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Liver Function and Cardiovascular Risks: Experimental Evidences on its Oxidative Nutritional Life Style Links

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Abstract

Background: *In aerobic organisms, oxygen metabolism produces Reactive Oxygen and Nitrogen Species (ROS/RONS) as byproducts. Within certain quantities they act as mediators and stimulators of vital functions but when their generation overreaches the cellular antioxidant defense, they react against important biomolecules and led to Oxidative Stress (OS), affecting organs and important functions. OS affects liver, the resulted dysfunctions also affect other organs, increasing cardiovascular risk (CVR).*

Methods: *Using blood analysis, we followed the effects of induced OS on the organ functions of stressed mice and their recovery by antioxidant nutritional therapy with probiotics (yeast, selenized yeast, vitamin B12 and cranberry). We also evaluated the evolution of liver and cardiovascular functions in young men predisposed to liver, cardiovascular, kidney and diabetes diseases, combining a diet supplemented with probiotics and physical training. In stressed mice probiotic complemented diet resulted in better defense and recovery to OS. In individuals, nutritional therapy combined physical activities helped improve liver and cardiovascular function parameters. The combined nutritional and training strategy in young individuals allows to prevent fatty liver disease and CVR.*

Conclusions: *OS is the most important pathogenic cause in liver diseases, and it's well correlated with cardiovascular risks and other organic dysfunctions.*

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Keywords: Oxidative Stress (OS), ROS/RONS, Liver Disease, Cardiovascular Risks (CVR), Nutritional Strategy, Blueberry

Introduction

The liver is a crucial organ for the vertebrate organisms due to the large number of functions it assumes, including macronutrient metabolism, biotransformation, regulation of blood circulative volume, support of immune and endocrine systems, lipid and carbohydrate homeostasis and reserves, immune system support, control of growth signaling pathway, detoxification of drugs and xenobiotic compounds, among others. The capacity of liver to form and store glycogens by the way of gluconeogenic pathway is essential to energetic supply of the whole organism. The liver also oxidases lipids and conserves their excess in a form of adipose tissues, regulates protein and amino acid metabolism, is in charge for the amino acid deamination, and protein degradation by urea metabolism [1,2]. For all these reasons liver dysfunction impacts in other important biochemical processes and in the health condition of the organism, including the increase of CVR [3]. One of the important factors affecting liver and its functionality is the Oxidative Stress (OS). Redox imbalance is a source of many liver disorders and diseases, particularly triggering inflammatory, metabolic and proliferative liver dysfunctions. In liver hepatocytes, ROS/RONS are primarily produced in the mitochondria, during respiration, and in the endoplasmic reticulum, of by the way of the cytochrome P450 enzymes. In normal conditions, aerobic cells dispose a wide antioxidant system for maintaining REDOX homeostasis, neutralizing free radicals and neutralize oxidants. The antioxidant system is composed by antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), thioredoxin (Trx), as well as non-enzymatic antioxidants such as vitamins, catechin, curcumin, and carotenoids. When antioxidant defense is overpassed, the excess of ROS/RONS affects the structure of many biological molecules at different levels: cells, tissues, organs and whole organism. OS alters the liver functions resulted in metabolic alterations metabolic alterations that have an impact on cardiovascular system and increase cardiovascular risk (CVR) [4,5]. However, specific risk score-based treatment procedures are lacking, probably because mechanisms behind the increased CVR in patients with FLD are complex, multifactorial and difficult to elucidate. We present the use of specific blood biochemical markers to study this relation in stress induced mice fed with antioxidant diet complemented with probiotics (yeast, selenized yeast, blueberry extract) during stress and during recovery period with encouraging results. We also evaluated the evolution in young individuals showed predisposition to develop Non-Alcoholic Liver Fatty Disease (NALFD) and Cardiovascular Risks (CVR), combining nutritional (yeast, vitamin B12, blueberry extract) and physical training strategies with positive results. In both studies we followed the dynamic of blood biochemical parameters liked to hepatic and cardiovascular functions [5]. It has been reported that ingestion of probiotic supplements brings the described beneficial effects on vertebrate organisms but synergic combinational effect has not been extensively evaluated [6]. Our main focus is to appeal to these combinational assessments to prevent liver disease and cardiovascular risk.

Oxidative Stress

Oxygen is essential but highly reactive molecule that during its metabolism may induce oxidative stress (OS) causing modification in biopolymers and cellular structures by the generation of large amounts of reactive oxygen and nitrogen species (ROS/RONS). Among these reactive species we can listed the following: ROS (superoxide anion (O_2^-), hydroxyl radical (HO^-), hydrogen peroxide (H_2O_2), peroxyl

(ROO−), reactive aldehyde (ROCH), singlet oxygen (O-2)) and RONS (nitric oxide (NO), nitrogen dioxide (NO2−), peroxynitrite (ONOO−), nitrosyl cation (NO+), nitroxyl anion (NO−), dinitrogen trioxide (N2O3), dinitrogen tetraoxide (N2O4), nitrous acid (HNO2), peroxynitrous acid (ONOOH), and nitryl chloride (NO-2Cl) (7). ROS/RONS are important as a part of both intracellular and intercellular signaling systems, whether nearby, between cells of the same organ or tissue, or distal, between different organs or tissues and systemically in whole organism allowing to perform important physiological functions coordinately (Figure 1).

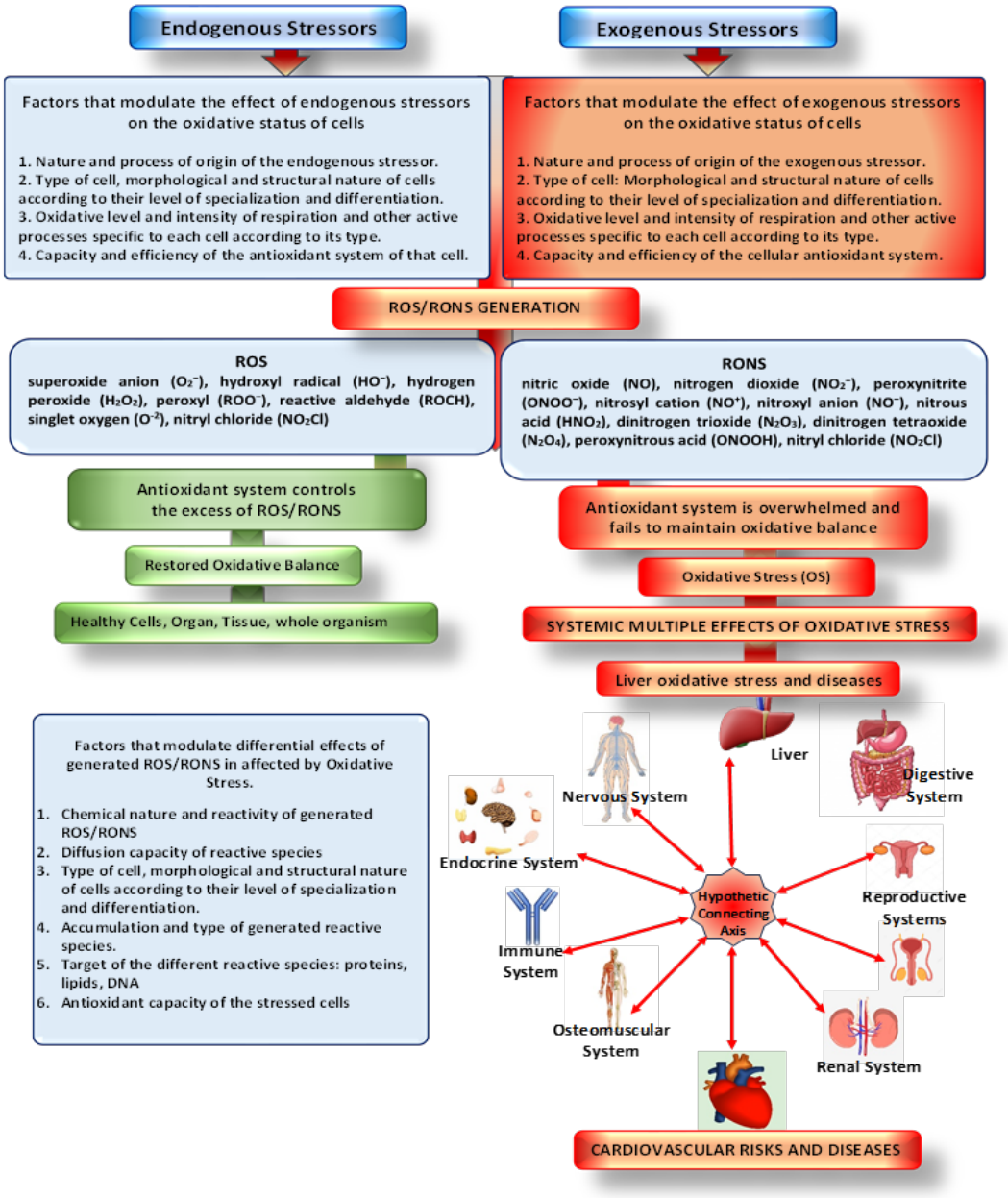

Figure 1: Oxidative stress has systemic effects.

Excess of ROS/RONS produces OS exceeds antioxidant capacity triggers oxidative stress with the affectation of functional integrity of cells, tissues, organs and whole organism. Cellular morphology, physiology, intensity of REDOX (particularly respiratory function) specific cell functions, capacity of the antioxidant system and gene expression pattern constitute important facts determining susceptibility of differentiated and specialized cells to stressors. On the other hand, different ROS/RONS species differ from each other due to their chemical nature, reactivity, diffusion capacity and type of biological compound they attack.

These reactive species are also an important inducer of cellular defense against invading microorganisms, coordination of metabolism, gene expression and the unleashing of growth or death processes [8,9]. When the generation of ROS/RONS exceeds certain limits and the cell antioxidant system in unable to keep REDOX homeostasis, many biological structures and molecules are attacked by ROS/RONS with the affectation of the functional integrity of cells, and causing dysfunction in tissues and organs and compromising the state of health of the entire organism [10,11].

