also be mediated by exogenous HSP60.

1.6.2 HSP90

HSP90 is a highly abundant and ubiquitous molecular chaperone playing an essential role in many cellular processes, such as cell cycle control, cell survival, hormone and other signalling pathways. It is important for the cell's response to stress and is a key player in maintaining cellular homeostasis (Jackson SE. 2013; Prodromou C., et al., 2003). Unlike the HSP60, the high constitutive levels of HSP90 observed in unstressed cells seem to play a vital role in modulating cellular protection through its interaction with other HSPs and various kinases. Recently, HSP90 was found to be a repressor of the double-stranded ribonucleic acid–dependent protein kinase R (PKR), and thereby keep NF-κB inactive (Malago JJ., et al., 2002).

As for HSP60, studies show that dietary supplementation with *Bacillus* could be used as an antibiotic alternative, as it modulates NF-κB signaling pathway, by regulating jejunal mucin-2 (MUC2), growth factor (GLP-2 and TGF-β2), and HSP (HSP60, HSP70, and HSP90) mRNA levels (Kan L., et al., 2021). In this experiment, up-expression of jejunal growth factors and HSPs, improves tissue repair and anti-stress host capacity.

In addition, overexpression of HSP90 in IECs reduces hypoxia-induced apoptosis. Notably, under physiological conditions, IEC apoptosis balancing IEC proliferation is the main mechanism responsible for intestinal epithelium turnover. However, excess IEC apoptosis could be pathogenic as the loss of IEC following apoptotic cell shedding could compromise epithelial integrity and increase host susceptibility to infection. Thus, it has been shown that through the phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt) signalling pathway, HSP90β exerts a cytoprotective effect against intestinal epithelial injury, through directly interacting with phosphorylated AKT (pAKT) and stabilizes it (Zhang S., et al., 2013).

Moreover, during host-microbe interactions, GM may bind to the host mucosa components, including HSP90 resulting in direct involvement in phagocytosis or bacterial activity (Singh KS., et al.,2018).

Research Aims

2. Objectives

The aim of my PhD project was the investigation of the connection between brain and intestine following the so-called brain gut axis. In particular, the project is centred on the protective effects of the probiotic *Lactobacillus fermentum* (*L. fermentum*) against oxidative stress damage and inflammation induced by EtOH consumption, using an animal model of alcohol abuse.

As mentioned above, chronic alcohol consumption induces damages to multiple organs, representing one of the most common causes of mortality worldwide (World Health Organization, 2018). Although the liver is mainly involved in the metabolism of alcohol, supporting the greatest degree of tissue damage, recent evidence has highlighted the role of the gut microbiota and its metabolites in the pathophysiology caused by excessive alcohol consumption (Meroni M., et al., 2019). Several studies have shown that alcohol-dependent subjects have increased intestinal permeability (known as *leaky gut*) and an altered composition and functionality of the intestinal microbiota, favouring the translocation of bacterial components of intestinal origin, such as lipopolysaccharides and endotoxin, from the intestinal lumen to the systemic circulation and to other organs (Samuelson DR., et al., 2019). In this way, endotoxin and non-metabolized alcohol reach the systemic circulation and other organs, including the CNS, leading to neurodegeneration. In fact, at the CNS level, alcohol affects several neurological pathways, causing significant changes in behaviour, cognitive and memory deficits. Furthermore, EtOH is also capable of inducing apoptosis and causing functional alterations in astrocytes, amplifying the neurodegeneration (Bishehsari F., et al., 2017; Huf F., et al., 2019).

Therefore, the use of probiotics could play a key role in the protection of alcohol damage, since they exert various gut health benefits, modulating of gut microbiota and

producing of bioactive metabolites. Among the large range of bacteria, the most common strains belong to Lactobacillus species (Kim BK., et al., 2017).

It is a Gram positive, optional aerobic bacterium that has antioxidant properties due to its ability to produce gluthatione, bacteriocins and biosurfactants (Hor YY., et al., 2019; Barone R., et al., 2016).

Starting from these assumptions, my doctoral thesis focused on the protective effects of the probiotic *L. fermentum* against oxidative stress damage and inflammation induced by chronic ethanol consumption, using an animal model of alcohol abuse. In fact, female 12-month-old mice (BALB/cAnNHsd) were orally fed with EtOH (96%) alone (n=5) or in combination with *L. fermentum* (10^9 CFU) (n=5) (groups 12 EtOH, and 12 EtOH-P, respectively), every day for twelve weeks. Both groups were compared with mice (n=5) fed with the standard diet (Ctrl) as per a previously calibrated experimental model (Barone R., et al., 2016).

At the end of the treatment, the mice were sacrificed and their brains and intestine were dissected, fixed and embedded in paraffin for histological analysis.

As better elucidate later, immunomorphological analysis was conducted to look for glia markers in the small intestine and cerebellum, since they are typically implicated in mechanisms pertaining to the effects of ethanol. Hematoxylin-eosin straining showed a *leaky gut* in the small intestine of mice treated with ethanol, characterized by altered barrier structure, and loss of the normal morphology of Purkinje cells in the cerebellum. Furthermore, immunomorphological and quantitative real-time PCR analyses were used to evaluate the effects of *L. fermentum* on the NF-κB signalling pathways and on the levels of cytokines and molecular chaperones. The levels of phosphorylated NF-κB were elevated in the 12 EtOH group but were lower in the 12 EtOH-P. *L. fermentum* induced downregulation of the TNF-α production and NF-κB activation via increased expression of the IκB-α. The probiotic induced the expression of the chaperones HSP60 and HSP90, by inhibiting IkB-kinase (IKK) and preventing NF-κB activation.

In conclusion, the experiments were conducted to identify a possible pathway explaining the protective role of the probiotic not only at the intestinal level but also in the cerebellum as a consequence of the connection between gut and brain.

The data obtained are summarized at the end of the thesis, while figure 23 is a representative explanation of the investigated molecules and of the working hypothesis I tried to demonstrate.

Materials and Methods

3.1 Animal model

The animal model was performed with female 12-month-old mice (BALB/cAnNHsd) obtained from Harlan laboratories S.r.l. (Udine, Italy). All animal experiments adhered to the recommendations in the guide for the care and use of laboratory animals set by the National Institutes of Health (NIH, Bethesda, MD). The animals were kept at a constant 12 :12 h light–dark cycle and had free access to food (4RF21 standard diet, Mucedola; Settimo Milanese, Milan, Italy) and water, which was the diet for the control (Ctrl) mice (n=5). For the experimental mice, EtOH 96% (Girolamo Luxardo, Torrella, Padova, Italy) was added to account for 15% of total calories. To allow for acclimation to the alcohol diet, the animals were given 5% EtOH-calorie content for 2 days, which was increased to 10% EtOH-calorie content for 2 days, followed by 15% EtOH-calorie content diet (Carr RM., et al., 2013 and 2014) for 12 (mouse group 12 EtOH, n=5) weeks. A calculation to increase the standard caloric need of the mice (14 kcal per day, 3.6 g of their food) with the addition of EtOH was made considering the caloric content of EtOH diluting it in water in a final volume of 100 µl. This volume was orally administered to the mice every day using a pipette. In another group of mice (12 EtOH-P, n=5) in addition to ethanol, they also received orally, using a pipette, *L. fermentum* probiotic (Bromatech S.r.l., Milan, Italy), 10^9 CFU (colony-forming unit) per animal in water, each day during the week, with two days off on weekends, until the end of the experiment. The probiotic was given 20 min after the EtOH administration to investigate its efficacy to counteract the deleterious effects of ethanol (**figure 9**). Food and water intake were measured every day, and body weight was measured three times weekly. The diet was controlled weighing the food to adjust it to the daily caloric needs of the mice. At the end of treatment, the mice were sacrificed 48 h following the last treatment and their brains and intestines dissected, fixed in

formalin and embedded in paraffin for histological and immunological analysis (Barone R., et al., 2016).

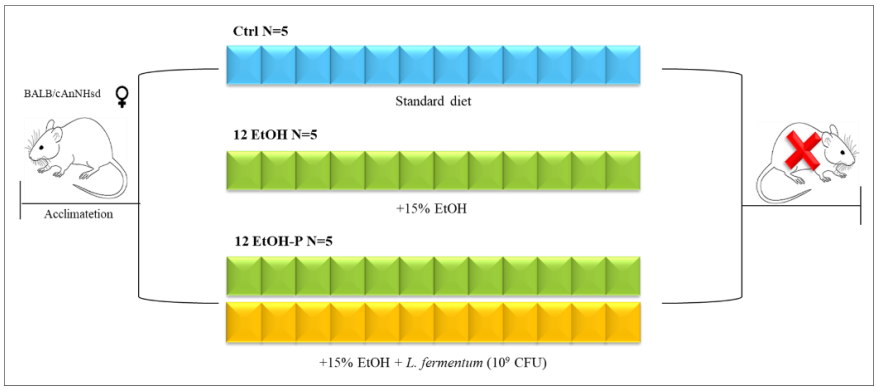

Figure 9. Experimental mice: animal model and diet (Barone R., et al., 2016).

3.2 Histopathology

Brain and intestinal sections with a thickness of 5 µm were obtained from paraffin blocks and stained with hematoxylin and eosin (H&E) for histological examinations. Sections were de-waxed in xylene for 10 min and rehydrated by sequential immersion in decreasing ethanol concentrations. Then, the sections were stained with hematoxylin-eosin and analyzed using a Leica DM5000 upright microscope (Leica Microsystems, Heidelberg, Germany). Brain and intestinal sections were examined by two independent observers in a blind manner, using coded slides and not knowing their source.

