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MICROAL

EFFECT OF MODERATE BEER CONSUMPTION WITH AND WITHOUT ALCOHOL ON THE INTESTINAL MICROBIOTA AND METABOLIC MARKERS

Dissertação para obtenção do Grau de Mestre em
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***MICROAL* - EFFECT OF MODERATE BEER CONSUMPTION WITH AND WITHOUT ALCOHOL ON THE INTESTINAL MICROBIOTA AND METABOLIC MARKERS**

O trabalho experimental foi executado na Faculdade de Ciências Médicas da Universidade NOVA de Lisboa, sob orientação da Professora Doutora Conceição Calhau e da Professora Doutora Diana Teixeira. A estreita colaboração com diferentes unidades de investigação, nomeadamente o CINTESIS, o Centro de Apoio Tecnológico Agroalimentar (CATAA) sediado em Castelo Branco, e a Ophiomics, sediada no laboratório central Germano de Sousa, em Lisboa, foi determinante para o sucesso desta investigação.

A candidata declara que contribuiu ativamente na realização do trabalho experimental, bem como na interpretação e discussão dos resultados apresentados desta dissertação.

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ABBREVIATIONS

AB	Alcoholic beer
ALAT	Alanine aminotransferase
ALP	Alkaline Phosphatase
ASAT	Aspartate aminotransferase
BFM	Body fat mass
BMI	Body mass index
CK	Creatine Kinase
CNS	Central nervous system
CPR	C Protein reactive
CVD	Cardiovascular disease
GGT	Gamma glutamyl transferase
GIT	Gastrointestinal tract
GLP1	Glucagon-like peptide 1
GPR	G protein receptors
HbA1c	Glycated haemoglobin
HDL	Hight density lipoprotein
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HPA	Hypothalamic-pituitary-adrenal
IXN	Isoxanthohumol
LDL	Low density lipoprotein
NAB	Non-alcoholic Beer
PYY	Peptide YY

SCFA	Short chain fatty acids
TLRs	Toll like receptors
VLDL	Very low density protein
XN	Xanthohumol
ZAB	Zero alcoholic beer

ABSTRACT

Our gastrointestinal tract (GIT) is colonized by trillions of microbes, especially in the gut. The gut microbiota plays a pivotal symbiotic role within the host, influencing key metabolic, physiological, and immune functions, which can be determinant for health and disease. Dysbiosis is referred as the unbalanced composition of the microbiota and has been reported in several pathologies such as the metabolic disease. Diet has been a factor strongly associated with the composition of the intestinal microbiota. Plant-based food, for instance, provides carbohydrates that can reach the colon intact and be metabolized by bacteria. Polyphenols are also present in those foods and have been reported to have a protective effect on human health. Most of the dietary polyphenols reach the colon intact, where the gut microbiota can metabolize them, to be absorbed and degraded to simpler phenolic derivatives and other metabolites. Fermented beverages, such as beer and wine can be much rich in polyphenols, beside, the occurrence of metabolic activity of microorganisms can change the nutritive and bioactive properties of those food matrices resulting in a myriad of beneficial by-products, attributing to them prebiotic features. Several studies have proved the beneficial effects of red wine, including in the intestinal microbiota, however, to date, nothing has been clarified about the effect of beer consumption on the gut microbiota. Beer is a fermented food rich in polyphenols originated in hop and in malt. This is a pilot study intended to observe the effect of moderate beer consumption, by testing individuals for metabolic markers and gut bacteria composition after four week of beer intake, with and without alcohol. Preliminary results show that beer intake produced effects on biochemical parameters and electrolytic markers, independently of their alcoholic content, and on microbiota in a level of alcohol dependence. This clinical trial will be further extended, and future results beer composition will allow a more conclusive description on the influence of beer intake in the human health.

Keywords: alcohol, beer, clinical trial, metabolism, microbiota, PREDIMED, polyphenols

RESUMO

O trato gastrointestinal é colonizado por triliões de micróbios, especialmente no intestino. O microbiota intestinal desempenha um papel simbiótico fundamental dentro do hospedeiro, influenciando as principais funções metabólicas, fisiológicas e imunológicas, determinantes na saúde e, portanto, na doença. A disbiose é referida como sendo o desequilíbrio da composição do microbiota e tem sido observada em várias patologias, tais como a doença metabólica. A dieta tem sido um fator fortemente associado à composição do microbiota intestinal. Alimentos de origem vegetal, por exemplo, fornecem hidratos de carbono que podem chegar intactos ao cólon e serem metabolizados por bactérias. Os polifenóis também estão presentes nesses alimentos e são recorrentemente retratados como protetores para a saúde humana. A maioria dos polifenóis da dieta chegam intactos ao cólon, onde o microbiota intestinal os metaboliza, transformando em metabolitos com atividade biológica e biodisponíveis. Bebidas fermentadas, tais como a cerveja e o vinho, ricas em polifenóis, que por ação da atividade metabólica dos microrganismos da fermentação, alteraram-se as propriedades nutritivas e bioativas dessas matrizes alimentares, no que resulta uma panóplia de subprodutos benéficos, atribuindo-lhes assim características prébióticas. Vários estudos comprovam os efeitos benéficos do vinho tinto, inclusive no microbiota intestinal, no entanto, até o momento, nada foi esclarecido acerca do efeito do consumo de cerveja no microbiota intestinal. A cerveja é um alimento fermentado, rico em polifenóis originados no lúpulo e no malte. Este é um estudo clínico piloto que pretende avaliar o efeito do consumo moderado de cerveja em marcadores metabólicos e na composição das bactérias intestinais após quatro semanas de ingestão de cerveja, com e sem álcool. Resultados preliminares mostram que a ingestão de cerveja produziu efeitos sobre alguns dos parâmetros bioquímicos e marcadores eletrolíticos, independentemente do seu teor alcoólico, e sobre o microbiota intestinal, num nível de dependência de teor alcoólico Esta investigação está ainda em curso e os resultados futuros da composição mais detalhada da cerveja permitirão uma visão mais detalhada acerca da influência da ingestão de cerveja na saúde.

Palavras-chave: álcool, cerveja, metabolismo, microbiota, PREDIMED, polifenóis

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CHAPTER I

Introduction

The gut microbiota

It's been long time established that human is not a lone self being. This relates not to other beings beside us but rather the inhabitants in ourselves. The human body surface and mucosa is colonized by trillions of microbes coexisting in a symbiotic relationship. Collectively they are referred to as the human microbiome, including eukaryotes, archaea, bacteria, and viruses [1][2]. In the gastrointestinal tract, specific regions condition distinct habitats that harbour most of our resident microbes, estimated to be most densely populated (0.15 kg of microbial biomass) in the gut, particularly by bacteria (99%) [3][4].

This intricate and mutually beneficial relationship has evolved over millennia based on selective pressures, both intrinsic and extrinsic, that shape each species appearance in host, in number, and behaviour [5].

Development of gene-sequence-based metagenomic methods has led to major advances in defining the total microbial population of the gut [3]. Estimates of the number of bacterial species present in the human gut vary widely among studies, however, the composition of microbial community in the human gut it's been found to be fairly stable at the phylum level, with Bacteroidetes and Firmicutes being the most populous bacterial phyla, constituting more than 90% of the gut microbiota, followed by the phyla Actinobacteria and Verrucomicrobia [4][6]**.**

The colonization of the microbe gut community is suspected to start even in womb, but it is from birth were newborns are colonized by microbiome species present in the environment and especially from the mother [7]. Increasing diversity and compositional shifts continue gradually from infancy to adulthood, with greatest variation observed among adults, although family members tend to harbour more similar communities than unrelated individuals [8]. During aging it's often

seen diversity loss and compositional shifts associated to general debilitated health states but also to diet changes, constrained lifestyle and intake of medication [9].

The first enlightenments on human gut microbiota revealed the extent to which different aspects of the microbiota can be beneficial to the maintenance of the intestinal wall integrity, preventing microbial translocation into the body, and assisting in obtaining energy from the undigested diet. In the past decade focus on human gut microbiota brought to light important, yet complex features of this organisms influencing key metabolic, physiological and immune functions between individuals, which can be determinant for health and disease [10].

Gut microorganisms play a pivotal symbiotic role in helping humans access calories from the nutrients that pass through the digestive tract without undergoing metabolic transformation, such as plant-derived dietary fibres and complex carbohydrates, most of which cannot be degraded by human digestive enzymes [11]. Specific bacteria transforms these indigestible food components by fermentation within the intestinal lumen, into short chain fatty acids (SCFAs) (figure 1), primarily propionate, butyrate, and acetate acids, typically found in a proportion of 1:1:3 respectively [6], as its main end products together with various gases (hydrogen, methane and carbon dioxide) [12]. For example, propionate is mainly produced by Bacteroidetes, whereas the production of butyrate is dominated by Firmicutes [6]. Other bacterial end products include lactate, ethanol, succinate, formate, valerate, caproate, isobutyrate, 2-methyl-butyrate, and isovalerate [13].

Approximately 90% of the SCFAs present in the intestinal lumen are absorbed, generating high concentration of SCFAs in blood plasma. For example, the acetate entering the portal circulation is used as substrate for hepatic lipogenesis and cholesterol biosynthesis [14][15], while propionate is used as a substrate for gluconeogenesis, lipogenesis, and protein synthesis [16]. Exception for butyrate that is almost completely metabolized by colonocytes contributing for the maintenance of colonic homeostasis [16]. Thus, some studies show butyrate as playing a role in positively interfering in the synthesis of the mucin layer, reinforcing the colon barrier defence, and by doing so, creating an obstacle to proinflammatory compounds and uptake of antigens [14].

SCFAs stimulates multiple hormonal and neural mechanisms that suppress appetite and energy intake. SCFAs activate G-protein receptors (GPRs), identified as GPR41 and GPR43 receptors (also named FFAR2 and FFAR3) [16], that stimulate the release of anorexigenic hormones like ghrelin, leptin, cholecystokinin (CCK), glucagon-like peptide 1 (GLP1), and peptide YY (PYY), playing a role in energy intake regulation (figure 1) [17]. Both receptors are expressed primarily by the enteroendocrine L cells in colonic mucosa, but they are also expressed on immune cells, particularly on polymorphonuclear leucocytes, and thereby play an important role in the gut inflammatory response, signalling pathways to the production of cytokines and regulation of cell proliferation [14].

Another GPR for butyrate, GPR109a, discovered to be expressed in the apical membrane of both colonocytes and enterocytes as well as on colonic immune cells, has shown to exert immuno-

modulatory effect by suppressing the expression of nuclear factor-κB (NF - κB) and promoting the differentiation of colonic regulatory T cells [14][16][18]. SCFAs, particularly butyrate, can exert direct anticarcinogenic effects through GPR activation. GPR43 and GPR109a both act as tumour suppressors by inhibiting proliferation and increasing apoptosis in colon cancer cell lines [16]. SCFAs also can promote histone deacetylases' (HDACs) inhibition, particularly butyrate. HDAC are enzymes involved in the regulation of gene expression, thereby their inhibition is associated with cell cycle arrest, causing antiproliferative and proapoptotic effects [14][16][19].

The microbiota is also involved in the production and absorption of vitamins and micronutrients. The presence of SCFAs may help in the absorption of minerals such as calcium, by increasing their solubility and increasing the expression of calcium binding proteins [20]. Specific gut bacteria are also known for synthesise a large number of vitamins of the B group, like riboflavin, biotin, nicotinic acid, pantothenic acid, pyridoxine and thiamine, vitamin K, and carry out bile acids transformation into secondary bile acids, responsible for solubilize dietary fats to promote their absorption [6][21]. Furthermore, resident microbiota keeps a play role in prevention of colonization by pathogens per direct competition for nutrients and through its recognition by toll-like receptors (TLRs) involved in immune response, that trigger the expression of anti-microbial peptides controlling the intestinal barrier penetration [22].

Toxic metabolites and a lower availability of SCFAs are recognize as potential causes for all these mechanisms to be disrupted, as a result of an altered microbial composition known as dysbiosis [14][16]. It is widely accepted that dysbiosis of the gut microbiota, as a disrupting factor, interferes with host's metabolism and has a role in pathological conditions [23]. Therefore, the resilience of the healthy microbiota protects us from dysbiosis-related diseases [24].

Given the complex interplay between the microbiome, host and the ecosystem, a certain amount of the host's diary intakes (diet and medication), as well as host's genetics and interactions with environmental factors, are expected to shape the composition of the microbial community or even cause perturbations and trigger microbiome shifts, with great impact on our physiology [25].

By understanding the taxonomic and functional diversity of the microbiota it's possible to use association to dissect host-gut microbiota interactions and demonstrate causal roles of the gut microbiome in disease development. The sequencing-based assessment of microbial communities in human fecal material has linked alterations in gut microbiota composition to disease and chronicle conditions [26].

Initiatives like The Human Microbiome Project (HMP) consortium, Metagenomics of the Human Intestinal Tract (MetaHIT) and the MiBioGen consortium intend to compile the human gut microbiome and establish a population-scale framework to study the influence of human genetics on gut microbiota. Collaborative effort aim to assemble population-level cohorts worldwide, expecting to grow harmonization upon the microbiome data and provide full information on human gene-

microbiome associations by using genome wide association studies (GWAS) meta-analyses [1][10][27].

The Gut-brain axis

There is a large body of evidence supporting the concept of the “the gut-brain axis”, a bi-directional communication network between the gut and brain [28]. Signals from the brain can Influence the gastrointestinal tract (GIT) functions, such as motility, secretion and mucin production, as well as immune functions, including the modulation of cytokine production by cells of the mucosal immune system, and conversely, visceral messages from the GIT can influence brain function, with great impact on human psychologic behaviour [22].

Although signalling pathways are not fully understood, the design of this intricated communication system involve central nervous system (CNS), the enteric nervous system (ENS), sympathetic and parasympathetic branches of the autonomic nervous system (ANS), neuroendocrine signalling pathways, and neuroimmune systems [29].

Several proposed mechanisms of action include a direct neural route through the vagus nerve (figure 1). Other potential mechanisms of action include a humoral route over neurotransmitter modulation. The humoral components of the gut-brain axis consist of the hypothalamic–pituitary–adrenal axis (HPA), the enteroendocrine system and the mucosal immune system [30].

The HPA is responsible for coordinate stress responses, resulting in the release of stress hormones. Stress responses can be caused by increased circulating pro-inflammatory cytokines, subsequent to microbiome dysbiosis conditions, that can sensitize the HPA axis to stress-induced activation, and can also increase anxiety- and depressive-like behaviours [29]. The stress hormones released, like the cortisol (figure 1), might influence bacterial gene expression or signalling between bacteria, altering the microbial composition and its activity [28].