Pluricellular organisms are composed by tissues and organs with highly differentiated and specialized cells as a result of long evolutive process. In animals, particularly vertebrates, there are organs belong to main vital systems: brain (central nervous system), spinal cord (peripheral nervous system), liver with its multifaceted activity (system endocrine), kidneys (excretion system), heart (cardiovascular system), among others. The nature of the targeted by ROS/RONS cells in different tissues and organs can determine their susceptivity to different stressors. The most important that determines mentioned events are cellular specific morphology, physiology, intensity of respiratory function, specific functions, antioxidant capacity and induced specific gene expression patterns. In addition, different ROS/RONS species differ each other due to their chemical nature, reactivity, diffusion capacity and type of biological compound they attack (Figure 1). Liver is particularly sensitive to OS and if the antioxidant defense of the hepatocytes is surpassed the generated ROS/RONS inflammation and poor liver function [12-15]. This dysfunction causes functional changes in important biological processes, including gene transcription and expression, protein modification, lipid peroxidation, cell apoptosis, and hepatic stellate cell activation among others organic alterations [13]. Activation of the apoptotic or necrotic process can even cause the death of hepatocytes. These lethal processes take place through different metabolic pathways, including degradation of mtDNA, strand breaks, mutagenic base lesions in ROS-damaged mtDNA, alteration in enzymatic patterns, lipid peroxidation of cellular membranes and hepatoxicity [14-16]. The role of oxidative stress in liver diseases has been extensively explored and still a matter of permanent research. The interest in this is not limited to hepatic diseases where OS is the etiological origin, but also in cases where liver malfunction impacts on the physiological condition of other organs and systems, exacerbating other diseases. This fact is well understood because of liver multifunctionality and its role in the maintenance of homeostasis and metabolic processes in animal but also in all vertebrates [16,17]. Such pathologies as alcoholic liver disease (ALD), non-alcoholic liver disease (NAFLD), steatosis or fatty liver disease (FLD), hepatic fibrosis (HF), hepatic encephalopathy (HE) and hepatocellular carcinoma (HCC) are crucial to increase cardiovascular risks (CVR) as well as the risks of the emergence and aggravation of other pathological processes and diseases in other vital organs and organic systems [18,19]. Results obtained in animal models and clinical trials demonstrated that prolonged oxidative stress in liver caused inflammation and development of multiple diseases independently of the illness etiology [15,16,20].

Oxidative Stress and Liver Functions

In hepatocytes, OS causes the accumulation of lipids in the liver increasing the probabilities to develop severe liver diseases such as NAFLD and also increasing CVR mainly through the metabolic disorders they cause. (Figure 2).

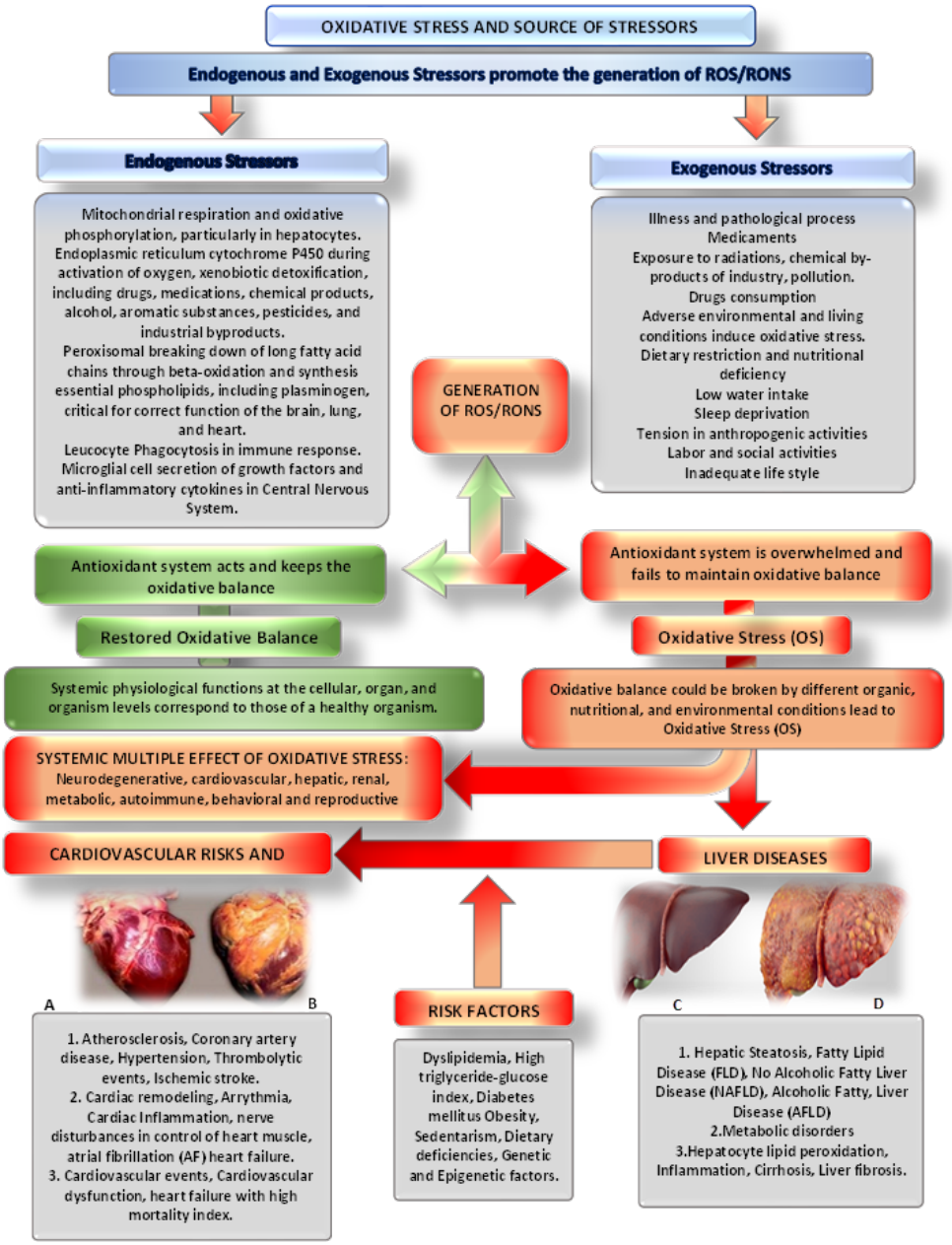

Figure 2: Liver damage by Oxidative Stress increases cardiovascular risk.
Liver diseases and their subsequent impact on metabolic imbalance frequently cause cardiovascular outcomes. Oxidative Stress induces liver dysfunction causing metabolic alterations that in turn create cardiometabolic abnormalities that considerably increase cardiovascular risk. Patients with alcoholic and nonalcoholic hyperlipidemia, are prone to suffer prothrombotic, proinflammatory and procoagulative events closely linked with Cardiovascular Risk.

The relationship between OS and liver inflammation have been studied evidencing that both events are well correlated and damage liver condition leading to hepatic illness [20,21]. Gross antioxidative therapy showed unsatisfied outcomes in many human clinical trials, although the existence of interdependence between oxidative stress, inflammation and liver diseases are firmly established [18]. To abord more specific treatment it's necessary to block only specific pathway that are the cause of detrimental effects in stressed organisms, but this fact forces to establish such specific pathway and implement suitable therapies. A failed therapy can have adverse results than desired, worsening oxidative conditions, intensifying inflammatory processes, and leading to health deterioration [22-24]. ROS/RONS can activate redox-sensitive transcription factors,

inducing changes in the normal gene expression and protein synthesis, altering signaling pathways in important enzymes, such as cellular kinases, phosphatases, and even in the expression of transcription factors, involved in regulation of cellular cycle, proliferation, differentiation and apoptosis [25-27]. In hepatocyte mitochondria, OS produces secondary inhibition of lipids β-oxidation, promoting the accumulation fatty acids in liver, increasing risk to develop Fatty Liver Disease (FLD). Other important process in hepatocyte is the degradation of purine mainly driven by a cytosolic xanthine oxidase (XO) that regulates the conversion of hypoxanthine to uric acid, with generation of large amounts of superoxide anions as a byproduct. The synthesis of XO is induced by 4-HNE (4-Hydroxynonenal) an α, β-unsaturated hydroxyalkenal produced by lipid peroxidation, and trend to cause ROS-mediated hepatic damage [28-29]. Lipid peroxidation of fatty acids, is another process that implies a generation of large amount of ROS/RONS by the way of microsomal cytochromes CYP2E1 and CYP4A [30]. Incidentally CYP2E1 can be upregulated by existence of free fatty acids and also in case of existence of insulin resistance, a fact that indicate the correlation between diabetes and liver OS-mediated damage [31]. Experimental and clinical reports evidence the relationship between the increment of superoxide production with the activity of NADPH oxidase. In terms of inflammation, it was formerly established the infiltration of various types of serum pro-inflammatory cells, such as neutrophils, macrophages, T helper cells, natural killer T (NKT) cells, and natural killer (NK) cells. In steatosis, inflammatory mediators, such as TNF-α, IL-6, and IL-1β are found to be upregulated, contributing to different metabolic alterations including insulin resistance and liver damage. Kupffer cells (KCs), resident liver macrophages critical for liver function that are the first innate immune cells protecting liver from bacterial infections, react to lipid peroxidation inducing the synthesis of TNF-α and Fas- L L. Both molecules are members of the tumor necrosis factor (TNF) family and act, through activation of independent mechanisms, as initiators of apoptosis. In the initial phase of Fatty Live Disease (FLD), the immune resident cells, as KCs and dendritic cells, react to early signs of liver damage by generating proinflammatory cytokines, as IL-1β, and chemokines, as chemokine ligand 2 (CCL2) in a recurrent spiral-type cycle (19). ROS/RONS cause lipid peroxidation, resulting in the generation of 4-HNE and MDA, which can passively diffuse to the outside of cells, increasing the amount of proinflammatory cytokines and activating hepatic stellate cells, causing inflammation [32-33]. It is known that the levels of markers of lipid peroxidation and oxidative DNA damage, like 4-HNE and 8-hydroxydeoxyguanosine, are well correlated with the depth of inflammatory, necrosis and fibrosis processes [34]. Described processes promote also the release of proinflammatory cytokines, leading to neutrophil chemotaxis and lesions of NASH. ROS/RONS activate NF-κB signaling pathway, leading to the synthesis of TNF-α and the upregulated TGF-β, IL-8, IL-6, and Fas-L. TGF-β, IL-8, and 4-HNE promote the neutrophil infiltration during liver inflammation causing more generation of infiltrated neutrophils and other immune cells produce more inflammation and mitochondrial dysfunction increasing OS-mediated liver disease [35,36].