3.3 Immunofluorescence

For immunofluorescence, brain and intestine sections were incubated in the antigen unmasking solution (10 mM tri-sodium citrate, 0.05% Tween-20) for 8 min at 75°C

and treated with a blocking solution (3% BSA in PBS) for 30 min. Afterwards, the primary antibody (anti-GFAP, mouse monoclonal 1:50; anti-S100β, rabbit polyclonal 1:250; anti-FABP7, mouse monoclonal 1:50; anti-HSP60, mouse monoclonal 1:50 and anti-HSP90, mouse monoclonal 1:50; anti-pNF-κB, rabbit polyclonal 1:50; anti-IkBα, rabbit polyclonal 1:50; anti-TJP1, rabbit polyclonal 1:50) was applied and the sections were incubated in a humidified chamber overnight at 4 °C (table 1). Then, the sections were incubated for 1 h at room temperature with a conjugated secondary antibody (anti-mouse ATTO-488; anti-rabbit ATTO-647; anti-goat and anti-rabbit IgG–FITC), while nuclei were stained with DAPI (1:1000) for 8 minutes. Finally, the images were captured using a Leica Confocal Microscope TCS SP8 (Leica Microsystems).

3.4 Immunohistochemistry

Immunohistochemistry was performed on 5 μm thick sections of paraffin-embedded intestinal tissue, obtained with a cutting microtome. The sections were dewaxed in xylene for 30 min at 60°C, rehydrated, at room temperature, by sequential immersion in a graded series of alcohols, and transferred into water for 5 min. Subsequently, the serial sections were incubated in antigen unmasking solution (10mM tri-sodium citrate, 0.05% Tween-20, pH 6) for 8 minutes at 95°C and, afterwards, immersed in acetone at -20°C for 8 min to prevent the detachment of the sections from the slide. All subsequent reactions were conducted at room temperature. After a wash with PBS (Phosphate Buffered Saline pH7.4) for 5 min, sections were immunostained, using using Histostain®-Plus 3rd Gen IHC Detection Kit, which utilized the labelled biotin-streptavidin methodology. The primary antibodies used were anti-S100β, rabbit polyclonal 1:400, anti-GFAP, mouse monoclonal 1:100; anti-HSP60, rabbit polyclonal 1:300; anti-HSP90, mouse monoclonal 1:200; anti-MMP9, rabbit polyclonal 1:100; anti-pNF-κB, anti-rabbit polyclonal 1:100 (table 1). Appropriate positive and negative (isotype) controls, were run concurrently. Nuclear counterstaining was carried out using hematoxylin (Hematoxylin aqueosus formula, N. Cat. S2020, DAKO). Finally, the slides were prepared for observation with coverslips with an aqueous mounting

solution. The observation of the sections was performed with an optical microscope (Leica DM 5000 B) connected to a digital camera (Leica DC 300F).

TABLE 1. Antibodies

Primary Antibody	Overview	IHC	IF
Glial Fibrillary Acidic Protein (GFAP)	Sc-33673 Mouse Monoclonal Santa Cruz Biotechnology, Dallas, USA	1:100	1:50
S100β-calcium-binding protein (S100β)	Rabbit Polyclonal	1:400	1:250
Phospho-proinflammatory nuclear factor (pNF-κB)	Sc-33020 Rabbit Polyclonal Santa Cruz Biotechnology, Dallas, USA	1:100	1:50
Heat Shock Protein 60 (HSP60)	Ab-13532 Mouse Monoclonal Abcam Cambridge, UK	1:300	1:50
Heat Shock Protein 90 α/β (HSP90)	Sc-13119 Mouse Monoclonal Santa Cruz Biotechnology, Dallas, USA	1:200	1:50
Fatty Acid Binding Protein 7 (FABP7)	AT1D1 Mouse Monoclonal OriGene Technologies, Germany	-	1:50
Matrix Metalloproteinase 9 (MMP9)	Sc-10737 Rabbit Polyclonal Santa Cruz Biotechnology, Dallas, USA	1:100	-
Tight Junction Protein 1 (TJP1)	C82740 Rabbit Polyclonal Sigma-Aldrich, Germany	-	1:50
NF-kappa-B inhibitor alpha (IκBα)	Sc-203 Rabbit Polyclonal Santa Cruz Biotechnology, Dallas, USA	-	1:50
Interleukin 6 (IL-6)	P620 Rabbit Polyclonal Invitrogen, Carlsbad, USA	-	1:50

3.6 RNA preparation and Quantitative Real Time Polymerase Chain Reaction (RT-PCR) Assay

Total RNA was extracted using the Tri-reagent (SigmaAldrich) according to the manufacturer's instructions, from cry preserved intestinal tissues of control and treated mice. RNA concentration in the samples was determined spectrophotometrically. Reverse transcription was performed using the ImProm-II Reverse Transcriptase Kit (Promega, Madison, WI, USA). qRT-PCR analysis was performed using GoTaq qPCR Master Mix (A6001, Promega). All samples were run in triplicate. A threshold cycle (CT) was observed in the exponential phase of amplification, and quantification of relative expression levels was performed with standard curves for target genes and the endogenous control (GAPDH). Geometric means were used to calculate the ΔΔCT (delta-delta CT) values and expressed as $2^{-\Delta\Delta CT}$. Complementary deoxyribonucleic acid (cDNA) was amplified using the Rotor-gene 6000 Real-Time PCR Machine (Qiagen GmbH, Hilden, Germany), using primers indicated in Table 2.

TABLE 2. Primers used for qRT-PCR

Primers	Sequence
Glyceraldehyde-3-phosphate dehydrogenase (GADPH)	F: 5′-CAAGGACACTGAGCAAGAGA-3′ R: 5′-GCCCCTCCTGTTATTATGGG-3′
Tumor necrosis factor (TNF-α)	F: 5′-CCCCCAGTCTGTATCCTTCT-3′ R: 5′-TTTGAGTCCTTGATGGTGGT-3′

Results

4.1 Effect of ethanol consumption and *L. fermentum* administration on intestinal barrier structure

To detect the effects of EtOH on intestinal damage and the protective role of *L. fermentum*, I studied several mechanisms underlying the EtOH-induced barrier dysfunction, including direct damage to epithelial cells. The H&E stain conduced on small intestine showed a "*leaky gut*" in the 12 EtOH group, characterized by altered barrier functions and structure (**figure 10**). In the Ctrl group, the intestine appeared normal with intact epithelial barrier, organized enterocytes, and no signs of inflammation. However, long-term EtOH ingestion has been reported to induce histological changes in small intestinal mucosa in 12 EtOH, showing long villi, due to alcohol-induced gut leakiness. In the 12 EtOH-P (±500µm) villi are longer than the Ctrl (±380µm) but shorter than the 12 EtOH (±600µm) (**figure 10A**). In particular, the analysis showed that the villus length decreased significantly in 12 EtOH-P, compared with 12 EtOH ($p<0,001$) (**figure 10B**). Furthermore, mice treated with only EtOH showed a loss of ephitelium in the tips of the villi, compared with 12 EtOH-P. Additionally, we examined the alterations in the number of goblet cells (black arrows), one of the major components of the extrinsic barrier producing mucin. As showe in the figure 10C, 12 EtOH displayed a decreased number of goblet cells than 12 EtOH-P and Ctrl groups, leading to less mucus secretion.

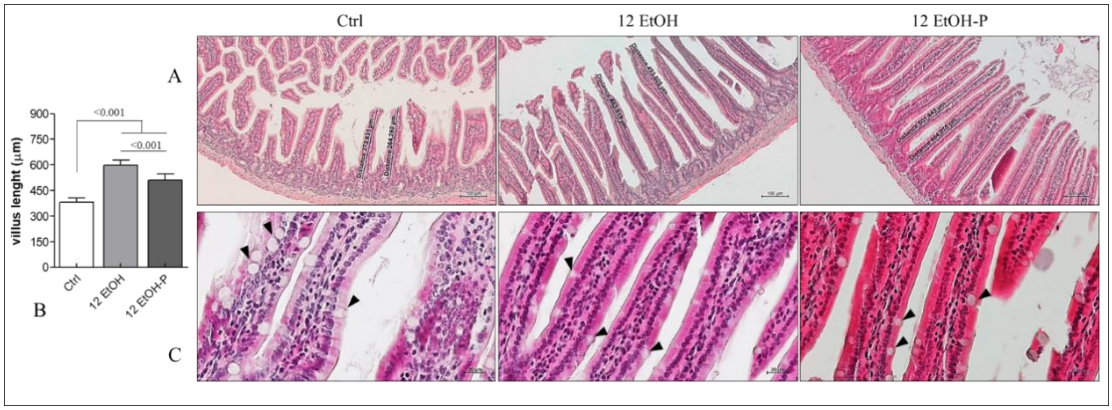

Figure 10. Histopathology of the small intestine. H&E-stained tissue sections of small intestine from mice fed with a standard diet (Ctrl), ethanol (12 EtOH) and ethanol and *L. fermentum* probiotic diets (12 EtOH-P). **A.** The images identify the three components of the mucosa: epithelium, lamina propria and muscolaris mucosae. **B.** The analysis showed the villi length. The bars indicate the degree of the villi length in the small intestine of Ctrl, 12 EtOH and 12 EtOH-P diet. One-way analysis of variance, Bonferroni post hoc test. Magnification 10X. **C.** The images show the components of the small intestine wall. In the intestinal villi of 12 EtOH, note the minor presence of the mucous-secreting globet cells (black arrows).

4.2 Effect of ethanol consumption and *L. fermentum* administration on the intestinal barrier integrity

To determine the effects of ethanol and of *L. fermentum* administration on the integrity of the intestinal barrier, the expression level and subcellular localization of the matrix metalloproteinase 9 (MMP-9) and ZO-1 were evaluated through an immunohistochemistry and immunofluorescence analysis (**figure 11-12**).