On the other side, enteroendocrine cells (EECs), can be stimulated trough bacterial by-products, such as SCFAs, to produce hormones that regulate appetite, as previously described [28]. Further, EECs are involved in the production of serotonin (5-HT) [22], a neurotransmitter implicated both in regulating GIT functions, like motility and pain perception, as in the brain, regulating mood and cognition (figure 1) [24]. Gut microbiota may also play a crucial role in the regulation of systemic 5-HT levels through the metabolism of tryptophan, its precursor [31]. Plus, several bacterial strains are able to synthetize gamma-aminobutyric acid (GABA) through the decarboxylation of l-glutamate, both acting as inhibitory and excitatory neurotransmitters respectively, with reciprocally modulating activity in human CNS [32]. GABA is reported as having immunomodulatory properties and is also involved in behavioural modulation (figure1) [31].

The immune system also plays an important intermediary role in this dynamic equilibrium between brain and gut. Chronic inflammatory sates can be induced via cytokine release from mucosal immune cells and have been related to a variety of depressive like behaviours, including sleep

and appetite disturbances, on the other hand, promotion of a healthy gut microbiota and probiotic therapy, has been linked with reversion of these clinical conditions, furthermore, exposure to stress and anxiety-like behaviour can impact the microbiota community profile by altering the relative proportions of the main microbiota phyla (figure 1) [29]. Several lines of evidence emerged primarily from animal studies supporting the suggestion that gut microbiota influence depressive symptoms states [33], oxidative stress [34], and anxiety-like behaviour [35]. Experiments in this field used several approaches, including germ-free mice, induced neurotoxicity, antibiotic-induced dysbiosis, fecal transplants and probiotic therapy [31].

Alterations in this communication might be vulnerable to environmental factors, such as stress and the use of antibiotics [34]. The high co-morbidity between stress-related psychiatric symptoms such as anxiety, with gastrointestinal disorders including irritable bowel syndrome (IBS) and inflammatory bowel diseases (IBD) like Crohn's disease (CD) and ulcerative colitis, is associated with dysbiosis and inflammation [28][31]. It has been suggested that impairment in the gut microbiota as part of brain-gut-axis, activates mucosal immunity leading to loss of epithelial layer (protective barrier) leading to dysmotility and hypersensitivity in IBS patients [36]. Joossens *et al.* investigated fecal samples of patients with CD and observed a dysbiosis-like pattern on those patients, along with lack of butyrate-producing capacity in conjunction with mucin degradation [37]. Clinical evidence is mounting to support the role of probiotic intervention in reducing the anxiety and stress response in IBS patients [31].

Gut microbiota and metabolic diseases

Obesity incidence rates are now above 20% in most Western countries and represent a major public health burden [17]. The consumption of more energy-dense, nutrient-poor foods containing high levels of sugar and saturated fats in combination with reduced physical activity are generally the most pointed reasons for obesity increasing in developed countries.

In short, obesity is an accumulation of excess body fat as a consequence of a lifestyle associated to the large ingestion of energy exceeding the amount expended, resulting in a positive energy balance and increasing the risk of developing chronic diseases such as the metabolic syndrome and related comorbidities [38]. The Metabolic syndrome is associated with an abnormal metabolism of glucose and lipids owing from a disorder of the energy storage and utilization, resulting in a cluster of metabolic disturbances like glucose intolerance, insulin resistance, dyslipidaemia, and hypertension. Furthermore, it is strongly associated with type-2 diabetes (T2D), cardiovascular disease, chronic kidney disease, and overall mortality [39]. Obesity-related diseases are now one of the leading causes of preventable death worldwide [40].

Usually, the parameters indicating a person is obese are centred in increased adiposity and increased waist circumference [41]. A crude population measure for obesity is the body mass index (BMI) calculation. Other parameters highly associated with obesity include hypertension, dyslipidaemia, insulin resistance, low-grade inflammation, and hormonal imbalances [42].

Obesity has been associated with phylum-level changes in the microbiota, reduced bacterial diversity, and altered representation of bacterial genes and SCFAs concentrations (figure 1) [8], on the other hand, metagenomic studies have shown that improved metabolic health is associated with a relatively high microbiota gene content and with an increased microbial diversity [43].

Mice's gut microbiota targeted by a transition to high-fat, high-sucrose (HF/HS) diets showed a disproportion across the most abundant phyla (Firmicutes and Bacteroidetes) [44]. Relative proportion of Bacteroidetes is decreased in obese people by comparison with lean people, and this proportion increases with weight loss [45]. *Akkermansia muciniphila* was identified as a candidate microbiota influencing dietary response with observed striking effects on weight gain, adiposity, plasma lipids and insulin resistance [44].

Results of adiposity measures in a cohort of 3666 twins were crossed with their fecal microbiome available profiles. Results showed that fecal microbial diversity and specific members of the human fecal microbiota are strongly associated with obesity-related phenotypes, specifically abdominal adiposity. Results also show that android/gynoid ratio is highly heritable in this same cohort and confirm high heritability estimates for the remaining adiposity phenotypes [46]. Hypothesis that both the composition of the gut microbiota and host genetic background might partially dictate dietary response was also confirmed with experiments between different mouse strains [44][47].

Human gut fecal samples from 94 participants have shown differences in colonic fermentation between lean, and overweight/obese (OWOB). OWOB participants had higher concentrations in total SCFAs, and individually [48].

Gut microbiota may influence adiposity and weight-gain through several interdependent pathways, including increase energy harvest from diet, generation of metabolites such as SCFAs, satiety through the brain-gut axis, alterations in the metabolism of carbohydrates and lipids, metabolic endotoxemia, and low-grade inflammatory responses within the host (figure 1) [49].

The gut microbiome capacity to harvest energy from the diet further increases monosaccharide uptake from the gut lumen to the liver, which, along with SCFAs, act as substrates for de novo lipogenesis in the liver. This effect is complemented by the action of lipoprotein lipase which facilitates the storage of triglycerides as fat in the adipocytes causing expansion of the adipose tissue (figure 1) [39]. Furthermore, the liver secretion of the fasting induced adipocyte factor (Fiaf), a protein that antagonizes the effects of lipoprotein lipase, ultimately preventing the storage of triglycerides as fat, is supressed in overnutrition conditions [50]. In turn, adipocytes are involved in the synthesis and release of peptide hormones (adipokines) implicated in glucose homeostasis. Leptin is one of those adipokines known for repress food intake and promote energy expenditure, improvement of insulin sensitivity in muscles and reducing intra-myocellular lipid levels through a combination of direct activation of adenosine monophosphate-activated protein kinase (AMPK) [51]. AMPK acts to regulate energy homeostasis primarily via stimulation of fatty acid oxidation,

ketogenesis, glucose uptake, and insulin secretion while simultaneously inhibiting cholesterol and triglyceride synthesis along with lipogenesis. Additionally, the microbiome seems to have a suppressive effect on AMPK activity, thereby predisposing the host to accumulation of the excess fatty acids and insulin resistance [50].

SCFAs, besides serving as a source of energy, also play a role on the development of glucose homeostasis by modulating the hepatic metabolism of carbohydrates and lipids with inhibitory effect on glycolysis and, in contrast, stimulation of lipogenesis or gluconeogenesis [38], and by promoting satiety through the brain-gut axis. For example, in high fat fed rodents, Increased acetate production lead to activation of the parasympathetic nervous system, resulting in hyperphagia (due to increased ghrelin secretion) and increased energy storage as fat, leading to obesity, hypertriglyceridemia and insulin resistance (due to increased glucose-stimulated insulin secretion) [52].

Adiposity and body weight can be modulated via SCFAs' receptors GPR41 and GPR43 in processes involving hormone appetite regulation (figure 1). Levels of PYY, a key hormone involved in the elevation of intestinal transit rate and reduction in energy harvest, were decreased in genetically modified mice deficient in GPR41 [53]. On the other hand, SCFAs may prevent obesity via activation of GPR43. Normal diet GPR43 -/- deficient mice gained weight, whereas HFD-fed mice overexpressing GPR43 remained lean. Increased levels of GPR43 inhibit insulin signalling in adipocytes and fat accumulation in white adipose tissue [54]. Besides, GPR43 activation by SCFAs promotes the release of GPL-1 by intestinal enteroendocrine L cells, thereby leading to insulin release and stimulating glucose tolerance [49].

Dysbiosis has been associated with pro-inflammatory events, suggested by the reduction in butyrate-producing bacteria and the increase in mucin degrading bacteria. These characteristics potentially impair the gut integrity causing low-grade inflammation [42]. Chronic low-grade inflammation has been considered as a crucial event in the development of obesity-related insulin resistance, metabolic syndrome, and T2D. The inflammatory response associated with these conditions is thought to be mediated by excessive infiltration of macrophages and T cells in metabolic tissues, especially adipose tissue, and liver, and dysregulation of immune cells, leading to increased adipocyte production of several pro-inflammatory cytokines, including tumour necrosis factor (TNF)-α, interleukins (IL)-1 and IL-6 and reduce insulin sensitivity [39][43].

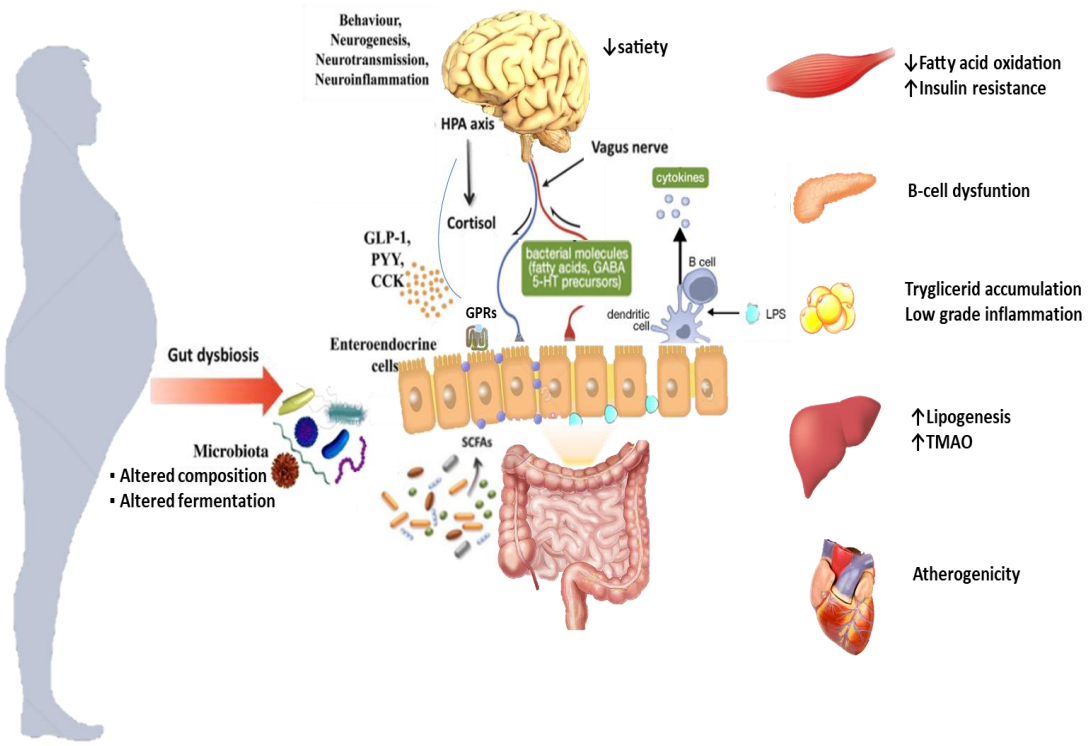

Figure 1 Gut-brain axis interactions and host energy harvest regulation influencing peripheral organs. Pathways involved in bidirectional communication between the gut microbiota and the brain, adapted from Thakur et al [55], Including endocrine (cortisol), immune (cytokines), and neural (vagus and enteric nervous system) pathways. The hypothalamus–pituitary– adrenal axis (HPA) regulates cortisol secretion, affecting immune cells, gut permeability, and barrier function, and change gut microbiota composition. Conversely, the gut microbiota and probiotic agents can alter the levels of circulating cytokines and modulate systemic tryptophan levels, influencing brain function. In addition, short-chain fatty acids (SCFAs) and GABA are neuroactive bacterial metabolites that can also modulate brain and behaviour. Simultaneously, mechanisms by which gut microbiota regulates host energy harvest, by activation of G protein receptors (GPR) to release anorexigenic hormones and influencing metabolic processes in peripheral organs, adapted from Tremaroli et al.[56].

Lipopolysaccharide (LPS) is a virulent factor (endotoxin) located at the outer membrane of Gram-negative bacterial species and have a major role in both acute and chronic infections [42]. Alterations in gut permeability can promote bacterial translocation over the intestinal wall or partly via bacterial capsule fragments that can enter the bloodstream leading to increased circulating LPS levels (Figure 1). This phenomenon, known as metabolic endotoxemia, can be followed by low-grade inflammation, insulin resistance and, ultimately, obesity, T2D [49], and non-alcoholic fatty liver disease (NAFLD) [57].

Another mechanism involving metabolic endotoxemia, is related to the expression of cannabinoid receptors that can be made via the LPS receptor signalling system, increasing the tone of endocannabinoid (eCB) system in plasma and adipose tissue [58]. The eCB system is suggested to be a mediator of communication between the adipose tissue and the gut microbiome. Activation of eCB system impacts the gut barrier integrity and increases LPS levels, meanwhile, the disruption in the regulation of eCB system tone is influenced by increased LPS levels and stimulates adipogenesis [59].

Recently, it has been studied the mechanistic pathway that links toll-like receptors (TLRs) signalling with diet, gut microbiota, host immune system, and insulin resistance. TLRs are structures of the so-called innate immune system that have been demonstrated to increase blood glucose and nonesterified free fatty acids (FFAs) [50]. Dietary fatty acids and enteric LPS can act as ligands of TLRs and activate the innate immune system [60]. TLR-5 was associated with the induction of inflammatory cascade and consequently inflammatory transcription of various cytokines and inflammatory mediators, resulting in a low-grade inflammatory state associated with obesity [50]. Activation of TLR4 by FFAs increases the gene expression of inflammatory cytokines, such as TNF-α and IL-6, in macrophages and adipocytes with direct impact on the pathology of β cell dysfunction and T2D [39].