Metabolism is an interconnected maze of multiple pathways of physiological and biochemical processes, assuming that effects on one particular pathway also impact others. Just as an effect on a particular organ or tissue influences others and the entire organism. Following this criterion, liver damage obviously increases cardiovascular risk [15,16,37,38]. Increases in alcohol consumption cause Alcoholic Fatty Liver Disease (AFLD) and together with hepatic cirrhosis and Non-Alcoholic Fatty Liver Disease (NAFLD) become the most common causes of Chronic Liver Disease (CLD). It has been found that cardiovascular disease is the major cause of death in CLD [39,40]. Cardiovascular risk factors such as atherosclerosis, hypertension, coagulation problems, cardiac dysfunction and pulmonary hypertension increase as liver function deteriorates with the exacerbation of liver function problems related especially to metabolic problems [40,41]. As with many diseases, both pathological conditions also share risk factors that impose an adverse condition on liver function, including those of a pharmacochemical nature, due to the toxicity of a specific drug or pharmacological substance; genetic, such as enzyme deficiency and genetic malformations of the organs. Other important factors that precondition hepatic dysfunction may have epigenetic origin, the cause of which is more difficult to detect but is manifested in the clinical history of the individual's

previous generations [43]. All these conditions lead to oxidative stress, systemically altering all the physiological functions of the organism and in liver cause not only hepatic but metabolic disfunction and led to increase CVR, with Fatty Liver Disease (FLD: NAFLD and AFLD) [44]. The relationship between liver disorders and cardiovascular risk is evident not only because both of them share many causes and risk factors, such as obesity, physical sedentary lifestyle, poor quality nutrition, lipidemia alterations, type I and II diabetes, oxidative stress, among others, but also in the fact that the CVR increases to the extent that liver function deteriorates. In general, Fatty Liver Disease (FLD) associated with metabolic dysfunction and oxidative stress (OS) is a matter of growing concern to public health in exacerbating by today's intense life, poor quality nutrition, exposition to different harmful and stressful environmental agents, consumption and abuse of drugs, medications and unhealthy products and sedentary style of life. FLD associated with dysfunctional metabolism has an increasing global prevalence of 25%, and with a high probability of evolving into other more serious diseases such as cirrhosis, metabolic syndrome, hepatocellular carcinoma, which results in a drastic increase in cardiovascular risk (CVR) [45,46].

Oxidative Stress and its Effect on Hepato-Cardiovascular Dysfunction

Liver diseases and their subsequent impact on metabolic imbalance frequently cause metabolic alterations that in turn create cardiometabolic abnormalities and CVR (Figure 2) [47]. Patients with alcoholic and nonalcoholic hyperlipidemia, frequently suffer prothrombotic, proinflammatory and procoagulative events closely linked with CV risks [48-50]. NAFLD increases CVR through complications such as coronary arteries disease (CAD), subclinical atherosclerosis, cardiac arrythmias, heart dysfunction, prothrombotic condition and other functional alteration of cardiovascular system and all these factors can further contribute to an increased CVR and lead to cardiovascular disease [51,52]. Pathogenic liver diseases also can increase CVR in a multifactorial manner, which include inflammation processes, adipokines, intestinal dysbiosis, oxidative stress as well as psychological disorders including anxiety and depression [53,54]. Among liver diseases the main metabolic disorder associated hepatic dysfunctions is the Metabolic-dysfunction Associated Steatotic Liver Disease (MASLD) that includes both Non-Alcoholic Fatty Liver Disease (NAFLD) and Alcoholic Liver Disease (AFLD). NAFL, characterized by three overlapped steps: liver steatosis, excess fat in the liver; non-alcoholic steatohepatitis (NASH), including liver inflammation and hepatocyte degeneration; and the final step where hepatic dysfunction evolutes to fibrosis, cirrhosis and to hepatocarcinoma [55,56]. NAFLD increases CVR through complications such as coronary arteries disease (CAD), calcification of coronary arteries, subclinical atherosclerosis, cardiac arrythmias, heart dysfunction, prothrombotic condition and other cardiovascular risk factors that induce cardiovascular events and mortality [51,52]. Pathogenic liver diseases also can increase CVR in a multifactorial manner, which include inflammation, adipokines, intestinal dysbiosis, and psychological disorders including anxiety and depression (Figure 3) [53,54].

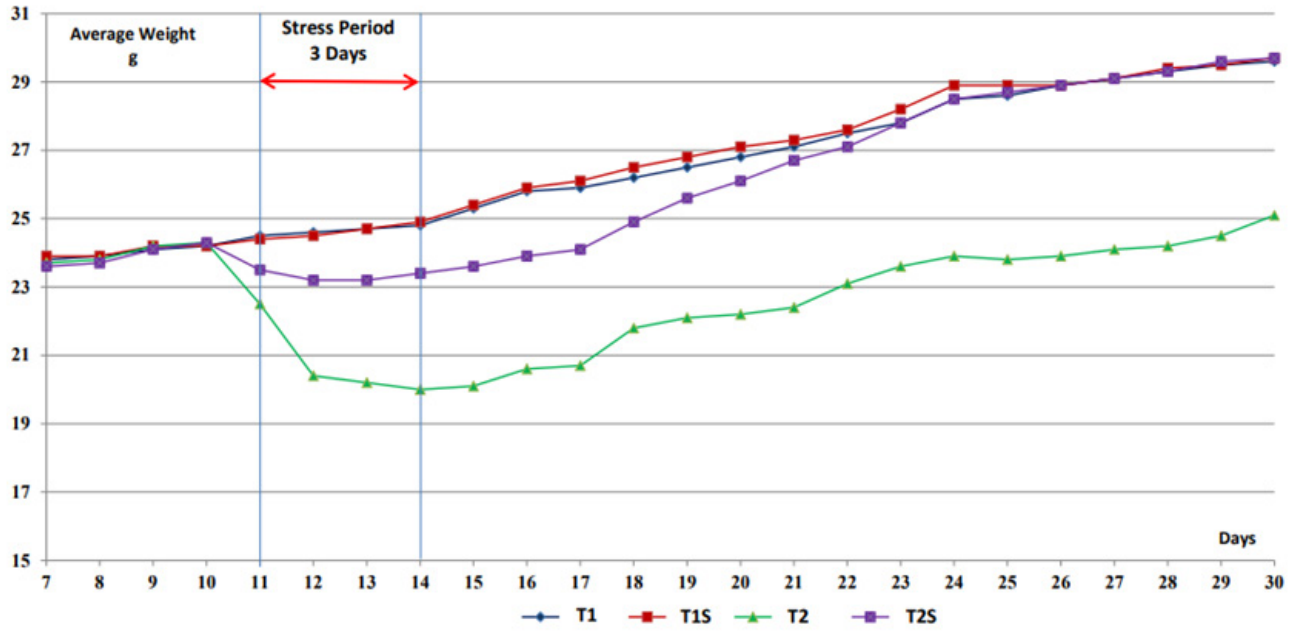

Figure 3: Effect of antioxidant diet in stressed mice.
Dynamic of Weight gain week old C57BalC mice subjected to induced environmental stress and treated with different antioxidant diets. The treatments correspond to: T1-normal diet not stressed, T1S-noemal diet and stressed, T2-aupplemented diet not stressed, T2S supplemented diet stressed. The best response to induced stress and the best weight recovery was observed in the group that received supplemented diet previously and after stressed period.

Specific and General Biomarkers Linking Hepato-Cardiovascular Dysfunction

Liver and heart health problems can be asymptomatic, and without any visible manifestation of disease is only possible to detect by clinical studies. Otherwise, the symptoms will be visible only when the pathology scaled to a serious health problem. Regular tests and checkups are crucial to observe the health condition and to prevent cardiovascular dysfunction [57]. Due to the complexity of liver functions, efforts have been made to develop noninvasive a series of test and to establish biological markers to detect liver dysfunctions. These biological markers help predict whether liver disease is developing and whether its repercussions pose risks to other vital organs and systems, including CVR [58]. The best way to study the liver function and its implications is to combine the blood chemistry tests, measurement of liver stiffness and the use of either imagen tests like ultrasound- or magnetic resonance–based elastography analysis. The traditional blood markers measuring the levels of certain enzymes and proteins in serum is a suitable method that can indicate how it influences in the whole organism. The main blood biomarkers are: Alanine aminotransferase (ALT), aspartate aminotransferase (AST) Alkaline phosphatase (ALP) and y-Glutamyl transferase (GGT), serum bilirubin, prothrombin time (PT), the international normalized ratio (INR), serum total protein and serum albumin. These parameters can help to established an area of the liver where damage would be located and if something outside the normal ranges is observed, more specific tests can be used. Combined independent data in form of indexes allow to standardize criteria about the nature of liver dysfunction and damage. For example, the International Normalized Ratio (INR) is useful to follow the evolution of patients under anticoagulant treatment through prothrombin time. and it is particularly useful to follow. The elevations in ALT and AST disproportion to elevations in alkaline phosphatase and bilirubin denote hepatocellular disease, elevation in alkaline phosphatase and bilirubin in disproportion to ALT and AST would characterize a cholestatic pattern [59]. In addition to these blood tests, it is advisable

to complement them with imaging studies for a comprehensive and accurate diagnosis. The most recurrent imagen methods are ultrasound, nuclear magnetic resonance (NMR) or nuclear magnetic imaging (NMI).

Fatty Liver Disease (FLD: AFLD and NAFLD) can be present in three progressive overlapping stages: liver steatosis (excessive fat accumulation), liver steatohepatitis (liver inflammation and hepatocyte degeneration) and liver fibrosis (fibrosis, cirrhosis and to hepatocarcinoma) [55,56]. Each of these stages has characteristic values for both serum biomarkers and typical images. A comprehensive analysis of the results allows to stablish a more accurate diagnosis of hepato-cardiovascular axis. Parameters as Fatty Liver Index, Hepatic Steatosis Index and Triglyceride Glucose Index help to determine Liver Steatosis and to follow the effect of medical treatments to avoid the progression of liver disease to the steatohepatitis stage. In this second stage where dysfunction and impairment of liver function become more evident, there are other biomarkers and parameters that reflect not only the accumulation of fat but also the degeneration of hepatocytes and the evolution to more chronic and irreversible damage to the liver, such as apoptosis, changes in the metabolism of fats and carbohydrates, liver inflammation and consequent alteration of inflammatory mediators. At this stage, alterations in common blood hepatic parameters become evident and levels of Alanine aminotransferase (ALT) and aspartate aminotransferase (AST), Alkaline phosphatase (ALP) and y-Glutamyl transferase (GGT), serum bilirubin, albumin and total serum protein become out of normal ranges. At this stage inflammation causes pain in the patient [60]. The third stage is a NASH-related fibrosis and the main biomarkers used are: Fribrosis-4-index, fibrosis score, BARD score, Hepamet fibrosis score, AST/platelet ratio index and FORNS index some newest test can be added (Fibrometer, Fibrotest, Enhanced liver Fibrosismainly due to the metabolic. The risk factors for fatty liver disease and cardiovascular disease are common: shared: obesity, elevated blood glucose levels until de veloping prediabetes and diabetes mellitus, hypertension, dyslipidemia. In addition, oxidative stress risk actors for liver and cardiovascular disease are caused for enhanced by unhealthy lifestyles where inadequate nutrition, consumption and ingestion of harmful substances, sleep deprivation, sedentary lifestyle and little physical activity are predominant habits [61,62]. All this leads to the deterioration of cardiovascular activity causing arteriosclerosis, directly linked to coronary artery diseases, ischemic stroke; cardiac arrhythmia, MF heart failure and serious life-threatening cardiovascular clinical events due to poor condition of the cardiovascular system [63,64].

