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Dr. Alessio Ardizzone

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Efficacy of a novel therapeutic, based on natural ingredients and probiotics, in a murine model of multiple food intolerance and maldigestion

Supervisor:
Chiar.ma Prof. ssa Emanuela Esposito

Coordinator: **Chiar.ma Prof. ssa Nunziacarla Spanò**

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TABLE OF CONTENTS

ABSTRACT

Patients with hypersensitive gut mucosa often suffer from food intolerances associated with an inadequate gastrointestinal function that affects 15-20% of the population. Current treatments involve elimination diets, but require careful control, are difficult to maintain long-term and diagnosis remains challenging. This study aims to evaluate the beneficial effects of a novel therapeutic of natural (NTN) origin containing food-grade polysaccharides, proteins, and grape seed extract to restore intestinal function in a murine model of fructose, carbohydrate, and fat intolerances. All experiments were conducted in 4-week-old male CD1 mice. To induce food intolerances mice were fed with either a high-carbohydrate diet (HCD), high-fat diet (HFD), or high-fructose diet (HFrD) respectively. After two weeks of treatment, several parameters and endpoints were evaluated such as: food and water intake, body weight, histological score in several organs, gut permeability, intestinal epithelial integrity, and biochemical endpoints. Our results demonstrated that the therapeutic agent significantly restored gut barrier integrity and permeability compromised by every food intolerance induction. Restoration of intestinal function by NTN treatment has consequently improved tissue damage in several functional organs involved in the diagnosis of each intolerance such as the pancreas for HCD and the liver for HFD and HFrD. Taken together, our results support NTN as promising natural care in the non-pharmacological strategy for the recovery of intestinal dysregulation, supporting the well-being of the gastrointestinal tract.

CHAPTER ONE: INTRODUCTION

1. INTRODUCTION

Food intolerances are non-immunological disorders that occur after consuming a specific food or one of its components [1]. Food intolerances are very common, affecting about 15-20% of the population in industrialized countries [2]; despite their wide diffusion, their etiopathogenesis is not yet fully known.

A thin line exists between the different food hypersensitivity disorders that are not always easily distinguishable. Among these, food allergies and food intolerances are often overlapped.

The two disorders have key pathophysiological differences that result in different diagnostic approaches and therapeutic options [3].

In food allergy, an abnormal immune response to food typically occurs within minutes or hours after its ingestion and can lead to severe allergic reactions [3]. While in food intolerances no immunological component is involved, indeed the individual reacts to food ingestion with gastrointestinal dysfunctions like abdominal pain, bloating, flatulence, and altered bowel microflora [3].

Moreover, it is important to cite the non-coeliac gluten sensitivity (NCGS), still considered an unclear and controversial event that triggers gastrointestinal and/or extra-intestinal symptoms due to gluten ingestion in the absence of coeliac disease and wheat allergy. The condition affects a small portion of the population, around 0,63-6%, making the pathophysiology difficult to understand [4, 5].

It has been understood that the excessive ingestion of a certain food predisposes the patient to develop a sensitization towards it [6].

In this context, given the greater consumption of industrial food in Western countries, food intolerances and maldigestion have increased exponentially in recent years [7-9],

Diet is an important factor that determines the well-being of the individual, relatedly, it is proven that changes in lifestyle and dietary composition constitute a predisposing factor to developing various diseases [10].

In particular, high carbohydrate intake, consisting mainly of food with a high glycemic index, has harmful metabolic effects [11, 12] that may lead to the development of diseases such as gastrointestinal disorders, metabolic syndrome and may increase the risk of cardiovascular disease (CVD) [13, 14] as well as a greater predisposition to diabetes mellitus [14].

Similarly, unhealthy fatty meals, with a high content of saturated fatty acids, represent a predisposing factor to obesity, CVD [15], gastrointestinal disease [16], and dyslipidemia [17]. Moreover, steady consumption of a high-fat diet, known to reduce intestinal barrier function [18] is also closely related to the development of metabolic diseases including non-alcoholic fatty liver disease (NAFLD) [19].

In addition, a large body of evidence warns of the risks of excessive fructose consumption [20, 21].

Fructose is a molecule classifiable as a 6-carbon monosaccharide, which is present naturally in a wide range of foods such as fruits, vegetables, and honey [22]. In the last 40 years, its use as a food sweetener has grown exponentially [23]. Consequently, its higher consumption in the population has led to an increase in fructose malabsorption and intolerance, which has often been associated with unexplained bloating, belching, distension, gas, abdominal pain, or diarrhea [24].

Based on what was previously disclosed, high sugar or lipid intakes can worsen the patient's quality of life, promoting metabolic changes and dysregulating the homeostasis of the gastrointestinal tract.

Certainly, in the multifaceted etiology of these food disorders, intestinal barrier disruption has a significant role. The alteration of the intestinal barrier, following a non-balanced diet, affects the metabolic machinery responsible for digestion and absorption of nutrients [25, 26].

In fact, a lack of balance in the function of the intestinal epithelial cells that maintain the microbiota environment can lead to an uncontrollable immune reaction as well as bacterial overgrowth causing different disorders such as autoimmune and metabolic diseases [27].

Thus, hypersensitivity of intestinal mucosa leads to a partial or total loss of the ability to digest, altering nutrients sensing in the digestive tract [28]; in which also epithelial tight junctions (TJs) loss plays a key role [29, 30].

Hence, it is reasonable to assume that patients suffering from food intolerances need a solution to restore the integrity of the intestinal barrier.

Currently, non-pharmacological treatment options are considered to be of great support to traditional therapy, improving health status and proving to be safe and effective in managing the symptoms of various intestinal disorders [31] and food intolerances too.

In this regard, several scientific findings [32, 33] highlighted how non-pharmacological interventions, such as the administration of exogenous oral enzymes such as β-galactosidase or xylose-isomerase, proved to be effective in reducing the symptoms of lactose intolerance [32] and fructose intolerant patients [33] respectively.

In addition, probiotics [34] and also many compounds of natural origin [31] have been shown to be remarkably effective in counteracting gut dysbiosis and intestinal injury in the field of gastroenteritis [31, 35], Crohn's disease [36], and ulcerative colitis [37]. However, the effectiveness of probiotics is limited due to the variability of microbiota and response to modulation attempts, and the diversity of probiotic strains used [38].

Consequently, at the present time, elimination diets remain the accepted way of dealing with food intolerances, but they are not easy to follow and are often related to nutritional deficiencies. For these reasons, the discovery of other therapeutic options is necessary.

Given the need for new therapeutic options to treat food intolerances, this study aimed to evaluate the beneficial effects of a novel therapeutic of natural origin (NTN) for the treatment of food intolerances.

NTN contains probiotics and natural compounds that are considered a remarkable and effective nonpharmacological option in counteracting gut dysbiosis and intestinal injury. Indeed, *Lactobacillus acidophilus* and *Lactobacillus reuteri* are gram-positive bacteria, which provide valid support in the equilibrium of intestinal microflora, normalizing the passage of stool as well as stool consistency in subjects suffering from intestinal disorders [39]. Acacia and Pea protein are natural compounds exercising an emollient and soothing action of the digestive tract thanks to their high fiber content [40, 41]. In particular, Acacia, mainly composed of complex polysaccharides, resists digestion in the upper gastrointestinal tract, thus reaching the large intestine which can induce an increase in Bifidobacterium spp [40]. Similarly, Pea protein modulates intestinal bacteria activities [41]; thus, both natural compounds are able to improve the gut mucosal barrier and gut homeostasis. β-galactosidase, thanks to its enzymatic activity, result helpful in the case of galactose-containing carbohydrate intolerances as shown by pre-clinical and clinical studies [9]. NTN contains also grape seeds extract, a suitable source of proanthocyanidins, with valuable antioxidative properties. This natural compound has been revealed to improve intestinal health by reverting plasma bacterial endotoxins to basal levels [42]. Therefore, considering the beneficial properties of the aforementioned compounds in providing intestinal relief, we assessed NTN in multiple murine models of food intolerances: carbohydrate, lipid, and fructose.

CHAPTER TWO: FOOD INTOLERANCES

2. FOOD INTOLERANCES

2.1 Definition

Food intolerances are adverse reactions to food that determine a state of suffering of the organism following the ingestion of food or one of its components [3].

Food intolerances are still one of the most controversial areas of medicine in which the mechanisms underlying the pathology as well as the clinical symptoms are not always clear; moreover, given the lack of reliable diagnostic tests, the diagnosis is often late.

As a result of this complex clinical picture, there are often conflicting views on the management of these disorders and their social impact.

2.2 Etiopathogenesis

The causes of food intolerances are not yet fully known. The incomplete knowledge of the etiopathogenesis of these clinical conditions, the lack of data on their real epidemiology, as well as the absence of a gold standard for their diagnosis, make the overall picture difficult to understand [43].

The most consolidated hypotheses include a genetic and familial predisposition, intestinal infections, and certainly, a key role is attributed to the dietary patterns and lifestyle factors adopted by the patient.

There are key pathophysiological differences between food allergies and food intolerances (summarized in Table 1), resulting in different diagnostic strategies and therapeutic options according to the involvement of the immune system or not [3].

In this regard The American Academy of Allergy Asthma and Immunology (AAAI) has proposed a widely accepted classification that uses the generic term "adverse reaction to food", then distinguishes between allergies and intolerances: allergies are mediated by

immunological mechanisms; in intolerances, however, the reaction is not caused by the immune system [44].

A similar definition was issued in 2010 in an Expert Panel Report sponsored by the National Institute of Allergy and Infectious Diseases (NIAID). In fact, according to AAAI food allergy was defined as “an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food” and food intolerances as “non-immune reactions that include metabolic, toxic, pharmacologic, and undefined mechanisms” [45].

However, this distinction and the diversity of pathologies are not embodied in the public perception, thus, adverse reactions that can occur following food intake are often overlapped, misunderstanding the two different pathogenetic mechanisms.

Table 1 – Differences between Food allergies and Food intolerances

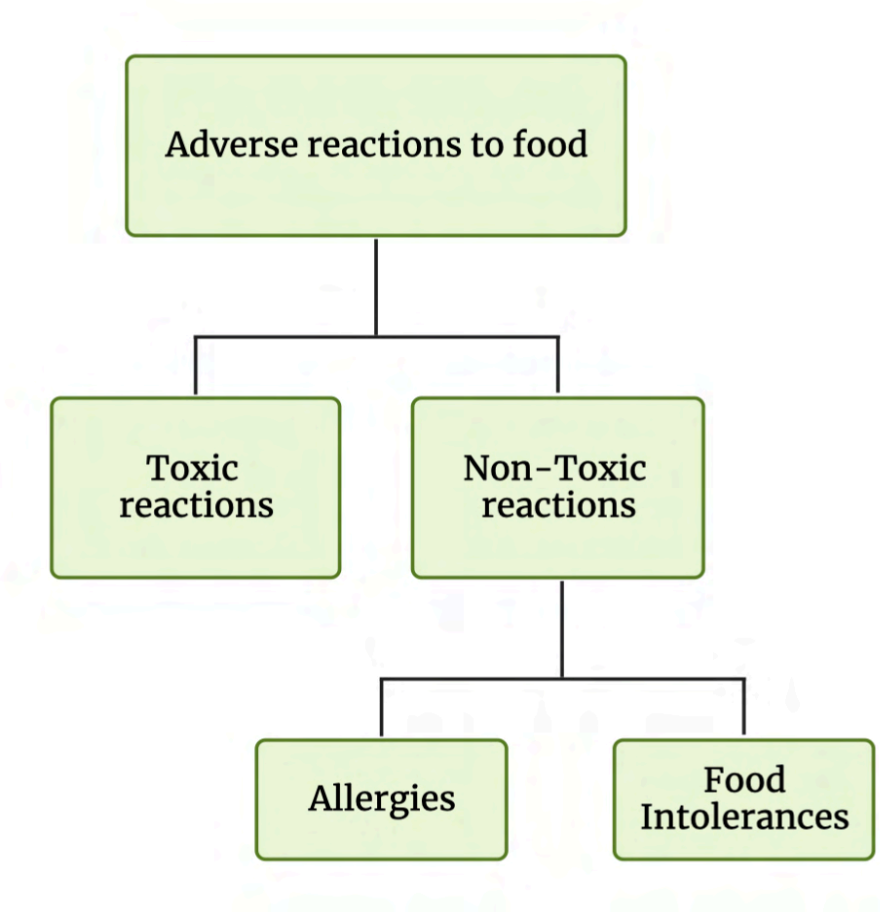
ALLERGIES	FOOD INTOLERANCES
IgE dependent reaction	IgG dependent reaction
After a sensitization phase, the allergic forms are connected to the production of IgE	Food intolerances depend on the production and accumulation in the body of IgG
Non-dose dependent reactions	Dose dependent reactions
The triggering of an allergic reaction in a predisposed subject is independent of the amount of allergen with which the subject comes into contact	The triggering of a food intolerance reaction derives from a sort of intoxication and due to frequent and repeated food intake
Rapid onset of symptoms	Late onset of symptoms
Allergic symptoms are immediate, symptoms appear within a short time after food, usually 24 hours. It is therefore easier to identify the cause of the reaction, with a clear cause-effect relationship. The allergic reaction can be extremely serious, even leading to anaphylactic shock.	Symptoms are mediated, less intense, often chronic in nature and above all tend to appear after some time from food intake (48-72 hours). If not recognized and treated, over time they can create the conditions for insidious ailments.

2.3 Classification

Over the years, different classifications of adverse food reactions have been proposed. One of the most accepted in the scientific world was redacted by the European Academy of Allergology and Clinical Immunology (EAACI) [46]. In particular, this classification

introduces a distinction between toxic and non-toxic reactions (represented in Flowchart 1).

Toxic (or poisoning) reactions are caused by the presence of toxins in the food and depend exclusively on the amount of toxic food ingested. For example, the toxic reactions that develop following the ingestion of mushrooms belong to this category. Otherwise, non-toxic reactions depend on the individual's susceptibility and are divided into allergies and intolerances (see Flowchart 1).



Flowchart 1. General classification of food adverse reactions

2.3.1 Toxic reactions

Toxic reactions, including food intoxication and food toxinfection, do not depend on individual susceptibility to a particular food [46, 47].

Food intoxication develops following the ingestion of water or food contaminated with pathogens. Once infiltrated into the food, in suitable conditions, these agents are able to produce toxins capable of affecting human health [48].

Most common food intoxications are represented by Clostridium botulinum and Staphylococcus aureus bacteria or synthetic compounds present in nutrients (e.g., insecticides, fertilizers, antibiotics, and metals), but the intoxication can be also caused by natural harmful substances (e.g., the solanine contained in the Solanaceae, the hydrocyanic acid present in some types of almonds, the erucic acid found in the oil of the rapeseed and the myristicin in the nutmeg) [49, 50].

Consuming tuna or mackerel stored for a long time can also lead to food intoxication, generally known as a scombroid syndrome [51]. This toxic reaction presents symptoms like allergies but really is related to the formation and accumulation of histamine as well as other biogenic amines in spoiled food; therefore, there is no immunological reaction in the affected subjects [52].

Food toxi-infections are caused by food contamination due to pathogenic microorganisms [53]. Unlike food intoxication, microorganisms, in addition to causing disease, can continue to produce toxins even after being ingested by the host organism.

The most common symptoms involve the gastrointestinal system including nausea, diarrhea, vomiting, and abdominal pain.

The diagnosis is made following the assessment of the clinical picture and following targeted diagnostic tests aimed at identifying the pathogen (or its toxins) in the patient's stool.