Dysbiosis has been reported in cardiovascular pathologies, such as atherosclerosis, hypertension, and heart failure. Recently, gut microbiota-derived metabolites trimethylamine and trimethylamine N-oxide (TMA/TMAO) have been reported to impact host physiology during the pathogenesis of CVD (figure 1) [61]. Gut microbiota metabolizes the TMAO precursor, TMA, from dietary choline, phosphatidylcholine, and L-carnitine. TMA is readily absorbed and travel through the portal circulation to the liver to be oxidized into TMAO by hepatic enzyme flavin monooxygenase 3 (FMO3). FMO3 expression can be up-regulated by bile acids via nuclear receptor farnesoid X receptor (FXR) activation [49]. The gut microbiota-driven TMA/ FMO3/TMAO pathway is proven to be key regulatory in metabolism of cholesterol and inflammation. Increased plasma levels of TMAO have been reported in endothelial dysfunction and accelerated vascular inflammation, plus, becoming a new biomarker in diagnosis of CVD [62].

Whether dysbiosis of the microbiota is a cause or a consequence of the onset of disease, is therefore likely to exacerbate the progression of the disease and affect the type of strategies needed to restore symbiosis [6].

Diet and gut microbiota:

Plant-based food and polyphenols

Aside from breast milk, the overall diet is a major factor that strongly influences the composition of the intestinal microbiota (figure 2) [11][13][27][48].

Cross-sectional studies carried out in several population-based cohorts explored human gene-environment interactions with respect to gut microbiome composition and have identified nutrition and medication, as major influencing factors. Zhernakova *et al*. sequenced the gut microbiome of 1,135 participants from a Dutch-based cohort and observed significant associations between the gut microbiome and various intrinsic, environmental, dietary and medication parameters, and disease phenotypes. 126 factors collectively were entitle to explain 18.7% of the variation in human gut microbiome composition, 60 of them being dietary related [63]. In another metagenomic study, two independent cohorts of 914 individuals (PopGen) and 1,115 individuals (Food-Chain Plus; FoCus) were enrolled to provide fecal samples. Diet was significantly associated with the landscape of the human gut microbiome and explained 5.79% of the variation in gut microbiome, age accounted for 4.74% in the combined cohort, followed by BMI, smoking and sex, 3.79%, 2.14% and 1.79%, respectively [64].

Hypothesis that the microbiota of the human gut can respond rapidly to large changes in diet is supported by evidence by a large set of studies. Yet, people can have individualized responses to a particular change in diet owing that to the individualized nature of their gut microbiota [43].

Ten days of a controlled-feeding intervention with a low-fat/high-fiber or high-fat/low-fiber diet into healthy volunteers resulted in immediate changes in the gut microbiome composition, however, significant changes in phyla proportions were not observed [65].

Comparison between animal and plant-based diet also produced different results in gut microbiota. The plant-based diet altered gut microbiota composition, but the changes in bacterial clusters were much less compared with what it was seen during the animal-based diet [39]. For instance, a diet containing a high amount of animal protein, amino acids, and saturated fatty acids was correlated with the Bacteroides enterotype. On the other hand, a high intake of carbohydrates and simple sugars was linked to the Prevotella enterotype [27].

All these results express the influence that diet patterns may have in the gut microbiome composition and how shifting of nutrients available for gut bacteria favour the growth of specific species in detriment of others [43].

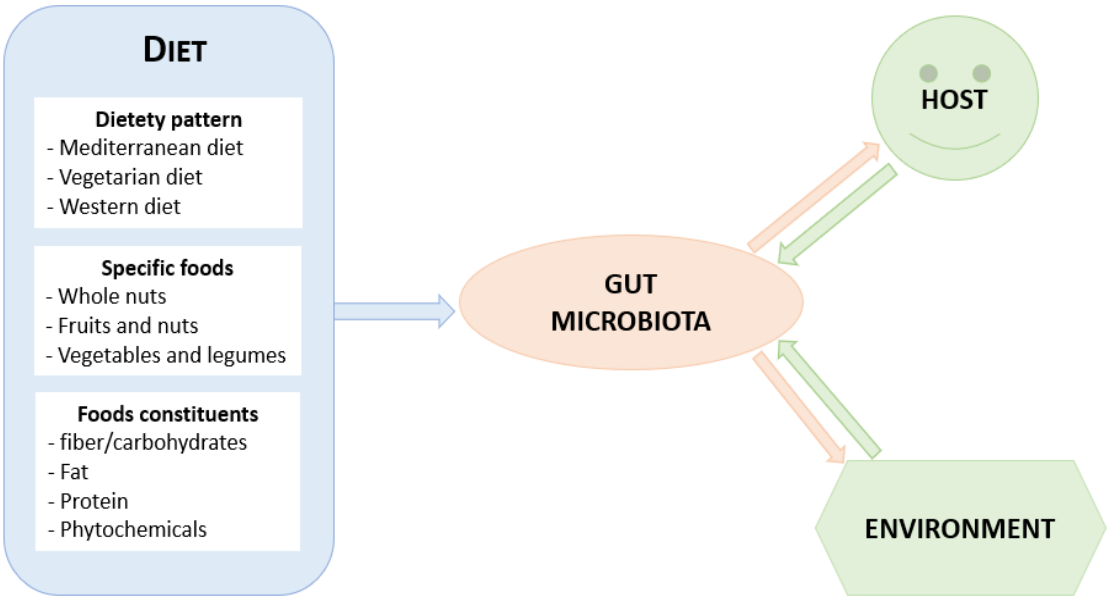

Figure 2 Factors influencing the composition of the human gut microbiota, with special focus on diet, adapted from Graf *et al* [66].

The majority of nutrients used by gut microbiota are plant-derived. Polyphenols are secondary metabolites in plants. A plant-based diet provides the intake of this compounds which have been associated to health benefits related to the cardiovascular function, modulation of oxidative stress, and inflammation [62]. Several studies also characterize the polyphenols as potent inhibitors of microorganism growth. According with that trait, influence of polyphenol intake on the composition and activity of the non-pathogenic gut microbial community become a subject of interest [20].

Polyphenol metabolism starts in the mouth and proceeds along the gastrointestinal tract, however, most of the dietary polyphenols reach the colon intact, where the gut microbiota can metabolize them, thus releasing aglycones that might, to a certain extent, be absorbed and degraded to simpler phenolic derivatives and other metabolites [67]. Dietary polyphenol's biotransformation on the human gut is dependent on its structure and on the existence of the specific microbial species to perform the necessary transformation giving origin to more stable bioactive compounds able to be absorbed at enterocyte and colonocyte levels and to be incorporated into the blood stream to exert their protective effect [68][69]. Polyphenols can step in through direct uptake in the intestine but also upon interaction with the gut microbiota for example by modification of the microbial composition and metabolic activity [67][69].

Effects of dietary polyphenols of black tea and red wine grape extract were observed in vitro gut microbial ecosystem, namely simulator of the intestinal microbial ecosystem (SHIME). Results in the antimicrobial effects of polyphenols showed a selective reduction in bacterial numbers. In

contrast, other bacterial groups appeared to be stimulated in the presence of the existent polyphenols. The selective effects of polyphenols suggest that species that become more abundant during intervention express metabolic routes for polyphenol degradation or are more resistant to these compounds [69].

Fermented food

Since prehistoric times humans have been used fermentation methods as a mean to preserve food with enriched sensorial palate, improved digestibility, and sometimes an euphoriant effect due to the presence of ethanol [70]. Fermenting a food, such as yogurt and cultured milk, wine and beer, sauerkraut and kimchi, is basically to submit that food to the action of microorganisms that will transform it enzymatically in a desirable way for human consumption [71].

The metabolic activity of microorganisms can change the nutritive and bioactive properties of food matrices resulting in a myriad of beneficial by-products that, otherwise weren't available. Furthermore, fermentation can result in the removal of toxic or undesirable food constituents [72].

The fraction of microbes that survives through the human digestive tract potentially increases the numbers of microbes in the diet introducing new microbes into the indigenous intestinal microbiota [72]. This microbial exposure can contribute to a larger diversity in gut microbial ecosystem, acting as probiotics. Nevertheless, it’s a fact that the limitation of microbial exposures in Western societies lead to development of sanitation procedures that, usually at the end of the fermentation, eliminate most of the living organisms present in food [72]. Fermented beverages like wine and beer are usually submitted to such processes, but still an amount of evidence suggests they may be an important constituent of the Mediterranean diet [71].

Scalation in research begun when observations of low incidence of coronary heart disease were seen in populations with rich saturated fats diet, a well-known risk factor for CVD. Fermented beverages consumption, such as wine, were noted as a regular food intake for those populations and several studies stablished an inverse association between their consumption and mortality from cardiovascular diseases (“French paradox”). The bioactive compounds present in wine were identified and scientific efforts were made to elucidate the mechanisms of their action. Among them, phenolic compounds proved exerting biological activity *in vivo*. Controlled clinical trials taken so far provided information on wine consumption and its positive correlation with lipid metabolism and the endothelial function, and the inflammatory process. Alcohol and phenolic content in wine were largely associated with this positive effects on human metabolism [73].

Beer and other alcoholic beverages may have played a key role in cementing human societies through the social act and rituals of drinking [74]. Humans have been brewing and drinking alcoholic beverages for millennia, and our gut microbiota may have evolved to tolerate both regular and highly variable levels of alcohol consumption [75].

The human body can completely metabolize the alcohol. The action of the alcohol dehydrogenase (ADH), the enzyme performing the metabolism of ethanol, starts in the stomach and continues in the liver, where the ADH has a much greater affinity for alcohol converting it into the toxin acetaldehyde, later converted into acetate by the Acetaldehyde dehydrogenase (ALDH) [76]. Although the liver is considered the primary site for ethanol metabolism, extrahepatic organs are also equipped with the enzyme ADH to metabolize ethanol, including the intestinal mucosa [77].

Individuals differ substantially in their bodily response to alcohol. Various factors will play a role, including, sex, body weight, general state of health, amount of activity, and whether the alcohol is being consumed on its own or alongside food. Nevertheless, ADH seems to deal with all the alcohol in moderate healthy drinkers [76].

Moderate or 'low-risk' drinking is defined by the National Institute on Alcohol Abuse and Alcoholism as no more than 7 drinks/week for women or 14 drinks/week for men, at non-binge levels (<0.08 g/dL blood alcohol concentration; NIAAA 2018) [75].

Moderate alcohol consumption in adults has been appointed as a healthy perk for the cardiovascular system [78][79], by contrast, heavy alcohol consumption is pathological and associated with increased morbidity and mortality attributed to the direct toxicity of its metabolites (acetaldehyde). Alcohol abuse is also associated with profound shifts in gut community [80], inflammation and hyperpermeability [81].

The mechanisms by which alcoholic beverages may exert their beneficial actions involve lipid regulation and systemic anti-inflammatory effects. However, fermented drinks, like red wine or beer, provides additional benefits to those of other alcoholic beverages probably due to its higher polyphenolic content [82]. The health benefits associated with the Mediterranean diet, which combines moderate wine and beer consumption with a diet rich in fruits, vegetables and whole grains, suggests that polyphenols may have synergistic effects with compounds found in other groups of foods [82].

Queipo-Ortuño *et al.* evaluated the effect of a moderate intake of red wine in healthy volunteers, with and without alcohol, on the modulation of the gut microbiota composition and reported potentially favourable changes in the gut microbiome possibly due to prebiotic benefits associated with the high polyphenol content of red wine[83]. Limited data are available concerning the human biodisponibility of beer polyphenols [84]. There are some data from case-control studies on beer intake association with cardiovascular risk protection [85]. No clinical data is available regarding to the beer consumption effects on human gut microbiota.

Beer production by-products and health

It is believed that grain-based fermented drinks, like beer, are originated from the regions where grain cultivation first flourished – the Fertile Crescent, Mesopotamia and Egypt, where a great variety of beverages became available from grains shaped by climatic and cultural peculiarities [86]. Barley becomes to be the dominant cereal in brewing because it was the easiest to malt, although other cereals could replace it or be used as a supplement. The production of hopped beer, rather than other herbs, occurred mainly from the necessity to provide a wholesome and stable beverage, regarding the preservative aspects of hop as an additive. Overtime, that become particularly important for sea voyages, and beer trade along the shores of Northern Europe [87] where cold conditions inhibited the development of viticulture [88].

At first, brewers used a top-fermentation process in which the yeast would flocculate up to the surface of the fermenting wort and were accommodated to higher fermentation temperatures (15°C to 25°C). In the 15th century, experiments with storing beer in cool mountain caves allowed the beer to gain a rich, full-bodied texture and taste, with the use of low temperatures (4°C to 10°C) in which fermentation occurred at the bottom of the vat [88]. Beer made by the bottom-fermentation method was usually aged to give it a milder taste and clearer appearance and it was named “lager”, a derivative of the German word *lagern*, which means “to store” [89].

It was often practice carrying out multiple extraction of the same grist in order to yield beers of different strengths. “Strong beer/ale” was fermented using wort drawn from the first mash, with weaker beers derived from the second and third mashes [88]. These latter brews (table or small beer) were everyday drinks consumed by all classes and ages [90]. The boiling and the hopping were inadvertently water purification techniques and it was universally recognised that it was safe to drink beer. In the Great Britain for instance, even infants drank low alcohol beer. The nutritive value of the beer, additional to its safety dimension when compared to water which purity was unreliable, were aspects appointed by physicians [76]. In fact, fermented beverages, such as beer, occur in Egyptian old documents, ship’s cargo manifest and in World War II nutritional status papers as nutritional food [76].

The regions of Bohemia and Bavaria in Central Europe were the focal point for the development of modern brewing [89]. The decree limiting brewing ingredients to hops, barley, malt and water known as the Bavarian Purity Law (“*Reinheitsgebot”*, literally “purity order”) of 1516 and aligned later ordinances defined unambiguously the term ‘beer’, regulated brewing techniques, stated the price of this beverage, and established administrative measures to ensure a sufficient supply and a satisfactory quality [86]. These measures influenced beer production until this day.

From the middle 19th century, the introduction of instrumentation and the development of refrigeration equipment made quality control more certain from batch to batch, and permitted opera-

tions to continue even during hot weather [91]. The Industrial Revolution brought large-scale production, with London being the capital of the first industrialized nation scoring changes in the scale of operation of breweries [88].

French chemist Louis Pasteur's work helped establish practices that greatly enhanced output – the use of pasteurisation enabled longer-lasting beer. A major boost in beer production was achieved when Professor Emil Chr. Hansen, a Danish mycologist of the Carlsberg Laboratory, isolated the yeasts responsible for fermentation. The professor also developed methods for growing yeast cultures that were free of other contaminating yeasts or bacteria. In 1883, Hansen supplied the first single-cell yeast culture to Carlsberg Breweries in Copenhagen [70].