Fatty Liver Index (FLI) is an index that is evaluated in ranges from 0 to 100 using Body Mass Index (BMI) and waist circumferences, Triglyceride level in serum (TG) and glutamyl transferase (GGT) concentration. BMI is measured in kg/sqm, surrogating measure for body adiposity and considering weight and height of studied person. Waist circumference reflex a higher risk of FLD, particularly NAFLD when persons is not obese but have large waist circumference. A waist circumference larger than 40 inches for men or 35 inches for women could mean that you have an increased risk of heart disease, stroke, or type 2 diabetes. High levels of serum triglycerides are associated with FLD, pancreatic and liver disfunctions whereas high levels in Serum GGT (gamma-glutamyl transpeptidase) is usual marker for liver function. All primary data from serum analysis and measurements are used to calculate FLI according to standard formula. If the obtained result score 30 or less mean hepatic steatosis and FLD is not present whereas scores 60 or more indicate that FLD is present in the studied person [56,65]. FLI is a suitable FLD biomarker, particularly to detect NAFLD. Different studies controlled for age, sex, obesity, consume of alcohol and tobacco products, presence of dyslipidemia and hypertension, in adult populations this index show well correlation of Hepatic steatosis Index (HIS) and Fatty Liver Indexes (FLI) with Cardiovascular Risk (CVR) [66]. The NAFLD Liver Fat Score (NAFLD-LFS) is other important biomarker to determine if the patient carries metabolic syndrome, DM2, fasting serum insulin, AST and ALT. Al these measure parameters are integrated in formula to solved to obtain the value of NAFLD-LFS index [67]. Another steatosis marker is the Hepatic Steatosis Index (HIS). This index takes in to consideration both liver transaminases (AST and ALT), Body Mass Index (BMI), female sex and existence of Diabetes mellitus type 2 (DM2). The re sult of calculation is a curve graphic and the derivate from this curve represent the

(AUC) and when NAFLD-LFS is higher than 1.257 is a clear indication of Cardiovascular Risks (CVR). If NAFLD-LFS is higher than -0.640 the correlation to cardiovascular disease (CVD) is unadjusted [68]. This correlation seems to be more evident in lean patients than in obese ones [68]. Insulin resistance, a health condition that does not trigger any symptomatology, and for this reason Triglyceride-Glucose Index (TGI) was created. A patient with insulin resistance has a necessity to secrete more insulin than usual to keep adequate blood sugar levels. This condition increases the risk of developing prediabetes and diabetes DM2 if it isn't discovered and reversed [68]. If TG index 4.49 the patient is diagnosed with insulin resistance. Triglyceride and glucose level in blood are usually taken to test metabolic and liver functions but also is recognized as cardiovascular risk biomarker [69,70]. This biomarker is associated with cardiovascular risk and death, coronary artery disease, coronary angiography in patients with FLD (NAFLD) independent from sex, age, and other cardiovascular risk factors such as hypertension and diabetes mellitus [70]. For diagnosis of steatohepatitis (NASH) stage other biomarkers has been developed to complement imaging test [53]. This stage is characterized by hepatocyte apoptosis, live inflammation, dysfunctional carbohydrate and lipid metabolism and deterioration of liver oxidative balance.

Hepatocyte apoptosis is related with the presence of Cytokeratin 18 (CK-18) that is released to blood during hepatic injury and hepatocyte apoptosis. CK-18 can be detected using specific monoclonal antibodies able to detect two antigens, M30 and M65. Patatin-like phospholipase (PNPLA3) and glucokinase regulatory protein (GCKR) can be used as a biomarker to NASH too [71]. The three parameters previously described found to be well correlated with cardiometabolic disorders and cardiovascular risk factor and disease [72]. To follow the chronic inflammatory process at this stage is usually to test high sensitivity C-reactive protein level (hs-CRP) allowing to distinguish NASH from NAFLD [73]. High level of hs-CRP protein is consisted with the calcification of coronary arteries andis also well correlated with metabolic-associated atty liver disease, other cardiovascular risk factors, cardi ovascular events and mortality [74]. Another group of proteins related to the inflammatory process characteristic of NASH and that can be used as markers of this stage are: tumor necrosis factor (TNF-alpha), released by hepatocytes in response to lipid deposition; interleukins (IL-1-beta, IL-6), related to inflammation, severe atherosclerosis, NAFLD and NASH. In animal models was observed that a reduction of these cytokines also reduces cardiovascular events [75,76].

In NAFLD patients that evolute to NASH score of ≥ 4 a significantly higher levels of lipid peroxidation (LPO) were observed. ROS/RONS-mediated attack provokes lipid peroxidation, resulted not only in fat accumulation, but also in structural affectation of cell membranes and their physiological functions. Lipid peroxidation is caused when hydrogen atom is abstracted from an unsaturated fatty acid by the reactive ROS/RONS, and this is just the initial step of a chain reaction that results in the disruption of cellular membrane and release high reactive metabolites are generated more reactive species, maximizing this destructive process that may cause apoptosis or other lethal mechanisms resulted in cellular death [77]. Followed this process the stellar cells are activated promoting the overexpression of proinflammatory cytokines, cell necrosis, apoptosis and the development of fibrosis [78]. It is important to elucidate the molecular mechanisms underlying the basal inflammation present in liver in individuals affected by NASH.

Liver produces hormone named Fibroblast Growth Factor 21, (FGF-21) that is linked with NAFLD and cardiovascular diseases, the highest level of such hormone was found when patients suffer both diseases, NAFLD and CVD (77). In the last stage of NAFLD some NASH-linked fibrosis markers have been established, including Fibrosis-4 Index (FIB-4), NAFLD Fibrosis Score (NFS), BAED Score, AST to Platelet Ratio Index (APRI), Forns Index and Hepamet Fibrosis Score [79]. The index for Liver Fibrosis FIB-4 is calculated with a formula used to determine NAFLD/NASH status and the level of fibrosis in NAFLD. The FIB4 index was developed in 2006 as a noninvasive method to diagnose liver fibrosis and combines aspartate aminotransferase (AST-GOT) levels, alanine aminotransferase (ALT-GPT) levels, platelet count, and age (79, 80). A higher FIB-4 score indicates more advanced liver fibrosis and its scores are well correlated with cardiovascular risk even in patients without NAFLD or AFLD (80). The FIB-4 index is well

correlated with cardiovascu lar risk even in patients without NAFLD or AFLD. Higher FIB-4 conduce to higher CVR and this direct correlation is true regardless of the existence of other risk factors [80]. Other important biomarker is the NAFLD Fibrosis Score (NFS), an index used to predict the advanced fibrosis in patients affected by NAFLD or AFLD and higher values of NFS have been related to Coronary Artery Calcium (CAC) progression; CAC is a marker of atherosclerosis, an important condition of cardiovascular risk and this reason. Higher CAC determination is directly correlated with higher cardiovascular risk, whereas lower CAC is associated with a low risk of cardiovascular events [81]. The BARD score is also a result of an algorithm combining three basic sources: Body Mass Index (BMI) and relation AST/ALT values. This biomarker used to predict advanced fibrosis in patients with NAFLD [12,82]. Platelet Ratio Index (APRI), one of the non-invasive liver fibrosis serum markers, can predict cardiovascular risk (CVR). This fibrotic test uses the data of AST values and platelets as a tool originally to predict liver fibrosis and cirrhosis in viral hepatitis patients but has been validated to evaluate metabolic syndrome and to predict cardiovascular risk (CVR) [83]. APRI significantly is well correlated with CVR and its elevated values imply a significant increase in CVR for both genders but specially in females. The Framingham Risk Score (FRS) is a sex-specific algorithm used to estimate the 10-year cardiovascular risk of an individual and is well correlated with APRI. This correlation also exhibits the axis between liver disease and cardiovascularrisks [83]. FORS Index and Hepamet Fibrosis Score (HFS) are also non-invasive tests used to test fibrosis severity and also to explore the cardiovascular risks [84]. The FORNS Index is a biomarker used to predict liver fibrosis in patients with chronic hepatitis C, taking into account the platelet count, gamma-glutamyl transferase (GGT), cholesterol levels and age [84]. This index is well correlated with many cardiovascular risks (CVR) and it is a predictor of cardiopulmonary diseases [85]. Hepamet Fibrosis Score (HS) isthe same type algorithmic index, a product of applied formula incorporating several parameters: gender, age, BSA, platelets, insulin levels and diabetes status, and it was established for diagnosis in advanced liv er fibrosis [86,87]. The use of algorithmically complex indexes as biomarkers help to accurately analyze primary data obtained by blood test and biometrical noninvasive diagnostic methods. At the same time, it demonstrates the close relationship between liver diseases, where oxidative stress is the main pathological process that causes it, and cardiovascular risk. The selection of which biomarkers are most suitable for assessing the state of health of the liver and the level of cardiovascular risk that this implies will depend on the direction in which it is considered necessary to direct the exploration of a specific clinical pathology.