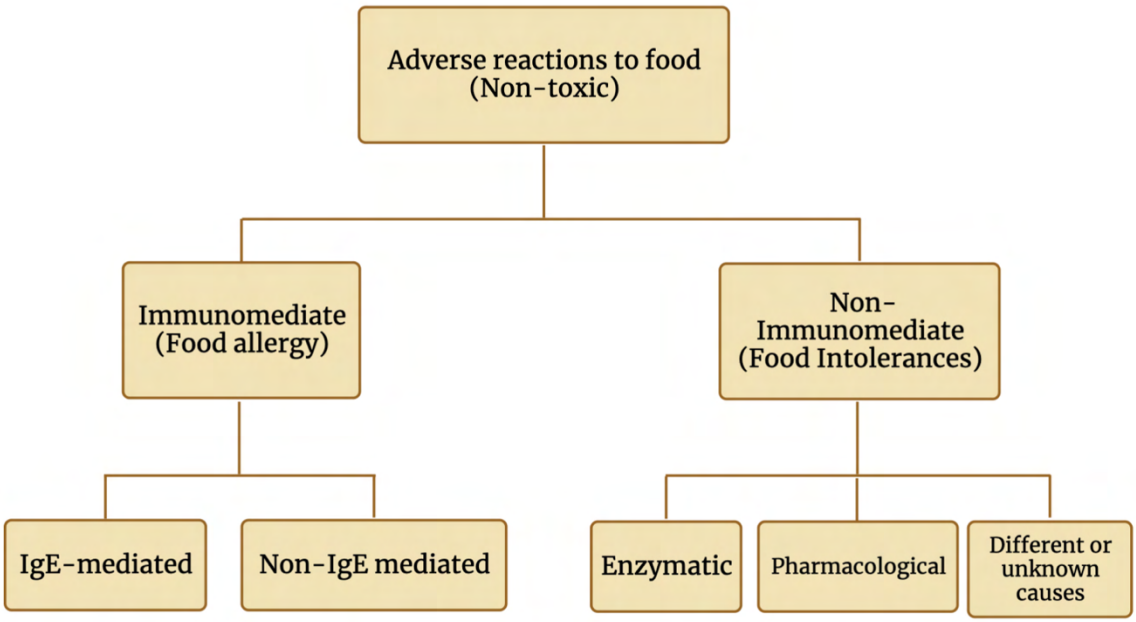
In most cases, the diagnosis of food toxi-infection can be traced back to Campylobacter, Salmonella, Listeria, and Escherichia coli bacteria [54-57]. Foodborne infections are a major challenge for global health, in this regard, the World Health Organization (WHO) has provided several reports with guidelines intended for public health professionals and food inspectors, also recommending the implementation of main rules of hygiene [58].

2.3.2 Non-toxic reactions

Non-toxic food reactions, also called food hypersensitivity reactions, depend on individual predisposition toward specific compounds present in food [59].

Non-toxic food reactions are usually divided into immune-mediated reactions defined as food allergies, and non-immune-mediated reactions known as food intolerances [60].

In 2004, The European Food Safety Authority (EFSA), which provides independent scientific advice on food-related risks, drew up an official classification of food intolerances and allergies, now commonly adopted by the scientific world [61] (see Flowchart 2).



Flowchart 2. Classifications of non-toxic reactions

As shown in Flowchart 2, food intolerances have different etiologies through which is possible to sort as follows:

2.4 Enzyme Intolerances (metabolic intolerances)

This type of food intolerance is determined by the body's inability to digest certain nutrients because of the absence or reduced enzymatic activity responsible for their metabolization [7].

Usually, enzyme deficiency is already present at birth, leading to the development of various symptoms related to the transformation of nutrients such as carbohydrates or proteins. Nevertheless, in some cases, the enzymatic alteration appears over time or develops as a result of diseases.

2.4.1 Lactose intolerance

The most frequent enzyme intolerance is lactose intolerance, the sugar present in the milk of all mammals [62].

In this type of intolerance, the consumption of milk and dairy products causes a non-allergic reaction accompanied by gastrointestinal disturbances such as bloating, abdominal pain, and diarrhea.

The pathology develops following the progressive loss of a specific enzyme called “lactase”, a protein located in the intestinal mucosa, capable of splitting the disaccharide lactose into two smaller subunits, the monosaccharides glucose and galactose [62].

Since the absence of the lactase enzyme in the intestinal villi, the body is unable to digest lactose which, consequently, reaches the colon in quantities greater than the absorption capacity of the intestinal wall. The permanence of lactose inside the intestinal lumen causes fermentation, thus determining the development of gastrointestinal signs.

2.4.2 Galactosemia

Galactosemia is a rare genetic metabolic disease caused by the absence of an enzyme responsible for the metabolism of galactose [63].

Galactose is a simple sugar present in free form in breast milk, while in cow's milk this monosaccharide is associated with glucose, forming the disaccharide lactose [64]. This serious hereditary disease causes an accumulation of galactose in the blood, which leads to serious complications such as enlarged liver, kidney failure, cataracts, and brain damage that occur from the first days of life [65].

2.4.3 Favism

Favism is an inherited genetic disease characterized by the absence of the enzyme glucose-6-phosphate-dehydrogenase (G6PD), necessary for the metabolism of glucose, in red blood cells [66]. Patients suffering from this enzyme deficiency, following the ingestion of peas or broad beans, undergo severe hemolysis which consequently causes anemia and jaundice [67].

2.5 Pharmacological Food Intolerances

Pharmacological food intolerances are due to the presence of pharmacologically active components in the ingested food (like fish, chocolate, and fermented products) or substances added to foods (food additives) such as dyes, flavor enhancers, preservatives, natural and artificial flavors [68].

This category includes biogenic amines such as histamine, tyramine and phenylethylamine which due to their presence in certain foods have the ability to trigger pharmacological intolerances following ingestion [69].

Xanthines, which include caffeine, theophylline, and theobromine, are contained in drinks based on coffee, chocolate, tea and guarana, and similarly to biogenic amines, they can induce intolerance reactions [70].

In fact, these substances thank to their pharmacological activity can stimulate the central nervous system (CNS), kidneys, and heart.

Therefore, in subjects with particular intolerances, xanthines induce vomiting, tachycardia, and headache [71].

Once the diagnosis of intolerance has been ascertained, diet therapy consists of the temporary or permanent exclusion of foods containing these molecules.

2.6 Food intolerances with indefinite causes

Food intolerances with an indefinite cause are due to a psychological or psychosomatic response, as in the case of food aversion or repulsion (food adversity) [71].

The reactions of food additives also fall into this category and can be defined as indefinite intolerances.

Generally, additives used in food production are limited to the legal dosage and are recognized as safe products.

However, some individuals may still show intolerance to glutamate or certain food dyes by experiencing symptoms such as asthma, rhinitis, hives, itching, and migraine. The exact mechanisms of these intolerances have yet to be fully understood.

2.7 Secondary Food intolerances

In this typology, food intolerances are the result of underlying diseases.

Secondary food intolerances commonly involve the gastrointestinal tract, as occur in individuals suffering from inflammatory bowel disease (IBD), gastritis, gastroesophageal reflux, etc. [72].

2.8 Symptomatology

As a result of the intestinal dysregulation the symptomatology of food intolerances is mainly gastrointestinal, but not only.

Symptoms include bloating and abdominal pain, diarrhea, vomiting, weight loss, blood in the stool, and rarely other organs are affected [1].

If extraintestinal complications occur, these may include skin manifestations (rash, eczema, and itching) and neurological manifestations (migraine and dizziness) [73, 74] as summarized in Table 2.

Table 2 – Main symptoms of food intolerances

Gastrointestinale disorders	Diarrhea / constipation, bloating, irritable bowel syndrome (IBS), gastritis, reflux ...) Associated malabsorption and / or nutrient deficiencies
Skin symptoms	Eczema, psoriasis, rashes, keratosis pilaris, urticaria
Neurological symptoms	migraines, headaches, memory problems Chronic fatigue, mood swings and depression

Symptoms related to food intolerances, in some cases, can become chronic, giving rise to the development of other intestinal diseases such as IBD [75].

2.9 Complications

The complications that arise from an unsuitable dietary lifestyle are numerous and are often not clinically distinguishable due to the GI similar symptoms.

In fact, the symptoms associated with these diseases, such as diarrhea, constipation, or abdominal pain can overlap between gluten-related diseases and other food intolerances as well as being similar to other intestinal diseases, such as irritable bowel syndrome (IBS) [76].

For IBS patients the diet could be considered an alternative therapeutic strategy. Indeed, the diet in IBS plays a critical role whereby physicians need to assess eating patterns and diet in IBS patients [75]. Relatedly, it was well established that IBS patients have a higher perceived food intolerance, which in turn can trigger nutritional concerns [77, 78].

It has been estimated that about 80% of patients with IBS need dietary advice aimed at eliminating food-triggering intestinal discomfort [79].

Especially, in the last decade, carbohydrates and gluten have been identified as triggering compounds for IBD disorders and food intolerances [76, 80, 81].

Concerning this, The United Kingdom National Institute for Health and Care Excellence (NICE) recommendations indicate, as first-line treatment in IBS general lifestyle and dietary advice, the consumption of regular meals avoiding the suspected trigger foods, and as second-line treatment the low-FODMAP diet [82].

However, despite the many studies conducted, the mechanisms by which food intolerances overlap with IBS, and vice versa, still remain a field to be explored.

Some studies seem to suggest an interplay between FODMAP, NCGS, and IBS [83, 84].

A more detailed evaluation of the multiple factors contributing to food sensitivity in IBS patients could provide further useful evidence. Specifically, considering the most recent

investigations the interplay between food-specific antibodies, carbohydrate malabsorption, and gluten sensitivity should be deepened.

2.10 Diagnosis

After excluding the presence of a food allergy, the diagnosis of food intolerances is made through food exclusion. The investigation consists in identifying the suspicious food, eliminating it from the daily diet and then gradually reintroducing it after a short period (exclusion diet) [3].

Usually, in the case of food adverse reactions, the symptoms disappear during the period of food exclusion, reappearing when it is reintroduced into the diet.

Once food intolerance has been ascertained, specific diagnostic tests are carried out, although some of these are considered to be of poor scientific reliability and clinical efficacy. Specifically, cytotoxic test, Alcat test, Vega test, IgG4 dosage, hair analysis, iridology, bioresonance and kinesiological test, pulse test, electrical tests, auricular heart reflex, and DRIA are recognized as tests without scientific validity [1, 85-89].

Some of these are described below.

2.10.1 IgG4 (immunoglobulin G4) dosage

IgG4 serological tests are continuously adopted for the diagnosis of food-induced hypersensitivity, representing a growing market [87].

Specifically, the test is performed on IgG4 present in the blood, evaluating their variation for different foods. The test can be performed on any age group, and a large-scale screening is usually done for hundreds of food products using enzyme-linked immunosorbent tests and radio-allergo-sorbent tests [87].

In many patients the serum samples show positive IgG4 results without clinical symptoms corresponding to food intolerances, these results together with the lack of convincing scientific evidence disprove the validity of these tests [87].

2.10.2 DRIA test

This test verifies the alteration of muscular effort during hypersensitive states; therefore, it is based on a kinesiological reflex of the human organism.

The patient is placed on a seat having an ankle strap, after which, foods suspected of causing food intolerances are administered to the patient sublingually [89].

The test result is based on the muscle traction exerted by the patient, which is measured by a dynamometer [89].

However, food intolerance disorders would appear not to cause changes in the patient's muscle strength, therefore the test is considered unscientific.

2.10.3 Antigen Leukocyte Cellular Antibody Test (ALCAT)

The test is performed through a simple blood sample, which will be put in contact with food, dyes, or additives to be investigated.

The reading of the result is possible thanks to a computerized device that has the ability to measure the variations of immune blood cells in contact with the tested substances, thus identifying any adverse responses [86].

While this test also does not have solid scientific validity, it has been approved for commercialization by the FDA [86].

2.10.4 Bioresonance test

It is also called "energetic" therapeutic method. This method is based on the false idea that the human body emits energy wavelengths and frequencies after ingesting particular foods [88].

Through a special tool, it would be possible to filter the pathological electromagnetic waves and treat food intolerances. Again, no scientific evidence has proven the test's effectiveness.

2.10.5 Vega test

The Vega test is a non-invasive diagnostic technique. The test is carried out using a special "energy" tool whose operating principle has not yet been well defined. During the analysis, the patient holds the instrument in his hand, the tip of which is placed on the skin. The vials containing the food extracts are inserted inside the instrument. If during the test of a particular vial there is a "drop in energy" it means that the organism is weakened, so the patient should eliminate the food as it causes food intolerances. The test has no scientific basis [85].

2.10.6 Cytotoxic test

The cytotoxic test involves the analysis of the leukocyte reaction against the food suspected of causing food intolerances [1]. This analysis is performed directly on leukocyte cells using an optical microscope. During the observation of leukocytes, a different degree of reaction to the food extract is attributed on the basis of a score ranging from 1 to 4 (1: normal, 2: swelling, 3: vacuolation, 4: rupture). Therefore, the cytotoxic

test is based on the scientific basis that the alteration of white blood cells would act as a "spy" of food hypersensitivity. In fact, this assessment is very subjective and may differ between assessors. In addition, changes in white blood cells are due to multiple factors, including changes in pH, temperature, and osmolarity of the blood, which should be taken into consideration [1].

The consultation of unqualified personnel could lead to severe diets, which can conduct the patient to serious food deficiencies essential for the organism.

Otherwise, specific tests to recognize probable food intolerances are constituted by serological tests (blood tests) and breath tests, accepted as “conventionally validated tests” [74, 90].

2.10.7 Serological tests: ELISA method

The ELISA kit is a diagnostic test widely accepted by the scientific community in the diagnosis of IgG-mediated food intolerances. The method is based on the dosage of antibodies in the blood of the IgG class using Enzyme-Linked Immunosorbent binding; the dosing of antibody levels is possible thanks to the presence of food antigens in the wells of the plate [91].

The evaluation of the result takes place through a spectrophotometric reading system, which allows you to evaluate the intensity of the colorimetric reaction of the ELISA kit, expressed as a percentage.

Therefore, the percentage results for each food will be indicated in the patient report. The test is also useful in highlighting the patient's food reactivity towards fungi, yeasts, and molds.

2.10.8 Diagnosis of lactose intolerance

The diagnosis of lactose intolerance is generally simple and easily understood from the symptoms, which are always dose-dependent [92].

If lactose intolerance is suspected, a breath test is useful. The test allows for ascertaining lactose intolerance through the presence of hydrogen (H_2) in the exhaled air [93].

The test is easy to perform and primarily involves the assumption of a dose of lactose followed by the analysis of the exhaled air after approximately 4 hours. The presence of the hydrogen peak in the exhaled air indicates the fermentation of the lactose not absorbed by the intestinal bacterial flora. The test has a recognized scientific validity; however, it should be noted that some intolerant patients are negative breath tests despite showing specific symptoms.

2.10.9 Diagnosis of fructose intolerance

Two forms of fructose intolerance are known: one on a genetic-hereditary basis and the other with a multifactorial component.

- **<u>Dietary fructose intolerance</u>**

  Dietary fructose intolerance has an intestinal etiopathogenesis, in fact, it is caused by the insufficient capacity of the intestine to absorb the fructose load ingested with food [94].

  As for lactose intolerance, the symptoms are dose-dependent, making the diagnosis simple and feasible through a specific breath test.

  Fructose intolerant patients will have to eliminate foods such as fruit, sucrose, and corn syrup from their diet.

However, if the patient has a "mild" type of intolerance, she can eat vegetables without complications; otherwise, a patient with "severe" fructose intolerance cannot take even a few grams of monosaccharide, so she must eliminate any food that is a source of fructose. In some cases, there may be an overlap between dietary fructose intolerance and reduced tolerance to FODMAPs (Fermentable Oligosaccharides, Disaccharides, Monosaccharides, and Polyols), scientific studies aim to deepen this correlation [94].

- **Hereditary fructose intolerance**

  Hereditary fructose intolerance is a rare autosomal recessive genetic disorder caused by liver deficiency of an enzyme called “fructose-1-phosphate aldolase” [95].

  Symptoms include hypoglycemia, sweating, confusional states and kidney damage [96]. In the long term, the disease also leads to gastrointestinal diseases and postprandial hypoglycemia [97].

  A liver biopsy is required for diagnosis, while healthy carriers can be identified through a genetic screening [95].

2.10.10 Diagnosis of non-celiac gluten sensitivity

Non-celiac gluten sensitivity does not meet the diagnostic criteria for celiac disease. In fact, this intolerance can be confirmed or not only through a gluten-exclusion diet [98].

Non-celiac gluten sensitivity does not lead to celiac-like complications, although the long-term effects are not yet known.

Actually, the pathophysiology of non-celiac gluten sensitivity is largely unclear, and there are contrasting data on the trigger of this condition [99].

2.11 Epidemiology

As reported by Zopf et al. [2], food intolerances referred to non-toxic and non-immunologically mediated clinical disorders. Food intolerances are much more commoner than immunologically mediated allergies or toxic disease mechanisms.

Scientific data reported a high percentage of food intolerances (around 15% to 20%) compared to food allergies (around 2% to 5%) [2].

However, data are not always reliable since the patient's diagnostic evaluation results very difficult, and often the symptomatology of several food disorders or allergies is overlapped [100].

Therefore, a detailed immunological investigation should always be performed to detect whether or not a food allergy is present. In addition, the frequent symptomatology overlaps between carbohydrate malabsorption, histamine intolerance, atopic disease, and food allergy should be considered. This would be useful in deciphering and distinguishing the various food diseases and therefore having a clearer and more truthful epidemiological picture [46, 101-103].