From the 1880s, brewers in United states developed a new style of lager using readily available cereals like corn and rice as adjuncts. The cereals used were unmalted and gelatinized before addition to the malt mash. Along with the development of an accelerated brewing process where storage time was minimized, and filtration was used for clarification [88].

Succession of economic and social influential factors (such as World War I, and World War II) in common beer producer countries, as well as the low need for investment, led to the development of brewing across the world instead of the declining in beer consumption, confirming that beverage to be one of a favourite. Beer is embedded within social and cultural traditions defining, in part, the diet of many Europeans. Similar to wine, beer consumption is also associated with social events but with more informal occasions and is more likely to be chosen by the younger generations. For the Portuguese population, beer consumption is mainly associated with lunch and dinnertime and also largely consumed outside home [92]. With over 90% market share, lager beer is by far the most popular beer style globally [93].

From a nutritional perspective this is an interesting beverage to study since patterns of consumption determine whether it affects health and well-being on a positive or negative way [92]. While wine is frequently featured as an alcoholic beverage with potential health benefits, consumers are often confronted with beer common misconceptions, like the development of the "beer belly" [88].

Four raw materials are required for manufacturing beer: malt (usually malted barley), hops, water, and yeast. Other raw carbohydrates that may be added are considered as adjuncts (not essential). Knowledge of these raw materials properties and their effects on the brewing process and final product provides the basis for their handling and processing.

Malting and brewing (figure 3) are both designed to maximise the extraction and digestion of starch and protein from barley, yielding a highly fermentable extract that is known as wort. The processes are also designed to eliminate materials that can have an adverse effect on beer quality. The vast majority of the chemical constituents of beer are derived either directly from the malted barley, adjuncts, water and hops, or are produced through the metabolism of yeast during the alcoholic fermentation of wort [76]. Focus will be devoted on detailing potential healthy constituents and its source in beer (Table 2).

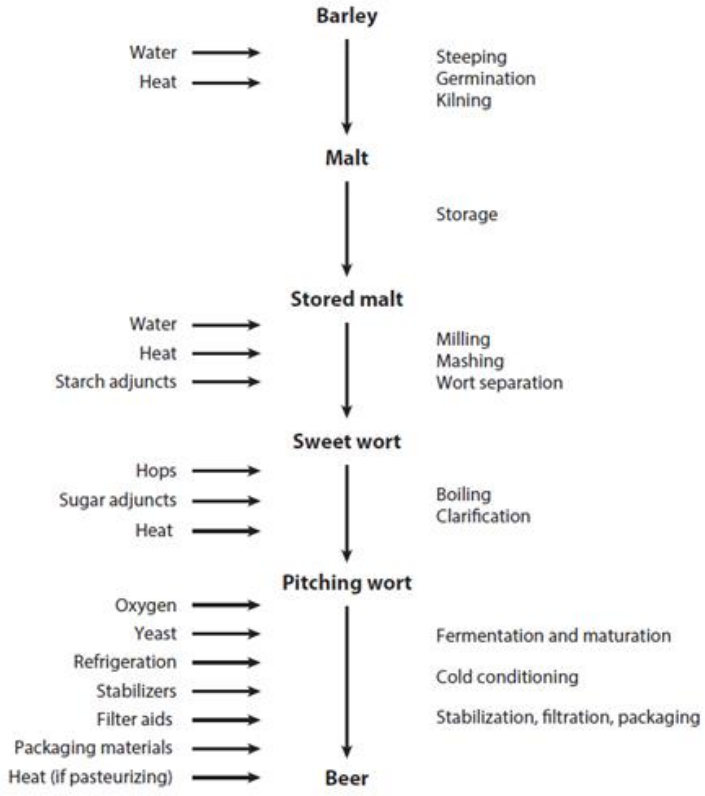

Figure 3 An overview of malting and brewing steps,

adapted from Bamforth [94]

Malting

Malting is essentially, a controlled and limited germination of the barley grains, to obtain sugars accessible for hydrolysis in the brewing process. The process is meant to trigger metabolism and synthesis of the enzymes required for degrade the grain cell walls and protein components making it more soften to milling, and to modify the grains starches, and yield a wide range of sugars meant to be fermented [88].

The process of malting comprises three primary steps: steeping, germination, and kilning. Steeping the barley in cool water is designed to increase grain moisture level allowing the grain to start germinating, producing roots and embryonic shoots [88]. During germination, the amylases that break down the starch are activated, and these are important for the subsequent mashing process. Specifically, the β-amylase enzyme is very important during mashing because it attacks gelatinized starch to produce maltose, the main sugar (45-60%) of brewers' wort, and maltotriose [76][88].

If allowed to continue, a new barley plant would be formed, but the process is arrested by kilning, which is the use of progressively increase temperatures over the range of 50°C to 200°C. Grains modification continues until excessive water loss stops enzymic action. The germinated cereal grains that have been dried through this process are referred as malt. The more intense the kilning process, meaning high level of temperature range, the darker the malt that is produced and the more roasted, coffee-like, and smoky are the flavour characteristics developed. The process will, therefore, determine the different malt types existing to brewing. Colour development results from reactions between sugars and amino acids (Maillard reaction) of the malt to form melanoidins [76][88]. The choice, amount, and combination of malt types will create a great variety of characters in beer. Essentially, malts used for making very pale lager-style beers are kilned quite gently. Lager and pilsner malts have low colours of about 2 °EBC (European Brewery Convention) units resulting from low temperatures of kilning, around 80°C, and, as a result, have high enzyme activities [88].

The malting process is responsible for modifications in the composition of barley, involving changes and degradation of endogenous phenolic compounds. Some authors have demonstrated that the contents of phenolic compounds in malt are usually higher than in barley, but proportions of the different groups are nearly identical, suggesting that a better extraction of flavonoids and phenolic acids in malt is possible after kilning [95]. Carvalho *et al.* analysed the content of single polyphenols in 10 different varieties of barley and corresponding malts. In barley samples, the main phenolics identified were (+)-catechin, vanillic acid, caffeic acid, p-coumaric acid and ferulic acid. In malt, vanillic acid was not identified, however, epicatechin, sinapic acid and traces of cinnamic acid were identified. Catechin was the phenolic in higher concentrations both in barley and malt, followed by ferulic acid. The analysed phenolic compounds can be divided into three main categories: benzoic acid derivatives (gallic acid, protocatechuic acid and vanillic acid), cinnamic acid derivatives (caffeic acid, coumaric acid, ferulic acid and sinapic acid) and flavan-3-ols ((+)-catechin and (−)-epicatechin) [95].

Hops

The Hop plant (Humulus lupulus L.) belong to the family *Cannabaceae*. It is a perennial plant (it loses the aerial part during the Winter) and is described as dioecious (feminine and masculine plants appear), however, only the female plants form the inflorescences, commonly called cones, within which the yellow lupulin glands develop and originate secondary metabolites [88].

According to their physicochemical properties, secondary metabolites are often categorised into three fractions: the hop resins (hard and soft resins), the hop oil, and hop polyphenols. The proportion of soft resins and similarly ratios of other components, particularly within the oil fraction, can be used to determine the acceptability and quality of a variety, as well as comparing with those of other varieties [88].

Hops are vital to the organoleptic qualities of beer, including taste and flavour, despite quantitatively the hop affords a minor fraction of the overall composition of beer, [76]. Hops are the source of bitterness (from the hop soft resins - alpha-acids or humulones and beta-acids or lupulones) and aroma (from the essential oils - mostly terpenoids) used in the brewing industry [96]. Furthermore, they have bacteriostatic activity, that together with other key factors like the very low pH (typically in the range 3.8–4.6), lack of oxygen, minimal levels of residual nutrients such as sugar and amino acids, the content of ethanol and perhaps the presence of some other antimicrobial constituents such as polyphenols, contribute to the beer resistance to spoilage [76].

During the wort-boiling step of the brewing process, the secondary metabolites are transformed by isomerisation/degradation, oxidation and reduction reactions of the hop acids, into desired components with bittering, aromatising, and preservative function [97]. Undesirable residues can be found in hops, also, resins and oils present in whole hops are susceptible to oxidative degradation, therefore, hop processed forms such as pellets or extracts result in a much more concentrated, homogeneous, reliable material for use in the brewery industry [88].

The hard resins account for about 20% of the total resins and are characterised as prenylated chalcones and Prenylated flavonoids, with xanthohumol being the major constituent. Other hop polyphenols consist in a complex mixture of aromatic carboxylic acids and non-prenylated flavonoids, including proanthocyanidins and flavonol glycosides, mostly in the hop cone petals and footstalk and not in the lupulin, with the exception for the prenylflavonoids. Hops may contribute up to about one third of the total polyphenols in beer (Table 2) [98].

Xanthohumol, Isoxanthohumol and 8-Prenylnaringenin

Hop-derived prenylflavonoids are becoming targets of interest due to their potentially biological effects. Among them, xanthohumol (XN), isoxanthohumol (IXN) and 8-prenylnaringenin (8-PN).

Xanthohumol is a chalcone compound that is almost unique to hops. Presence in hop female inflorescences has been estimated at levels around 0.95% of their dry weight [99]. Conventional brewing will introduce XN to the wort, however, the brewing process causes thermal isomerization of XN into the prenylated flavonoid IXN, also further losses occur during fermentation and filtration [100], therefor, the amount of XN represents only a small percentage of the hop-enriched products. Nevertheless, beer consumption is still the most significant dietary source of XN, and the other prenylflavonoids and their concentrations depend on its form of use in the brewing process [99]. For example, xanthohumol-rich beers may contain about 3.4 mg of XN/L, while in commercially available ones is usually found less than 0.2 mg of XN/L (Table 1) [100], which may be not sufficient to cause the disease-preventive effects [99]. A range of brewing studies has been carried out using XN enriched hop products but generally only a small increase of the XN content is observed in conventionally produced filtrated beers.

XN has gained great attention because of its multiple health-promoting properties recently evidenced in several investigations. Test results *in vitro* suggest that the hop prenylated flavonoids

are potent and selective inhibitors of human cytochrome P450, phase I enzymes involved in carcinogen activation process [101]. XN was proven to reduce reactive oxygen species (ROS) and dysfunctional lipid metabolism in rats, which may contribute to ameliorate metabolic syndrome [102]. Recently, Miranda *et al.* showed the antiobesity effect of XN on a mouse model. Results revealed a reduction in plasma LDL-cholesterol, IL-6, insulin and leptin levels by 80%, 78%, 42%, and 41%, respectively, compared to the control group, at doses of 30 and 60 mg/kg/day, corresponding to a human equivalent dose of 350 mg/day for a 70 kg person [103]. Similar results were previously found in obese induced rats, then treated with XN. The highest XN dose (16.9 mg/kg) exerted beneficial effects on body weight and glucose metabolism in obese male rats [104]. These findings suggest that XN holds promise as a therapeutic agent for treating obesity and dysregulation of glucose metabolism, conditions associated with metabolic syndrome.

Isoxanthohumol's presence in hop cones is very low, around 0.008% in the dry weight [105]. This compound become exclusively abundant in beer, as a result of thermal isomerization of xanthohumol. This is reason why IXN is considerate to be an efficient biomarker of beer consumption [106].

IXN was represented in a group of compounds tested both *in vitro* and *in vivo* as potential inhibitors of pathological angiogenesis associated with a chronic inflammation state and oxidative stress. The studies confirmed that IXN, and XN exerted anti-angiogenic effects proven to be useful as a therapeutic agent against inflammation and angiogenesis associated pathologies [107].

IXN also influences several key stages of colorectal carcinogenesis observed in colon cancer cell line Caco-2, by exerting cytostatic/cytotoxic and antigenotoxic effects. In human HT-115 colon carcinoma cells used as a model for colon metastasis, IX reduces proliferation by increasing the G2/M and sub-G1 cell cycle fractions [108][109].

The 8-PN is considered the most potent phytoestrogen isolated until now, which appearance in beer (in small amounts) is largely because of isomerization during wort boiling of its precursor, desmethylxanthohumol [110]. Though, its concentration in beer is very low, studies based on the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) showed that hop prenylflavonoids pass unaltered through the stomach and small intestine, and demethylation of IXN into 8-PN (up to 80% conversion) occurs in the distal colon, mediated by local microbiome, and to a lesser extent, converted by enzymes associated with cytochrome P450 in the liver [105]. Thereby, depending on interindividual variability of intestinal bacteria, intestinal 8-PN concentrations can be increased [111].

Table 1 XN and IXN contents in commercial beers,

Beer type	Xanthohumol	Isoxanthohumol
US major brands		
Lager/pilsner	0.034 (4.0)	0.50 (1.3)
Lager/pilsner	0.009 (1.8)	0.68 (0.1)
Lager/pilsner	0.014 (4.1)	0.40 (3.0)
Lager/pilsner	–	–
Imported beers		
Imported stout	0.34 (2.4)	2.10 (0.4)
Imported lager	0.002 (15)	0.04 (3.2)
Imported pilsner	0.028 (8.8)	0.57 (1.2)
Imported pilsner	0.012 (3.5)	1.06 (0.3)
Other beverages		
Non-alcohol beer	0.003 (14)	0.11 (1.9)

adapted from Stevens [110].

Brewing

Brewing is nowadays conducted in well-designed and highly hygienic facilities, for the most part fabricated from stainless steel. In the brewery, the malted grain must first be milled and then mixed with hot water in a process called mashing. At this temperature the granules of starch are converted into a form that is much more susceptible to digestion by the amylases developed during malting, that start to act once the gelatinisation of the starch has occurred in the mash tun [76].

The liquid portion of the mash, known as wort, is recovered in a filtration operation, and transpose to the kettle where it is boiled. Many brewers add some adjuncts at this stage. Adjuncts are starch source materials employed for brewing (but not essential for beer production) for several reason such as to enhance beer foam stability, to impart elements of beer characteristics like colour, flavour, or body, or to produce beer at lower cost — adjuncts generally cost less than barley malt and can be an alternative source of fermentable extract, used to replace a portion of the barley malt [88]. Most brewers at this point, also introduce at least a proportion of their hops (usually extracts or pellets) [76].

After the precipitate produced during boiling has been removed, the hopped wort is cooled and pitched with yeast. Fermentation is complete when the desired alcohol content has been reached and when unpleasant compounds, developed during fermentation, have been mopped up by yeast [76].

In traditional lager brewing the ‘green beer’ is matured by several weeks of cold storage, a process called “aging”, used to produce a stable, quality product suitable for filtration and packaging.