Study of Antioxidant Nutritional Assessment to Prevent Liver Damage and Cardiovascular Risks Preparation of a Probiotic Supplements

It is known that ingestion of probiotic supplements brings beneficial effects to all organisms, including vertebrate, but synergic combinational effect has not been commonly evaluated [6]. Our main focus is to study these combinational effects using the results obtained in a series of preliminary studies (Data not shown). We conformed two probiotic supplements, basically composed by yeast extract, or by its product, and raw dehydrated extract of blueberry (Vaccinium corymbosum L.). We designed probiotic supplement for the first experiment with stressed C57/BalC and other with human individuals with antecedents of NAFLD, blood hypertension, and other factors promoting NAFLD and increasing NAFLD. We also used a selenized yeast extract in the experiment with mice, but it was not taken in the composition of probiotic supplement using in human individuals for safety considerations (88). The probiotic antioxidant diet using in mice was prepared as follows. The standardNUTRICUBOS-LabChows (Agribrands Purina, Mexico) was used as a basic food (Normal Diet) for mice (T1). The probiotic-enriched (T2) was composed by basic food (Normal Diet) supplemented by probiotic mixture containing a combination of yeast (final concentration 30%: weight), selenized yeast extract (bioselenium, 0.015 % Se: weight) , powder of dehydrated raw blueberry extract (5% AP: eight) and Vitamin B12 (0.01 % VB12: weight) the rest of the was composed by the normal diet reported by NUTRICUBOS (64.9 % Normal Diet: weight), but in higher concentrations to compensate the volume corresponding to added complements. Both diets meet the established standards recommended for Mice US National Research Council (1995).The study in human individuals originally was a part of educative project in engineering education,

focused in the relationship between sustainability and character education. In part, the project focuses on health problems affecting the population, so we tried to explore whether by using a more active and healthy diet and lifestyle we could improve health in individuals. The students selected for this study were young students who had a family history of overweight, type II diabetes, NAFLD and cardiovascular disease. We tried to improve liver function and reduce cardiovascular risk. The volunteers were male students at the Higher Technological Institute of Irapuato (ITESI-TecNM, Guanajuato, Mexico) during the years 2012-2014, corresponding to studying years 2 to 5 semesters [89]. The probiotic was administered in the form of a dehydrated powder to be added to different types of previously prepared foods: smoothies, rice, and different meal. The dose of 5 gr consists in: 4.5 grams of yeast extract (90%: weight), 0.5 grams of dehydrated blueberry powder (5%: weight) and 0.8 micrograms of vitamin B12 are added, representing diary intake's dose. To facilize the intake of supplement a dosage spoon corresponding to daily intake. The chemical nature of the ingredients that make up probiotic supplements used for vertebrates, including humans, indicate that they are safe and harmless and do not represent any danger to animal and human health in any quantity and proportion [90-92].

Results

Example 1: Effects on different organic functions and beneficial effect of a probiotic-supplemented diet on the confrontation and recovery to OS in stress-induced C57/BalC mice

Objective

To observe the effect of OS in different physiological functions in stressed mice in evaluation of probiotic supplemented diet in the recovery period.

Material and Method

This study was performed in accordance with the Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research and the SynergiaBio Institutional Committee of Ethics, Animal Care, Ecology and Welfare [15, 16, 92, 93]. The diets and probiotic dietary supplement were designed and prepared according the established standards recommended for Mice US National Research Council [94].

Animal Care, Husbandry and Probiotic Testing

Six-week-old female C57/BalC mice were housed in temperature controlled (22-25ºC) on a 12h light/dark cycle (12h/12h) with access to water and food "ad libitium". A total of 60 animals were divided into 4 groups of 15 individuals each. First two groups were fed with a normal control diet without probiotic supplements (T1) and other two groups were fed with a diet enriched with experimental probiotic supplement (T2). The induction of oxidative stress in mice fed by environmental factors was carried out in 15 of mice fed with the normal diet (T1S) and 15 fed with the supplemented diet (T2S) for 72 hours, while the other two groups (T1, T2) remained un stressed. The induction of OS was performed submitting test groups (T1S, T2S) to environmental stress conditions during 72 hours. The OS induction was performed altering the periods of light/dark, deprivation of food, water, sleep disturbance, movement and change of place. The effect of OS was monitored using blood test to determinate the evolution of blood biochemical parameters markers related with specific liver, renal, cardiovascular, blood cell components, oxidative status and immune system during 30 days. The animals were weighed daily until day 30th. Blood tests were carried at the day 1 (prior to day exposure) and at the day 15th, 30th according with standard procedures [93]. Liver and renal functions were evaluated by measuring, serum albumin, urea, uric acid, creatinine, alanine transaminase, aspartate transaminase, alkaline phosphatase, serum glutathione peroxidase and serum total antioxidant capacity. Measurements were done using the following commercial kits: a) Serum Albumin (QuantiChrom™ BCG Albumin Assay Kit, BioAssay Systems, Hayward, CA, USA); b) Urea BUN (Mouse Blood Urea Nitrogen ELISA Kit, Creative Diagnostics, Shirley, NY, USA); c) Uric Acid (QuantiChrom™ Uric Acid Assay Kit, BioAssay Systems, Hayward, CA, USA); d) Creatinine (Mouse Creatinine CREA ELISA, Kamiya Biomedical Co., Seattle, WA, USA); e) Alanine Transaminase (EnzyChrom™ Alanine Transaminase Assay Kit, BioAssay Systems, Hayward, CA, USA); f) Aspartate Transaminase (EnzyChrom™ Aspartate Transaminase Assay Kit, BioAssay Systems, Hayward, CA, USA); g) Alkaline phosphatase (Mouse Alkaline Phosphatase (ALP) ELISA, Kamiya Biomedical Co., Seattle, WA, US); h) Serum GSH-Activity (Mouse Glutathione (GSH) Colorimetric Cuvette Detection Kit

(Innovative Research, MI, USA) and i) Plasma Total Antioxidant Capacity (Total Antioxidant Capacity Assay Kit, ABCAM, Cambridge, MA, USA). The immunological status of animals was followed up by using counting lymphocytes, neutrophils and monocytes. The production of some proinflammatory cytokines (Interleukin-2(IL-2), Interleukin 12 (IL-12) and gamma interferon (IFN-γ)) and anti-inflammatory (Interleukin 4 (IL-4), Interleukin 10 (IL-10)) cytokines by using commercial kits (AbCam, Cambridge, UK). Data analysis was performed as follows. One-way ANOVA followed by Dunnett's or Fisher's protected least significant difference multiple comparison testing in SPSS13.0 (SPSS, Chicago, IL, USA). When necessary, data were transformed for normalization and to reduce heterogeneity of variance p-values 0.05 or more were considered statistically significant [93].

Results

We evaluated antioxidant probiotic supplement composed by yeast extract, blueberry extract, vitamin B12 and selenized yeast extract in OS stressed mice [95,96]. The obtained results suggest that used probiotic-supplemented diet had a positive effect on antioxidant capacity, immunological, renal, hepatic, metabolic functions contributing to improve health and reduce liver and cardiovascular risk [97]. The evolution of hepatic (Alanine Transaminase, Aspartate Transaminase, Alkaline Phosphatase), renal (Urea BUN, Uric Acid, Creatinine) and Serum Oxidative Status (Serum Albumin, Serum Glutathione Peroxidase, Serum Total Antioxidant Capacity) functions were observed by blood tests and weighing during 30 days. The animals fed with diet supplemented with diet reached the parameters of control group and the end of experiment, showing a complete recovering (98). The blood parameters measured are altered in all stressed mice but the animals fed with supplemented diet (T2S) tend to normalize these values quickly compared to those that received the normal diet (T1S). control groups. Increases in proinflammatory interferon IFN-β were observed too. This type of interferon plays a key role in the innate immune response to infection, developing tumors and other inflammatory processes [99, 100, 101]. The evolution of weight gain also evidences the systemic positive effect of supplemented diet (T2S) (Tables 1, 2)) [102]. Cytokines are important players in regulating immune homeostasis thanks to the pleiotropic characteristics present in the majority of cytokines. Pleiotropy is defined as the capacity of one cytokine to exhibit diverse functionalities, in redundant overlapping manner with differentially activities. Cytokines are important for regulating immune homeostasis and have a variety of effects in different cells. Cytokine pleiotropy provoke induction of a wide range of functional activities in different cell subsets, including immunologic, hematopoietic, and pro-inflammatory processes. Interleukin 6 (IL-6) has a broad range of effects, stimulating antibody production, induces differentiation of naïve CD4 T cells into effector T cells, and activates vascular endothelial cells to produce IL-6, IL-8, and other immune related proteins (103). Another example is interleukin 10 (IL-10), that is the only cytokine that can both promote and downregulate Th2-dependent allergic responses. the type of cytokine that is produced but also but also in the entire pattern of cytokine expression, the proportion between its different types in the effect at the level of organs, system or physiological process in particular and type of cell in which the summative effect of the cytokines produced is evident. In addition, it's necessary to understand the protein determinants in the different cytokines that promotes specific processes in specific cells to elucidate aspects that are important to harness its potential as a therapeutic [104, 105].

Table 1: Dynamics of some Hepatic, Renal, and Oxidative Status in Stressed Mice.

Organic Function	Test	Diet	Day 1	s	Day 15	s	Day 30	s	Observed Tendency
Hepatic		T1	28.41	1.43	27.95	2.42	28.82	2.35	Stable normal ALT values
		T1S	28.18	2.35	69.32	2.92	38.44	2.37	Increase at day 15, incomplete recovery at day 30
		T2	27.63	2.37	27.64	2.38	28.09	2.52	Stable normal ALT values
		T2S	27.34	1.99	39.96	2.08	27.32	1.95	Increases at day 15, complete recovery at day 30
	Aspartate Transaminase (ASP U/L)	T1	68.34	1.52	67.41	2.15	68.42	2.54	Stable normal ASP values
		T1S	68.32	2.26	138.54	1.71	95.25	1.68	Increase at day 15, incomplete recovery at day 30
		T2	64.42	2.11	68..26	1.75	67.71	2.54	Stable normal ASP value
		T2S	65.73	2.82	82.42	2.21	66.69	2.41	Increases at day 15, complete recovery at day 30
	Alkaline Phosphatase (ALP U/L)	T1	41.62	2.28	41.15	2.05	38.57	2.78	Stable normal ALP values
		T1S	42.37	2.38	149.86	2.15	86.56	1.49	Increase at day 15, incomplete recovery at day 30.
		T2	43.88	2.03	41.67	2.35	40.43	2.83	Stable normal ALP values
		T2S	40.77	2.29	69.62	2.43	39.45	2.39	Increases at day 15, complete recovery at day 30.
	Urea (BUN mg/dL)	T1	21.41	1.51	21.86	1.74	21.92	1.51	Stable normal Urea values
		T1S	21.58	1.43	59.67	1.39	34.35	1.37	Increase at day 15, incomplete recovery at day 30.
		T2	21.25	1.74	20.31	1.68	21.87	1.41	Stable normal Urea values
		T2S	21.64	1.52	32.97	1.86	21.23	1.44	Increase at day 15, complete recovery at day 30
Renal	Uric Acid (UA mg/dL)	T1	0.16	0.02	0.17	0.04	0.17	0.03	Stable normal Uric Acid values
		T1S	0.15	0.03	0.38	0.03	0.32	0.03	Increase at day 15, incomplete recovery at day 30
		T2	0.16	0.03	0.17	0.02	0.16	0.05	Stable normal Uric values
		T2S	0.17	0.06	0.41	0.04	0.16	0.02	Increase at day 15, complete recovery at day 30
	Uric Acid (UA mg/dL)	T1	0.16	0.02	0.17	0.04	0.17	0.03	Stable normal Uric Acid values
		T1S	0.15	0.03	0.38	0.03	0.32	0.03	Increase at day 15, incomplete recovery at day 30
		T2	0.74	0.04	0.73	0.03	0.74	0.02	Stable normal Creatine values
		T2S	0.74	0.02	0.89	0.04	0.71	0.04	Increases at day 15, complete recovery at day 30