2.12 Therapeutic strategy

Several therapeutic strategies have been proposed over the years in addition to dietary restrictions and/or eliminations, which have always been considered extremely harmful to the body [45].

In the case of patients with lactose malabsorption, one of the proposed strategies has been to stimulate bacterial adaptation or tolerance through modifications in the gut flora using prebiotics or systematic ingestion of lactose-containing foods [104].

Furthermore, it was observed that a specific increase in beta-galactosidase activity could improve lactose digestion and reduce its fermentation products [105].

In this regard, several papers proved the beneficial effects of treatments based on enzyme replacement, through the administration of exogenous enzymes in the form of capsules/tablets before meals, confirming their effectiveness [106].

Particularly, new therapeutic options have been formulated, as reported by Savaiano and colleagues [105], RP-G28 is a novel galacto-oligosaccharide and can be classified as a lactose derivative. In their analysis, it was demonstrated that RP-G28 administration led about half of the patients involved to a complete resolution of abdominal pain both at the end of the trial and one month after.

Additionally, patients also reported improved long-term lactose tolerance, thus reintroducing dairy products after treatment [105].

In fecal exams, a notable upturn was found in lactose-fermenting bacteria such as *Faecalibacterium, Bifidobacterium,* and *Lactobacillus*. Consequently, the hypothesis that the alterations in the diet result in the intestinal and therefore fecal microbiome seems to be endorsed [107].

Thus, the use of probiotics should be always evaluated; in fact, although it does not represent a resolutive cure, still improves the management of some food intolerances-related symptoms, such as abdominal cramping, vomiting, bloating, flatulence, and diarrhea [108].

In the event of intolerance to sugars and carbohydrates, in addition to the use of probiotics and natural supplements (such as Acacia, Curcumin, etc.), a diet low in FODMAP is usually proposed.

The low-FODMAP diet should be followed by qualified personnel (dietician or nutrition biologist) and planned as a three-phase diet [109-111]:

- **first phase**: characterized by short-term (until 8-10 weeks) reduction in FODMAP intake.
- **second phase:** re-challenge to assess tolerance.
- **third phase:** long-term maintenance, excluding only foods that caused specific symptoms during the second phase (re-challenge phase).

During the three phases, the patient must be continuously monitored and subjected to routine analyzes in order to avoid serious nutritional deficiencies.

2.13 Prevention and dietary lifestyle

Gut health is closely linked with food ingestion and the digestion system [112]. The latest research presented the main role of intestinal microbiota in regulating tolerance, both immunological and non-immunological, towards ingested substances [113, 114].

Relatedly, dysbiosis is often linked to a poor diet and could represent the source of inflamed intestinal mucosa as well as reduced tolerance to nutrients [75, 115].

Therefore, in daily life, clinicians advise following a balanced diet completed of all the micro and macronutrients as indicated in the Mediterranean diet, with the purpose to promote the growth and maintenance of the intestinal microbiota [116, 117].

In specific circumstances, based on medical suggestions, probiotic administrations (yogurt, kefir, miso, fermented cheeses) or supplements could be beneficial to promote the development of a favorable environment for digestion by rebalancing the intestinal flora and contributing to the prevention of food intolerances.

CHAPTER THREE: FOOD INTOLERANCES AND INTESTINAL DYSREGULATION

3. FOOD INTOLERANCES AND RELATED INTESTINAL DYSREGULATION

3.1 Absorption of Nutrients

The gut is a key element in the context of food intolerances [118]; therefore, the deepening of its functions can be useful in understanding the multifaceted pathological background of food intolerances as well as in the discovery of new therapeutic targets.

In fact, the intestine is the primary seat of digestion of all consumed nutrients along with the absorption of water, vitamins, and minerals [119].

These simultaneous processes of digestion and absorption begin in the duodenum and are completed in the rest of the small intestine, furthermore, the contributions of other organs such as the pancreas and liver are fundamental [120].

3.1.1 Sugars

Most of the carbohydrates in the diet are present in the form of polysaccharides, such as starch and cellulose from vegetal products or glycogen from animal products [121].

Carbohydrates can only be absorbed as monosaccharides, so most of the carbohydrates consumed must be hydrolyzed [122]. This is possible thanks to the synergistic action of the pancreas and GI tract.

Polysaccharides are digested by amylases present in saliva and pancreatic juice (salivary and pancreatic amylase respectively) [118].

Salivary amylase is able to digest polysaccharides only in the initial stages of digestion as it is subsequently inactivated by the acidic environment of the stomach [123].

Pancreatic amylase, on the other hand, continues the digestion of polysaccharides in the intestine [124].

The hydrolysis of carbohydrates to monosaccharides is completed by enzymes present on the brush border of the apical membranes of the absorbent cells that cover the intestine.

These enzymes include dextrinase and glucoamylase hydrolyzing dextrins, saccharase which hydrolyzes sucrose to glucose and fructose, lactase with the ability to hydrolyze lactose to glucose and galactose, and maltase which hydrolyzes maltose to two glucose molecules [118, 125].

Once the carbohydrates have been digested into monosaccharides, these are actively absorbed through the epithelial cells of the intestinal villi.

After passing over the epithelial cells, these molecules diffuse into the capillaries and are transported through the bloodstream into the general systemic circulation.

3.1.2 Lipids

The lipids consumed in the diet are mainly triglycerides (90%), phospholipids, and cholesterol [118].

The digestion of lipids is similar to the carbohydrate pattern, but with the substantial difference that these molecules are not soluble in water.

The class of enzymes responsible for the digestion of lipids is called "lipase" [126], of which it is possible to distinguish lingual lipase, gastric lipase, and pancreatic lipase [127-129].

Furthermore, the effective digestion of lipids is made possible thanks to the action of bile salts, which carry out their activities in the duodenum [130].

Thus, in this complicated process, there is strong cooperation between the digestive system and the hepatic circulation.

The lipid molecules, after being divided into fatty acids and monoglycerides, are absorbed by the enterocytes by simple diffusion.

From here, once aggregated into chylomicrons, they are transported by the lymphatic flow into the bloodstream and released for multiple functions including the development of adipose tissue [118].

3.2 Gut barrier

Understanding the physiological mechanisms of nutrient absorption highlights how this process is a cooperative machine in which the intestinal epithelium plays a pivotal role.

The gut barrier consists of four layers comprising luminal intestinal alkaline phosphatase (IAP), the mucus layer, the epithelial cell layer, and the antibacterial proteins [26, 131]

However, this barrier can also be distinguished in terms of functionality in the extracellular or luminal, cellular, immunological, and intestinal vascular barrier [26].

3.2.1 Intestinal epithelial barrier

The intestinal epithelial barrier is formed by a single cell layer of epithelial cells and has the function of limiting transcellular transport.

Inside this monolayer, specific cells like goblet and Paneth cells supply additional factors to the gut barrier by producing mucus and antibacterial proteins respectively.

Furthermore, paracellular transport between intestinal epithelial cells is also restricted by intricate spatial arrangements of specialized proteins [26, 131].

Physiologically, a functional and undamaged intestinal barrier restrains the infiltration of bacteria or bacterial products, thus preventing intestinal and systemic inflammation [132, 133].

Contrarily, the disruption of the gut epithelial cell barrier causes an increase in bacterial translocation, including the diffusion of infective bacteria into the lamina propria, favoring subsequently the onset of pathogenic states.

3.2.2 Intestinal Epithelial cells: regulators of barrier function

The intestinal epithelial layer is extremely dynamic and characterized by a considerable turnover rate, in fact, the intestinal epithelial cells are rapidly regenerated and replaced within 48-96 hours [134, 135].

The maintenance of this renewal of the cellular layer represents a fundamental step in the tight regulation, in order to avoid an imbalance in intestinal homeostasis [136, 137].

As stated, the intestinal epithelial monolayer has a heterogeneous composition in which different types of specialized epithelial cells can be identified, such as enterocytes, Paneth cells, goblet cells, endocytes and micro-folded cells; each of these cell types fulfilling a distinct function [27].

In this variety, the most representative are intestinal epithelial cells or enterocytes, whose central function is the safeguarding of the epithelial barrier integrity [138, 139].

Paneth cells are located at the base of the crypts and produce antimicrobial peptides like α-defensin, which prevent microbial access to the intestinal lumen [27].

Mucus, trefoil peptides and resistin-like molecule β are secreted by the goblet cells, and they are essential for both protection and reparation of the epithelial layer in addition to having a noteworthy role in epithelial homeostasis [140-142].

Endocytes control entering antigens and microfold cells secrete IgA, which together with goblet cells, exhibit bacterial antigens to dendritic cells.

Intestinal epithelial cells are also qualified for phagocytosing bacteria, thus counteracting bacterial toxins.

The function of recognizing bacteria-derived molecules, known as prokaryotic-associated molecular patterns, is expressed through the support of Toll-like receptors (TLRs) on the

cell surface and through the nucleotide-binding oligomerization domain-like receptors in the cytoplasm [143].

This joint action stimulates defense mechanisms by the secretion of anti-microbial peptides [144].

Intestinal epithelial cells also support a “two-way” exchange with the underlying immune cells to set the inflammatory reaction versus bacterial toxins [145].

The epithelial layer forms, conjunctly with the mucosal layer and specialized cells, an intricately structured and rigorous barrier continuously inspected by immune cells to create an immune-silent environment.

All of this contributes to the creation of a polarized stratum, which found a tight barrier constituting intracellular tight junctions, adherent junctions, and desmosomes.

3.3 Tight junctions (TJs)

As already mentioned, the fundamental role of intestinal epithelial cells is the preservation of barrier integrity, which permits the absorption of essential ions, nutrients, and water but limits the entry of bacterial toxins and pathogens [145].

In particular, the epithelial layer allows the transport of molecules through three main pathways:

- the **trans-cellular pathway:** this pathway involves passive diffusion across cell membranes.
- the **carrier-mediated pathway:** in this case, there is a carrier/receptor-mediated transcellular pathway.
- the **paracellular pathway:** in this case, there is a passive diffusion that occurs between the spaces of the adjacent cells.

Tight epithelial junction (TJ) proteins represent the most apical component of intracellular epithelial junctions. At the intestinal level, TJs perform different functions, but especially they "seal" the paracellular space between intestinal cells, strictly limiting the transport of hydrophilic molecules [146-148].

More specifically, the "gate and fence function" attributed to TJs allows the paracellular transport of certain solutes and molecules but precludes the intramembrane transport of proteins, lipids, and peptides of microbial origin [27, 149].

Therefore, any alteration in TJs can be harmful to the organism, and as proven by several scientific studies, it can be fertile ground in the pathogenesis of multiple diseases, including GI diseases [26, 150].

3.4 TJs Classification

The TJs class is constituted by various transmembrane and cytosolic proteins among which Occludin, Claudins, Zonula Occludens (ZO), Tricellulin, Cingulin, and Junctional Adhesion Molecules (JAM).

These proteins are able to form a complex architecture both thanks to the interaction between them but also thanks to the interface with the cytoskeleton [151].

Furthermore, with the exception of Cingulin and ZO, most of these are integral membrane proteins present in the paracellular spaces between cells.

The cytoskeletal linking proteins Cingulin and ZO act together with the cytoplasmic peripheral membrane proteins such as Occludin, Claudin and JAM establishing powerful cross-links; in addition, they also cooperate with the membrane cytoskeleton constituted of F-actin and myosin.

Summarizing, TJs, synergistically with intracellular signaling proteins, stimulate a wide range of cellular activities [152] which precisely have the function of:

- support the integrity of the intestinal barrier
- rate-limiting paracellular permeability
- program the rapid opening and closing of the barrier in case of injuries and other signals
- continuously transmit signals to the individual cellular components, regulating the enhancement or modulation of the intestinal barrier.

Over the last ten years, there has been a noteworthy research effort to characterize the properties of TJs as well as to study their function in the context of human health and related pathological conditions.

Considerable progress has been made in understanding the mechanisms related to TJs, elucidating their structure, multifunctional role, and biological crosstalk.

3.4.1 Occludin

Among TJs, Occludin was the first identified protein [153]. In the basal epithelium, this protein is extensively phosphorylated at serine and threonine residues [154]. Within the intestinal barrier, Occludin has multiple roles, in particular, it provides structural integrity, also representing an integral component in the barrier function of TJs [155].

These assumptions have been confirmed by numerous preclinical studies [156, 157], which have shown a direct correlation between the expression of Occludin and barrier properties.

Interestingly, Occludin KO mice exhibited complex histological phenotypes, accompanied by states of chronic inflammation and a faulty epithelial barrier as well as hyperplasia in the gastric epithelium [158].

Severely impaired Occludin expressions were also observed in pathological models of inflammatory bowel disease, thus suggesting once again the critical role of Occludin in maintaining barrier integrity [159-161].

Collectively, these findings highlighted the complex functions of Occludin, further suggesting that the mechanisms by which Occludin regulates TJ should be more investigated by well-designed future studies.

3.4.2 Claudins

Claudins are another major class of TJs proteins and have the ability to regulate the paracellular space [151, 162]. To maintain paracellular integrity, a physiological balance is required between the various claudin isoforms, each of which has a different role [163]. The integrity of the intestinal barrier is highly influenced by claudin expressions, so their alteration, depending on the type of dysregulated claudin isoform, can lead to pathological states [164].

Regarding this, it has been proved that the downregulation of claudin-5 and claudin-8 is extremely correlated with a worsening of the barrier integrity [165].

Differently, in diseases such as IBD, claudin-2, a TJ protein required for the formation of paracellular water channels, is over-expressed in damaged epithelial tissues, causing inflammatory states [151, 165, 166].

3.4.3 ZO

Peripherally to the membrane are present the ZO proteins, ubiquitously expressed in epithelial and endothelial cells [167].

Among the various isoforms of ZO, we have ZO-1, ZO-2 and ZO-3. All three subtypes interact with different cellular proteins through a series of protein-binding domains, including: the SH3 domain, the PDZ domain, and the leucine-zipper domain [168, 169]. Furthermore, ZOs are essential for the formation of scaffolds and the connection of other TJ proteins to the cytoskeleton [170].

3.4.4 Other TJs

Another TJ protein implicated in maintaining intestinal barrier integrity is Junctional Adhesion Molecule A (JAM-A). JAM-A (-/-) mice have been observed to have increased barrier permeability with high bacterial translocation and increased polymorphonuclear leukocyte infiltration, even if animals didn't present spontaneous colitis [171, 172].

Unfortunately, in this interesting scenario, the functions of other TJ proteins and their exact biological mechanism remain still, for the most part, unknown.

3.5 TJs and their biological cross-talks

Despite the mechanism of TJ regulation is nowadays unclear, the most recent scientific findings elucidated the crosstalk of different signaling pathways in regulating the formation and dysregulation of TJs [173].

In this biological tool, numerous molecules involved in these signal transduction processes like small GTP-binding proteins and tyrosine kinases, such as c-Src, c-Yes as well as protein kinase C (PKC), have been correlated with TJs, thus denoting their pivotal function in the maintenance of TJs integrity and proper assembly [174-176].

Moreover, growing evidence emphasized the role of cytokines as influencing factors in the modulation of various TJs proteins during pathological conditions [177, 178].

About this, it is well-known the main role of Tumor necrosis factor-α (TNFα), interferon-γ (IFN-γ), and interleukins in the regulation of TJ integrity [177].

TNFα is a relevant factor in the internalization of caveolin-1 mediated Occludin, thus leading to a high intestinal permeability [179].

On the other hand, it has been proved that Occludin upregulation is capable of reducing the increase in intestinal permeability induced by cytokines, restoring intestinal homeostasis [179].

Other biological mechanisms are involved in the regulation of TJs, in particular some scientific data highlight the interplay between the NF-κB signaling pathway and the transcription of proinflammatory species such as TNFα [180].

It was probed how inhibition of NF-κB translocation protected mice against colitis symptoms, thus demonstrating its function in controlling the barrier activity of gut epithelial cells [181, 182].

Otherwise, the modulation of epithelial permeability by IFNγ is not yet clear and is therefore still under examination.

Nevertheless, it would emerge that IFNγ can alter actomyosin cytoskeletal interaction with TJs proteins [183-185].

It also appears that IFNγ may stimulate an upturn in intestinal barrier permeability, by under-expressing ZO-1 and Occludin in a dependent manner on the adenosine monophosphate-activated protein kinase (AMPK) pathway and regardless of cellular energy levels [186].

Thus, considering the damaging effect on intestinal integrity of both TNFα and IFNγ, their co-presence results in the dysregulation of TJ proteins [187].

Although the specific kinases involved in occludin phosphorylation are yet to be fully investigated, it has been estimated that PKC may be one of the key factors in the field of TJs.