As showed in Figure 11, immunoreactivity for MMP-9 increased in the epithelial cells of the small intestine belonging from mice treated only with ethanol compared to the control and to 12 EtOH-P (**figure 11**).

ZO-1 immunoreactivity decreased after EtOH consumption compared with the control (**figure 12**). In particular, in the small intestine of control mice, ZO-1 was localized to epithelial tight junction, lying at the apical compartment of cell-cell junctions. In contrast, this ordered appearance of ZO-1 was disrupted in the small intestine of 12 EtOH, indicating that EtOH consumption impairs intestinal tight junction barrier. Instead, *L. fermentum* treatment up regulated ZO-1 immunoreactivity compared with the EtOH group (**figure 12**).

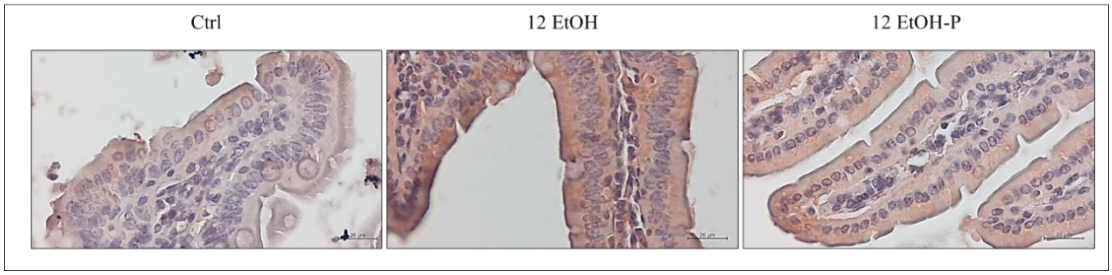

Figure 11. Immunohistochemistry of MMP-9 on small intestine slices. The immunohistochemistry reaction was conducted on small intestine slices of mice fed with a standard diet (Ctrl), ethanol (12 EtOH) and ethanol and *L. fermentum* probiotic diets (12 EtOH-P). Immunohistochemistry reaction shows MMP-9 was strongly induced in the 12 EtOH. Magnification 63X.

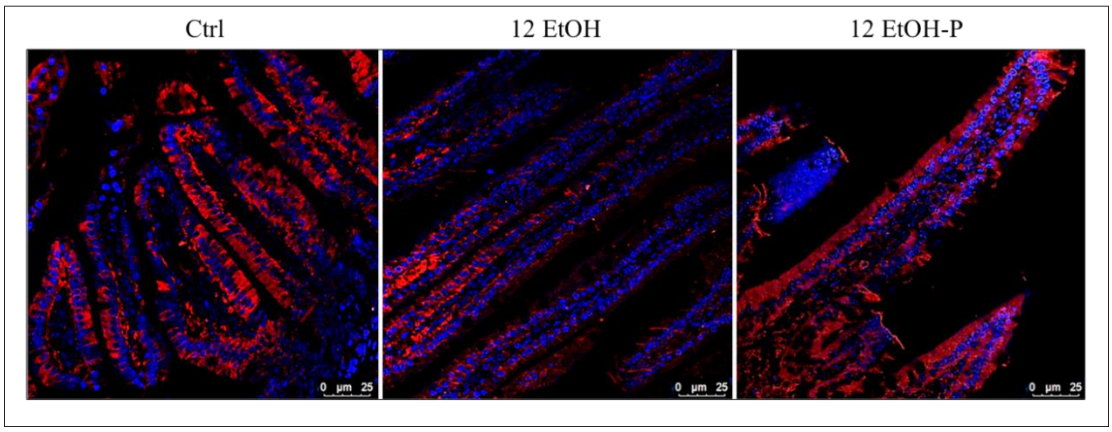

Figure 12. Immunofluorescence of ZO-1 on small intestine slices. The immunofluorescence reaction was conducted on small intestine slices of mice fed with a standard diet (Ctrl), ethanol (12 EtOH) and ethanol and *L. fermentum* probiotic diets (12 EtOH-P). In Ctrl, ZO-1 was localized to epithelial tight junction, lying at the apical compartment of cell-cell junctions. In contrast, this ordered appearance of TJP-1 was disrupted in the small intestine of 12 EtOH. Instead, *L. fermentum* treatment up regulated ZO-1 localization compared with the EtOH group. Magnification 40X.

4.3 Ethanol consumption and *L. fermentum* administration induced glial fibrillary acidic protein (GFAP) and anti-S100β-calcium-binding protein (S100β) expression in the small intestine

As astrocytes and microglia in the CNS, enteric glial cells (EGCs) participate in the regulation of inflammatory events in the gut. For this reason, the immunohistochemistry reaction was conducted on small intestine slices to identify the ENS, using anti-GFAP (**figure 13A**) and anti- S100β antibodies (**figure 13B**). Both glia markers were found elevated in 12 EtOH compared to 12 EtOH-P and Ctrl groups. Glia markers labelling was observed in the *lamina propria*, at the base of the cryps, and in the myenteric and submucosal plexuse.

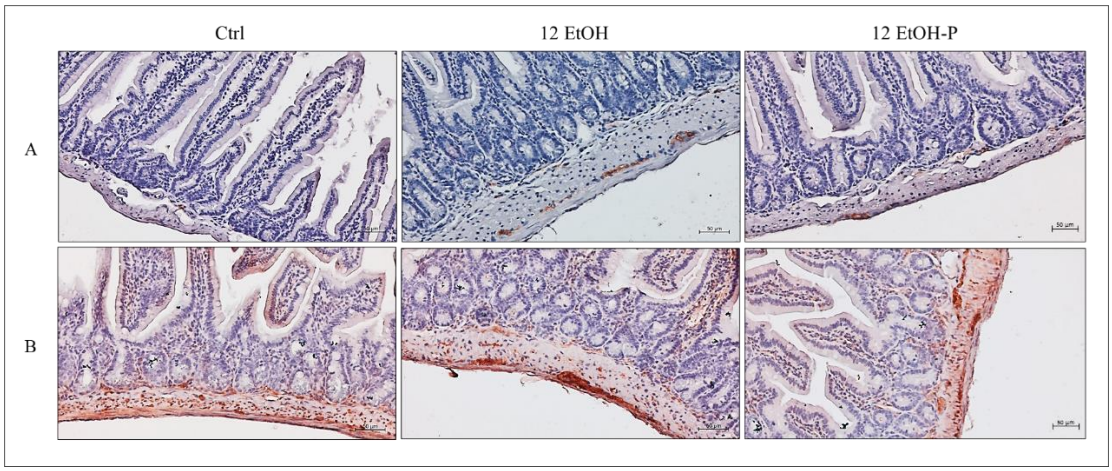

Figure 13. Immunohistochemistry of GFAP and S100β on small intestine slices. The immunohistochemistry reaction was conducted on small intestineslices of mice fed with a standard diet (Ctrl), ethanol (12 EtOH) and ethanol and *L. fermentum* probiotic diets (12 EtOH-P) to identify the ENS, using antiGFAP (**A**) and anti-S100βantibodies (**B**). Glia markers labelling was observed in the lamina propria, at the base of the cryps, and in the myenteric and submucosal plexuse. Magnificarion 20X.

4.4 Small intestine belonging from *L. fermentum*-fed mice showed the decrease of pNF-κB and IL-6 immunoreactivity and the increase of IκB-α

In order to elucidate the pathway involved in the possible anti-inflammatory effects of *L. fermentum*, I studied the expression of pNF-κB and IL-6 (**figure 14 and 15A**). In particular, the immunohistochemistry reaction conducted on small intestine slices showed that in 12 EtOH the tips of villi were strongly positivity for the pNF-κB,

indicating the presence of inflammation as a consequence of a probably immune response caused by a translocation of bacteria components. Instead, the pNF-κB expression level was diminished in 12 EtOH-P (**figure 14**). Moreover, the immunofluorescence for IL-6 revealed that the immunoreactivity was lower in Ctrl and 12 EtOH-P groups compared with12 EtOH (**figure 15A**).

Finally, to better understand how the probiotic may regulate NF-κB activity, I investigated the distribution of its inhibitor IkB-α. As showed in figure 15B, IkB-α tissue distribution decreased in 12 EtOH compared with the control and 12 EtOH-P (**figure 15B**).

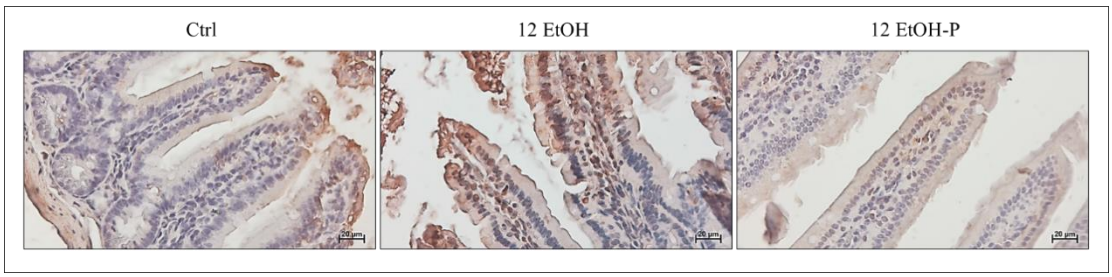

Figure 14. Immunohistochemistry of the pNF-κB on small intestine slices. The immunohistochemistry reaction was conducted on small intestine slices of mice fed with a standard diet (Ctrl), ethanol (12 EtOH) and ethanol and *L. fermentum* probiotic diets (12 EtOH-P). Immunohistochemistry reaction shows pNF-κB was strongly induced in the 12 EtOH. Magnification 40X.