Filtration is often conducted to clarify the beer as well as to contribute to the microbiological stability of many beer types. However, filtration can remove other important compounds critically important to the mouthfeel, flavour, and aroma profile, as much as many of the possible healthy compounds in beer, like polyphenols. Usually, the two main types of filter in use are kieselguhr and perlite, once they leave no residue in the beer [76].

Fermentation in Lager beer production

Saccharomyces own several properties including fast growth, efficient glucose repression, good ability to produce and consume ethanol, and a tolerance for several environmental stresses, making them a perfect model for domestication [112].

The handling of the yeast is key to the efficiency of brewery fermentations and to the quality of the final beer, therefor, is important to preserve the vitality and viability of the culture in order to retain its predictably behave. Yeasts usually are propagated from a laboratory culture to full-scale pitching, and stored for reuse while maintaining its phenotypical homogeneity [88] [113].

Traditionally, lager beer is produced by bottom-fermenting yeasts at fermentation temperatures between approximately 5°C and 15°C [88]. Studies identify the *Saccharomyces pastorianus* used in lager beer production (syn. *Saccharomyces carlsbergensis*) as a domesticated species created by the fusion of a Saccharomyces cerevisiae ale-yeast with a cryotolerant *S. eubayanus* sp. nov. [74]. Besides de difference in fermentation temperatures and yeast flocculation (top or bottom), one major distinguishing difference between ale and lager strains concerns to the capability of lager yeasts to ferment melibiose, a reducing disaccharide formed by an alpha-1,6 linkage between galactose and glucose. lager yeast possess the MEL genes to produce the enzyme α-galactosidase (melibiase), different from ale strains that do not produce α-galactosidase and therefore are unable to utilize melibiose [88].

Beer yeast obtains energy by two metabolic pathways: respiration (in the presence of oxygen) allowing the yeast to grow, and fermentation (in absence of oxygen). Wort fermentation in beer production is largely anaerobic, however, limited oxygen must be made available to the yeast to achieve balanced fermentations. Ensuring the correct yeast oxygenation is necessary for good yeast growth and production of desired flavour compounds, for example, oxygen is an essential nutritional element for the biosynthesis of ergosterol and unsaturated fatty acids [114].

The principal objective of wort fermentation is to consistently metabolize wort constituents to produce beer with satisfactory quality, stability, and drinkability. For that, yeast uses the wort sugars like sucrose, fructose, and glucose representing around 20%, maltose being the most abundant sugar, around 60%, and maltotriose representing around 20%, together with dextrin material and nitrogen to produce alcohol, carbon dioxide (CO_2), new yeast cells, and a plethora of flavour compounds [115].

The uptake pattern of sugars usually follows the route of simplest sugars (the monosaccharides glucose and fructose) first, followed in increasing order of complexity by disaccharides (maltose) and trisaccharide (maltotriose). Both complex sugars are hydrolysed to glucose units by the α-glucosidase system, however, uptake and hydrolysis of maltose and maltotriose from the wort is dependent on the glucose concentration. When the glucose concentration is high the MAL genes are repressed. Yeast also requires nitrogen, mainly in the form of amino acids, vitamins and minerals for optimum growth and fermentation. Zinc and magnesium are essential for yeast fermentation acting as a cofactor for many yeast enzymes, including the zinc-metalloenzyme alcohol dehydrogenase, the terminal step in yeast alcoholic fermentation. Magnesium is important for key metabolic processes and is directly involved in ATP synthesis. Like zinc, magnesium also protects the cell from stress. Copper, iron, and calcium also are needed for the cellular homeostasis [114].

The formation of excretion products, like ethanol, depends on the overall metabolic balance of the yeast culture, and is influenced by several factors, like incubation temperature, adjunct level, wort pH, buffering capacity, wort gravity, oxygen, and pressure [88]. There are several other compounds found in beer, resulting from fermentation, such as carbon dioxide, glycerol, higher alcohols, fatty and organic acids, esters, aldehydes, ketones, vicinal diketones and sulphur compounds which give character to the beer [90]. There are, however, some flavour compounds that are unpleasant and need to be reduced or removed [88]. A level of CO_2 is left remaining in the beer to give it appropriate carbonation and effervescence [88].

Alcoholic *vs* non-alcoholic beer

The non-alcoholic beer or alcohol-free beer, production is an effort to appeal to consumers who would appreciate its flavour attributes without the alcohol constraints, being strategically positioned between beer and soft drinks [116]. Non-alcoholic drinks or very low alcohol drinks become more present nowadays, partly as a result of drinking and driving concerns (social and legislative), general health concerns such as the consumption during pregnancy, abstinence and religious reasons [92].

In most of the EU countries terminology is not consensual for beers with diminished alcohol content. They are often divided into low-alcohol beers (LABs) with no more than 1.2% alcohol by volume and alcohol-free beers (AFBs) containing ≤ 0.5% alcohol by volume, also frequently addressed as non-alcoholic beers (NABs) [116]. In this dissertation, terminology on beer labelling's will be according to common designation, as non-alcoholic beer (NAB) ≤ 0.5% alcohol by volume, and beer with 0.0% alcohol by volume shall be named as zero-alcohol beer (ZAB).

Strategies to produce NABs or ZABs can be divided into physical and biological processes. Biological approaches take place when limiting ethanol formation during the beer fermentation. They can be performed in traditional brewery simply by the method of arresting fermentation at some point, for example by cooling down the system, and hence do not require additional equipment

(this is method most used); by purchasing genetically modified yeasts, which fermenting performance is design for the purpose; or, by changing the mashing process and limiting the levels of the fermentable sugars. There are also production processes using continuous fermentation with immobilized yeast and requiring special equipment. These processes limit the formation of ethanol but not to a level of zero content. If that specification is required, they must be, therefor, complemented with other physical technics in order to achieve that purpose. Also, this methods of beer dealcoholisation usually affect the sensory perception of the final product in a negative sense [116].

By interfering with the fermentation process, the formation of some specific compounds is expected not to happen, or to happen in a lesser degree. The same outcome is expected when using physical methods for separate alcohol from beer, as certain compounds usually present in beer are more likely to be loss, although many processes include their recovery [116].

The so-called physical methods are based on gentle removal of alcohol from regular beer and require considerable investments into the special equipment for alcohol removal. It can be employed thermal technics, such as evaporation and vacuum rectification, or by membrane, including dialysis or reverse osmosis [116][117].

Thermal dealcoholisation is the simplest method but may present major disadvantages related to the use of temperatures, even if lower when pressure is applied, resulting in loss of volatiles. Vacuum rectification makes use of a vacuum column for dealcoholisation and allows the separation of the aroma compounds in a vacuum degasser, which are later, recovered into the dealcoholized beer in an extent of 6% to 20% of the original beer [116][117]. Both dialysis, and reverse osmosis are membrane processes that differ in applied pressures and temperatures, membrane materials and their structures. The principle is based on the semipermeable character of the membranes [116][117]. Dialysis technic makes use of the concentration gradient of compounds between beer and dialysate by means of diffusion, based on the principle of the counter current flow. The concentration of all the dissolved substances on both sides of the membrane try to come into equilibrium [117]. In reverse osmosis, the beer (concentrate) is passed through a semipermeable membrane and the alcohol–water mixture (permeate) permeates the membrane selectively when transmembrane pressure substantially exceeds the osmotic pressure of beer. The first phase is the concentration of the original beer by removing permeates. Subsequently during the diafiltration phase the permeate removed from beer is quantitatively replaced by demineralized water until a desired alcohol content has been achieved. This process is industrially unfeasible for obtaining ethanol concentrations below 0.45%, due to the increasing consumption of diafiltration water [116]. Improvements in recovery techniques are mostly directed towards the recovery of the aromatic profile, yet, most studies are not enlightening regarding other compounds, such as polyphenols.

Overall, some of the processes eligible to brew a high-quality lager beer, are designed to eliminate less flavoured compounds and, by drag, end up eliminating other compounds that could be of interest in a health point of view.

Table 2 - Relevant compounds that can be found in filtrated beer

	Phenols	Dietary fibres	Minerals	Vitamins	Organic and fatty acids
Hop (25% poly-phenols)	· Xanthohumol · Desmethylxantho-humol · Isoxanthohumol · Catechin · Proanthocyanins				
Malt (75% miner-als; 75% poly-phenols)	· Ferulic Acid · Catechin · p-Coumaric acid · p-Hydroxybenzoic acid · Sinapic acid - vanillic acid	· β-glucan · Arabinoxylan	· Phosphate · Nitrate · Sulfate · Chloride · Sodium · Potassium	· Folate · Riboflavin · Pantothenic acid · Niacin	
Water (25% min-erals)			· Magnesium · Silicon · Calcium · Sodium		
Yeast					· Lactic acid · Pyruvic acid · Succinic acid · SCFAs (butyric, acetic, propanoic)

Aims

This dissertation focusses on the influence of moderate beer consumption, with and without alcohol, on intestinal microbiota and metabolic markers in male healthy volunteers, based on preliminary results on “Microal” clinical trial.

2

CHAPTER II

Methodology

Our study was a double-blinded, three-arm parallel-group, randomized controlled study to evaluate the effect of the consumption of beer with or without alcohol (5.20%, 0.5% max., and 0.00 %; V/V), for 4 weeks, on healthy individuals. This study followed a methodology already published by Queipo-Ortuno *et al* [83].

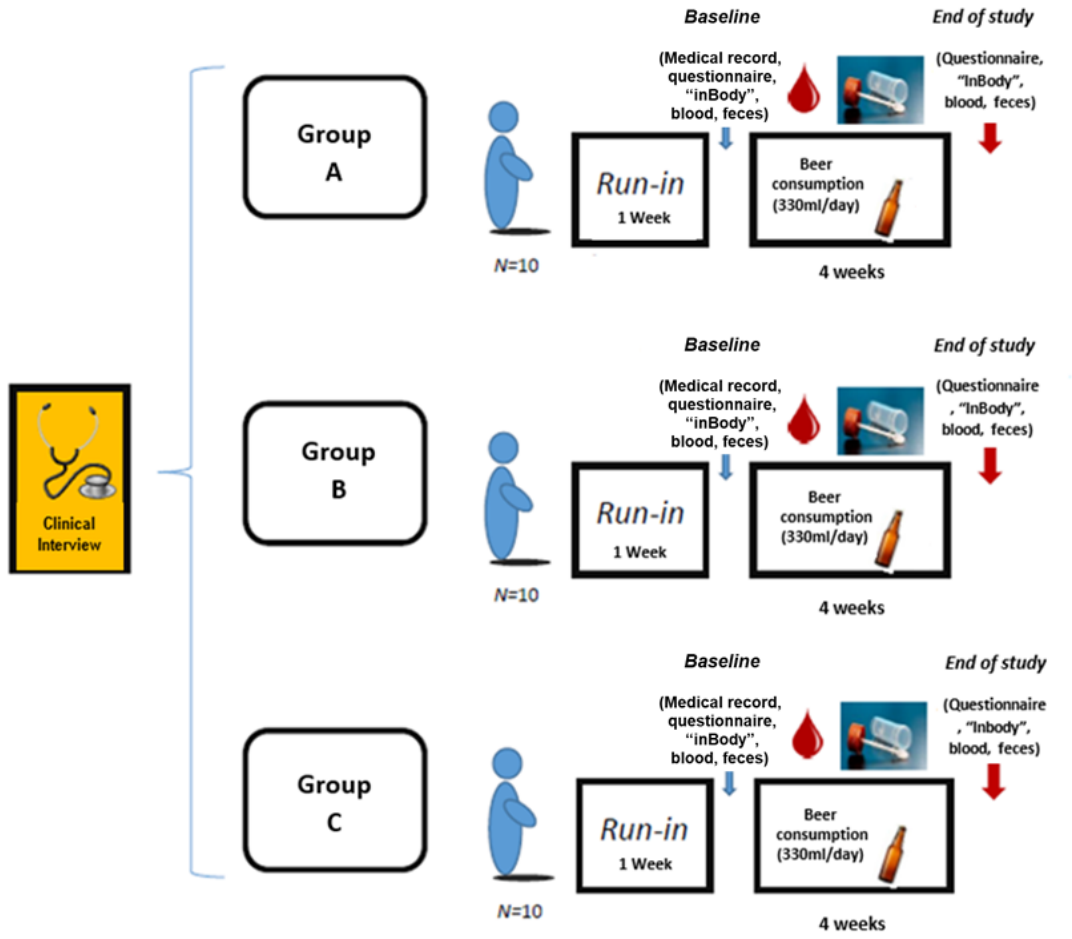

Figure 4 Study design, adapted from the protocol study sent to approval to the ethical committee

Selection of participants

Subjects were recruited from Lisbon metropolitan area, through advertising via media channels, online social groups, and newspapers (figure 5). Subjects were contacted by e-mail or phone contact according to the order of their voluntary registration. Volunteers were first submitted to a medical interview intended to apply the inclusion and exclusion criteria to enroll the subjects. Full information about the procedures and the aim of the study was given at this point. All the participants elected for the study gave written informed consent. The clinical protocol was approved by Ethics Committee of the Nova Medical School. The clinical trial was conducted from March 2018 to May 2018, in accordance with ethical principles of the Declaration of Helsinki, international law and Good Clinical Practice guidelines; it was registered in ClinicalTrials.gov Database, reference NCT03513432.

Inclusion and Exclusion criteria

The study involved healthy adult men aged 18-65 years, moderated alcohol consumers, capable of provide informed written consent. The participants were not receiving treatment for diabetes, hypertension, or dyslipidemia our any other relevant metabolic disease, nor did they have been diagnosed with any acute or chronic inflammatory diseases with relevant effect on gastrointestinal system, infectious diseases, viral infections, cancer, or a previous cardiovascular event at study entry. They have not reported any alcohol or substance abuse related ailment and they had not received any antibiotic therapy, prebiotics, probiotics, or vitamin supplements or any other medical treatment influencing intestinal microbiota during the 4 weeks before the start of the study or during the study (including the run-in period).

Beer

Three commercial beer types were used in the study: alcohol (% V/V): 5.2 – alcoholic beer (AB), alcohol (% V/V): max. 0.5 – non-alcoholic beer (NAB), and alcohol (% V/V): 0.0 – zero-alcohol beer (ZAB), supplied by Super Bock group.

Randomization

Eligible volunteers were to be randomized into 3 intervention groups (ratio 1: 1: 1). Enrolled volunteers were invited to select a random sealed envelope containing a number (1 to 10), and a letter (A, B or C) correspondent to the beer tag. The intervention starting day was chosen from an interval of week days, according to the participants availability and not according to the group order.

Beers were labelled by an external investigator as A, B or C according to their alcoholic content, and the link between letters and type of beer was not established for the rest of the study group investigators.