Especially, Occludin, after being phosphorylated, cooperates with ZO-1 and other TJs proteins. In this framework, inflammatory pathological conditions or elevated reactive oxygen species (ROS) levels could lead to alterations in the phosphorylation pattern such as an increase in tyrosine phosphorylation.

These processes can lead to an alteration of the protein-protein interactions of Occludin with other TJs such as ZO-1, ZO-2 and ZO-3, disrupting the membrane integrity [188].

Among the most qualified hypotheses, it is supposed that the increase in oxidative stress causes intestinal permeability by the phosphorylation of the tyrosine of the Occludin as well as by the redistribution of Occludin, ZO-1, E-cadherin, and β-catenin from the intracellular junctions [189].

However, although some reports theorized the function of c-Src family kinases in H_2O_2-induced tyrosine Occludin phosphorylation, many of the tyrosine kinases involved in the phosphorylation process continue to be largely unknown [155, 190].

Although studies in this field have elucidated some of the mechanisms of TJ regulation, in vivo studies that describe their role in pathological conditions are lacking. Many studies have clarified some of the regulatory mechanisms of TJs, although their participation in pathological conditions is still unclear.

Despite advances in identifying the indisputable role of post-translational phosphorylation of TJs proteins, future studies are crucial to decoding the kinases and phosphatases involved, so as to have a better understanding of their physiological mechanisms.

3.6 Intestinal barrier integrity and pathological conditions

Recently, considerable importance has been attributed to the intestinal barrier function in the etiopathogenesis of various diseases.

Indeed, it has been disclosed that the intestine regulates the well-being of the whole organism through its biological links even with distal organs like the brain (gut-brain axis) or other gastric organs like the liver or pancreas.

In particular, the dysfunction of intestinal barrier integrity was demonstrated in both intestinal and systemic diseases, including IBDs, autoimmune diseases, and other metabolic disorders [191].

Nonetheless, scientific opinion is still doubtful whether the loss of barrier integrity is the cause or consequence of the aforementioned diseases.

Whereby, it is essential to recognize the contributing factors to the loss of intestinal barrier integrity in pathological conditions such as food intolerances.

Considering the main role of intestinal epithelium in pathological conditions, the identification of TJ proteins as new pharmacological and non-pharmacological targets could allow the design of new therapeutic strategies for the treatment of a broad spectrum of human diseases [173].

CHAPTER FOUR: NOVEL THERAPEUTIC COMPOSITION

4. NOVEL THERAPEUTIC COMPOSITION

The growing use of natural treatments has favored the greater attention to medicinal and officinal plants, in order to study and focus their benefits on human health.

There are many compounds of plant origin, deriving from the ecosystem, which are used in the therapy of many diseases or adjuvants in supportive therapy.

Phytotherapics, food supplements and medical devices constitute a great source of nutrients, such as vitamins and minerals, amino acids, essential fatty acids, fibers, etc.

Specifically, in the last few decades, medical devices represented a valid support in the non-pharmacological strategy of many pathologies, improving the therapeutic care of patients.

Also, for the treatment of indigestion and food intolerance, natural compounds, as well as probiotics, were clinically proven to provide intestinal relief while maintaining the equilibrium of intestinal flora.

In particular, in this study we assessed the beneficial properties of NTN composed of *Lactobacillus acidophilus* and *Lactobacillus reuteri* both supporters of gut flora [39], Acacia and Pea protein with relief action on the intestinal tract [40, 41], β-galactosidase useful for carbohydrate intolerances [9] and grape seeds extract having precious antioxidative activities.

4.1 Lactobacillus acidophilus

Lactobacillus acidophilus, also known as L. acidophilus, or Acidophilus, is a type of gram-positive rod-shaped bacterium that occurs naturally in our intestines [192].

L. acidophilus belongs to a group of bacteria called lactic bacteria (or lactobacilli) for their ability to transform sugars into lactic acid and hydrogen peroxide which have the ability to inhibit the growth of dangerous bacteria in the intestine [193, 194].

Therefore, L. acidophilus represents one of the best-known probiotics as a beneficial microorganism for our health and is able to protect against infections [195].

In fact, it is considered that foods and food supplements containing this lactobacillus are able to promote the balance of gut bacteria, reducing harmful bacteria, which could thrive in the intestine due to specific disease or antibiotic therapy [196].

Although L. acidophilus has often been analyzed as a potentially useful treatment for diarrhea caused by the bacterium Clostridium difficile [197].

Otherwise, recent studies highlighted positive effects on the organism, and in particular on the digestion process as well as on the gut health and specifically on the balance of the intestinal flora, thanks to its bacteriostatic capacity preventing the proliferation of pathogenic entero-bacteria [198].

Furthermore, there would seem to be a possible correlation between the lower risk of tumor onset and the action of L. acidophilus [199].

In this regard, future studies will focus on the ability of L.acidophilus to eliminate or inactivate chemicals that would be carcinogenic.

Since lactic acid bacteria are used in the food industry for the production of many foods, L.acidophilus is present in some food such as yogurt, kefir and buttermilk [200] and also in soy fermentation products such as miso and tempeh [201].

Certainly, the number of live organisms present in these foods varies widely due to the differences in the various manufacturing processes.

L. acidophilus supplements are also commercially available in many formulations such as capsules, tablets, preparations for drinks, pearls, chewable pods, or liquid form. Based on

the preparations available in pharmacy they can contain a single strain, several strains, or a mix of several bacterial species.

4.2 Lactobacillus reuteri

Lactobacillus reuteri (L. reuteri) is a Gram-positive bacterium of the Lactobacillaceae family and it is one of several species that coexist in the enteric tract known as the microbiota [202]. This community is made up not only of bacteria but also of yeasts, parasites and viruses, involved in various positive functions in a balanced way, in a condition called “eubiosis”, and synchronized within the intestinal ecosystem [203].

Isolated for the first time in 1962, L. reuteri is also involved in the balance of the intestinal flora and is therefore identified as one of the fundamental probiotics for intestinal well-being [204]. L. reuteri (principally ATCC55730 and DSM17938) is not degraded in the gastric environment, thus reaching the small intestine, where it creates viable bacterial colonies [205].

Furthermore, L. reuteri does not carry acquired and/or transmissible antibiotic resistance, making it an ideal candidate for long-term administration.

The beneficial effects of L.reuteri are largely attributable to the production of reuterin, a broad-spectrum antimicrobial substance capable of inhibiting the activity of Gram-positive or negative pathogenic bacteria such as C. Albicans, C. glabrata, C. krusei or C. parapsilosis, but also fungi and parasites [206-209]. Moreover, reuterin decreases tumor growth in colorectal cancer, consequently restoring microbial dysbiosis [210].

In addition to restoring the balance of the intestinal microbiota and modulating intestinal permeability, L. reuteri exerts a valuable immuno-inflammatory modulation by reducing

the expression of pro-inflammatory mediators such as IL-1β, IL-8, TNF-α, etc. and by increasing anti-inflammatory cytokines like IL-10 [211-214].

As reported by the scientific literature data, among its most important clinical applications there is the treatment of colic and diarrhea in infants [215]. For this reason, both the EFSA (European Food Safety Authority) and the FDA (Food and Drug Administration) have recognized the safety profile of this lactic ferment, authorizing its use for the child [216].

4.3 Acacia gum

Gum Arabic, a natural gum also known as Acacia gum, is a substance exuded from Acacia trees, a genus of plants belonging to the Mimosaceae family [217, 218].

In the last few years, Acacia gum has emerged as a new prebiotic fiber. As a soluble dietary fiber obtained from the stems and branches of Acacia Senegal and Acacia Seyal, it is mainly composed of complex polysaccharides [219]. As revealed by several in vitro [220, 221] and human studies [222], Acacia has the ability to resist digestion in the upper gastrointestinal tract, reaching the large intestine in which induces a Bifidobacterium spp increase [219]. Nonetheless, the health benefits for humans are not totally well identified.

It is known that Acacia gum can promote satiety, helps to lose weight and reduces blood cholesterol levels [223, 224].

Moreover, Acacia fiber supplementation produced the implementation of the genus Asaccharobacter in female rats, a single species reported to be a powerful equol producer [225].

Therefore, as has been reported, subjects receiving the Acacia fiber-enriched supplement may benefit from equol's health-promoting benefits on osteoporosis, prostate cancer, and cardiovascular diseases [226, 227].

Furthermore, clinical tests reported as Acacia gum was able to produce a higher increase in both Bifidobacteria and Lactobacilli in fecal samples [222]. Collectively, the intestine-specific benefits of Acacia gum could be classified into decreasing potentially pathogenic gastro-intestinal microorganisms, improvements in SCFA production and maintenance of gastrointestinal pH, changes in bowel function, reduction of GI discomfort, and maintenance of fecal nitrogen content [228].

Thus, Acacia supplementation could be considered an advantageous aid against gastrointestinal and metabolic disorders.

4.4 Pea protein

Pea is one of the most cultivated legumes in the world with a global overall production of 13.5 million metric tons in more than 90 countries [229].

Particularly, Pea proteins, deriving from Pisum sativum L., are a precious resource of high-quality vegetable protein in the human diet [41]. Pea proteins are usually recognized as hypoallergenic, and numerous literature data emphasized their beneficial effects on human health [230, 231].

In particular, these positive outcomes are associated with the antioxidant [232], antihypertensive [233], anti-inflammatory [234], lowering cholesterol [235], and modulating intestinal bacteria activities [236] of Pea protein.

Moreover, the application of Pea protein in the food industries attracted much consideration thanks to its numerous functional properties such as solubility, water- and oil-holding abilities, and emulsifying, foaming, and gelling properties [41].

The regular nutritional intake of foods containing pea protein or powdered pea extracts may have a promising potential to decrease the risk of specific chronic diseases, thus resulting in benefits for human health, especially in the field of IBD [237].

4.5 β-galactosidase

The enzyme β-galactosidase is generally known as “lactase”, the lactose hydrolyzing enzyme. When this enzyme is lacking the overexpression of lactose in the gut cause tissue dehydration while decreasing calcium absorption, all of this triggers gastrointestinal symptoms such as diarrhea, flatulence, and cramps [92, 238, 239].

Lactose intolerance resulting from β-galactosidase deficiency precludes the consumption of dairy products in intolerant subjects [240].

This food disorder affects about 70% of the world’s adult population [241]; and in particular, its prevalence in Western countries varies from 4 to 50% [242, 243].

This enzyme is widely employed in food-processing industries. Indeed, in addition to the production of lactose-free products for lactose-intolerant individuals, β-galactosidases are also used to solve whey disposal issues on the commercial scale [244].

β-galactosidase derives from various sources such as microbial, vegetable, and animal origins [245]. However, it has been noted that microbial sources usually show higher productivity resulting in lower production costs [245].

Furthermore, the choice of the type of b-galactosidase also depends on the required reaction conditions [245]. In the case of bacterial β-galactosidases, it has been shown that

a pH between 2.5 and 5.4 is optimal, thus allowing their use for the hydrolysis of acid whey [246].

On the other hand, β-galactosidase from yeast has optimal activity at a pH between 6 and 7, which makes it more suitable for the hydrolysis of milk and sweet whey [247].

Evaluating the positive outcomes following β-galactosidase administration and its implication in many pathologies, its pharmaceutical formulations are a helpful digestive aid [248, 249].

4.6 Grape seeds

Grape is a fruit possessing multiple beneficial properties. The consumption of this fruit is often recommended in diets, however, in addition to grapes, grape seeds are also known to be favorable components for human health [250].

Indeed, grape seeds represent a considerable source of catechins and procyanidins as well as rich in vitamins, fiber, and polyphenols [251, 252]. A large piece of evidences highlighted the properties of grape seed extract such as antioxidant, anti-inflammatory, antidiabetic, anti-obesity, anticancer, anti-aging, and antimicrobial activities [253-257].

Particularly, Katsuda et al. demonstrated of grape seed extract reduced the intracellular growth of ROS, confirming its natural antioxidant capability and free radical scavenger action [253].

Moreover, grape seed extract ameliorated inflammatory status and hyperglycemic levels associated with obesity [255], also suppressing the body weight increase in C57BL/6J mice in a model of HFD-induced obesity [258].

It has also been proven how grape seeds extract counteracted inflammation by regulating cytokines levels, such as C-reactive protein, IL-6, and TNF-alpha [259].

Furthermore, in the field of oncology, grape seeds extract prevents tumorigenesis, exhibiting chemo-preventive abilities against different forms of cancer [260, 261]. Likewise, beneficial effects after the administration of grape seeds extract were seen in Alzheimer's disease and other neurodegenerative disorders [262-264].

Interestingly, grape seed extract can also influence the gastrointestinal tract.

Scientific findings elucidated the capacity of grape seeds extract to suppress DSS-induced colitis by improving the intestinal barrier, diminishing oxidative stress, regulating inflammatory cytokines and gut microbiota [254, 265]. These protective roles were also confirmed in the framework of IBD; grape seed extract decreased gut inflammation, improving TJs proteins and gut microbiota [257].

Therefore, considering these assumptions, grape seeds have a precious potential for the development of food supplements useful against different human disorders, especially for metabolic, IBD, and other gastrointestinal diseases.

CHAPTER FIVE: AIM OF THE THESIS

5. AIM OF THE THESIS

In recent decades, the consumption of industrial foods has grown considerably all over the world. This factor has been related to the onset of obesity and other metabolic diseases. In fact, between the late 1980s and the present day, the number of food-related disease subjects has greatly increased.

In this context, the most common disorders are obesity as well as food allergies and intolerances.

In addition to conventional drugs, natural substances can also be a valuable aid, providing additional support in the management of many intestinal and metabolic diseases thanks to their mucosal protectors and gut regulators activities.

Therefore, my Ph.D. project aimed to evaluate the action of natural compounds and probiotics in different animal models of food intolerances, in order to analyze their beneficial effect on intestinal regulation and functional organs for carbohydrate digestion, lipid accumulation, or fructose metabolism.

CHAPTER SIX: MATERIALS AND METHODS

6. MATERIALS AND METHODS

6.1 Materials

Standard diet (SD), high-carbohydrate diet (HCD), high-fat diet (HFD) or high-fructose diet (HFrD) were purchased from Envigo (Milan, Italy). The product containing Acacia Senegal (L.) Willd, tyndallized *L. acidophilus*, tyndallized *L. reuteri*, Pea protein, Grape seed extract and β-galactosidase was kindly provided by DEVINTEC SAGL (Lugano, Switzerland). The human doses shown in Table 3 were converted to mouse doses based on the body surface formulation [26]. The total dose of NTN administered to each mouse was: 37 mg/kg. Unless otherwise stated, all compounds employed in this study were obtained from Sigma–Aldrich (Poole, UK). For oral administration, NTN was dissolved in saline and given to the mice three times a day by oral gavage.

Table 3. NTN formulation. The table indicates the NTN components and the relative dosage. Doses were converted on the basis of mouse body surface formulation.

INGREDIENTS	QUANTITY (mg)
Acacia senegal (L.) Willd. (gummi)*	100
L.acidophilus tyndalized	10
L.reuteri tyndalized	7
Pea protein	50
Grape seed extract	50
β-galactosidase	13

6.2 Animals

Male CD1 mice (Envigo, Milan, Italy) at 4 weeks of age were used. Mice were housed in a controlled environment (22 ± 2 °C, 55 ± 15% relative humidity, 12 h light/dark cycle), with food and water ad libitum. Before this study, the animals were kept in a quarantine

area for one week. During this period, they were observed daily. In addition, a numbered tag placed through the edge of the right ear identified the animals selected for the study. Animal experiments are in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (DM 116192) as well as EU regulations (OJ of EC L 358/1 12/18/1986) and ARRIVE guidelines.

6.3 Experimental design

At the end of the quarantine week, the animals were carefully examined to evaluate their suitability for the study, and randomly divided into several experimental groups to induce the specific food intolerances.

6.3.1 HCD induction

For the induction of carbohydrate intolerance, mice were fed *ad libitum* with HCD (Table 4) diet for 5 weeks [266]. Control animals were fed *ad libitum* with a SD.

Table 4. Macronutrient composition of HCD

	HCD
Weight content (g/kg)	
Milk proteins	140.0
Starch	622.4
Sucrose	100.3
Soy Oil	40.0
Minerals	35.0
Vitamins	10.0
Cellulose	50.0
Choline	2.3
Energy content (%)	
Protein	14.7
Carbohydrate	75.9
Fat	9.4
Energy density (kJ/g)	15.95
Food quotient	0.946

Experimental groups

Group 1: SD; mice were fed with a SD plus vehicle for 3 weeks (N = 4);

Group 2: SD+NTN; mice were fed with a SD for 3 weeks plus oral administration of NTN for the next two weeks (N = 8);

Group 3: HCD; mice were fed with an HCD for 3 weeks plus oral administration of vehicle for the next two weeks (N = 8);

Group 4: HCD+NTN; mice were fed with an HCD for 3 weeks plus oral administration of NTN for the next two weeks (N = 8);

6.3.2 HFD induction

For the induction of lipid intolerance, mice were fed *ad libitum* a HFD (60% kcal derived from fat) for 14 weeks [267]. Control animals were fed *ad libitum* a SD.