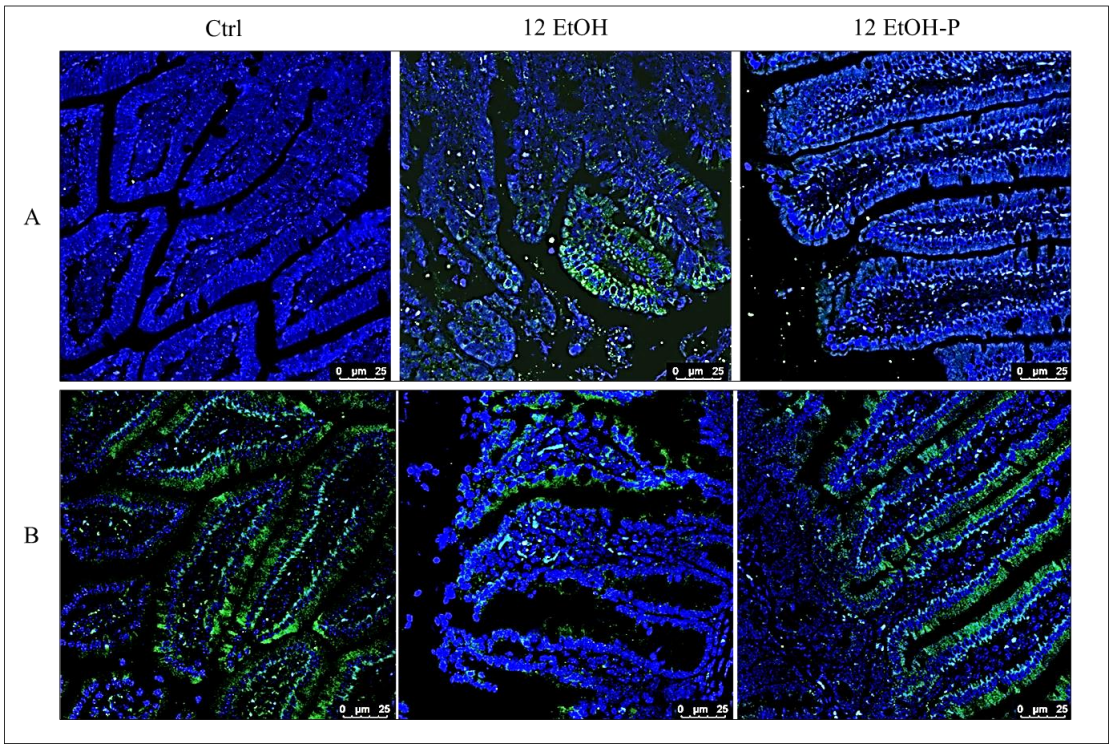

Figure 15. Immunofluorescence of IL-6 and IkB-α on small intestine slices. The immunofluorescence reaction was conducted on small intestine slices of mice fed with a standard diet (Ctrl), ethanol (12 EtOH) and ethanol and *L. fermentum* probiotic diets (12 EtOH-P). **A.** In 12 EtOH, IL-6 was localized to epithelial tips **B.** Immunofluorescence reaction for IkB-α showed a decreased in 12 EtOH. Magnification 40X.

4.5 *L. fermentum* induced the down-regulation of TNF-α gene expression level

In order to complete the pathway involved in the possible anti-inflammatory effects of *L. fermentum*, I examined the gene expression levels of TNF-α on small intestine tissues by RT-PCR analysis. TNF-α gene expression level decreased significantly in 12 EtOH-P, compared with 12 EtOH ($p<0,03$), suggesting that TNF-α is downregulated by the protective effect of the probiotic against damage caused by alcohol abuse (**figure 16**).

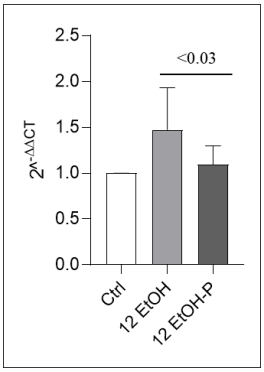

Figure 16. TNF-α gene expression level in small intestine of mice fed with a standard diet (Ctrl), ethanol (12 EtOH) and ethanol and *L. fermentum* probiotic diets (12 EtOH-P). The bars indicate the degree of the cytokine's gene expression normalized for the reference genes, according to the Livak method ($2^{-\Delta\Delta CT}$), in the small intestine of Ctrl, ethanol diets 12 EtOH, and ethanol and probiotic 12 EtOH-P diet mice. Ratio pro-inflammatory cytokines levels/GAPDH levels as a reflection of TNF-α decrease (mean±s.d.). One-way analysis of variance, Bonferroni post hoc test.

4.6. Effects of *L. fermentum* administration on HSP60 and HSP90 immunoreactivity in the small intestine

Since several members of HSPs are able to act against oxidative stress damage and inflammation, I conducted experiments to investigate the HSP60 and HSP90 roles (**figure 17-18**). In particular, the immunohistochemistry analysis showed an overexpression of both HSP60 and HSP90 in the small intestine of mice treated with *L. fermentum* (12 EtOH-P) compared to 12 EtOH.

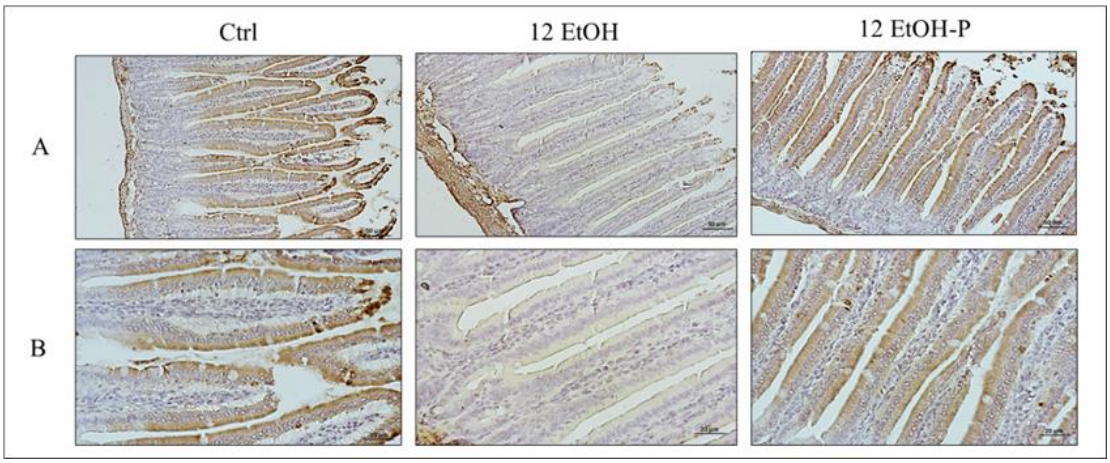

Figure 17. Immunohistochemistry of HSP60 on small intestine slices. The immunohistochemistry reaction was conducted on small intestine slices of mice fed with a standard diet (Ctrl), ethanol (12 EtOH) and ethanol and *L. fermentum* probiotic diets (12 EtOH-P) to identify the HSP60 expression. Magnification 20X (**A**) and 40X (**B**).

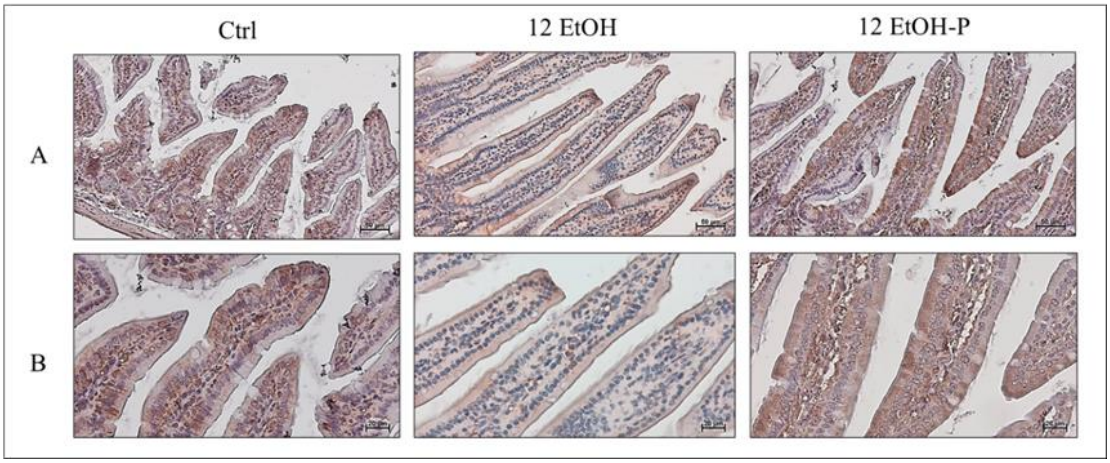

Figure 18. Immunohistochemistry of HSP90 on small intestine slices. The immunohistochemistry reaction was conducted on small intestine slices of mice fed with a standard diet (Ctrl), ethanol (12 EtOH) and ethanol and *L. fermentum* probiotic diets (12 EtOH-P) to identify the HSP90. Magnification 20 (**A**) and 40X (**B**).

4.7 Effect of ethanol consumption and *L. fermentum* administration on the cerebellum

As EtOH exposure causes cerebellar dysfunction, H&E staining was conducted on cerebellum of brain slices to identify if there were any morphological changes in the experimental groups (**figure 19**). In particular, the cerebellum of 12 EtOH fed mice showed the presence of vacuolization in the area presenting white matter, probably caused by lipid peroxidation. Furthermore, in the 12 EtOH-P, the presence of vacuoles decreased after treatment with the probiotic (**figure 19B**). In addition, the H&E staining of cerebellar cortex of the EtOH-treated groups (12 EtOH and 12 EtOH-P), showed a loss of the normal cellular morphology of PCs. In particular, many PCs were observed without a prominent nucleolus or well-defined nuclear membrane, losing their characteristic pyriform shape (**figure 19C**). This fact could result in modifications of the structure and function of other brain regions that receive cerebellar inputs.