Serum Oxidative Status	Serum Albumin (BSA mg/dl)	T1	2.87	0.15	2,76	0.18	2.75	0.14	Stable normal BSA values
		T1S	2.71	0.14	3,88	0.12	3.32	0.15	Increase at day 15, incomplete recovery at day 30
		T	2.83	0.11	2,72	0.15	2.69	0.24	Stable normal BSA values
		T2S	2.88	0.13	3,99	0.11	2.58	0.11	Increases at day 13, complete recovery at day 30
	Serum Glutathione Peroxidase (GSH-Px nmol/ml)	T1	28.55	1.09	28.18	1.34	29.31	1.81	Stable normal GSH-Px values
		T1S	28.82	1.45	18.14	1.35	19.21	1.38	Decrease at day 15, incomplete recovery at day 30
		T2	27.73	1.04	28.73	1.68	27.89	1.21	Stable normal GSH-Px values
		T2S	27.91	1.22	17.82	1.62	27.67	1.43	Activity decrease at day 15, complete recovery at day 30
	Total Antioxidant Capacity (TAC nmol/L)	T1	0.99	0.02	0.95	0.03	0.96	0.04	Stable normal TAC values
		T1S	0.98	0.04	0.65	0.04	0.79	0.03	Decrease at day 15, incomplete recovery at day 30
		T2	0.97	0.03	0.78	0.05	0.98	0.04	Stable normal TAC values
		T2S	0.96	0.02	0.95	0.03	0.97	0.03	Decrease at day 15, complete recovery at day 30

Dynamic of liver, mice subjected to oxidative stress conditions were monitored by blood biochemical tests from peripheral blood of mice C57/BalC subjected to stress. T1: normal diet not stressed; T1S: normal diet stressed; T2: supplemented diet not stressed; T2S: Supplemented diet stressed. Animals fed with supplemented shown a complete recovery diet from induced oxidative stress at the end of experiment.

Table 2. Dynamics of Immunological Function in Stressed Mice.

Function	Test	T	Day 1	s	Day 15	s	Day 30	s	Observed Tendency
Leucocytes Cells x 103/µL	Lymphocytes Cells x 103/µL	T1	10.31	0.73	10.68	0.62	10.65	0.43	Without significant changes
		T1S	10.87	0.47	6.41	0.63	8.19	0.45	Decrease at day 15, incomplete recovery at day 30
		T2	11.42	0.68	13.34	0.68	13.25	0.55	Without significant changes
		T2S	11.84	0.62	14.55	0.72	12.08	0.53	Increment at day 15, complete recovery at day 30
	Neutrophils Cells x 103/µL	T1	5.85	0.31	5.77	0.29	5.86	0.39	Without significant changes
		T1S	5.72	0.39	5.12	0.36	5.29	0-35	Decrease at day 15, incomplete recovery at day 30
		T2	5.76	0.34	5.99	0.42	5.86	0.47	Without significant changes
		T2S	5.84	0.41	6.77	0.43	5.88	0.47	Increment at day 15, complete recovery at day 30

	Monocytes Cells x 103/µL	T1	0.75	0.17	0.74	0.17	0.77	0.15	Without significant changes
		T1S	0.72	0.13	0.56	0.15	0.61	0.16	Decrease at day 15, incomplete recovery at day 30
		T2	0.73	0.16	0.70	0.15	0.72	0.16	Without significant changes
		T2S	0.72	0.12	0.51	0.12	0.75	0.14	Increment at day 15, complete recovery at day 30
Phagocytosis assay (%)	Phagocytosis in Monocytes (%)	T1	36.09	1.52	38.55	1.29	37.49	1.32	Without significant changes
		T1S	35.68	1.71	24.21	1.53	30.63	1.51	Increment
		T2	35.92	2.12	37,95	1.21	39.93	1.46	No Increment
		T2S	35.15	1.66	59.04	1.45	72.05	1.41	Increment
	Phagocytosis in Macrophages (%)	T1	33.21	1.31	35.57	1.45	35.23	1.51	Without significant changes
		T1S	35.88	1.22	67.34	1.05	78.16	1.42	Increment
		T2	36.34	2.13	65.32	1.42	77.16	1.24	Increment
		T2S	36.02	1.12	75.72	1.14	95.86	1.33	Highest Increment
Cytokines pg/mL	Interferon α (IFN-α). pg/mL	T1	360.65	2.32	355.39	2.09	369.69	1.23	Without significant changes
		T1S	349.43	1.87	372.46	1.79	420.37	1.45	Increment
		T2	351.26	1.92	389.23	1.48	451.34	1.32	Increment
		T2S	248.38	1.37	402.21	1.65	480.34	1.42	Highest Increment
	Interferon β (IFN-β). pg/mL	T1	201.32	1.76	208.23	1.89	210.45	1.43	Without significant changes
		T1S	202.15	2.01	243.23	1.76	259.15	1.49	Increment
		T2	201.97	1.54	229.32	1.95	240.27	1.28	Increment
		T2S	199.45	1.59	261.32	1.67	299.49	1.36	Highest Increment
	Interferon gamma (IFN-γ). pg/mL	T1	130.19	1.27	141.21	1.15	149.25	1.54	Without significant changes
		T1S	141.31	1.79	187.95	1.38	312.55	1.67	Increment
		T2	132.76	1.32	157.87	1.72	258.59	1.94	Increment
		T2S	139.12	1.48	189.21	1.47	320.02	1.59	Highest Increment
	Interlukin 2 (IL2) pg/mL	T1	210.19	1.32	200.52	0.13	231.49	0.59	Without significant changes
		T1S	221.31	0.79	289.92	1.62	312.55	0.63	Increment
		T2	213.54	1.48	238.65	1.52	289.27	0.86	Increment
		T2S	211.26	0.64	291.15	1.66	350.09	0.77	Highest Increment
	Interlukin 4 (IL4) pg/mL	T1	283.81	1.12	296.45	1.31	306.68	1.09	Without significant changes
		T1S	281.23	1.28	351.2	1.39	425.82	1.46	Increment
		T2	285.37	1.76	380.02	1.96	530.95	1.31	Highest Increment
		T2S	279,97	1.43	360.02	1.52	430.95	1.31	Increment
	Interlukin 10 (IL10) pg/mL	T1	359.51	1.41	373.65	1.46	361.43	0.75	Without significant changes
		T1S	348.23	.1.49	537.69	1.51	654.31	0.65	Increment
		T2	339.45	1.29	450.45	1.48	510.32	1.25	Increment
		T2S	360.23	1.55	550.05	1.39	760.24	0.59	Highest Increment

	Interlukin 12 (IL12) pg/mL	T1	322.45	1.05	327.31	1.61	339.67	1.25	Without significant changes
		T1S	315.86	1.32	436.57	1.47	532.48	1.37	Increment
		T2	331.22	1.59	441.38	1.18	468.31	1.42	Increment
		T2S	310.98	1.21	450.26	1.61	640.23	1.29	Highest Increment

Immunological function was followed using blood biochemical tests from peripheral blood of mice C57/BalC subjected to stress in stressed mice after environmental stress induction and during period of recovery. T1: normal diet not stressed; T1S: normal diet stressed; T2: supplemented diet not stressed; T2S: Supplemented diet stressed. Animals fed with supplemented shown a complete recovery diet from induced oxidative stress at the end of the experiment.

Example II. Effect of combinational therapy and lifestyle intervention in young persons predisposed to hepatic and cardiovascular risk

Objective

Evaluation of a diet enriched with probiotics combined with physical exercises in patients with a history and predisposition to fatty liver disease and cardiovascular risk through monitoring of organic functions through blood chemistry analysis.

Material and Method

A group of volunteers aged between 17 to 19 years old were selected to test probiotic and antioxidant dietary complements generated according to previous studies [15, 92, 93]. The selected individuals were students of Irapuato Institute of Technology (ITESI). We selected those who presented some family antecedents of clinical history and preconditions for probable development of Fatty Liver Disease (FLD) and cardiovascular risk (CR). Two test groups were conformed: those who had some excess body weight (OWO) and those who were skinny but with abdominal fat accumulation (SAF). The groups were made up of 34 volunteers in the overweight group and 35 in the second for, a total of 69 volunteers who reached the end of the study. Each group followed a flexible diet limited the intakes of carbohydrate and fat consumption according to characteristics of each individual. Physical conditioning program, was designed for both groups (OWP and SAF) complementing the effect of probiotic supplemented diets. After first year 14 individuals in OWO and 20 ones in SAF, some participants decided to complement conditioning training by other sports discipline by themselves. The overweight group (OWO) underwent a low carbohydrate but flexible diet, low fat and higher in proteins. For skinny individuals (SAF) the diet focused primarily on lowering these abdominal lipid reserves. Diet was moderately low in carbohydrates and lipids, but higher than those diet for individuals in OWO group, and high proteins intakes. In this group the exercises were aimed to eliminate abdominal fat during the first 6 months, and then the calisthenic training was similar to those established for the group of overweight individuals. Due to the heterogeneity among the participants, and the small number we considered not working the data statistically. Blood chemistry tests and BMI were used to follow the initial health status and different organic functions, were followed using clinical laboratories test every year.

Results

Blood chemistry tests show positive evolution of evaluated parameters in the majority of case the levels were for renal, hepatic, immunological, metabolic function as well as for the status of cardiovascular risk (CR) when compared with the accepted clinical standard values. (Table 3, 4). In general, the evaluated parameters are within normality, although in some cases they are already slightly outside the reference standards. In the case of the WOW group, the average levels of glucose, LDH, gamma-GGT, ST and ALT as well as the AST/ALT ratio are slightly outside the standards. However, regarding the AST/ALT index, its value must be taken into account if the levels of these liver enzymes are at high levels, which is not the case. Regarding the

cardiovascular risk parameters, the levels of cholesterol, HDL, LDL, LDL/HDL index, triglycerides, total lipids and alkaline phosphatase are within or close to the limit of the reference standard. In other cases, it was possible to reverse the unusual variations to enter within the values considered normal. In the SAF group, we have a similar situation with the values of glucose, LDH, AST, ALT elevated or close to the limits established by the reference standard in liver activity, while in relation to cardiovascular risk the values of cholesterol, HDL LDH, triglycerides, total lipids and alkaline phosphatase are close to or above those set by the reference standard. In case on stressed mice, we can observe increasing of IL-12 and IL-10 consistent with alterations in immune systems under oxidative stress. Because of its double function of a proinflammatory cytokine and an immunoregulatory factor, IL-12 plays a key role in the resistance to infections, particularly those related with bacterial infections and intracellular parasites, against which phagocytic cell activation and Th1-mediated responses are particularly effective (Table 4) [100, 104, 105]. This evidences a situation where it is possible to progress to a condition that causes the development of NAFLD if corrective treatment is not performed. In the tables with the results of the experiments we observe an improvement in the evaluated parameters until they reach the normal levels established for a healthy organism.