Experimental groups

Group 1: SD; mice were fed with a SD plus vehicle for 14 weeks (N = 4);

Group 2: SD+NTN; mice were fed with a SD for 12 weeks plus oral administration of NTN for the next two weeks (N = 8);

Group 3: HFD; mice were fed with an HFD for 12 weeks plus oral administration of vehicle for the next two weeks (N = 8);

Group 4: HFD+NTN; mice were fed with an HCD for 12 weeks plus oral administration of NTN for the next two weeks (N = 8);

6.3.3 HFrD induction

For the induction of fructose intolerance, Mice were fed ad libitum a HFrD for 15 weeks [268, 269] (30% in drinking water). Control animals were fed *ad libitum* a SD.

Experimental groups

Group 1: SD; mice were fed with a SD plus vehicle for 15 weeks (N = 4);

Group 2: SD+NTN; mice were fed with a SD for 13 weeks plus oral administration of NTN for the next two weeks (N = 8);

Group 3: HFrD; mice were fed with an HFrD for 13 weeks plus oral administration of vehicle for the next two weeks (N = 8);

Group 4: HFrD+NTN; mice were fed with an HFrD for 13 weeks plus oral administration of NTN for the next two weeks (N = 8);

At the end of the experiments, animals were sacrificed, and tissues were surgically removed and processed for histological examinations and biochemical analyses. In addition, blood of each mouse was collected for further biochemical assay.

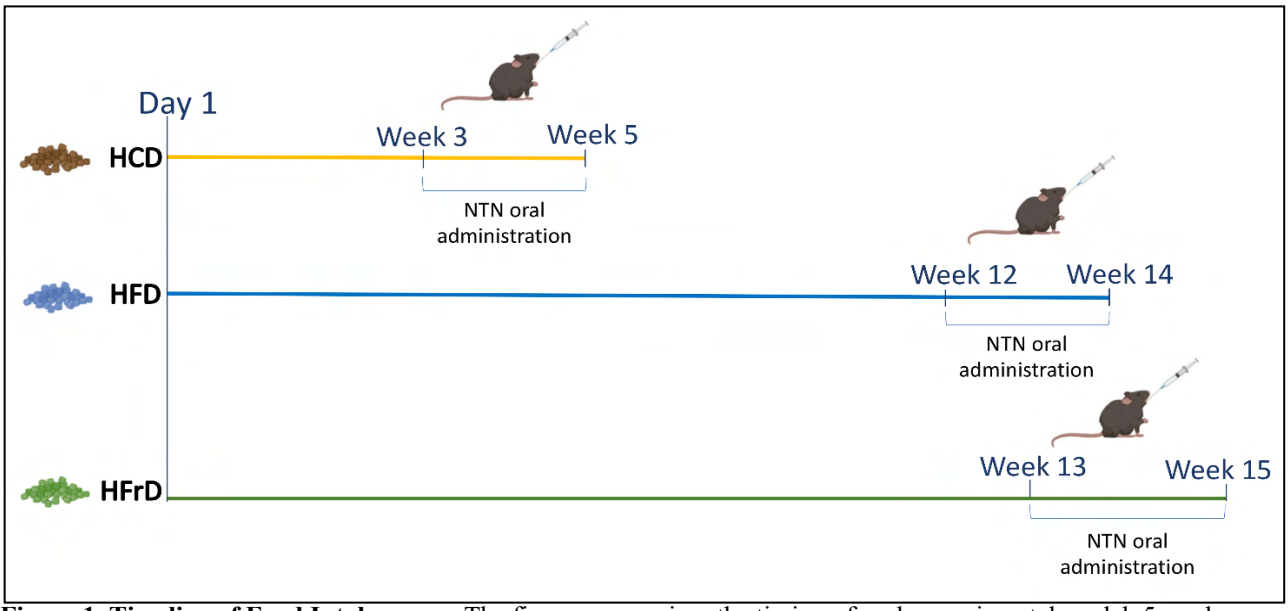


Figure 1. Timeline of Food Intolerances. The figure summarizes the timing of each experimental model. 5 weeks for carbohydrate intolerance (the administration of NTN was conducted in the last two weeks); 14 weeks for lipid

intolerance (the administration of NTN was carried out in the last two weeks); 15 weeks for fructose intolerance (the administration of NTN was carried out in the last two weeks).

6.4 Histological Evaluations

Histological analyses were performed as previously described by Casili et al. [270] and reported below. Immediately after the sacrifice of the animals, samples were fixed in 10% (*w/v*) PBS-buffered formaldehyde solution at 25 °C for 24 h. After dehydration, samples were included in paraffin. Tissue sections of 5µm were stained with Hematoxylin/Eosin (H&E, Bio-Optica, Milano, Italy) to evaluate histological alterations of pancreas, abdominal adipose and intestine tissues in HCD; liver and abdominal adipose tissues in HFD; liver and intestine tissues in HFrD. The results of histological examinations were displayed at 10x magnification (100 µm scale bar). All the histological analyses were performed in a blinded manner.

6.5 Immunohistochemical localization of ZO-1 and Occludin

Immunohistochemical localization of TJs in HCD and HFrD tissues were done as previously described by Campolo et al. [156]. Slices were incubated at room temperature overnight with the following primary antibodies: anti-ZO-1 (Santa Cruz Biotechnology sc-33725, 1:100 in PBS, *v/v*) and anti-occludin (Santa Cruz Biotechnology sc-133256; 1:100 in PBS, *v/v*). After primary antibody incubation, sections were washed in PBS and incubated with secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h. The reaction was revealed by a chromogenic substrate (DAB), and counterstaining with Nuclear Fast Red (Bio Optica, Milan, Italy). For a graphic display of the densitometric analyses, the % of positive staining (DAB brown staining) was measured by computer-

assisted color image analysis (Leica QWin V3, Newcastle, UK). The percentage area of immunoreactivity (determined by the number of positive pixels) was expressed as % of total tissue area (red staining) within five random fields at a 40x magnification [271]. For immunohistochemistry, 20x (50 μm scale bar) and 40× (20 μm scale bar) were shown. Immunohistochemical studies were performed in a blinded fashion.

6.6 Gut permeability

FITC-dextran was used to measure the intestinal permeability in HCD, HFD and HFrD animals.

Mice were fasted for 6 hours, after which FITC-dextran was administered by gavage (500 mg/kg body weight, 125 mg/ml). Subsequently, 100 μl of blood was collected from the caudal vein after 1h and 4h. The blood was centrifuged at 12,000 × g for 5 min at 4° C. The plasma concentration of dextran was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The standard curve was created by diluting FITC-dextran in untreated plasma diluted with phosphate-buffered saline (1:1, v/v).

6.7 Plasma insulin and glucose levels

In the HCD study, blood was collected from the tail vein of each mouse and subsequently centrifuged for 10 min, at 3000 x g, 4°C; plasma stored at −20°C for assay of insulin and glucose levels as previously reported [266, 272]. Plasma glucose was measured spectrophotometrically using commercially available colorimetric kits (Aspen

Laboratories Pvt. Ltd., New Delhi, India) and expressed as plasma glucose (mg/dL) levels. Plasma insulin was detected using an enzyme-linked immunoassay.

6.8 Analysis of liver weight

Hepatic steatosis was evaluated as previously described by Tao et al. [273]. At the end of HCD experiment, the weight of livers was measured through analytical balance (Bel engineering balance; Monza, Italy) to evaluate the effects of the high carbohydrate intake on lipid hepatic accumulation.

6.9 Quantification of NEFA and TG

Lipid tolerance test was performed based on Peterson et al. [267]. Fasted mice were IP-injected with 20% emulsified Intralipid (10 mL/g of body weight Sigma Aldrich), mimicking the sudden rise of plasma lipids in response to food intake. Sera was collected via tail bleed using a MicrovetteH CB 300 (Sarstedt) at 0, 1, 2, 3, and 5 h post-injection. Serum levels of non-esterified fatty acids (NEFA) and triglycerides were quantified using kits from Wako and Infinity Triglycerides, respectively.

6.10 Statistical analysis

Experimental data are expressed as mean ± standard error of the mean (SEM) of N observations, in which N represents the number of animals studied. In the experiments involving histological evaluations, images are representative of at least three independent experiments. In order to reach the minimum number of mice required for every

technique, an ANOVA (fixed effects, omnibus, one-way) was defined “a priori” with the G-power software. This statistical test supplies a professional method to analyze the sample size required to make the experiments. Data analysis was performed with One-Way and Two-Way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. Only a *p*-value less than 0.05 was considered significant.

CHAPTER SEVEN: RESULTS

7. RESULTS

7.1 Effects of NTN administration on body weight, food intake, pancreas tissue damage and glucose-insulin levels in HCD mice

The weight gain that occurs during NTN intake is due not to a physiological or metabolic consequence of monosaccharides or disaccharides , but to a modification of sugar intake resulting from an alteration in energy balance [274] that alter hunger-satiety continuum, thus facilitating carbohydrate consumption in the absence of energy needs [275].

After 5 weeks of HCD, the mice showed a moderate weight gain and an increased food intake compared to the control group (Figure 2A and 2B); NTN treatment was able to reduce body weight already after one week of treatment (week 4 in the graph 2A) in mice fed with HCD as well as to restrain carbohydrates consumption (Figure 2A and 2B).

A high dietary carbohydrate intake results in elevated circulating glucose levels and hyperinsulinemia [276] as well as pancreatic β cell dysfunction, thus leading to poor management of the glycemic load [277]. In relation to this, we analyzed tissue integrity of the pancreas by H&E staining to evaluate the morphological changes after HCD.

In the pancreas of HCD mice was found a significant increase of tissue damage, accompanied by moderate hyperplasia of the islet of Langerhans and neutrophilic infiltration (Figure 2E, histological score 2G) compared to the control group (Figure 2C, histological score 2G). However, NTN administration significantly improved the pancreas tissue architecture (Figure 2F, histological score 2G), a feature correlating also with the better management of glycemic and insulin parameters, a notable feature of carbohydrate intolerance. In fact, following the HCD diet, we assisted in a marked increase in both glucose (Figure 2H) and insulin (Figure 2I) plasma levels compared to SD; conversely, the two-week treatment with NTN reduced considerably both parameters.

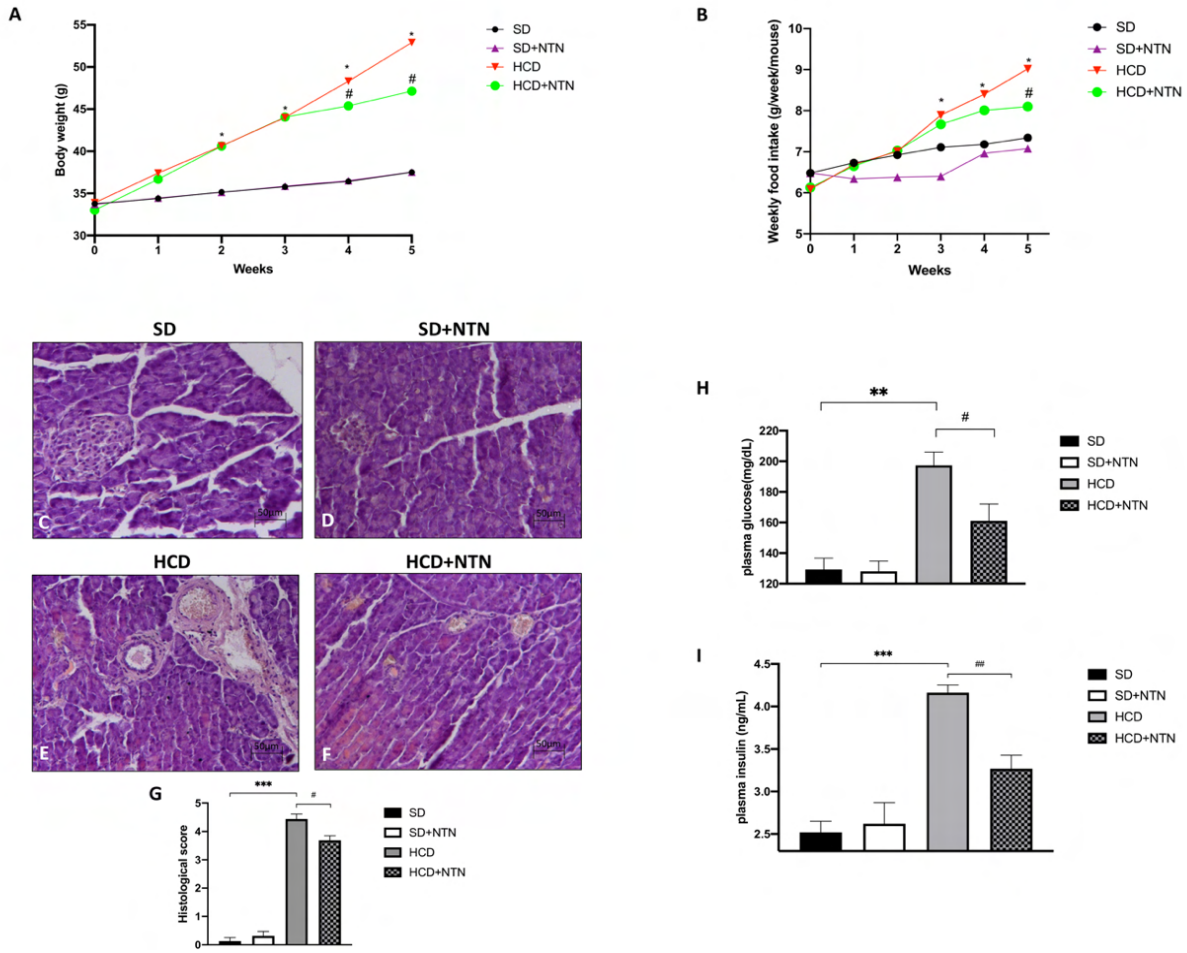


Figure 2. Effects of NTN on body weight, food intake, histological damage of the pancreas and glucose-insulin levels in HCD intolerant mice. A slight increase in body weight and food intake was detected in HCD-mice compared to the control group (A, B); NTN administration reduce both parameters in HCD mice (A, B). Extensive neutrophil infiltration and tissue damage were observed in mice fed with HCD (E, G) compared to SD animals (C, G). Administration of NTN was able to significantly counteract the extent of tissue damage and neutrophil infiltration in HCD mice (F, G). NTN administration reduced both glucose and insulin levels (H, I). Data are representative of at least three independent experiments. Values are means ± SEM. One-Way and Two-Way ANOVA test. * $p < 0.05$ *vs SD;* ** $p < 0.01$ vs. SD; *** $p < 0.001$ vs. SD; # $p < 0.05$ vs. HCD; ## $p < 0.01$ vs. HCD.

7.2 Effects of NTN administration on abdominal adipose tissue damage and steatosis in HCD mice

Histopathological evaluation of carbohydrate intolerant mice displayed a significant increase of tissue damage and neutrophil infiltration in white abdominal adipose tissue

(Figure 3C, histological score 3E) compared to SD mice (Figure 3A, histological score 3E); NTN supplementation appreciably restored architecture of the abdominal adipose tissue (Figure 3D, histological score 3E). A decrease in adipose content was also found in the liver; in fact, the administration of NTN was able to significantly reduce hepatic steatosis caused by excessive calorie intake (Figure 3F).