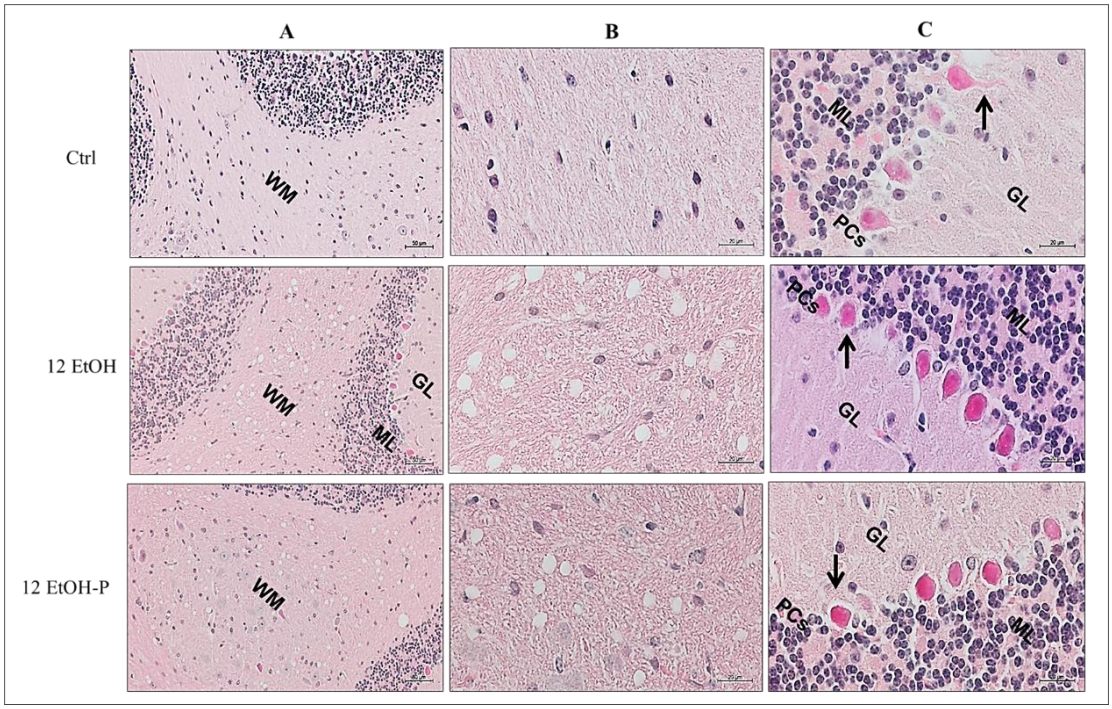

Figure 19. Histopathology of the cerebellum. H&E-stained sections of cerebellum slices of mice fed with a standard diet (Ctrl), ethanol (12 EtOH) and ethanol and *L. fermentum* probiotic diets (12 EtOH-P). **A.** In general, the cerebellum is divided into the following histological layers: the molecular layer (ML), Purkinje Cells layer (PCs), granular layer (GL) and the subcerebellar white matter (WM). Magnification 20X. **B.** Morphological analysis showed the presence of vacuolization in the area presenting white matter in the cerebellum of mice treated with ethanol (12 EtOH and 12 EtOH). Magnification 20X. **C.** In addition, the H&E stain of cerebellar cortex of the ethanol-treated groups (12 EtOH and 12 EtOH-P), showed a loss of the normal cellular morphology of PCs. In particular, many PCs were observed without a prominent nucleolus or well-defined nuclear membrane, losing their characteristic pyriform

shape. Magnification 63X. WM: White matter; ML: Molecular layer; GL: Granular Layer; PCs: Purkinje cells

4.8 Effect of ethanol consumption and *L. fermentum* administration on astrogliosis

The consequence of EtOH exposure on glia cell activation and vacuole formation in the cerebellum were investigated using GFAP, S100β and fatty acid binding protein 7 (FABP7) immunolabeling (**figure 20**). EtOH-exposed mice presented GFAP positive astrocytic area in central white matter, where vacuolization was found. Therefore, increases in the numbers of GFAP+ processes would be a consequence of astroglisosis, leading to astrocyte hypertrophy. GFAP protein expression levels decreased in the cerebellum of 12 EtOH-P compared to the 12 EtOH group, suggesting the protective role of *L. fermentum* against the alteration of these glial changes (**figure 20A**). Regarding S100β expression level, the protein was strong in 12EtOH and 12 EtOH-P, compared to the Ctrl. The S100β expression level increase in the soma of Bergmann glial within the Purkijnie cell layer of ethanol-treated mice versus the Ctrl group, leading probably to neuronal degeneration (white arrows) (**figure 20B**).

Finally, FABP7 is abundantly expressed in several type of glial cells, such as astrocytes and oligodendrocyte, due to gliosis after EtOH treatment. In the Ctrl, FABP7 was present in a unique population of glia of molecular layer, as normal cerebellum condition, and it was increased in a subset of reactive astrocytes. In fact, not only the strongest immunostaining for FABP7 in the 12 EtOH was present in the Bergmann glia, but also it was positive in the cytoplasm of some neurons and in the fibres of white matter, in the same area in which the vacuolization was observed (**figure 20C**).

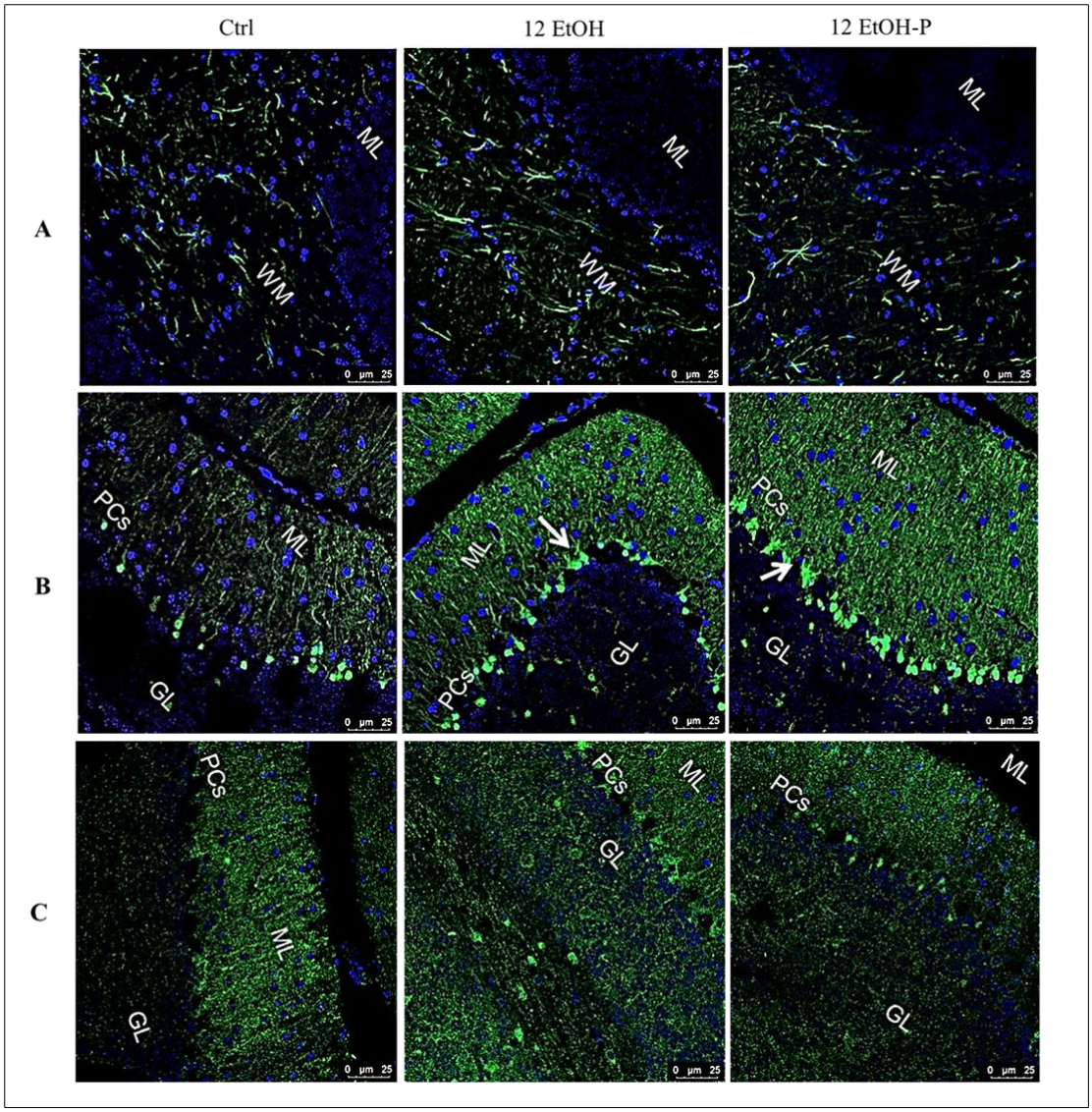

Figure 20. Immunofluorescence of GFAP, S100β, FABP7 on cerebellum slices. To study the astrogliosis, the immunofluorescence reaction was conducted on cerebellum slices of mice fed with a standard diet (Ctrl), ethanol (12 EtOH) and ethanol and *L. fermentum* probiotic diets (12 EtOH-P) to identify the glia cells, using anti- (GFAP) (**A**) and anti-S100β (**B**) and anti-FABP7 antibodies (**C**). Magnification 40X. WM: White matter; ML: Molecular layer; GL: Granular Layer; PCs: Purkinje cells

4.9 The cerebellum belonging from *L. fermentum*-fed mice showed the decrease of pNF-κB

To demonstrate if the effects registered in the small intestine may be translated at the central level, I investigated pNF-κB tissue distribution on cerebellum slices by

immunofluorescence (**figure 21**). The immunoreactivity for pNF-κB was low in the control group while increased in the 12 EtOH in the area of white matter and in the granular layer (**figure 21A-B**). Instead, pNF-κB distribution levels decreased in 12 EtOH-P in the same regions.