Figure 5 Advertising copy for recruitment of trial participants

Intervention

After selection, volunteers went a run-in period (1 week), in which they were instructed to not change their physical activity levels and maintain their dietary pattern, followed by 1 period of 4 weeks' intervention with the dietary supplement (NAB (0.5% alcohol max.), ZAB (0.0% alcohol), or AB (5.2% alcohol)), 330mL (1 bottle) each day, preferably at dinner, according to their group. The subjects were asked not to change their dietary and lifestyle habits and to avoid over consumption of alcoholic beverages during the study.

Compliance

Volunteers adherence to the study (daily consumption of beer) was monitored through self-reported questionnaire for beer consumption frequency at the time of each weekly beer collection. A final questionnaire was applied to participants asking if there were changes made in alcohol consumption, diet pattern and physical activity during the study and their perception about the type of beer they had consumed (if with or without alcohol).

Evaluation methods

Each participant provided 2 different fecal samples: a first baseline sample after the run-in period and a sample at the end of the intervention study. Blood samples were also collected under 12-hour overnight fasting conditions, at baseline and after the intervention period. At baseline, PREDIMED questionnaire [118] was applied to frame participants dietary pattern. Also, at baseline, a structured questionnaire was adapted to provide additional data on social-demographic, alcohol consumption habits and physical activity. Anthropometric measures were conducted using the ®InBody770, USA, at baseline and at the end of the study.

Outcome measures

Microbiota

Participants were supplied with the EasySampler complete stool collection kit, already containing RNAlater- Stabilize and protect RNA with immediate RNase inactivation (Sigma-Aldrich) for RNA stabilization and storage. Instructions were given for the sample collect being made in the same day as the delivery. Fecal samples were received, registered, and immediately stored at 80ºC until analyzed.

<u>DNA extraction</u>

The NZY Tissue gDNA Isolation Kit (NZYTech, Lisbon, Portugal) was used to extract genomic content from the stool samples with some alterations to the protocol. The samples were weighted to 170-200mg, homogenized with TE buffer (10 mM Tris/HCl; 1 mM EDTA, pH 8.0) and centrifuged at 4000 x g for 15 min. The supernatant was discarded, and the pellet was resuspended in 350 μL of buffer NT1. After an incubation step at 95 °C for 10 min, samples were centrifuged at 11000 x g for 1 min. Then, 25 μL of proteinase K were added to 200 μL of the supernatant for

incubation at 70 °C for 10 min. The remaining steps followed manufacturer's instructions. The DNA concentration was assessed by absorbance at 260 nm, with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the purity was estimated by determining the A260/A280 ratio.

Sequencing

An Ion 16S Metagenomics Kit was used to amplify the hypervariable regions of the 16S rDNA gene from bacteria isolated from fecal DNA. This kit employs two primer sets targeting the V2-4-8 and V3-6, 7–9 hypervariable regions, paired with Environmental Master Mix v2.0. Following manufacturer's instructions, PCR amplifications were prepared with 1 µL of the extracted fecal DNA diluted in purified water, to obtain a final 1.5 ng/µL concentration. Two separate pools for each sample using the two primer sets were run on a Veriti™ 96-Well Thermal Cycler (AB Applied Biosystems™, Thermofisher Scientific, US). PCR conditions for construction of all sequencing libraries consisted of one initial cycle at 95°C for 10 min; 25 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 20 s; and a final incubation at 72°C for 7 min. Following amplification, PRC pools from the two reactions were combined, and PCR purification was performed by using the AMPure XP magnetic beads (Agencourt, Beckman Coulter, Inc., Indianapolis, IN), and eluted in 6.5µL of nuclease free water. PCR amplification products (amplicons) were assessed by running aliquots of the reactions on the 2200 TapeStation System (Agilent). This procedure allowed determining how well sample preparation has gone for next generation sequencing, and the calculation of DNA input for library preparation. Results of DNA fragments are represented as bands out on a mini electrophoresis system, with a DNA concentration value associated. Strong amplicon bands at the expected size ranges were detected in all samples, indicating that reaction inhibition was absent or minimal. Pooled amplicons from each sample were diluted in purified water and a concentration of 2.5 ng/µlL was achieved. The sample amplicons were end-repaired using the Ion Plus Fragment Library kit following the manufacturer's instructions and barcoded according to sample assignments using in conjunction the Ion Xpress Barcode Adaptors 1–16 kit (Thermofisher Scientific, US). Repaired amplicon products were purified using AMPure XP magnetic beads and eluted in 6.5µl low Tris-EDTA (low-TE) buffer. Subsequently, Platinum PCR Supermix High fidelity kit (Thermofisher Scientific, US) was used to amplify the repaired amplicons and construct the library, followed by another purification step using the AMPure XP magnetic beads. Each DNA library was diluted (1:10) in Nuclease free water and quantified by using 2200 TapeStation System (Agilent) to evaluate the sample quality. Finally, next-generation sequencing of the 16S rRNA gene fragment libraries was performed on an Ion PGM System (Life Technologies) using Ion PGM 400 sequencing reagents and Ion 318v2 chips following the manufacturer's instructions.

Sequencing was performed at Ophiomics, in Germano de Sousa central laboratories, Lisboa.

Blood and biochemical analysis

Venous blood samples were collected into one serum separator tube, one EDTA containing tube, and one falcon tube. Samples were processed and analyzed in an outsourced certified clinical laboratory (NP EN ISO 9001:2015) - (BMAC, Lisboa) for the biochemical outcomes variables.

The falcon tube blood sample was centrifuged at 3200 RPM, for 15 minutes at room temperature, and a serum aliquot was immediately stored in a special cool transport container (at <- 18°C) for LPS quantification.

Serum concentrations of lipoproteins were measured by spectrophotometry of total cholesterol, triglycerides, HDL, and LDL. Glucose metabolism was accessed by measuring fasting glucose through spectrophotometry method, insulin was determined through quimioluminescence method and Glycated Hemoglobin through HPLC method. The hepatic function was evaluated by measuring the levels of transaminases through spectrophotometry method for Aspartate Aminotransferase (ASAT) and Alanine Aminotransferase (ALAT), Gama-Glutamyl Transferase (GGT), Alkaline Phosphatase and Albumin. The inflammatory marker C-reative protein (CRP) was determined through immunoturbidimetric assay, and total Creatine Kinase cardiac marker was measured through spectrophotometry method.

The insulin resistance was estimated using the "Homeostasis Model Assessment-Insulin Resistance" (HOMA-IR), applying the formula [((glucose/18.01) × insulin) / 22.5]; considering the variables fasting glucose (mg/dL) and fasting insulin (µU/mL) [119].

LPS quantification in plasma was performed using the Chromo-Limulus Amebocyte Lysate (Chromo- LAL) reagent (Associates of Cape Cod, Inc.., Falmouth, MA, USA). Briefly, serum samples were diluted 1:50 in ultrapure water (Merck Millipore, Billerica, MA, USA) and heated for 15 min at 70ºC. Samples and Chromo-LAL were incubated at 37ºC for 40 min and absorbance was read every 20 seconds at 405 nm in a spectrophotometer.

Lifestyle parameters

A complete medical record was obtained, including data on alcohol intake, smoking habits, anthropometric measures (weight, height, waist circumference, hip circumference and BMI calculation). All information was converted into data by using the SPSS statistics program (version 22 software, SPSS Inc. Chicago, IL).

Characterization of beers under study

Chemical analysis of the three beer's composition of minerals were performed at the Centro de Apoio Tecnologico Agro-alimentar (CATAA), Castelo Branco.

Determination of minerals

The beers were first degassed (50 mL) in an ultrasonic bath (Elma, S300H) for 10 minutes. An approximate amount of 2 g was weighed, and the exact weight was recorded in order to quantify the mineral content of the samples. For digestion, 6 mL of nitric acid specific for analysis of trace metals (Prolabo 69%) and 2 mL of hydrogen peroxide (Prolabo 30%) were added. The sample was then digested in a microwave (Milestone, Ethos One). After completion of the digestion, ultrapure water was added to the samples until the final volume of 50 mL was reached. Calcium, sodium and potassium elements were quantified by flame atomic absorption spectrophotometry (Thermo Scientific, ICE 3000). While phosphorus and magnesium by Inductively Coupled Plasma Optical Emission Spectrometry, ICP-OES (Horiba Jobin Yvon, Activa M). The protocol was elaborated end executed in CATAA.

Statistical analysis

Statistical analysis was performed including all participants who finished the study and complied to the diary beer intake. The numeric results at baseline and after intervention are expressed as means ±SDs. Intervention effects within intervention group were calculated as the difference between the changes from baseline measures and after the 4-wk intervention period by using the non-parametric Wilcoxon signed-rank test with statistical significance set at a 2-sided p value. A linear mixed model for repeated measures with compound symmetry as covariance structure was used to compare intervention changes between groups. Compound symmetry was used, instead of the unstructured structure because it resulted in the best fit according to a likelihood ratio test. Intervention and time were included as fixed variables. Statistical significance was set at a *P* value < 0.05.

Table 3 - Trial Chronogram

Tasks	March/2018	April/2018	May/2018	jun/18	July/2018	Aug./2018	Sept./2018
Disclosure	X						
Enrollment	X						
Medical Interviews	X						
Run-In	X	X					
Collecting samples at baseline	X	X					
Intervention (4 weeks)	X	X	X				
Collecting samples at study final		X	X				
Biochemical Analysis (blood samples)	X	X	X				
DNA extraction (fecal samples)				X			
DNA sequenciation					X	X	
Statistical Analysis						X	X

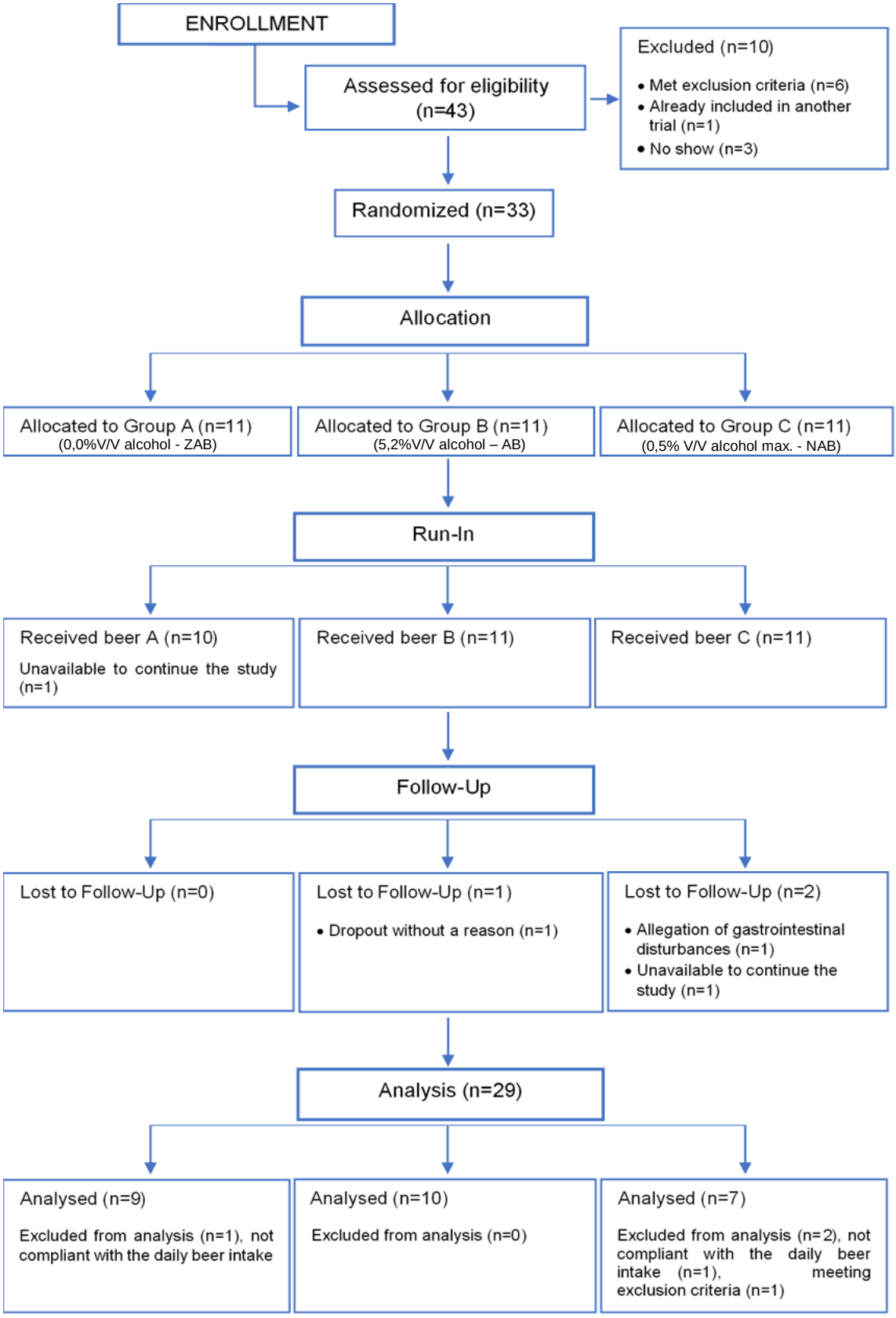

Figure 6 Trial flow-chart

Results

Baseline Characteristics

Of the 43 subjects screened to assess eligibility to participate in the study protocol, 33 were randomly assigned, and 29 completed the intervention. Of those, 26 were included in the statistical analysis (Figure 6). Three individuals were excluded from statistical analysis: two were not compliant with the daily beer intake, established at a minimum of 75% of adherence, and one presented biochemical results compatible with the exclusion criteria (Hb A1C > 6.5% - Diagnosis of diabetes (Norma DGS 033/2011)). Statistical outcomes were reproduced from a universe of n=9 for group A, n=10 for group B, and n=7 for group C (Figure 6).

Participants in the study were healthy men, with a mean age of 34 years (range: 19-58 years), BMI (kg/m^2) between 16.6 and 38.7 (1 underweight, 11 normal weight, 8 overweight and 6 obese), and body fat mass (BFM %) between 4% and 58% (11 within limits and 15 out of limits). The Kruskal-Wallis test ($P > 0.05$) showed that individuals had similar distribution for the referred parameters between groups (group A, B and C).

Results of participants PREDIMED questionnaire showed a mean score correspondent to “medium adhesion” to the diet in all groups. The Kruskal-Wallis test ($P = 0.139$) showed that the diet pattern was similar between groups.