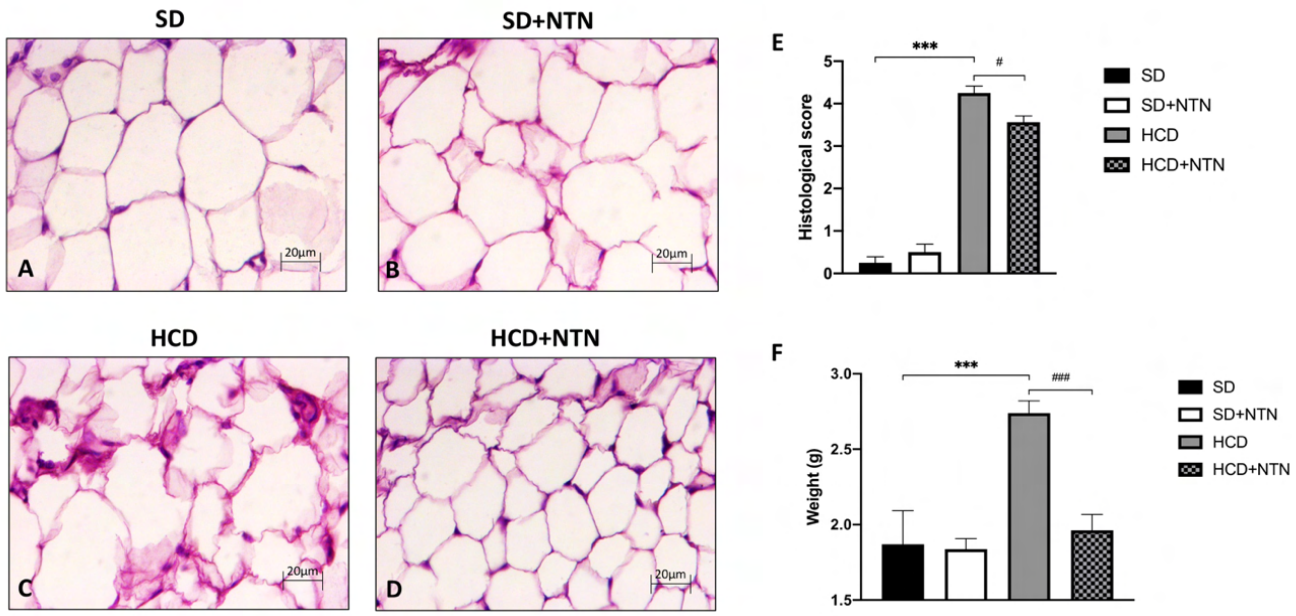


Figure 3. Effect of NTN on adipose abdominal tissue and liver in HCD mice. HCD mice showed a significant tissue injury in adipose tissue (C, E), on the contrary, SD mice showed no tissue damage (A, E). NTN restored physiological parameters, thus reducing neutrophil infiltration and adipocytes size (D, E). In addition, NTN was able to decrease liver weight (F). Data are representative of at least three independent experiments. Values are means ± SEM. One-Way ANOVA test. *** $p < 0.001$ vs. SD; # $p < 0.05$ vs. HCD; ### $p < 0.001$ vs. HCD.

7.3 Effects of NTN administration on intestinal tissue damage and permeability

Excessive consumption of carbohydrates leads to intestinal disorders characterized by intestinal dysregulated morphology, accompanied by high intestinal permeability and loss of tissue epithelial integrity [278, 279]. A significant increase in intestinal tissue damage

and neutrophil infiltration was observed in carbohydrate intolerant mice (Figure 4C, histological score 4E) compared to the control group (Figure 4A, histological score 4E). NTN administration significantly improved tissue architecture of the intestine counteracting the extent of intestinal tissue damage and neutrophil infiltration due to HCD (Figure 4D, histological score 4E).

Furthermore, to evaluate the barrier protective properties of NTN we assessed gut permeability with a Transelectrical Epithelial Resistance (TEER) test.

A marked increase in gut permeability was observed in mice fed with HCD compared to mice fed with SD (Figure 4F). NTN, after two weeks of treatment, significantly reduced the increase in the paracellular FITC-dextran flux induced by HCD, proving to be a good regulator of gut permeability (Figure 4F).

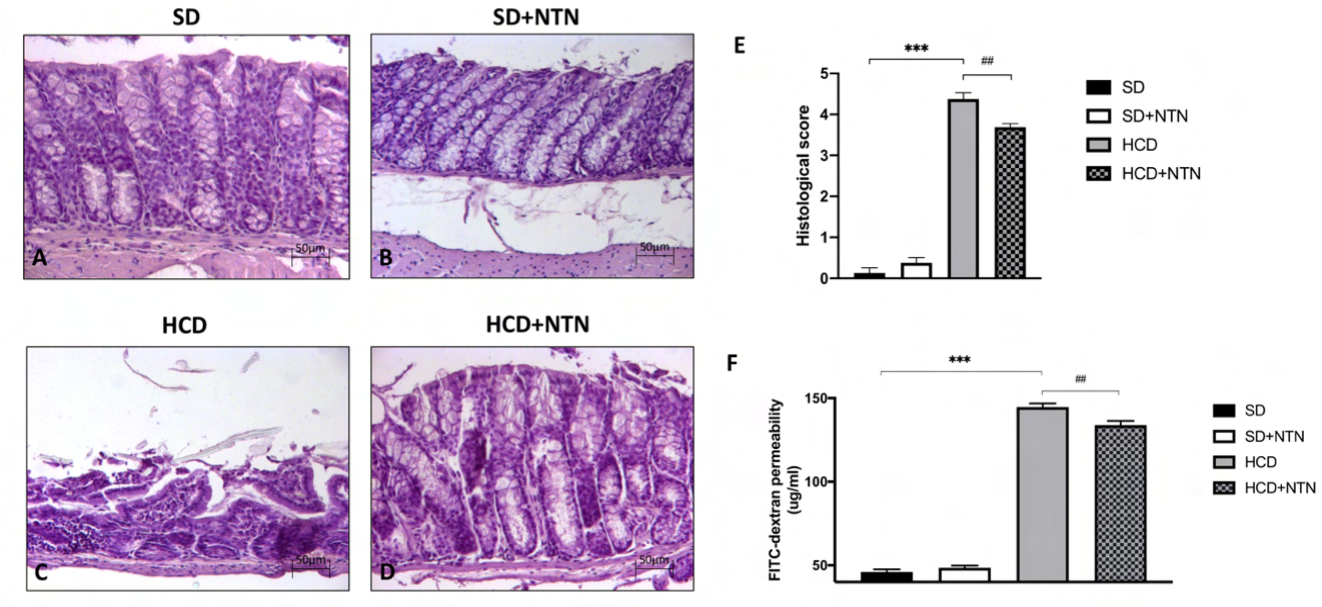


Figure 4. Effects of NTN on intestinal tissue damage and permeability in HCD mice. Neutrophil infiltration and tissue damage was observed in mice fed with HCD (C, E) compared to SD (A, E). Administration of NTN was able to significantly counteract the extent of intestinal tissue damage and neutrophil infiltration in HCD mice (D, E). FITC-dextran permeability assay of HCD mice jejunum exposed a marked increase of intestinal permeability; NTN exerted an important protective barrier effect (F). Data are representative of at least three independent experiments. Values are means ± SEM. One-Way ANOVA test. *** $p < 0.001$ vs. SD; ## $p < 0.01$ vs. HCD.

7.4 Effects of NTN administration on intestine epithelial integrity in HCD mice

TJs are multiprotein intercellular junctions adjacent to the apical ends of the paracellular spaces [280]. The main components are ZO-1 and Occludin, which among their main functions, regulate cellular permeability and barrier intestinal function [281]. Consequently, their dysregulation is often associated with bowel disease [281].

To evaluate the beneficial effect of NTN on intestinal epithelial integrity we estimated ZO-1 and Occludin expression through immunohistochemical analysis. Mice fed with HCD displayed a significant decrease in ZO-1 (Figure 5C, histological score 5E) and Occludin expressions (Figure 5H, histological score 5J) compared to control mice (Figure 5A and 5F respectively, histological score 5E and 5J). While the two weeks of treatment with NTN notably improved the integrity of the intestinal barrier, promoting the increase in the expression of ZO-1 (Figure 5D, histological score 5E) and Occludin (Figure 5I, histological score 5J).

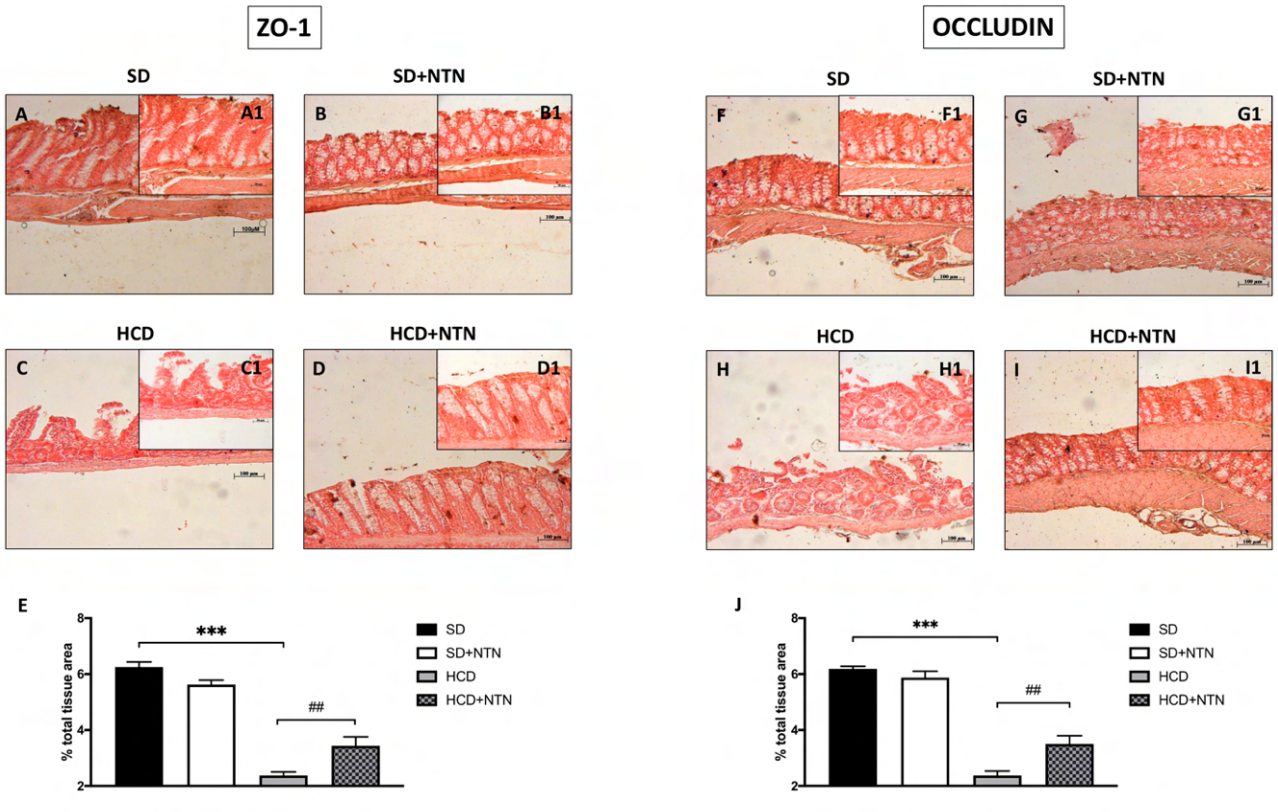


Figure 5. Effects of NTN administration on intestine epithelial integrity in HCD mice. A high percentage in the expression of ZO-1 (A, E) and Occludin (F, G) were found in intestinal tissues of SD mice, conversely HCD decreased such expressions (C, E, H, J). NTN has appreciably restored the levels of ZO-1 (D, E) and Occludin (I, J). Data are representative of at least three independent experiments. Values are means ± SEM. One-Way ANOVA test. *** $p < 0.001$ vs. SD; ## $p < 0.01$ vs. HCD.

7.5 Effects of NTN administration on body weight, food intake, liver tissue damage, lipid tolerance parameters and gut permeability in HFD mice

As demonstrated by several *in vivo* studies, excess dietary fat induces significant body weight gain [282, 283]. These assumptions demonstrate a link among increased fat depots, weight gain, and liver damage. In fact, liver injury probably aggravates the metabolic syndrome, supporting that not only the amount of calories is important in the induction of weight gain or metabolic syndrome, but other factors may be involved as well. Our data showed a substantial increase in body weight of HFD-fed mice compare to the control group (Figure 6A). NTN-two weeks treatment appreciably reduce weight gain already from the first week of treatment (Figure 6A). No significant variations were found in weekly food intake (Figure 6B).

Through H&E staining we evaluated liver tissue integrity. Mice fed with HFD demonstrated an accentuated hydropic degeneration and steatosis that was diffusely distributed throughout all areas of the hepatic acinus (Figure 6E, histological score 6G) compared to control mice (Figure 6C, histological score 6G).

On the other hand, lipid intolerant mice treated with NTN showed a meaningful reduction in hydropic degeneration and steatosis (Figure 6F, histological score 6G).

A physiological increase in non-esterified fatty acids (NEFA) and triglycerides (TG) plasma levels is usually observed after an intake of a high-fat meal [284, 285].

Hence, to determine whether the HFD-fed and NTN treated mice differ in their capacity to handle acute lipid challenge we performed a lipid tolerance test.

A significant increase in circulating NEFA and TG levels was observed in mice fed with HFD compared to SD-fed mice (Figure 6H and 6I respectively). NTN treated mice demonstrated a significantly greater capacity to clear an acute rise in NEFA and TG in response to emulsified lipid infusion compared to untreated mice (Figure 6H and 6I respectively).

Furthermore, as described by Tanaka et al. [286] HFD-derived free fatty acids increase sensitivity to intestinal damage; therefore, we have analyzed the barrier protective properties of NTN by assessing intestinal permeability with a Transelectrical Epithelial Resistance (TEER) test in HFD mice. A marked increase in gut permeability was observed in mice fed with HFD compared to mice fed with a SD (Figure 6J). However, NTN two-week treatment significantly reduced gut permeability (Figure 6J).

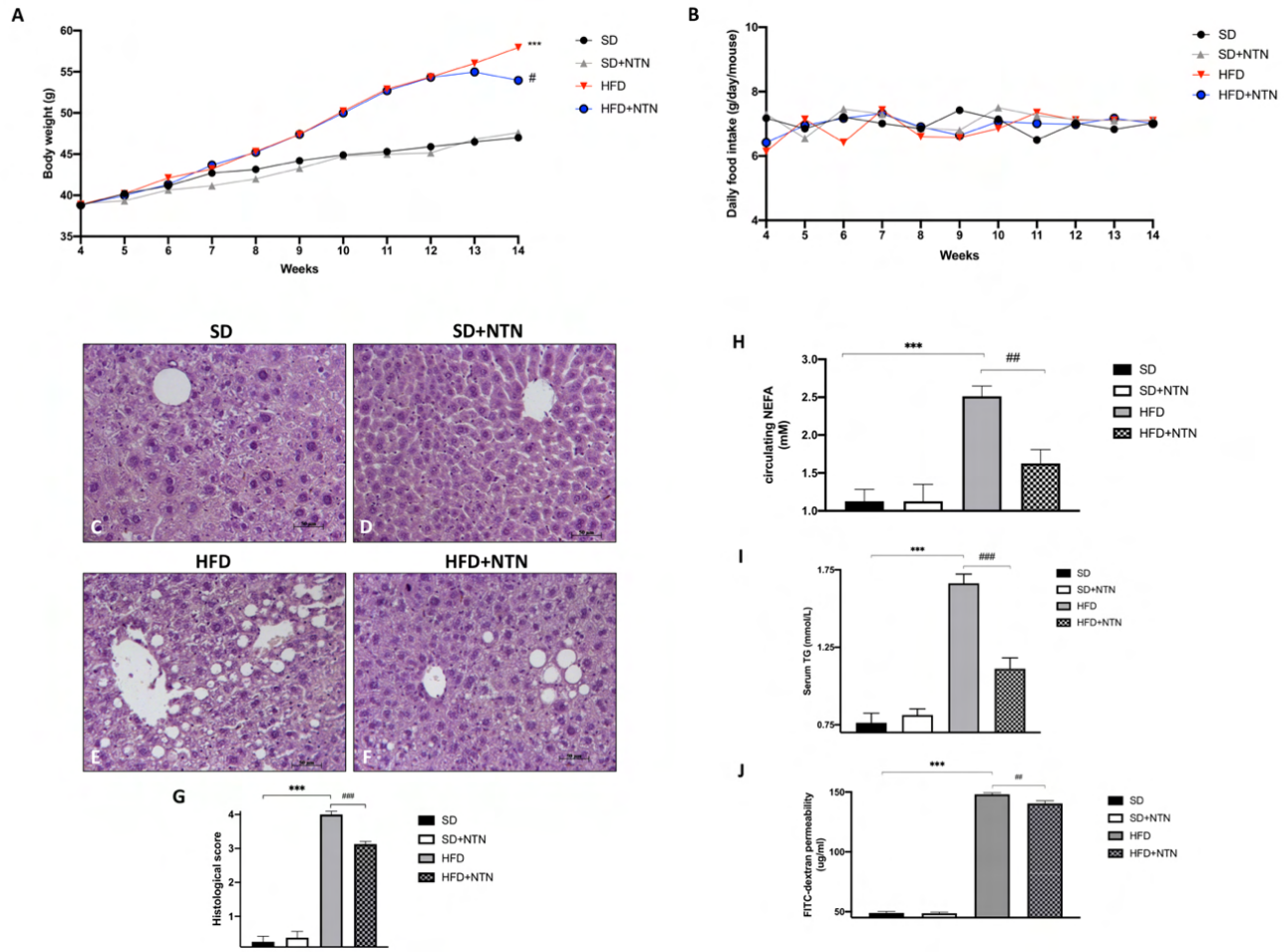


Figure 6. Effects of NTN on liver tissue, fat mobilization and gut permeability in HFD mice. HFD-fed mice shown a significant increase in body weight, compared to sham group (A); NTN considerably decreased weight gain (A). No significant differences were detected in mice food intake (B). Significant hydropic degeneration and steatosis were observed in mice fed with HFD (E, G) compared to SD (C, G). Administration of NTN was able to significantly counteract the extent of liver damage (F, G). In addition, NTN administered mice decrease NEFA and TG levels compared to HFD mice (H, I). FITC-dextran permeability of jejunum was very low in SD mice (J). Contrarily, after HFD, mice displayed an increased intestinal permeability that was reduced by NTN administration (J). Data are representative of at least three independent experiments. Values are means ± SEM. One-Way ANOVA test. *** $p < 0.001$ vs. SD; # $p < 0.05$ vs. HFD; ## $p < 0.01$ vs. HFD; ### $p < 0.001$ vs. HFD.