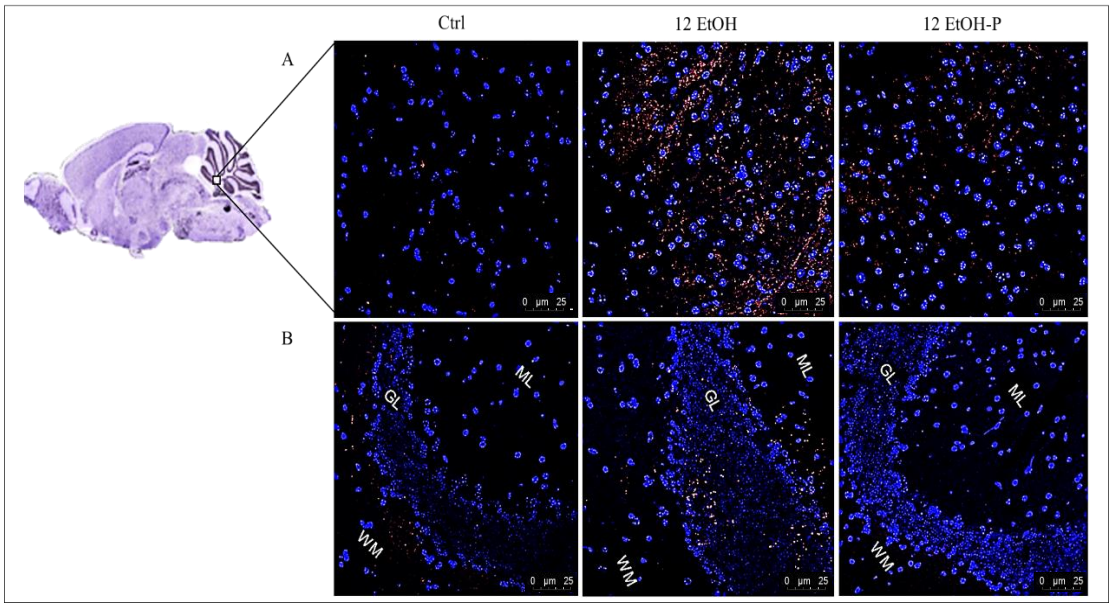

Figure 21. Immunofluorescence of pNF-κB on cerebellum slices. To demonstrate that reactive astrogliosis induces activation of the NF-κB signalling pathway in astrocytes, the immunofluorescence reaction was conducted on cerebellum slices of mice fed with a standard diet (Ctrl), ethanol (12 EtOH) and ethanol and *L. fermentum* probiotic diets (12 EtOH-P) to identify thei pNF-κB expression in white matter (**A**) and granular layer (**B**) of cerebellum. Magnification 40X. WM: White matter; ML: Molecular layer; GL: Granular Layer; PCs: Purkinje cells. (Paxinos G. and Franklin KB., 2019)

4.10 Effects of *L. fermentum* administration on HSP60 and HSP90 immunoreactivity in the cerebellum

Brain slices were used to conducted immunofluorescence analysis for HSP60 and HSP90 as markers of oxidative stress (**figure 22**). As in the gut, both protein levels increased in 12 EtOH-P compared with 12 EtOH (**figure 22A** and **22B**). In particular, the proteins seem to be present in the neuropil and in the cell body of neurons.

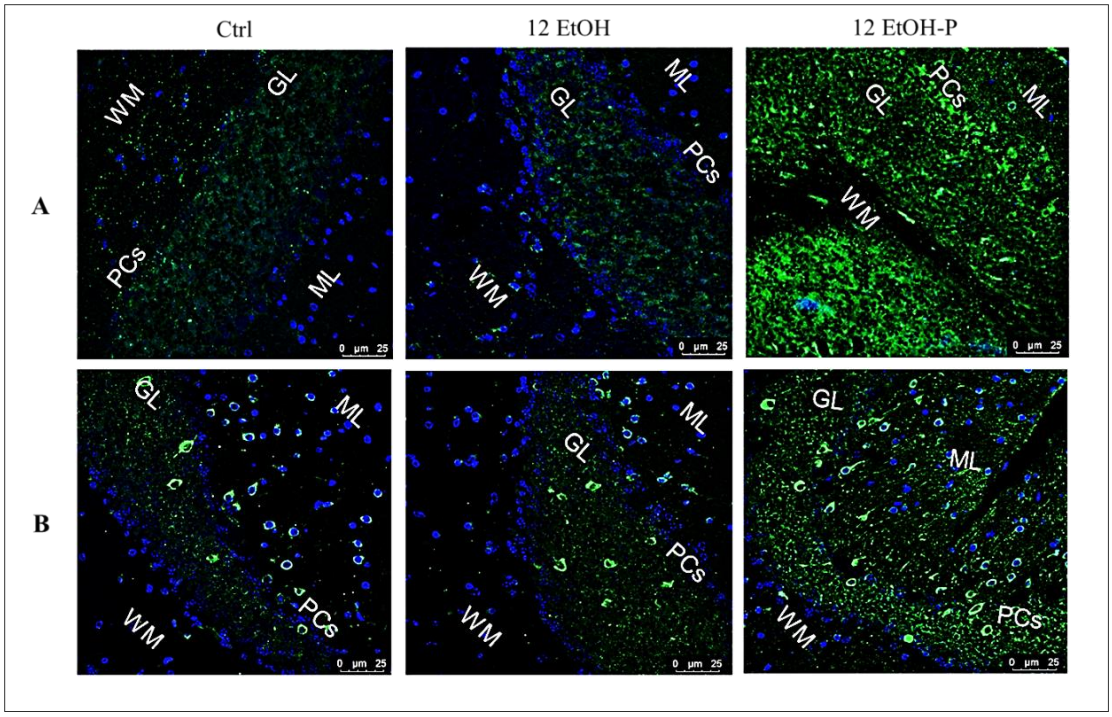

Figure 22. Immunofluorescence of HSPs (HSP60, HSP90) on cerebellum slices. The immunofluorescence reaction was conducted on cerebellum slices of mice fed with a standard diet (Ctrl), ethanol (12 EtOH) and ethanol and *L. fermentum* probiotic diets (12 EtOH-P) to identify the HSP60 (**A**) and HSP90 (**B**) expression. Magnification 40X. WM: White matter; ML: Molecular layer; GL: Granular Layer; PCs: Purkinje cells

Discussion

The digestive system and the brain are intimately connected to each other, constituting the so-called gut-brain axis (Foster JA., et al., 2017). However, due to the symbiosis between the host and gut bacteria, many papers have reported the definition of microbiota-gut-brain axis (Ding JH., et al., 2020; Suganya K., et al., 2020). Indeed, from birth to death, we are colonized by a vast, complex and dynamic consortium of microorganisms which, due to the presence of nutrients, mainly occupy the intestine. So, the microbiota communicates with the host in different ways, such humoral signalling molecules and hormonal components (Gentile F., et al., 2020; Hooper LV., et al., 2001; Sánchez B., et al., 2017).

The gut microbiota is composed of four bacterial phyla and in general, the phylotype composition differs considerably from person to person depending on many factors, such as the maternal microbiota, psychophysical state, and nutrition as alcohol abuse. Although the liver is mainly involved in the metabolism of alcohol, supporting the greatest degree of tissue damage, several studies have shown that alcohol abuse induces dysbiosis, altering the composition and functionality of intestinal microbiota (Bode C., et al., 2003; Le Chatelier E., et al., 2013; Leclercq S., et al., 2017; Parlesak A., et al., 2000; Samuelson DR., et al., 2019). This circumstance can lead to an increased intestinal permeability, known as *leaky gut*, linking a glial activation of ENS. Therefore, several studies suggest the use of probiotics. Generally, probiotic bacteria are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill C., et al., 2014), possessing the ability to transiently colonize the GIT, increase the concentration of beneficial microbes, and thereby create a balance in the gut microbiota to the ultimate benefit of the host (Sherman, PM., et al., 2009). So, they could play a key role in the protection of alcohol damage, since they exert various gut health benefits, modulating of gut microbiota and producing of bioactive metabolites (Engen PA., et al., 2015).

Among the large range of bacteria, the most common strains belong to Lactobacillus species. It is a Gram positive, optional aerobic bacterium that has antioxidant

properties due to its ability to produce gluthatione, bacteriocins and biosurfactants (Barone R., et al., 2016; Grenham S., et al., 2011; Hill C., et al., 2014; Hor YY., et al., 2019; Kim BK., et al, 2017). These antimicrobial factors inhibit the growth and virulence of enteric bacterial pathogens and, in particular, lactic acid–producing probiotics, as Lactobacillus, may also exert antimicrobial effects on pathogenic organisms by reducing the local pH of the microenvironment in the gut lumen (Sherman, PM., et al., 2009).