Table 3: Clinical Precedents of Hepatic and Cardiovascular Risk Factors in Individuals included in the Dietary-Lifestyle Study.

General Characterization of individuals included in the study							
Group	Age (years)	Height Range (cm)	Weight Range (Kg)	Waist Circumference (cm)	Healthy Waist Circumference standard (cm)	BMI Average	Healthy BMI standard
Overweight and Obesity (OWO) n=34	18-23	170-185	84-95	90.9-124.3	Up to 101.6 cm (in male)	25.1-32	18.5-24.9
Skinny with Abdominal Fat (SAF) n=35	18-23	170-185	64-78	88.9-114.3	Up to 101.6 (in male)	15.5-18	18.5-24.9
Family Clinical Precedents							
Group	Overweight	NAFLD	Hepatitis (any type)	Diabetes type 1	Diabetes type 2	Hypertension	Heart disease
Overweight and Obesity (OWO) n=34	24	9	4	1	15	21	15
Skinny with Abdominal Fat (SAF)n=35	2	11	3	0	13	27	12

Parameters such as body mass index (BMI) and waist circumferences (WC) and family clinical antecedents were included. The number showed in Family Clinical Precedents represent the number of total clinical cases in each group.

Table 4: Evolution of Liver and Cardiovascular Parameters in Young Individuals Subjected to Dietary and Physical Training Therapy.

Liver Function and Glucose

Group	Year	Total Bilirubin mg/dL	Ref. mg/dL	Direct Bilirubin mg/dL	Ref. mg/dL	Glucose mg/dL	Ref. mg/dL	BSA g/dL	Ref. g/dL	ALP U/L	Ref. U/L	LDH U/L	Ref. U/L	γ-GGT U/L	Ref. U/L	AST U/L	Ref. U/L	ALT U/L	Ref. U/L	AST/ALT Index	Ref.
WOW n=34	0	0.78	< 1.2	0.12	0.09 to 0.3	120	55 to 99	4.05	3.9 to 5.1	58	40 to 130	235	135 to 225	80	9 to 75	49	< 40	42	< 41	1.16	< 1 NAFLD > 2 AFLD
	Year 1	0.55		0.11		98		4.15		60		190		25		29		28		1.03	
	Year 2	0.54		0.10		85		4.52		65		178		24		17		20		0.85	
	Year3	0.54		0.11		72		4.54		64		172		23		15		19		0.65	
SAF n=35	0	0.89		0.13		108		3.62		87		164		50		53		44		1.21	
	Year 1	0.55		0.13		85		4.22		60		193		35		49		43		1.13	
	Year 2	0.53		0.12		80		4.31		60		159		25		16		18		0.88	
	Year3	0.53		0.12		75		4.53		65		169		26		16		18		0.88	

Test	Meaning of the evaluated parameters	Out of reference standard	Close to the limit of the reference standard	More positive data at the end of study

Bilirubin	Bilirubin can be found in blood at low level; however elevated levels may signal liver disfunction.
BSA	BSA: High levels of albumin are a sign of dehydration and malnutritional conditions. Low albumin levels sign kidney and liver diseases as well as protein absorption dysfunction.
AST	Aspartate transferase (AST): High levels of AST indicate metabolic problems and liver damage.
ALT	Alanine transaminase (ALT): High levels of ALT indicate metabolic problems and liver damage.
γ-GGT	Gamma-glutamyl transferase (High levels in blood evidence liver disease or damage to the bile ducts
ALP	Alkaline phosphatase (ALP): High levels of ALP in your blood may indicate metabolic problems, liver disease or bone disorders.
LDH	Lactate dehydrogenase in high levels in blood sign tissue damage, and help to follow a progressive health condition in severe infection, cancers, gluconeogenesis or DNA metabolisms
AST/ALT	The ratio between these two liver enzymes AST and ALT indicated status of liver function. If AST/ALT < 1 means NAFLD, but if AST/ALT >2 means AFLD.

Cardiovascular Risks and Heart Disease

Group	Year	Cholest. mg/dL	Ref. mg/dL	Cholest. HDL mg/dL	Ref. mg/dL	Cholest. LDL mg/dL	Ref. mg/dL	LDL/HDL	Ref.	Triglycerides g/dL	Ref. g/dL	Highly Sensitive C Protein mg/dL	Ref. mg/dL	Total Lipids mg/dL	Ref. mg/dL	Serum Phosph. mg/dL	Ref. mg/dL	AIP	Ref.
WOW n=34	0	210	< 200	63	40 to 60	157	< 100	2.49	< 3.0	160	< 150	0.21	< 0.5	701	380 to 748	183	161 to 265	4.1	< 4.5
	Year 1	128		49		85		1.73		145		0.15		464		181		3.4	
	Year 2	122		47		83		1.76		110		0.1		374		187		3.2	
	Year3	124		47		82		1.74		111		0.1		370		191		3.2	
SAF n=35	0	215		65		167		2.50		151		0.45		750		193		4.6	
	Year 1	120		51		90		1.63		139		0.24		409		185		3.3	
	Year 2	123		50		86		1.76		117		0.1		385		189		3.1	
	Year 3	120		49		84		1.77		109		0.1		391		185		3.1	

Test	Meaning of the evaluated parameters	Out of reference standard	Close to the limit of the reference standard	More positive data at the end of study

Cholesterol HDL	High-density lipoprotein cholesterol. HDL picks up excess cholesterol in your blood and takes it back to your liver where it's broken down and removed from organisms.
Cholesterol LDL	High levels of low-density lipoprotein (LDL) can build up within the walls of blood vessels and narrow the ways. When clot can form and get stuck in the narrowed space causing a heart attack or stroke.
ARI	LDL Cholesterol (mg/dL) / HDL Cholesterol (mg/dL) in a biomarker indicates cardiovascular risk. A high atherogenic index indicates a higher risk.
Triglycerides	They are our main source of energy and are essential for good High levels of triglycerides can raise the risk of heart disease
Highly Sensitive C Protein	The levels of the Liver C-reactive protein (CRP) increase when there's inflammation. A high-sensitivity C-reactive protein (hs-CRP) test is very sensitive than a standard test.
AIP	The atherogenic index of plasma (AIP) is a novel biomarker consisting of the logarithmically transformed ratio of triglycerides to high-density lipoprotein (HDL)-cholesterol.
Phospholipids	Hyperphosphatemia is a major cardiovascular risk factor for death, cardiovascular events and vascular calcification and it's associated with a greater risk of death and cardiovascular events.

The data were obtained during performance of a three-year study in young individuals with tendencies to develop NAFLD and Cardiovascular Risk. The individuals consumed regularly dietary supplemented composed by dehydrated extract yeast and blueberry powder and combined this functional diet with physical exercises and clinical parameters were clearly improved at the end of study.

Discussion

The increasing prevalence of obesity, inadequate nutrition, sedentarism, increasing stressors promoting oxidative stress has made fatty liver diseases (FLD) and particularly nonalcoholic fatty liver disease (NAFLD) the most common chronic liver disease and growing health problem associated with an increment of cardiovascular risk (CVR). NAFLD and especially its chronic inflammatory, steatohepatitis (NASH) is considered the fastest increasing etiology, frequently ending in cirrhosis and hepatocellular carcinoma. It is obvious that oxidative stress, whether due to the action of internal or external stressors, makes this organ a preferred target and through its influence affects other vital systems of the organism [15]. A healthy diet, characterized by daily intake of foods improving the antioxidant capacity of the organism can reduced incidence of stressor diseases related to oxidation, prevent oxidative stress and OS-derived dysfunctions and diseases, modulate REDOX processes within the limits of the antioxidant capacity but without losing their vital role in cell signaling at the cell, tissue, organ and systemic levels. Probiotics, prebiotics, or symbiotic contribute to the welfare and health of the intestinal microbiota. They can be consumed in the form of raw vegetables and fruit, fermented pickles, or dairy products. One the most valuable source of such beneficial dietary components is yeast, which contributes with many nutrients, including proteins, vitamins, β-glucans among others, to the healthy diet [96, 106]. β-glucans are absent in vertebrate cells, and it is recognized to have overall stimulation effect on innate

and acquired immunity. In their absence vertebrates can be an easier target to foreign invasive and potentially pathogenic substances. Microbe-Associate Molecular Pattern (MAMPs) by Pattern Recognition Receptors (PRR) are crucial in the development and functioning of innate immune system and are mainly expressed by antigen presenting cells such as dendritic cells, monocytes, macrophages, natural killer cells, neutrophils, eosinophils and in epithelial cell of many tissues, including intestinal epithelial cells [16, 93]. MAMPs include different agents, bacterial lipopolysaccharides, mannose; nucleic acids, peptidoglycan, lipoteichoic acid from Gram positive bacteria, etc. This fact could explain the multifaceted action of yeast β-glucans. β-glucans have the ability to promote the growth of beneficial microorganisms such as Lactobacillus ssp. and Bifidobacterium ssp. in the gut microbiota, and also stimulate the immune system in mammals [106, 107, 108]. Overall probiotic effect of yeast β-glucans prevent oxidative stress and have hepatoprotective effects by improving antioxidant capacity [15, 16, 92]. The incorporation of vitamin B12 responds to the fact that we want to overcome the deficiency found in our probiotic complement.