7.6 Effects of NTN administration on intestine epithelial integrity in HFD mice

We investigated the effect of NTN on ZO-1 and Occludin expressions, by immunohistochemical staining, also in HFD model. Obtained results revealed a basal expression of ZO-1 and Occludin in the tissues of SD group (Figure 7A, histological score 7E and 7F, histological score 7J respectively); while the HFD group was characterized by a reduction of both TJs expression (Figure 7C, histological score 7E and 7H, histological score 7J respectively). NTN treatment was able to appreciably upturn ZO-1 and Occludin expressions (Figure 7D, histological score 7E and 7I, histological score 7J respectively), thus repairing the compromised intestinal permeability.

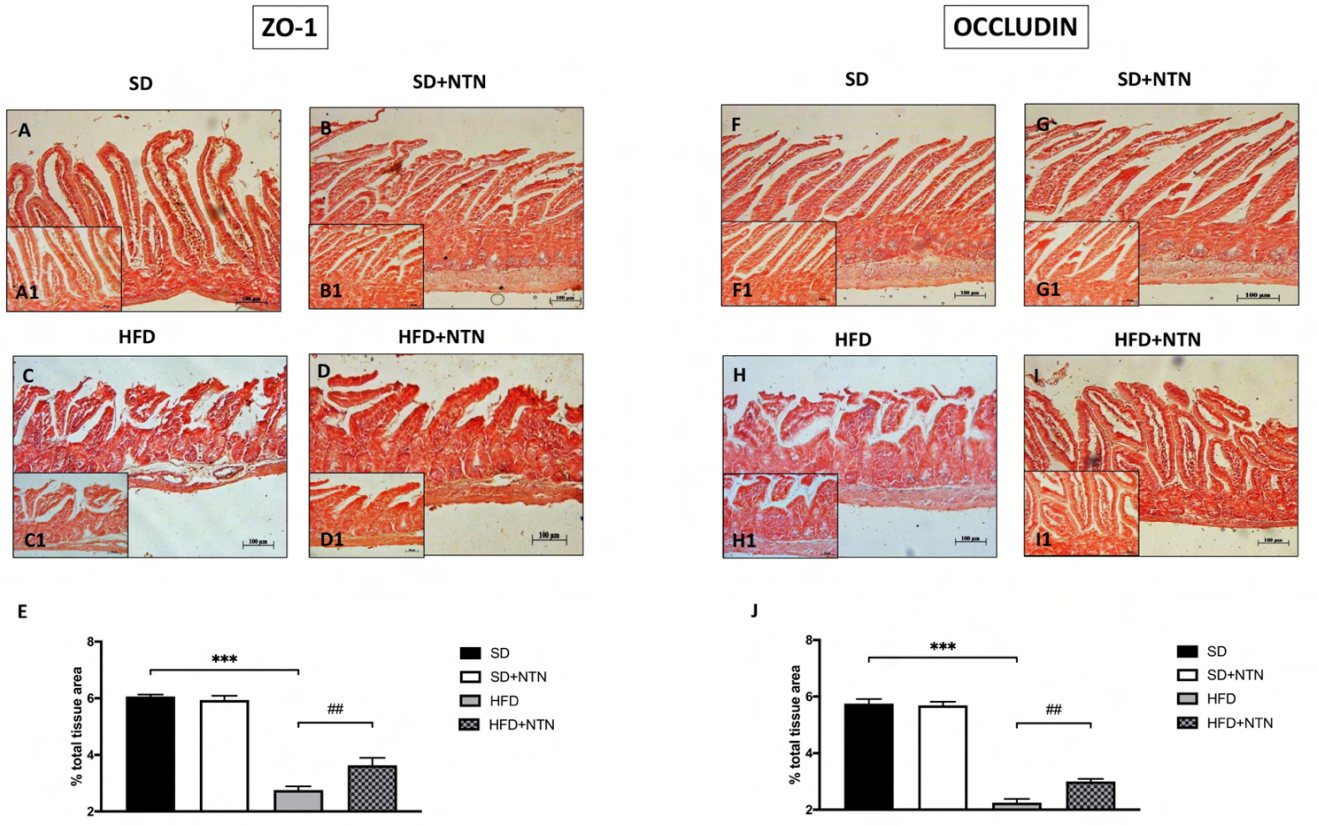


Figure 7. Effects of NTN administration on intestine epithelial integrity in HFD mice. High expressions of ZO-1 and Oc-cludin have been found in intestines tissues of the SD group (A, E and F, J respectively) compared to the HFD group (C, E and H, J respectively). The administration of NTN restored the expression of ZO-1 and Occludin proteins (D, E and I, J respectively). Data are representative of at least three independent experiments. Values are means ± SEM. One-Way ANOVA test. *** $p < 0.001$ vs. SD; ## $p < 0.01$ vs. HFD.

7.7 Effects of NTN administration on abdominal adipose tissue damage in HFD mice

Histopathological analysis of white adipose tissue from the abdomen was performed by H&E staining.

Lipid intolerant mice displayed a significant increase in the size of adipocytes and neutrophil infiltration (Figure 8C, histological score 8E) compared to control mice (Figure 8A, histological score 8E). Treatment with NTN significantly improved tissue architecture by reducing adipocytes size as well as neutrophil infiltration (Figure 8D, histological score 8E).

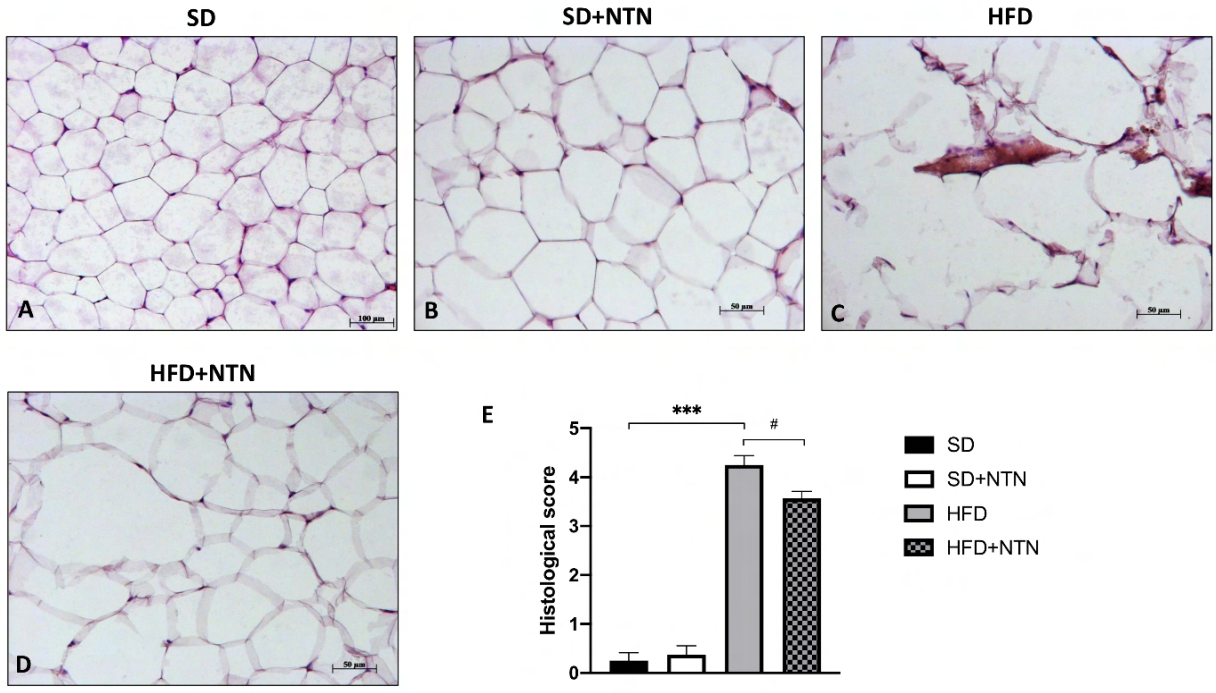


Figure 8. Effects of NTN on adipose damage in HFD mice. HFD led to a remarkable increase in neutrophil infiltration and adipocyte size (C, E) compared to SD mice (A, E). Administration of NTN was able to significantly counteract the extent of adipose tissue due to HFD (D, E). Data are representative of at least three independent experiments. Values are means ± SEM. One-Way ANOVA test. *** $p < 0.001$ vs. SD; # $p < 0.05$ vs. HFD.

7.8 Effects of NTN administration on body weight, food intake and liver tissue damage in HFrD mice

Although, HFrDs have been implicated in obesity via impairment of leptin signaling in humans, several *in vivo* studies [269, 287, 288] have invalidated these assumptions in mice.

This could be related to the higher mass-specific metabolic rate of mice, which might allow for greater tolerance to fructose consumption. In fact, the fructose may be oxidized to CO_2 and H_2O to a greater extent in mice than rats, without the deleterious effects of fructose metabolites shuttled into VLDL (very low-density lipoprotein) synthesis [288]

The results obtained from the analysis of body weight and water intake in HFrD mice did not show significant differences (Figures 9A and 9B), thus confirming that excessive fructose consumption is not directly related to body weight gain.

To evaluate the effect of NTN on liver tissue damage in fructose intolerant mice we carried out histological examination by H&E.

Mice fed with a HFrD significantly increased liver tissue damage (Figure 9E, histological score 9G) compared to control mice (Figure 9C, histological score 9G). NTN significantly reduced chronic inflammation, macrovesicular and microvesicular steatosis following a HFrD diet, improving liver tissue architecture (Figure 9F, histological score 9G).

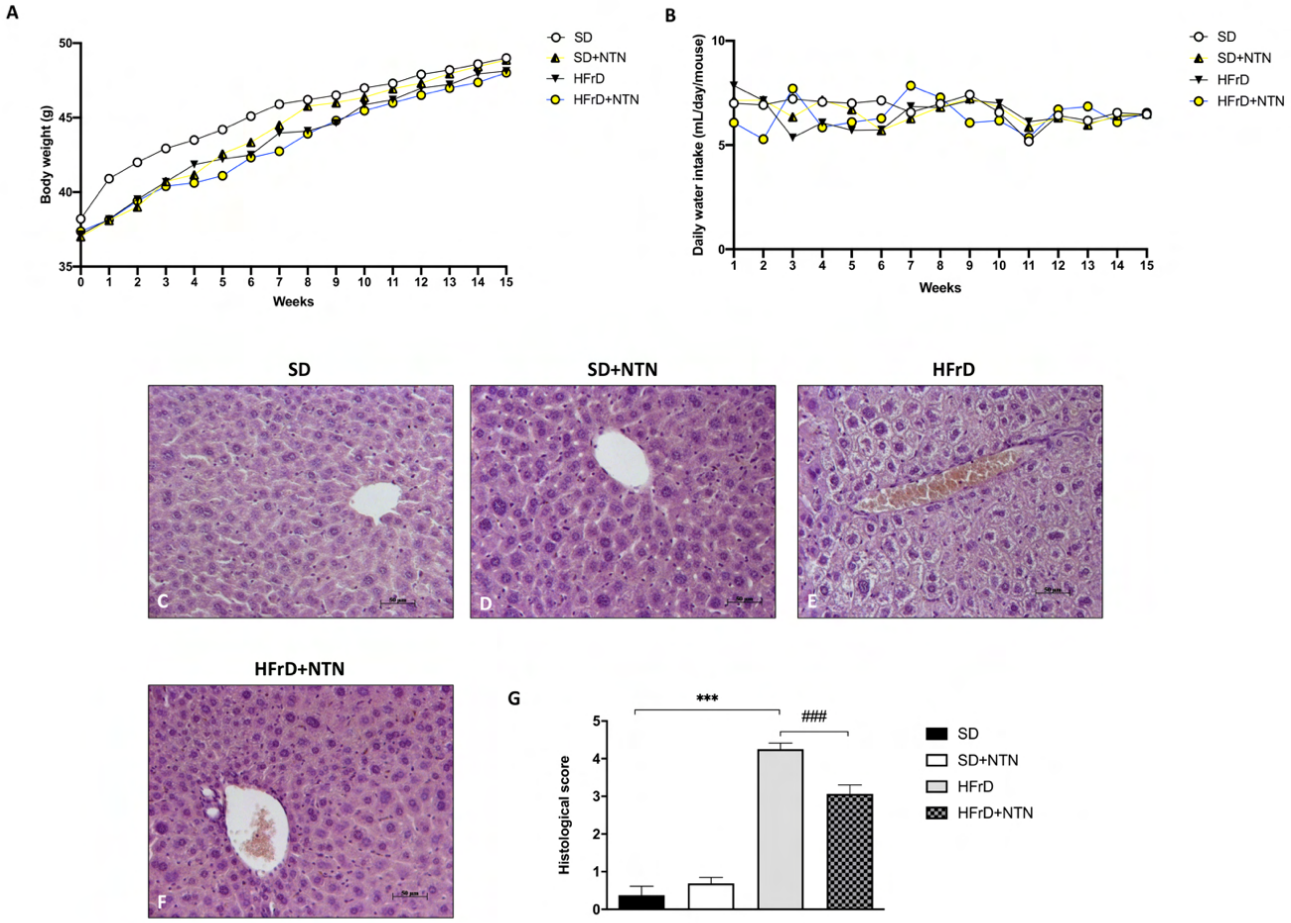


Figure 9. Effects of NTN on liver damage in HFrD. Significant macrovesicular and microvesicular steatosis was observed in mice fed with HFrD (E, G) compared to SD mice (C, G). Administration of NTN was able to significantly counteract the extent of liver damage in HFrD mice (F, G). Data are representative of at least three independent experiments. Values are means ± SEM. One-Way ANOVA test. *** $p < 0.001$ vs. SD; ### $p < 0.001$ vs. HFrD.

7.9 Effects of NTN administration on intestinal tissue damage and permeability in HFrD mice

Fructose intolerance is often associated with malabsorption and gastrointestinal disorders, including both increased intestinal motility and sensitivity, which overall lead to impaired bowel function [33, 289].

Therefore, we executed H&E staining to evaluate the effect of NTN on intestinal tissue damage in fructose intolerant mice.

Mice fed with a HFrD significantly increased intestinal tissue damage, as observed by the loss of lamina propria structure as well as inflammatory cell infiltration (Figure 10C, histological score 10E) compared to control mice (Figure 10A, histological score 10E).

NTN two-weeks administration significantly reduced neutrophilic inflammation and edema improving intestinal tissue architecture (Figure 10D, histological score 10E).

Moreover, elevated levels of fructose in the diet result in increased intestinal permeability [269]; thus, to assess the effect of NTN on gut permeability in fructose intolerant mice, we performed a FITC-dextran permeability assay.

A marked increase in gut permeability was observed in mice fed with a HFrD compared to mice fed with a SD (Figure 10F). NTN treatment significantly reduced gut permeability after two weeks of treatment (Figure 10F).