To study the EtOH effects and the protective role of the probiotic *L. fermentum*, here I used a mice model of chronic abuse of ethanol. The H&E stain conduced on small intestine showed a *leaky gut* in the 12 EtOH, characterized by altered barrier structure. In the Ctrl group, the intestine appeared normal with intact epithelial barrier, organized enterocytes, and no signs of inflammation. However, long-term ethanol ingestion induced histological changes in intestinal mucosa in 12 EtOH, showing significantlty longer villi than 12 Ctrl and 12 EtOH-P ($p<0,001$), with loss of epithelium on the surface. Additionally, mice treated with EtOH present alterations in the number of goblet cells, one of the major components of the extrinsic barrier producing mucin. In particular, 12 EtOH showed a decreased number of goblet cells than 12 EtOH-P and Ctrl groups, leading probably to less mucus secretion. In particular, these results are consistent with the findings that balanced and dynamic interactions among mucus layers, intestinal epithelial cells, microbiota, and host immune defence is essential for the maintenance of the intestinal mucosal homeostasis (Kim, YS. And Ho, SB., 2010). The disruption of the intestinal homeostasis results in the defective mucus barrier with increased permeability that results in inflammation and injury of the intestinal mucosal cells. Initially, mucin secretion is often associated with an increased mucin synthesis, resulting in chronic secretion that causes goblet cell depletion and a subsequent decrease in mucin synthesis. The syntheses of mucins are regulated by many bioactive factors, such as microbial products, toxins, cytokines, hormones/neuropeptides and growth factors (Theodoropoulos, G., and Carraway, KL., 2007). Several studies have shown that MUC2 has NF-κB binding sites in the promoter. Indeed, LPS and TNF-α increase MUC2 transcription through NF-κB activation mediated by PI3K/Akt pathways (Andrianifahanana, M., et al., 2006; Kim, YS. and Ho, SB., 2010).

Moreover, since tight junctions, between the epithelial cells, represent a structural basis constituting the mechanical barrier of intestinal mucosa, the morphological analysis was conducted to identify the expression of ZO-1 and MMP-9, an endopeptidase capable of degrading all components of the extracellular matrix (Cho YE., et al., 2018; Gumber S., et al., 2014 Li S., et al., 2020; Rempe RG., et al., 2016; Shigemori Y., et al.; 2006; Turner RJ., et al., 2016). While in Ctrl, ZO-1 showed a uniform distribution in intestinal epithelial cells, in 12 EtOH the protein decreased and slightly increased following the use of the probiotic. These data led me to hypnotize that EtOH and probably its metabolites, such as acetaldehyde, destroys intestinal epithelial TJ proteins, including ZO-1, causing poor intestinal barrier integrity. Furthermore, the increased immunoreactivity for MMP-9 in the epithelial cells of the small intestine belonging from mice treated only with ethanol supported the assumption of an increased intestinal permeability, caused by alcohol abuse in agreement with literature data (Pender SL., et al., 2006; von Lampe B., et al., 2000, Klarin B., 2008).

Since EGCs participate in the regulation of inflammatory events in the gut, immunohistochemistry reaction was conducted on small intestine slices to identify the ENS, using anti-GFAP and anti-S100β antibodies. Both glia markers are elevated in 12 EtOH group in comparison to the 12 EtOH-P and Ctrl groups. In particular, glia markers labelling was observed in the lamina propria, at the base of the crypts, and in the myenteric and submucosal plexus. The overexpression of glial markers is attributable to a release of microbial antigens, such as LPS, and therefore would be indicators of probable intestinal dysbiosis (Rao M., et al., 2018; Sharkey KA., 2015; Vergnolle N. and Cirillo C., 2018). In fact, these cells are relatively small, with a star shape, can express receptors for cytokines, neuropeptides, and neurotrophins, exerting functions in both the ENS and the immune system, and participating in the modulation of motility and secretion functions of the GIT. In addition, EGCs are necessary for the structural and functional integrity of the ENS, participating in the intestinal mucosal barrier and contributing to intestinal homeostasis (Christofi F. L., 2018; Giuffrè, M., et al., 2020).

As reported above, EtOH consumption has been associated with an altered intestinal barrier leading to dysbiosis, pro-inflammatory mediators and cytokine production

causing tissue injury, inflammation and apoptosis (Abrahao KP., et al., 2017). In this way, dysbiosis could promote the translocation of intestinal bacterial components, such as LPS and peptidoglycan (PGN), from the intestinal lumen to the systemic circulation and to other organs. Probably, the molecular mechanisms are associated with increased functionality of the NF-κB pathway. Normally, the inactive heterodimer NF-κB is bound to its beta inhibitor (IκB) in the cytosol. The activation of the inflammatory cascade causes phosphorylation and degradation of IκB, releasing NF-κB (Kim ML., et al., 2010; Nowak AJ., et al., 2020). Consequently, the activated transcription factor (pNF-κB) shifts to the nucleus and induces the expression of several factors including pro-inflammatory cytokines (Liu, T., et al., 2017; Ramakrishnan, S. K., et al., 2019; Tak PP. & Firestein GS., 2001; Yang, H., et al., 2014). For this reason, the immunohistochemistry and immunofluorescence reactions, conducted on small intestine, confirms that 12 EtOH exhibit a greater amount of immunoreactive cells for both pNF-κB and IL-6, indicating greater sensitivity to oxidative stress-induced inflammation. On the other hand, both proteins show a lower expression in 12 EtOH-P, probably thanks to the protective effect of the probiotic against oxidative stress.

Moreover, the activation of NF-κB could induce the transcription of inflammatory factors causing an increase in the expression of inflammatory cytokines and chemokines, which exploiting the systemic circulation, reach different organs, including the CNS. In this perspective, TNF-α production was assessed with RT-PCR in the intestine tissues of the mice. In particular, TNF-α gene expression level decreased significantly ($p<0,03$) in 12 EtOH-P, compared with 12 EtOH, suggesting that probiotic administration contribute to the beneficial effects on gut barrier integrity. In fact, TNF-α is a proinflammatory cytokine playing an important role in the progression of inflammatory responses in the GIT, causing an increase in intestinal permeability, which allows increased intestinal permeation of luminal antigens (Al-Sadi, R., et al., 2013; Ruder, B., et al., 2019; Watson, AJ., et al., 2009).

These alcohol-induced changes observed in the integrity of the gut barrier, including intestinal cytokine expression, may allow leakage of pathogen-associated molecules from the intestinal lumen into the systemic circulation and to other organs, including CNS (Al-Sadi, R., et al., 2009; Lowe, P. P., et al., 2018). In fact, at the CNS level,

alcohol affects several neurological pathways, causing significant changes in behaviour, cognitive and memory deficits. Furthermore, EtOH is also capable of inducing apoptosis and causing functional alterations in astrocytes, amplifying the neurodegeneration (Bishehsari F., et al., 2017; Huf F., et al., 2019; Oliveira-da-Silva A., et al., 2009; Santos R., et al., 2013). For this reason, the immunomorphological investigations were conducted on brain slices, focusing on cerebellum since it is one of the area's most affected by alcohol damage (de la Monte SM. and Kril JJ., 2014; Luo J., 2015). In particular, in 12 EtOH the cerebellum showed the presence of vacuolization in the area presenting white matter, probably caused by lipid peroxidation, which decreases in the mice treated with the probiotic (Bugiani M., et al., 2017; Yeung KY., et al., 1988; Zhou C. and Byard RW, 2012). Furthermore, the H&E stain of cerebellar cortex of the ethanol-treated groups (12 EtOH and 12 EtOH-P), showed a loss of the normal cellular morphology architecture of PCs. Many PCs were observed without a prominent nucleus or well-defined nuclear membrane, losing their characteristic pyriform shape (Luo J., 2015; Zhang GJ., et al., 2017). This fact could result in modifications of the structure and function of other brain regions that receive cerebellar inputs, amplifying the damage (https://neuropathology-web.org/chapter1/chapter1aNeurons.html).

In the cerebellum, severe vacuolation of cerebellar nuclei neurons and atrophy of PCs constitute the most striking lesions by alcohol consumption that could associated with impaired neurotransmission (Giannakopoulou A., 2017).

In addition, although neurons are the main conveyors of information between brain regions, it is known that the maintenance, survival and normal activity of neurons are fully dependent on the interaction with different types of glial cells (Miguel-Hidalgo JJ., 2018). Generally, these cells assist critically in the support of neurotransmission, survival of both neurons and other glial cells, supply of metabolites and brain injury repair. In the CNS, the main classes of glial cells are astrocytes, oligodendrocytes and microglia, which may or not perform different functions cooperatively by two or more cell types. For instance, astrocytes interact with neurotransmitter reuptake and metabolic support, oligodendrocytes with myelin formation around axons, and microglia with responses to injury and repair (Barres BA., 2008). Thus, if prolonged

alcohol exposure damages glial cells or disrupts their activity, serious disturbances of neuronal function are to be expected (Miguel-Hidalgo JJ., 2018).