All participants included in statistical analysis had normal fasting glucose at baseline, 1 was at borderline for fasting serum HbA1c (> 5.8% - < 6.5%) and 25 presented normal measures for fasting serum HbA1c (3.8% - 5.8%). As to the standard lipid profile, 18 had normal fasting total serum cholesterol (< 200 mg/dL), 7 had borderline-high fasting total serum cholesterol (200 – 239 mg/dL) and 1 had high fasting total serum cholesterol (> 240 mg/dL). Twenty participants had optimum fasting HDL serum cholesterol (> 45mg/dL), and 6 had low fasting HDL serum cholesterol (< 45mg/dL). As for triglycerides, 21 participants were within the acceptable range for normal fasting total serum values (< 150 mg/dL) and 5 had high fasting total plasma triglycerides (> 150 mg/dL). All test results showing alterations for glucose and lipid metabolism or possible impairment of hepatic, cardiac, and/or renal function were referred to the medical for clinical interpretation. A comprehensive metabolic panel of the 26 study participants that were included in the statistical analysis is listed below (Table 4), with the outcomes grouped according to the physiological evaluation measures and individual’s distribution on the study.

Table 4 - Participants characterization and biochemical profile at baseline

	Group A (ZAB)	Group B (AB)	Group C (NAB)
	Mean ± SD	Mean ± SD	Mean ± SD
Characterization of participants			
Age	34.0 ± 11.0	36.0 ± 13.0	30.0 ± 9.0
Weight	83.5 ± 24.9	78.2 ± 13.4	85.9 ± 14.9
BMI	26.3 ± 6.7	25.2 ± 3.7	27.5 ± 4.1
BFM	21.5 ± 17.4	14.3 ± 8.8	21.1 ± 10.0
Assessment of glucose metabolism markers			
Glucose	80.0 ± 9.0	83.5 ± 10.2	83.1 ± 6.9
HgA1C	5.1 ± 0.3	5.4 ± 0.4	5.3 ± 0.3
Insulin	7.6 ± 4.0	5.4 ± 2.3	8.7 ± 5.9
HOMA-IR	1.5 ± 0.9	1.1 ± 0.5	1.8 ± 1.3
Assessment of lipid metabolism markers			
Total Chol	179.9 ± 37.3	178.3 ± 36.5	188.9 ± 26.5
HDL	49.1 ± 12.4	50.3 ± 3.7	50.7 ± 7.7
LDL	104.6 ± 28.7	107.4 ± 28.4	119.7 ± 22.9
VLDL	26.2 ± 18.3	20.6 ± 16.1	18.4 ± 12.5
Triglycerides	132.1 ± 92.3	103.2 ± 81.4	92.0 ± 62.0
Assessment of inflammatory marker and cardiac function marker			
CRP	0.4 ± 0.6	0.1 ± 0.1	0.3 ± 0.2
CK	146.4 ± 40.5	309.5 ± 188.5	207.6 ± 147.2
Assessment of hepatic function markers			
Albumin	4.6 ± 0.2	4.6 ± 0.2	4.6 ± 0.2
ASAT	30.8 ± 5.8	31.6 ± 6.7	31.4 ± 7.3
ALAT	33.2 ± 12.5	35.6 ± 15.9	35.6 ± 18.3
ALP	74.6 ± 15.1	84.5 ± 22.7	92.4 ± 25.0
GGT	37.8 ± 23.3	24.8 ± 12.3	42.3 ± 39.1
Assessment of acid-basic balance electrolytes markers			
Cl^-	103.6 ± 2.7	103.1 ± 2.0	102.7 ± 0.8
Na^+	142.3 ± 2.1	142.3 ± 1.3	141.6 ± 1.1
K^+	4.4 ± 0.3	4.3 ± 0.3	4.7 ± 0.3
Assessment of Renal function markers			
Creatinine	0.9 ± 0.4	1.0 ± 0.1	1.0 ± 0.1
Urea	33.0 ± 6.7	37.8 ± 11.0	31.4 ± 6.0

Body mass index (BMI) (kg/m^2) normal range: 18.5 to 24.9; overweight: 25 to 29.9; obese: > 30; Body fat mass (BFM) (%); Glucose (mg/dL) 70 - 100*; Glycated haemoglobin (HbA1c) (%) 3.8 - 5.8*; Insulin (µu/mL) 6.0 - 27.0*; HOMA-IR < 2.33**; Total Cholesterol (Total Chol) (mg/dL) < 200*; HDL Cholesterol (mg/dL) > 45*; LDL Cholesterol (mg/dL) < 110*; VLDL Cholesterol (mg/dL) < 30*; Triglycerides (mg/dL) 50 - 150*; C-reactive protein (CRP) (mg/dL) < 0.50*; Creatine Kinase (CK) (U/L - 37º) < 170*; Albumin (g/dL) 3.5 - 5.2*; Aspartate aminotransferase (ASAT) (U/L - 37º) < 40*; Alanine aminotransferase (ALAT) (U/L - 37º) < 45*; Alkaline phosphatase (ALP) (U/L - 37º) 34 - 129*; Gamma glutamyl transferase (GGT) (U/L - 37º) < 60*; Cl- (mmol/L) 95-108*; Na+ (mmol/L) 136-145*;K+ (mmol/L) 3.5 -5.1*; Creatinine (mg/dL) 0.70 - 1.30*; Urea (mg/dL) < 50*

*Reference values

** Homeostasis Model Assessment index cut-off for insulin resistance for Portuguese population [119]

Microbiota

Table 5 Participants gut microbiota sequencing results at baseline

Baseline	Group A (n=9)	Group B (n=10)	Group C (n=7)
	Mean ± SD	Mean ± SD	Mean ± SD
Genus_not_identified	28.03 ± 11.76	34.06 ± 12.64	32.19 ± 13.40
Bifidobacteria	6.08 ± 6.78	3.28 ± 2.21	4.90 ± 3.59
Bacteroides	8.71 ± 5.06	13.44 ± 12.08	18.65 ± 7.25
Prevotella	21.41 ± 21.19	20.23 ± 18.56	2.11 ± 3.00
Blautia	1.50 ± 3.31	0.81 ± 1.34	0.53 ± 0.24
Clostridium	2.93 ± 1.71	2.69 ± 1.48	3.76 ± 2.61
Eubacterium	2.84 ± 1.66	2.19 ± 2.08	2.40 ± 1.20
Faecaliobacterium	9.18 ± 6.99	6.90 ± 3.84	7.76 ± 3.03
Lactobacilus	1.34 ± 1.46	1.32 ± 1.17	0.67 ± 1.04
Roseburia	3.07 ± 1.97	3.01 ± 2.73	5.97 ± 3.17
Ruminococus	1.94 ± 1.79	2.38 ± 1.71	1.90 ± 1.38
Bacteroidetes/Firmicutes	0.69 ± 0.44	0.85 ± 0.45	0.57 ± 0.17
Shannon_Index	3.13 ± 0.48	3.14 ± 0.42	3.34 ± 0.18

Results on microbiota are expressed as $\log_{10}$ 16s rRNA gene copies/2.5ng of DNA (table 5). The Kruskal-Wallis test was performed on the basis of the ratio Bacteroidetes/Firmicutes. The result (*P*=0,524) showed that individuals had similar Phyla distribution between groups at baseline. Diversity within subjects was accessed by determining the Shannon-diversity Index (SI) based on proportional abundances for the identified genus. Results demonstrate that for all groups, intra-diversity between subjects was high (SI≠0), revealing that, for most cases, there are

not specific bacteria genus dominance, or analysis was not able to find it. The ratio Bacteroidetes/Firmicutes show the Firmicutes Phylum as the predominant in all groups.

Participants compliance and perception of type of beer consumed

Weekly questionnaires revealed a satisfactory compliance to the study protocol. The daily consumption of beer was assessed for the three arms of the study. and the average compliance was 92.8% ± 7.7 for group A. 99.6% ± 1.1 for group B and 92.8% ± 9.4 for group C. There was a significative difference in compliance between groups (p= 0.047). with the B group showing higher percentage of adherence to the study.

Final questionnaire revealed the volunteers perception of the type of beer ingested. if with or without alcohol. and if without alcohol. which type. Details are represented on (Table 6).

Table 6 Participants perception of the of beer ingested

		n	%		n	%
Group A ZAB	"Alcohol"	1	11			
	"No alcohol"	**8**	**89**	"0.0%alcohol"	4	44
				"0.45%alcohol"	4	44
Group B AB	"Alcohol"	8	80			
	"No alcohol"	**2**	**20**	"0.0%alcohol"	1	10
				"0.45%alcohol"	1	10
Group C NAB	"Alcohol"	3	43			
	"No alcohol"	**4**	**57**	"0.0%alcohol"	2	29
				"0.45%alcohol"	2	29

Alcohol (% V/V): 5.2 – alcoholic beer (AB), alcohol (% V/V): max. 0.5 – non-alcoholic beer (NAB), and alcohol (% V/V): 0.0 – zero-alcohol beer (ZAB)

Final questionnaire also indicate that participants were compliant with the recommendation on maintaining their dietary, alcohol drinking, and physical activity habits with a “no” answer of 92.3%, 100% and 100%, respectively.
None of the participants reported adverse effects.

Composition of the three types of beer

Minerals

Composition of minerals for the three beer types was determined in duplicate. Results are listed in the table below.

Table 7 Beer mineral composition

	Na	K	Ca	P	Mg
	Mean (mg/mL)				
ZAB	3.6	63.0	5.2	43.3	10.7
AB	3.0	43.3	3.4	31.1	7.6
NAB	3.2	64.5	3.4	40.7	9.9

Alcohol (% v/v): 5.2 – alcoholic beer (AB), alcohol (% v/v): max. 0.5 – non-alcoholic beer (NAB), and alcohol (% v/v): 0.0 – zero-alcohol beer (ZAB)

Metabolic results after intervention

The anthropometric and biochemical variables of the participants are shown in Table 8.

As for the anthropometric measurements (body fat mass and body mass Index) and glucose metabolism assessment (fasting glucose, HbA1c, insulin and HOMA-IR), no differences were observed in none of the intervention groups, neither between groups; $P > 0.05$.

The electrolytes markers. sodium. and potassium. both displayed differences in the group intervened with the alcoholic beer (5.2% v/v). P=0.0473 and P=0.009 respectively. Sodium had a decreasing from baseline to post-intervention values. while potassium had increased values after the 4wk intake of the 5.2% alcohol beer. Those differences were not observed on the other groups. Significant P values (P=0.005 and P=0.008) between groups indicate differences in sodium and potassium measurements in time (pre- and post-intervention) but when comparing estimated marginal means between groups by using the same linear mixed model (data not shown). there was no statistically significant difference; $P > 0.05$.

There was a statistically significant increasing in blood total cholesterol after 4wk intake of 0.0% V/V alcohol intake. P=0.038. These alterations were not observed on the other groups. No other differences were seen for the remaining parameters of the lipid blood profile, namely, HDL, LDL, VLDL, and triglycerides.

Regarding to the hepatic profile of the participants, differences at baseline and post-intervention were observed in all groups. Alkaline phosphatase (ALP) recorded differences in all intervention groups (P=0.028; P=0.015; P=0.028; for group A, B and C, respectively). In all the groups the ALP values decreased after intervention. Albumin had statistically significant increased values (P=0.017 and P=0.037) in group A (0.0% v/v alcohol) and group C (0.5% v/v alcohol max.), while aspartate aminotransferase (ASAT) values slightly decreased in group C (P=0.046). Statistical analysis between groups showed significant P values for ALP and albumin (P=0.005 and P=0.008 respectively) in time, however. when pairing the groups for comparison, no statistically significant difference was observed; $P > 0.05$. The inflammatory and cardiac function markers did not show significative alterations pre- and post-intervention in neither groups.

Table 8 The effect of 4-wk beer intake (with and without alcohol) on cardiovascular, hepatic, inflammatory and metabolic outcomes

	Group A (n=9) - ZAB			**Group B (n=10) - AB**			**Group C (n=7) - NAB**		
	Baseline[1]	Post-Intervention[1]	*P* value within group[2]	Baseline[1]	Post-Intervention[1]	*P* value within group[2]	Baseline[1]	Post-Intervention[1]	*P* value within group[2]
BFM (%)	21.5 ± 17.4	21.6 ± 17.1	0.722	14.3 ± 8.8	13.9 ± 8.8	0.090	21.1 ± 10.0	21.1 ± 9.9	0.439
BMI (kg/m^2)	26.3 ± 6.7	26.1 ± 6.7	0.088	25.2 ± 3.7	25.2 ± 3.9	0.833	27.5 ± 4.1	27.4 ± 4.2	0.347
Glucose (mg/dL)	80.0 ± 9.0	78.7 ± 6.1	0.673	83.5 ± 10.2	81.0 ± 10.1	0.237	83.1 ± 6.9	80.6 ± 10.1	0.463
Hg A1C (%)	5.1 ± 0.3	5.1 ± 0.2	1.000	5.4 ± 0.4	5.2 ± 0.3	0.172	5.3 ± 0.3	5.3 ± 0.3	0.102
Insulin (µu/mL)	7.6 ± 4.0	8.8 ± 8.6	0.594	5.4 ± 2.3	5.8 ± 3.9	0.759	8.7 ± 6.0	8.2 ± 5.7	0.499
HOMA-IR	1.6 ± 0.9	1.8 ± 1.9	0.594	1.1 ± 0.5	1.2 ± 0.8	0.721	1.8 ± 1.3	1.6 ± 1.0	0.310
Sodium (mmol/L)	142.3 ± 2.1	141.2 ± 1.4	0.079	142.3 ± 1.3	141.3 ± 1.1	0.047*	141.6 ± 1.1	141.4 ± 1.4	0.564
Potassium (mmol/L)	4.4 ± 0.3	4.5 ± 0.4	0.172	4.3 ± 0.3	4.8 ± 0.4	0.009*	4.7 ± 0.3	4.7 ± 0.4	0.750
Total Chol (mg/dL)	179.9 ± 37.3	195.3 ± 37.1	0.038*	178.3 ± 36.5	178.2 ± 40.0	0.683	188.9 ± 26.6	193.3 ± 36.2	0.671
HDL (mg/dL)	49.1 ± 12.4	47.6 ± 12.8	0.212	50.3 ± 3.7	49.3 ± 3.6	0.766	50.7 ± 7.7	49.1 ± 6.7	0.246
LDL (mg/dL)	104.6 ± 28.7	114.7 ± 27.1	0.260	107.4 ± 28.4	112.0 ± 33.4	0.263	119.7 ± 22.9	124.7 ± 27.5	0.249
VLDL (mg/dL)	26.2 ± 18.3	34.8 ± 37.7	0.477	20.6 ± 16.2	16.9 ± 11.1	0.123	18.4 ± 12.5	19.4 ± 14.5	0.553
Triglycerides (mg/dL)	132.1 ± 92.3	174.2 ± 188.8	0.441	103.2 ± 81.4	84.4 ± 55.0	0.074	92.0 ± 62.0	97.0 ± 71.7	0.612
ASAT (U/L - 37º)	30.8 ± 5.8	30.9 ± 6.3	0.944	31.6 ± 6.7	54.9 ± 73.8	0.905	31.4 ± 7.4	27.6 ± 3.2	0.046*
ALAT (U/L - 37º)	33.2 ± 12.5	33.4 ± 17.3	0.406	35.6 ± 15.9	46.8 ± 32.9	0.779	35.6 ± 18.3	31.0 ± 15.4	0.176
ALP (U/L - 37º)	74.6 ± 15.1	71.4 ± 13.1	0.028*	84.5 ± 22.7	78.6 ± 17.4	0.015*	92.4 ± 25.0	83.9 ± 24.8	0.028*
GGT (U/L - 37º)	37.8 ± 23.3	47.6 ± 40.1	0.085	24.8 ± 12.3	28.0 ± 16.4	0.759	42.3 ± 39.1	39.4 ± 33.8	0.246
Albumin (g/dL)	4.6 ± 0.2	4.8 ± 0.2	0.017*	4.6 ± 0.2	4.6 ± 0.2	0.350	4.6 ± 0.2	4.7 ± 0.2	0.037*
CRP (mg/dL)	0.4 ± 0.6	0.5 ± 0.9	0.249	0.1 ± 0.1	0.2 ± 0.2	1.000	0.3 ± 0.2	0.4 ± 0.3	0.500
CK (U/L - 37º)	146.4 ± 40.5	204.6 ± 105.4	0.214	309.5 ± 188.5	1482.3 ± 3917.8	0.760	207.6 ± 147.2	153.6 ± 77.9	0.398