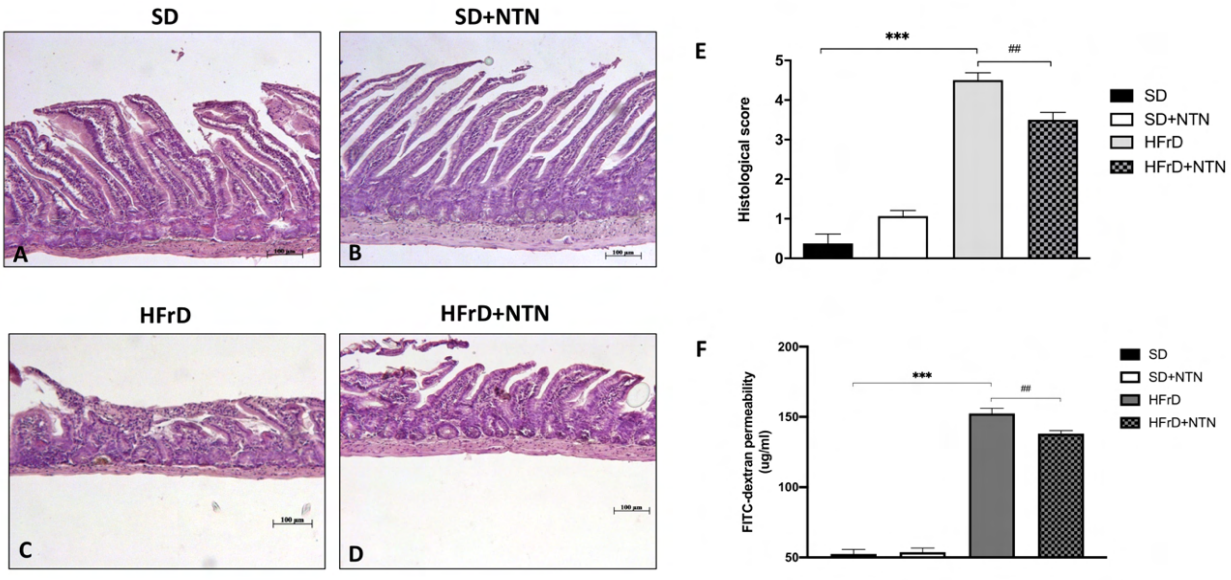


Figure 10. Effects of NTN on intestinal features in HFrD mice. Significant infiltration of inflammatory cells and tissue damage was observed in mice fed with HFrD (C, E) compared to SD mice (A, E). Administration of NTN was able to significantly counteract the extent of intestinal tissue damage (D, E). Gut permeability assay exhibited an evident increase

of intestinal permeability in HFrD jejunum compared to the SD group (F); NTN showed protective properties decreasing gut permeability (F). Data are representative of at least three independent experiments. Values are means ± SEM. One-Way ANOVA test. *** $p < 0.001$ vs. SD; # $p < 0.05$ vs. HFrD; ## $p < 0.01$ vs. HFrD; ### $p < 0.001$ vs. HFrD.

7.10 Effects of NTN on epithelial integrity in the intestines of HFrD mice

Chronic fructose intake is also associated with a loss of tight junction proteins, resulting in dysfunction of the intestinal barrier [290, 291].

In relation to this, we estimated the possible positive outcome of NTN on intestinal epithelial integrity through immunohistochemical localization of ZO-1 and Occludin.

Mice fed with a HFrD displayed a significant decrease in ZO-1 (Figure 11C, histological score 11E) and Occludin expressions (Figure 11H, histological score 11J) compared to SD-fed mice (Figure 11A and 11F respectively, histological score 11E and 11J).

The two-weeks treatment with NTN considerably improved the integrity of the intestinal barrier (Figure 11D and 11I, histological score 11E and 11J).

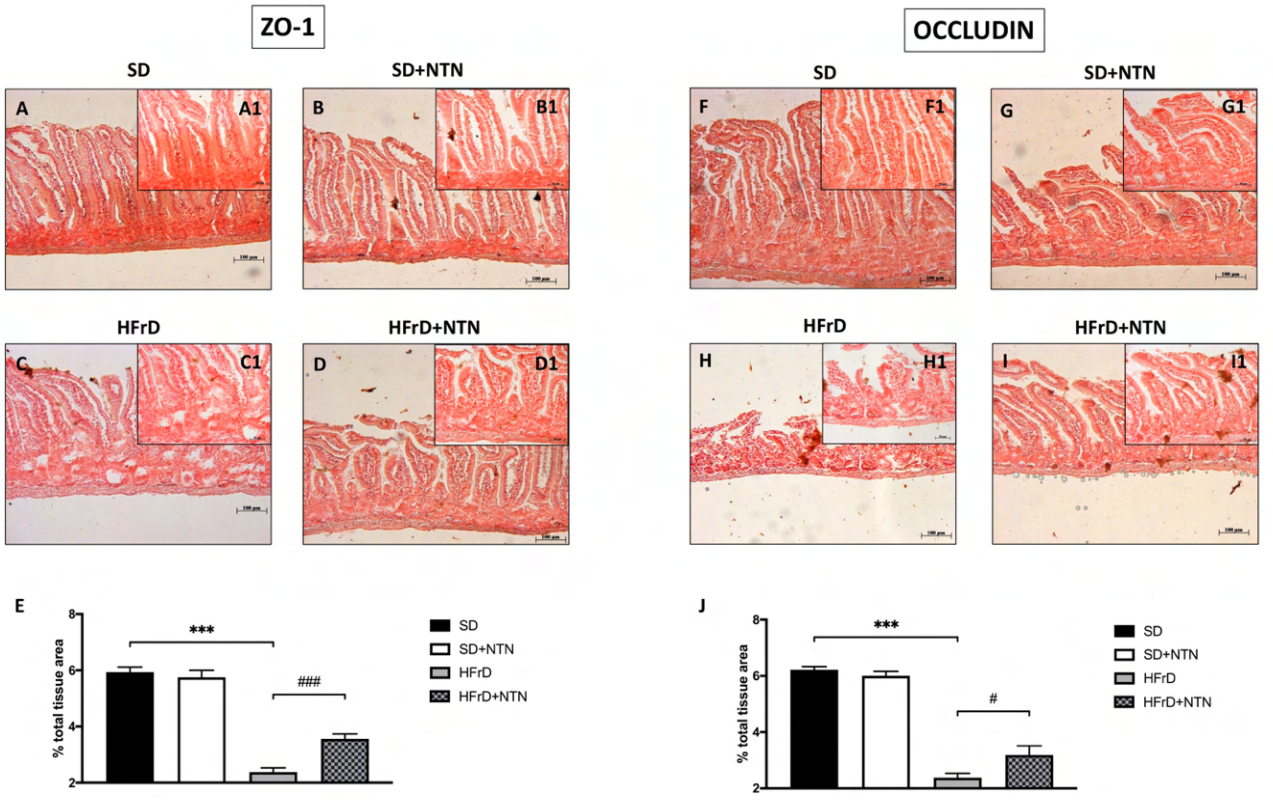


Figure 11. Effect of NTN on intestinal epithelial integrity in HFrD mice. Intestinal tissues of SD mice displayed high expressions of ZO-1 (A, E) and Occludin (F, J) proteins, contrariwise TJs expressions were reduced after HFrD (C, E, H, J). NTN two-week treatment significantly restored ZO-1 (D, E) and Occludin (I, J) expressions. Data are representative of at least three independent experiments. Values are means ± SEM. One-Way ANOVA test. *** $p < 0.001$ vs. SD; # $p < 0.05$ vs. HFrD; ### $p < 0.001$ vs. HFrD.

CHAPTER EIGHT: DISCUSSION

8. Discussion

Food intolerances refer to the difficulty in digesting certain foods; these disorders afflict a consistent percentage of the population, representing an influencing factor for the development of other pathologies such as irritable bowel syndrome (IBS) etc. [292]. In fact, up to 65% of IBS patients report that their symptoms are related to specific foods, these overlapped clinical signs make diagnosis even more difficult for food intolerances patients [293]. However, a sizable percentage of patients show gastrointestinal complaints, similar to indigestion, without a specific diagnosis making the management of symptoms, in the meanwhile, a priority. [3, 294].

It is important to underline that food intolerances are food disorders quite distinct from food allergies. In fact, in the case of food intolerances, the immunological component is not implicated, consequently, the individual does not react with an immune response but immediately replies to food ingestion with gastrointestinal and/or extraintestinal symptoms [3]. Although food intolerances are not life-threatening as in the case of food allergies, they still represent an uncomfortable condition for patients' quality of life. Following a comprehensive medical history including dietary and lifestyle evaluation, with a focus on potential food intolerances, patients with gastrointestinal symptoms are usually subjected to clinical examinations which may include blood and fecal tests, endoscopy and/or radiological imaging to rule out any organic disease or food allergy [1]. However, there are a limited number of clinically useful tests available to recognize specific food intolerances.

This unfavorably affects the patient's quality of life in terms of social activities and reduced dietary choices in order to achieve symptoms improvement [1].

Actually, food intolerances therapeutic options involve the adoption of eating plans that assume the elimination of specific foods from the diet, however, they are difficult to maintain long-term and often are not healthy approaches [3, 295].

Whereby, the discovery and subsequent employment of new therapeutic strategies could confer a new hopeful perspective on food intolerances.

The restoration of the integrity of the intestinal mucosa would represent a valid support to regulate nutrient sensing, thus helping to relieve food intolerances symptoms. Perturbation of gut barrier homeostasis can lead to increased epithelial permeability and dysbiosis of the microbiota, which has been recognized to play a key role in the pathophysiology of several gastrointestinal disorders [296].

In this regard, many researchers [297, 298] highlighted a strong connection between food hypersensitivity and intestinal disruption, suggesting this target as a promising therapeutic solution.

In recent years, new scientific findings [41, 299] promoted the effects of probiotics and dietary enzymes to help break down sugars in fructose and lactose intolerant patients. Alternative solutions that can reestablish the gut microbiota and promote gut homeostasis regardless of the food intolerances are needed.

Given these outcomes, we investigated the beneficial effect of a natural-based therapeutic in multiple murine models of food intolerances.

The intestinal mucosa represents the main tissue to investigate disease-related metabolism [300]. In particular, in the context of food intolerances, adverse reactions to food may cause the progressive alteration of the intestinal barrier, resulting in the development of a persistent inflammatory condition and impaired intestinal motility, sensitivity and permeability [28, 29].

Our results showed that the intake of HCD, HFD and HFrD led to a marked increase in intestinal permeability. Contrarily, treatment with NTN effectively provided rapid symptoms relief by restoring the compromised gut permeability in carbohydrate, lipid, and fructose intolerant mice within two weeks of treatment. It is widely known as an intact intestinal barrier is important to prevent the entry of endotoxins, microorganisms and undigested food particles while allowing physiological functions including but not limited to essential nutrients and water absorption to take place [29].

This physical barrier is held together by the TJs, such as Occludin that create bridges between intracellular ZO. Concerning this, the role of epithelial TJs is crucial to seal off gaps between cells and in maintaining gut homeostasis [148, 296].

Furthermore, several pre-clinical studies [281, 301] reveal that TJs breakdown is typical in many intestinal diseases, including food intolerances [269].

Our data confirmed, together with an alteration of the intestinal mucosal architecture, a TJ dysregulation due to HCD, HFD and HFrD. Nevertheless, NTN two-weeks treatment is proven to extensively recover intestinal tissue damage and restore Occludin and ZO-1 expressions in carbohydrate, lipid, and fructose intolerant mice. These positive outcomes are attributable to the modulation of intestinal bacteria activities and to protective barrier properties exerted on the intestinal mucosa [302, 303]; which led to a restoration of the intestinal epithelial barrier.

Interestingly, Do et al. [269] reported how the loss of intestinal permeability precedes lipid accumulation, which is subsequently associated with hepatic steatosis. In relation to this, other evidence [150, 304] supported the close correlation and cooperation between the gut and the liver, defined as gut-liver axis. In this reciprocal connection, the integrity of gut barrier plays a fundamental role in maintaining hepatic homeostasis [304]. More specifically, intestinal barrier function loss, due to TJs disruption allows the passage of

pro-inflammatory stimuli such as pathogen-associated molecular patterns (PAMPs) to the liver through the portal system promoting the progression of chronic liver diseases, such as cirrhosis ALD, and alcoholic liver disease (ALD), and Non-Alcoholic Fatty Liver Disease (NAFLD) [150, 305].

The results obtained from this study clearly confirmed an extensive increase in liver fat content following HCD, HFD and HFrD compared to SD. However, NTN two-week administration was able to promote a good recovery in steatosis in HCD-mice as well as in counteracting the accumulation of hepatic fat following a hyper lipidic diet, and to moderate hydropic degeneration of hepatocytes in fructose intolerant mice.

Recent findings [286, 306, 307] emphasized a crosstalk between intestinal epithelial damage and circulating free fatty acids (FFAs) concentrations. In fact, if on the one hand, the increase in HFD-derived free fatty acids produces "intestinal lipotoxicity", on the other hand, intestinal function is also involved in the regulation of plasma levels of FFAs [307, 308]. This thesis is supported by growing evidences that exposes how the intestine actively participates in the regulation of the lipid metabolism of the whole body through the regulation of nutrients, hormonal, metabolic and neural regulatory pathways [307]. On this basis, we looked at the main clinical markers of health or disease status of dyslipidemic patients, such as NEFA and TG.

Our data visibly revealed increased concentrations of both NEFA and TG in HFD mice. NTN two-weeks administration showed positive outcomes on lipid intolerance features, thus suggesting a good capacity to handle lipid load.

Visceral adipose fat (VAT) is a hormonally active tissue and possesses a unique biochemical profile that influences physiological and pathological processes in the human body, including metabolic processes [309]. Relatively, visceral obesity is associated with

several medical disorders such as metabolic syndrome [310], CVD [311] and a shortened life expectancy [312].

Moreover, several scientific data [313, 314] support how a high daily intake of fatty meals or refined sugars induced a progressive increase in white adipose tissue, especially in the intra-abdominal cavity.

Therefore, considering adipose tissue a useful biomarker of dietary fatty acid intake and carbohydrate excess consumption too [315, 316], we analyzed its morphological changes.

Consistent with what was previously mentioned, our study exposed how high caloric intake derived from HCD and HFD led to increased body fat, especially in abdominal visceral fat.

De facto, the obtained results showed an expansion in the size of adipocytes together with an increase in neutrophilic infiltration in white abdominal tissue of HCD/HFD mice, while NTN two-week treatment appreciably improved the architecture of the abdominal adipose by decreasing adipocytes size and infiltration of neutrophils in carbohydrate and lipid intolerant mice.

Hyperinsulinemia correlated with hyperglycemia is considered to be a sign of insulin resistance development [272], typical in carbohydrate intolerant patients [317].

Insulin is known to be a key hormone, secreted by β-pancreatic cells, which affects almost all organs in the body, including adipose tissue, liver, and the vascular system too [318].

In healthy subjects, insulin secretion is coordinated to circadian rhythms, which regulate the daily rhythm in glucose metabolism and whole-body insulin sensitivity [319, 320]. Otherwise, reduction in insulin sensitivity as well as its non-physiological fluctuations exposes the tissues to disruption of metabolic molecular pathways including glucose metabolism [321]. High carbohydrate intake also contributes to reduced insulin

sensitivity and poor management of glycemic control, thus highlighting the influence of the gastrointestinal tract on glucose metabolism [322].

Hence, considering the vital role of the insulin-glucose feedback loop in sugar blood control, we examined pancreatic tissue integrity and glycemic hallmarks in carbohydrate intolerant mice.

Our results confirmed a substantial increase in glucose-insulin levels and pancreatic islet hyperplasia following the HCD diet. However, NTN two-weeks treatment exerted beneficial properties, improving pancreatic tissue damage, and effectively regulating glycemic parameters in carbohydrate intolerant mice. In this context we speculated mucomimetic substances were also helpful in the management of carbohydrate intolerance, thus offering a new starting point for additional analyses.

CHAPTER NINE: CONCLUSIONS

9. Conclusions

In conclusion, the data obtained from the present study elucidate the many advantages provided by NTN administration, offering a new way on food intolerances management.

The beneficial effects deriving from this new natural-based product have been shown to contribute in restore intestinal mucosal barrier integrity and functionality, thus helping to relieve symptoms related to food intolerances. These benefits deriving from NTN result in a better management of glycemic dysregulation and lipid load as well as fructose intolerance features.

Therefore, considering these new insights, NTN could represent a promising natural support in the non-pharmacological strategy for patients suffering from food intolerances and intestinal permeability, improving their social relationships and their quality of life.

However, we are aware of the limitations of animal models in the translational reproduction of human metabolic disorders, especially in the field of food intolerances.

In fact, although rodent models replicate many aspects of human metabolic disorders, the main dissimilarities between species in basal metabolic rate, feeding behavior, fecundity, immune system, and gut microbiota composition should be considered.

Moreover, animal models are also influenced by the environmental conditions and the genetic background.

In this perspective, future evaluations of NTN in well-designed clinical trials could further deepen our knowledge of patient care in those suffering from one and/or multiple food intolerances.

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