For these reasons, the consequence of EtOH exposure on glia cell activation and vacuole formation in the cerebellum were investigated using GFAP, S100β and FABP7 immunolabeling (Cerutti SM., et al., 2000; Gonçalves CA., et al., 2008; Li, DR., et al., 2012; Sofroniew MV., et al., 2010). Mice treated with EtOH had an GFAP positive astrocytic area in central white matter, where vacuolization was found. Therefore, increases in the numbers of GFAP+ processes would be a consequence of astroglisosis, leading to astrocyte hypertrophy. Compared to the 12 EtOH group, GFAP protein expression levels decreased in the cerebellum of 12 EtOH-P, suggesting the protective role of *L. fermentum* against the alteration of these glial changes (Dlugos CA. and Pentney RJ, 2001; Kane CJ., et al., 2014; Rintala J., et al., 2001). Regarding S100β expression level, the protein was strong in 12EtOH and 12 EtOH-P, compared to the Ctrl. S100β were located in all layers except for the granular layer. The protein expression level increase in the soma of Bergmann glial within the Purkijnie cell layer of EtOH-treated mice versus the control group, leading probably to neuronal degeneration (Belozor OS., et al., 2019; Bluhm B., et al., 2015; Hachem S., et al., 2007; Rothermundt M., et al., 2003; Vig PJ., et al., 2006). Finally, FABP7 is abundantly expressed in several type of glial cells, such as astrocytes and oligodendrocyte, due to gliosis after EtOH treatment. In the Ctrl, FABP7 was present in a unique population of glia of molecular layer, as normal cerebellum condition, and it was increased in a subset of reactive astrocytes. In fact, not only the strongest immunostaining for FABP7 in the 12 EtOH was present in the Bergmann glia, but also it was positive in the cytoplasm of some neurons and in the fibres of white matter, in the same area in which the vacuolization was observed. Moreover, the high FABP7 expression level in 12 EtOH-P was probably caused by polyunsaturated fatty acids (PUFAs), produced by *L. fermentum*, that have important metabolic, structural and signal transducing roles (Boneva NB., et al., 2010). FABP7 a strong binder of PUFAs, is cytoplasmic proteins involved in fatty acid uptake, transport and metabolism, which is expressed in neural stem cells and astrocytes of the developing brain, suggesting a role in glial cell differentiation and proliferation of glial cells (Kipp, M., et al., 2011). Its increase confirms the protective role of the probiotic, as PUFAs are abundant in the developing

brain and are essential for normal brain development (Sharifi, K., et al., 2011). Several studies have suggested a negative correlation between low dietary intake of PUFAs and the risk of several neuropsychiatric diseases such as Parkinson's disease (de Lau, LM., et al., 2005), Alzheimer's disease (Morris, MC., et al., 2003), depression and other psychotic and mood disorders and developmental disorders like autism (Hedelin, M., et al., 2010; Kipp, M., et al., 2011). This confirms a probable correlation between the therapeutic effects of PUFAs caused by the probiotic and the damage induced by ethanol consumption. Regarding NF-κB, I observed that the constitutive form pNF-κB was found at low concentration in the white matter, near the granule layer in control mice. On the contrary, I detected a strong positivity in the same region in the cerebella belonging from ethanol fed mice. This observation, led me to suppose the presence of reactive astrogliosis and chronic inflammation of the white matter, as a consequence of brain injury caused by the EtOH consumption in agreement with data reported in literature (Saggu R., et al., 2016). Also, in the case of the cerebellum, the probiotic was able to decrease pNF-κB immunoreactivity confirming its anti-inflammatory activity even at the central level.

HSPs are induced in response to different type of stress such as oxidative stress and are implicated in many pathways critical for the maintenance of tissue omeostasis (Bellavia M., et al., 2013; Cappello F., et al., 2014; Marino Gammazza A., et al., 2016; Barone R., et al., 2016 and 2021). In this thesis, I investigated if HSP60 and HSP90 should be part of the mechanism involved in the protective effects of *L. fermentum* both at intestinal and brain level. As better elucidated in the result section, HSP60 is particularly expressed in the small intestine and cerebellum of 12 EtOH-P suggesting the protective role of *L. fermentum* against oxidative stress damage caused by alcohol consumption. Similarly, HSP90 rapidly increased in the intestine and cerebellum of mice treated with the probiotic.

It has been reported that in the intestine, induced HSPs help maintain tight junctions between IECs and promote barrier function (Kojima, K., et al., 2003; Petrof, EO., et., 2004; Tao, Y., et al., 2006). Then, *L. fermentum* treatment confers a degree of protection against oxidative stress injury, induced by alcohol consumption, both in the small intestine and in the cerebellum.

Although induction of HSP60 and HSP90 could be considered as cytoprotective effects of the probiotic, there is for sure something missing in the pathway that I investigated. So, *L. fermentum* inhibits NF-κB activation and HSPs, probably bind IkB-kinase (IKK), inhibiting it and preventing NF-κB activation (Dukay B., et al., 2019; Moura CS., et al., 2018; Petrof EO., et al., 2004; Thomas, CM. and Versalovic, J., 2010; Wang Y., et al., 2017). To confirm these assumptions, I conducted an immunofluorescence analysis on small intestine slices to detect IkB-α. The results showed that IkB-α levels decreased in 12 EtOH and have been restored in 12 EtOH-P compared to the control group, confirming the inhibition of NF-κB pathway.

The NF-κB pathway is one key signalling channel for activation of immune responses secondary to a variety of stimuli, and may represent a key "conversation node" between probiotics, the gut and CNS, following the so-called BGM axis (Thomas, CM. and Versalovic, J., 2010). Under non-stimulatory conditions, NF-κB is present in its inactive form in the cytoplasm, bound to the inhibitor molecule, IκB (Neish, A. S., et al., 2000; Petrof, EO., 2004 and 2009). When pro-inflammatory stimuli trigger signalling pathways, as alcohol consumption, IκB is phosphorylated and degraded. Once freed from IκB, NF-κB is able to migrate into the nucleus, bind target promoters and activate transcription of effector genes, influencing downstream cytokine secretion. Probably, *L. fermentum* prevents the degradation of IκB and the subsequent NF-κB translocation into the nucleus, resulting in decreased TNF-α (Lin, P. W., et al., 2009; Ma, D., et al., 2004; Zhang, L., et al., 2005).

This thesis has given special attention to the gut-brain axis and provided new insight especially regarding the involvement of HSP60 and HSP90 in the protection exerted by *L. fermentum* against oxidative stress and inflammation as well as in the investigation of the molecular mechanism that take place in the central nervous system as a consequence of the damage induced in the intestinal tract. The results could be useful to support the use of the probiotic in many inflammatory conditions to attenuate the consequences even at the central nervous system level.

Even if I obtained interisting results from the morphological analysis, due to the pandemic situation, it was no possible to have new groups of animals to increase the number of the examined specimens. This is a limitation of my study because it was no

possible to carry on also a biochemical and biomolecule examination, for a better quantitative analysis of the investigated markers.

Conclusion

This study showed that oral intake of *L. fermentum* protects against alcohol-induced *leaky gut* injury in mice, with consequent protection against inflammation also at the cerebellum level. The cellular and molecular mechanisms involved in this protection are probably associated with its ability to modulate NF-κB activation and the expression of TNF-α. Here a working hypothesis (**figure 23**).

The results indicated that *L. fermentum* exerted anti-inflammatory and cytoprotective effects, pointing to its possible application to treat intestinal inflammatory disorders.

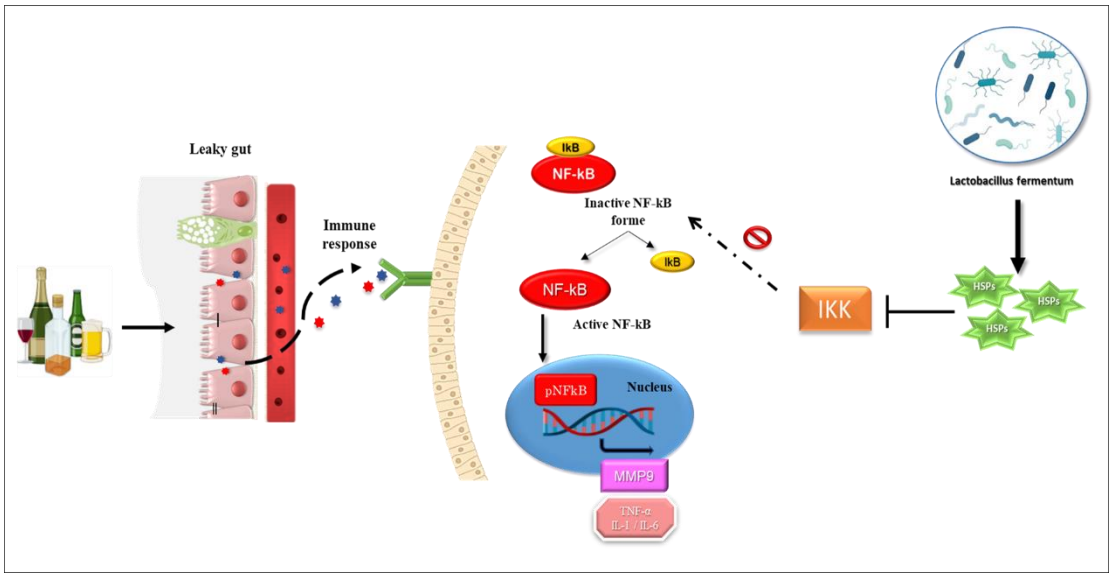

Figure 23. Hypothesis on *L. fermentum* molecular mechanisms. Alcohol-dependent subjects have increased intestinal permeability (known as *leaky gut*) and an altered composition and functionality of the intestinal microbiota, favouring the translocation of bacterial components of intestinal origin, such as LPS and endotoxin, from the intestinal lumen to the systemic circulation and to other organs (Samuelson, DR., et al., 2019). Probably, the molecular mechanisms are associated with increased functionality of NF-κB pathway. Normally, the inactive heterodimer NF-κB is bound to its beta inhibitor (IκB) in the cytosol. The activation of the inflammatory cascade causes phosphorylation and degradation of IκB, releasing NF-κB. Consequently, the activated transcription factor, pNF-κB, shifts to the nucleus and induces the expression of several factors including cytokines and chemokines as interleukins, which exploiting the systemic circulation, reach different organs, including the central nervous system (Nowak, AJ. and Relja, B., 2020). *L. fermentum* could be able to induce an increase in the HSPs expression, inhibiting IκB-kinase (IKK) (Moura, C. S., et al., 2018; Wang, Y., et al.,

2017). In this manner NF-κB cannot be activated and, consequently, HSPs exert an anti-inflammatory action, confirming the protective role of *L. fermentum* against inflammation caused by alcohol consumption.

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Here I am at the end of this thesis and these wonderful university years, in which I have matured as a person and as a professional in what is my greatest passion, Science. This thesis is the fruit of the readings, experiences and reflections I have gained over these years. I would like to dedicate these last pages to thank the people who have always supported me and been close to me. I would like these thanks to be a point of arrival, but also a point of beginning, because I believe that one never stops growing and I hope to be able to achieve new important goals in my life with all of them still by my side.

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