The consumption of raw fruits and vegetables is fundamental in the prevention and treatment of various diseases as well as the post-illness recovery. That is the case of blueberry, specie of the genus Vaccinium, a morphologically diverse genus which includes, 4250 species (33 types), grouped into 9 subfamilies and 125 genera distributed across Europe, the Americas, South, East and Central Africa, Asia [109, 110]. The most economically important and widely cultivated crop of Vaccinium genus is blueberry (Vaccinium corymbosum L.). Blueberry was domesticated and cultivated in North America, but today it´s present in the rest of the world and it's become an important crop and commercial commodity (The International Blueberry Organization (IBO), 2022). Blueberry is one of five healthy fruits recommended by the FAO, WHO and UN agencies due its many functional phytochemical compounds, including organic acids, as hydroxycinnamic and hydroxybenzoic acids, phenolics, anthocyanins, quercetin, kaempferol, resveratrol, minerals and vitamins [111, 112]. Among the properties of these bioactive compounds we can mention: improvement of antioxidant capacity, mitigation of anti-inflammatory processes, retardation of tumor-cell proliferation, neuroprotective effect, stimulation of cognitive brain function, improvement of hepatic, cardiovascular, renal and homeostatic functions, regulation of intestinal microflora, prevention of the development of obesity, type 2 diabetes, chronic liver inflammation, fatty liver disease, atherosclerosis, coronary heart disease and other cardiovascular risks [113, 114]. The most abundant vitamin in blueberry is ascorbic acid, vitamin C, an antioxidant that reduces the inflammation [115]. Ascorbic acid also affects inflammasome functioning through the stimulation of the thioredoxin-interacting protein (TXNIP), thus reducing both, ROS production and the expression of pro-inflammatory proteins, like interleukins 1β, 18 and caspase-1) and contribute to the development of neoplastic changes and atherosclerosis, increasing cancer and cardiovascular risks [115, 116]. Blueberries are rich in Poly Phenolic compounds (PP), mainly anthocyanins known for their antioxidant and cardiovascular protective and therapeutic effects, The same properties have found in polyphenolic compounds, including tannins The same properties have found in polyphenolic compounds, including tannins [115]. The type and content of anthocyanins depend up to the cultivar, 14 different anthocyanins were identified in 74 blueberry cultivars in southern China such as malvidin-3-O-galactoside, malvidin-3-O-glucoside and delphinidin-3-galactoside as the main components while another 36 were found in phenolics from northern highbush blueberry and one cultivar, "Elliott", showed the highest anthocyanin and free phenolic contents [117, 118]. The mode of action of the described effect is unclear, due that most of PP cannot be absorbed by mammalian animal [119]. Myricetin, also known as hydroxyquercetin, clearly has exhibited hepatoprotective effect through the down-regulation oxidative stress, cellular apoptosis, antitumor analgesic, antidiabetic inflammation and the increment of antioxidant capacity besides its partial regulation of sirtuin 1 and autophagic pathway [120]. The significant antioxidant activity of myricetin is attributed to the presence of three hydroxyl groups on ring B as compared to other flavonoids [121].

Many bioactive compounds can, directly or through secondary messengers, interact at DNA level with specific nuclear receptors (NRs). This is a family of transcription factors that regulate um metabolism, reproduction, inflammation, circadian rhythm and other physiological processes. These receptors are domains

that interact, in a specific manner, with all types of ligands and proteins, even when they are at very low concentrations and possibly this is one of the pathways that the active components of blueberry use to exert their regulatory influence as control agents of gene expression especially at the transcriptional level. NRs interact with specific ligands activating the expression of transcription factors, that subsequently control the expression of genes that regulate cell proliferation, differentiation and other important physiological processes. In hepatocytes, NRs sense changes in lipid metabolite levels and its dynamic producing distinct physiologic effects avoiding lipid accumulation in liver, preventing Fatty Liver Disease (FLD, NAFLD [122,123,124]. One example is the Peroxisome proliferator-activated receptors (PPARs) that are involved in regulating cell proliferation, differentiation and survival and as well as energy substrate metabolism [123]. Due to this interaction blueberry powder could mediate lipid metabolism, glucose metabolism, cell differentiation, and inflammation [124, 125]. Previous studies have demonstrated that dietary supplementation with blueberry powder increases bone mass in mice by suppressing PPARγ expression, which regulates marrow adipogenesis [126]. It is clear that elucidating the SNR-bioactive compound interaction and its subsequent effects is an interesting avenue for the development of medications and therapies that promote the biomedical use of blueberry [127].

In the case of mice, we observed the beneficial effects of Se incorporated to selenoproteins, but we rule out its use in humans in this moment because more information about its mechanism of action need to be elucidated for save use in human test [128]. The mode of action of selenoproteins is not completely elucidated but their probiotic and immune stimulatory effects, are known. More exhaustive researches could be conducted before its practical applications in human nutrition. In our both case of study we can observe the evolution of Alanine Transaminase (ALT), Aspartate Transaminase (AST), Alkaline Phosphatase (ALP) and Glutathione peroxidase (GSH-Px) indicate better liver function [129, 130]. Abnormal values of these parameters indicate a variety of pathophysiological conditions, including renal and hepatic malfunctions, liver inflammation and oxidative stress. Described evidences suggest that the probiotic-supplemented diet had a stimulatory effect on the proliferation of different leucocyte populations providing better protection against pathogens and infections and neoplastic diseases and reducing the likelihood of developing fatty liver disease (FLD), particularly NAFLD, and thereby reducing the risk of metabolic diseases, diabetes, and cardiovascular risk.

Probiotic-enriched and antioxidant nutrition along have limited effect and in many case the results obtained in animals and human patients are mixed of contradictories. It is obvious that the modern life implies exposure to stressful living and working conditions, often aggravated by the excessive intakes of alcohol and different types of drugs, provoking oxidative stress and detrimental results to health and particularly deterioration is observed in hepatic and cardiovascular functions. Liver damages by oxidative stress increase directly, thought metabolic alterations cardiovascular risk. Some of the factors that most frequently contribute to the emergence and rapid development of FLD are obesity and a sedentary lifestyle. a sedentary lifestyle. Lower physical activity, is directly linked to the rapid development and severity of fatty liver disease (FLD) [131]. Regular physical exercise is a lifestyle factor for a good health. Physical activities are considered a non-pharmacological or dietary mode to prevent, drive and treat many cardiovascular risks and diseases by the way of stimulation of different mechanisms and physiological processes, including the improvement of insulin sensitivity, stimulation of lipid metabolism, regulating autonomic function. Exercise stimulates the sympathetic nervous system and induces an integrated response from the body. This response maintains an appropriate level of homeostasis for the increased demand in physical, metabolic, respiratory, and cardiovascular efforts by the control of blood viscosity and pressure, controlling levels of endothelial nitric oxide avoiding hemodynamic shear stress (HSS) [132]. This is an important physiological stimulus in the regulation of nitric oxide release from the endothelium and when these mechanisms fail due to endothelial cell dysfunction, liver dysfunction, cardio-cerebrovascular risks, diabetes, and hemorrhagic events can arise [133]. The autonomic function is unconscious control of cardiac muscle work, smooth muscle contractions, exocrine and endocrine glands among other activities [134]. The physical training improves the number of mitochondria, increasing respiratory capacity of the cells, fatty acid oxidation,-blood vessels dilatation, improving myocardial per

fusion and reducing inflammation, atherosclerosis and regulation of diastolic blood pressure. and other cardiovascular risks CVR [135, 136].

The observed weight and muscular mass in SAB group individuals could be explained by the improvement in gut microbiota and bile acids through the pathways where Farnesoid X receptor (FXR) and Transmembrane G protein-coupled receptor (TGR5) are involved [136]. FXR is expressed in different organ and tissues but mainly in liver, intestines, white adipose tissue, adrenal glands, kidneys, and immune cells. This receptor plays a significant role in bile acid regulation and plays an important role in lipid metabolism. TGR5 promotes cAMP synthesis and activates the mitogen-activated protein kinase (MAPK) pathway2, involved blood glucose regulation through multiple pathways. For these reason ingestion of high caloric diet without regular physical activity can induce obesity in individuals [137]. The physical exercise stimulates increase of functional capillaries in the peripheral circulatory system, with consequent increase of cellular respiratory capacity [138, 139]. Muscular movements are a source of ROS/RONS by the way of respiratory function, it stimulates antioxidant capacity by enhancing the expression and activity of antioxidant enzymes [10, 140-144]. Physical exercise effects depend of multiple factors including: type of exercise, intensity-, duration- and type-dependent effects on antioxidant system of the cells [142-145]. But excessive physical exercise implies increases in oxidation rates and diminution of antioxidant capacity with a great damage to biopolymers [143]. By contrast low-to-moderate intensity exercise enhances the activity of antioxidant enzymes and other antioxidant mechanisms lowering the levels of ROS/RONS levels, improving adaptive responses and managing properly the oxidative stress (OS) [144-147].

The described processes are particularly evident in liver, for its role in metabolism, and cardiovascular systems. In liver physical exercise prevents fatty liver disease through by different ways, including increments in peripheral insulin resistance reduces the free fatty acids and glucose transformation for fatty acid synthesis and accumulation in the liver. In hepatocytes exercise stimulate fatty acid oxidation for covering of energetic demands and prevents mitochondrial and hepatocellular damage preventing NAFLD [37, 131, 132]. The antioxidant component of blueberries can reduce exercise-induced oxidative stress and muscle damage and upregulate adaptive processes in muscle recovery [147].

Conclusions

Obtained are interesting when it comes to reduce the causes and aggravating factors of liver diseases and cardiovascular risk in a context when unhealthy nutrition, obesity, sedentary life style, alcohol intakes and drugs consumption are a growing public health problem. Oxidative Stress (OS) is the most important pathogenic mechanism in FLD (NAFLD. AFLD). Yeasts as probiotics are a very valuable source of beneficial compounds, nutraceuticals, vitamins, minerals and beta glucans, among others. Blueberries are a source of various beneficial compounds and in our case, we use dehydrated blueberry powder. The combined nutritional supplements prepared for trials in oxidative stress induced mice is a multilateral two was directed interaction and beneficial effect of diet complemented by probiotic antioxidant supplement. In young individuals, with antecedents, tendencies of liver fatty disease and cardiovascular risk, the use of preventive and corrective assessment using antioxidant dietary supplement combined with physical exercises served to prevent development of fatty liver disease reduce cardiovascular risk by increasing antioxidant capacity and improving metabolic status. This association is needed as preventive method in both healthy and physically impaired individuals.

Our studies have some limitations that could affect the results and can limit the conclusions to the specific conditions. We have carried out our experiment with a small number of male individuals (69 persons) with an average age of 19-23 years. The individuals have different physical status, but this large variation in baseline health and physical status were limited with the conformation of two groups, skinny people with abdominal fat and overweight individuals, due to our interest focused on the prevention of fatty liver disease and cardiovascular risks. In all cases, the participants had a family history of fatty liver and cardiovascular risks in adults. Another factor that can alter the results is the way in which the probiotic is ingested, the daily dose can be mixed with different foods and that can alter the way in which the supplement acts on the microbiota, promoting it to a greater or lesser extent.

Author Contributions

De la Riva G.A. was the head and coordinator of the project, he has designed the experiment and was in charge of laboratory tests and redacted the present work. Moran Valdivia R. designed the composition and intake doses of probiotic supplements, he also contributes to the redaction of this manuscript, the conformation of the experimental models and the discussion of generated results. Jesus Aguilar Hernández A. J. designed the nutritional and training programs for each of the voluntary experimental groups in case study 2. García González R. prepared the probiotic supplement and analyzed the obtained data and results.

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Institutional Review Board Statement

Institutional review board (IRB) decision support the research and are agree with arrived conclusions in-hepatology 11: 1246-1255.

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