[1]Mean ± SD

[2] Wilcoxon signed-rank test

* *P* value < 0.05 within group

Microbiota results after intervention

Values on gut microbiota sequencing result from a combination of statistical analysis from selected regions (V2, V3, V4, and V6,7) of the 16s gene.

Analysis on gut bacterial sequencing results showed statistically significant alterations on the Ruminococcus genus after intervention for group B (*P*=0.022) (table 9).

The Firmicutes phylum maintain the predominance after intervention. The Bacteroides are the dominant genus in the Bacteroidetes phylum for all groups, while the Faecaliobacterium is the dominant genus in the Firmicutes phylum. The SI indicates that dominance of these genus is not statistically significant and diversity after intervention as not changed. As for the Prevotella values at baseline, those results significantly decreased after intervention, but not sufficient to cause statistically alterations within the groups where they showed increased values.

Table 9 The effect of 4-wk beer intake (with and without alcohol) on gut microbiota

	Group A (n=9)			Group B (n=10)			Group C (n=7)		
	Baseline[1]	Post-Intervention[1]	*P* value within group[2]	Baseline[1]	Post-Intervention[1]	*P* value within group[2]	Baseline[1]	Post-Intervention[1]	*P* value within group[2]
Bifidobacteria	6.08 ± 6.78	4.83 ± 3.56	0.678	3.28 ± 2.21	4.65 ± 4.58	0.333	4.90 ± 3.59	5.16 ± 3.22	0.866
Bacteroidetes	29.45 ± 14.58	29.69 ± 10.36	0.441	34.50 ± 10.82	28.64 ± 8.10	0.074	28.37 ± 2.45	31.35 ± 6.45	0.237
Bacteroides	8.71 ± 5.06	10.84 ± 5.95	0.208	13.44 ± 12.08	13.91 ± 11.41	0.721	18.65 ± 7.25	19.65 ± 11.30	0.866
Prevotella	21.41 ± 21.19	9.76 ± 14.86	0.225	20.23 ± 18.56	6.61 ± 8.29	0.080	2.11 ± 3.00	2.82 ± 3.66	0.180
Firmicutes	50.30 ± 16.18	49.58 ± 9.39	0.953	47.28 ± 14.51	50.01 ± 11.94	0.508	52.54 ± 9.84	49.67 ± 10.27	0.398
Blautia	1.50 ± 3.31	0.63 ± 0.46	0.953	0.81 ± 1.34	0.68 ± 0.59	0.507	0.53 ± 0.24	0.56 ± 0.29	0.753
Clostridium	2.93 ± 1.71	2.61 ± 1.33	0.441	2.69 ± 1.48	2.35 ± 1.18	0.285	3.76 ± 2.61	3.35 ± 1.59	0.398
Eubacterium	2.84 ± 1.66	4.26 ± 2.90	0.093	2.19 ± 2.08	2.18 ± 1.80	0.959	2.40 ± 1.20	1.94 ± 1.13	0.310
Faecaliobacterium	9.18 ± 6.99	6.95 ± 3.09	0.594	6.90 ± 3.84	5.38 ± 2.78	0.415	7.76 ± 3.03	7.30 ± 1.84	0.612
Lactobacillus	1.34 ± 1.46	0.64 ± 0.40	0.249	1.32 ± 1.17	1.63 ± 0.73	0.600	0.67 ± 1.04	1.75 ± 1.82	0.345
Roseburia	3.07 ± 1.97	2.58 ± 0.93	0.441	3.01 ± 2.73	3.57 ± 2.77	0.721	5.97 ± 3.17	4.42 v 3.32	0.128
Ruminococcus	1.94 ± 1.79	1.36 ± 1.21	0.139	2.38 ± 1.71	1.37 ± 1.02	0.022*	1.90 ± 1.38	2.25 ± 2.00	0.499
Shannon_Index	3.13 ± 0.48	3.27 ± 0.43	0.953	3.14 ± 0.42	3.33 ± 0.32	0.074	3.34 ± 0.18	3.40 ± 0.22	0.310

[1]Mean ± SD

[2] Wilcoxon signed-rank test

* *P* value < 0.05 within group

3

CHAPTER III

Results discussion

The effects of moderate beer consumption (with and without alcohol) on gut microbiota have never been described. Gut microbiota plays a pivotal role on lipids and glucose metabolism, as previously described in the first chapter. This is a pioneering study which main results can be regarded to future clinical trials and nutritional recommendations.

To evaluate the effect of four weeks moderate beer consumption on male healthy volunteers, an extended panel of physiological markers was assessed.

The three beer's characterization was also assessed by measuring their mineral content. Results can help us understand if beer dealcoholisation can be a major influence on their final composition. The three types of beer analysed (alcohol (% V/V): 5.2 – alcoholic beer (AB); alcohol (% V/V): max. 0.5 – non-alcoholic beer (NAB); and alcohol (% V/V): 0.0 – zero-alcohol beer (ZAB)) didn't have shown major differences in mineral content for sodium, potassium, phosphorus, calcium and magnesium, although there is a tendency for decreasing values for all minerals in the AB, particularly for potassium (Table 6). Those observations are not conclusive and further analysis in a larger sample of beers are essential to establish a relationship, however, considering the recovery technics of compounds when dealcoholized beer is being made (described in the first chapter), it is expected not to find differences in beer's minerals content.

In randomized clinical trials it's important to control confounding factors such as diet and exercise. In the current study volunteers were submitted to questionnaires intended to monitor nutrition and physical activity. Metabolic parameters results were discussed consistently, since variables were controlled trough accessing individuals' dietary patterns with the PREDIMED inquiry. Results showed similar dietary pattern between the arms of the study, and no changes were reported during the trial. According to these results, the changes observed post-intervention could be attributed the beer that volunteers were drinking.

The anthropometric measures (BFM and BMI) were not altered after de beer intake period for neither group. Results are in conformity with other studies who find no association between moderate alcohol drinking with those outcomes [120].

The glucose metabolism evaluation panel (fasting glucose, HbA1c, insulin and HOMA-IR) was not different between groups after the 4wk intervention period. In fact, glycaemic alterations are usually only seen in heavy drinkers with a poor nutritional status and/or with a liver or a metabolic disorder, like T2D. Similar observations were made for insulin resistance [121], although when alcohol is in a polyphenol rich matrix, like red wine, insulin and HOMA – IR levels decrease. These results were also seen for de-alcoholised red wine outcomes, suggesting that polyphenols play the major role in this decrease [122]. Despite the polyphenolic content of beer, the absence of those observations may be due to several aspects, such as the content of polyphenols being too low or not effective on this particular subject, or even the study duration being too short to produce alterations. Nevertheless, it is important to notice that a limitation to our study relates to the dealcoholisation process of beer may provoke a potential loss of non-alcoholic compounds such as polyphenols and other bioactive compounds, resulting in a different composition for the three beers. Xanthohumol and related prenylated flavonoids, polyphenols almost exclusively present in hop-derived products such as beer, have shown beneficial health effects, as exposed in the first chapter. Characterization of the three types of beer concerning their phenolic content, namely the XN and IXN, is warranted to verify the amount of polyphenols in beers of all the intervention groups. This will provide a better understanding upon the effects of XN and IXN, depending on the ethanol beer content. In light of Miranda *et al.* findings, regarding the positive relation between XN intake and glucose metabolism improvement [103], it will be of great interest to quantify the presence of these compounds in beer and evaluate their assimilation by testing for the presence of their metabolites.

Electrolytic balance tends to be very accurate at the vascular system level in order to maintain cell integrity. High sodium and low potassium levels have been linked to vascular disorders such as hypertension and coronary heart disease, [123] additionally, western diets have proven to be each lower in potassium and higher in sodium [124]. In contrast, beer has a high potassium content that could be interesting when included on an equilibrated diet. The study results revealed that both in group A and group C, there was no statistically significant difference in levels of sodium and potassium, between pre- and post-intervention. In group B (5.2% V/V alcohol), however, results show that sodium levels decreased significantly (P=0.047) while potassium levels increased (P= 0.009). Linear mixed model (data not shown) was applied to determine if there were significative differences between the groups. There was a significant difference in time (pre- and post-intervention) for both sodium and potassium levels (P= 0.005 and P=0.008, respectively), meaning that the 4wk period of beer intake was enough to produce effects. However, difference between groups was not statistically significant, which means that the magnitude of the effects

on group B was not of significantly different when compared to the other groups. Another interesting finding was that the potassium content on the alcoholic beer was the lowest between the three beers (Table 6), suggesting that even so, in that particular beer (with alcohol) the levels of potassium absorption could have been higher. To support this assumption, in related futures studies, dosing Na/K ratio in urine can be interesting, as well as keep in the register of blood pressure on the intervened individuals [125].

Lipid metabolism was assessed by measuring blood total cholesterol, cholesterol fractions and triglycerides. The cholesterol fractions (HDL, LDL and VLDL) and triglycerides did not alter significantly between baseline and after intervention for neither groups. Total cholesterol, however, significantly increased in group A (0.0% V/V alcohol) (P=0.038). Other study designed to evaluate the effects of alcohol and polyphenols from beer on atherosclerotic biomarkers in high cardiovascular risk men reported decreasing levels of HDL after the beer intake [126]. That observation was not verified in this study, yet, those results are reported on intervened cardiovascular risk men, suggesting that effectiveness on lipid metabolism may occur more sharply when lipid metabolism is compromised. Several study results associate moderate alcohol consumption with improvement on lipidic levels. Chiva-Blanch *et al.* when evaluating the effects of red wine polyphenols and alcohol on glucose and lipid metabolism concluded that moderate consumption of red wine and gin, but not de-alcoholised red wine, increased plasma HDL concentrations and decreased the LDL/HDL ratio, suggesting that the presence of alcohol was determinant on the effect [122]. A meta-analyses on wine, beer and spirit drinking related to fatal and non-fatal cardiovascular events also evidenced that both for wine and beer consumption there's a J-shaped significant inverse association with vascular risk [127]. Contrastingly, a recent systematic analysis from the Global Burden of Diseases, Injuries, and Risk Factors Study concluded that no level of alcohol consumption improves health. The study supports that potential beneficial traits decurrent of moderate alcohol consumption do not overcome several other health-related harms associated to alcohol intake and alcoholic behaviour on general consumers [128]. Either way, our study duration of 4 weeks may not represent the potential beneficial effects on lipid metabolism for long-term moderate beer consumption, weather with or without alcohol, and/or our population size is too small to establish a relationship.

Considering the possibility for the volunteers to consume an alcoholic drink during the study, liver enzymes were measured to evaluate the hepatic function at baseline and determine if there was a potential harm effect inflicted on any of those participants by drinking the AB. Results showed no significative alterations in all participants. Results post-intervention showed a significant decrease in ALP levels for all the groups (P=0.028, group A; P=0.015, group B; P=0.028, group C). Results are consistent with another study which relates the alcohol's dose-dependent effects on markers of liver function, reporting lower levels of ALP in healthy alcohol moderate consumers [129]**.** In our study decreased levels were also observed in groups drinking 0.5% V/V max., and 0.0% V/V alcohol, suggesting that other components in beer may contribute to the diminishing

values in ALP. ALP is an ecto-enzyme present in liver, bone, and intestinal epithelial cells. The circulating ALP is predominantly of hepatic and bone origin and altered levels can have several explanations; therefore, results cannot be interpreted isolated. A former study investigating the modulation of ALP activity in vascular smooth muscle cells by polyphenols rich beverages, concluded that “lager” type beer had a stronger inhibitory effect on ALP activity than “stout” type beer, although the last one theoretically having a larger polyphenolic content [130]. Considering these findings, in future studies it would be interesting to deepen this relation and investigate if this is a single polyphenol dependent effect or a synergistic effect between other compounds present in beer.

Based on metagenomic analyses of human fecal samples in individuals from three continents, a clustering data model proposed a stratification in the form of three major distinct community taxonomic patterns or “enterotypes”: Bacteroides, Prevotella, and Ruminococcus [3]. It was observed in many studies that gut community composition in healthy adults does not change substantially over long periods of time indicating a generally stable ecosystem and enterotype stability [3]. Each enterotype is supposed to cover a set of possible gut microbiota species that may have evolved to function optimally as a community [11]. Standardized “enterotyping” can be useful to reduce complexity in microbiome characterization and provide general data on health status. Bacterial DNA from stool samples of the participants was sequenced to observe the biodiversity of gut bacteria on those individuals. Analysis of the microbiota generated complex and relatively stable and unique profiles for each individual.

In our study bacterial clusters evaluation to establish the predominance of enterotypes between the groups was not a priority. The analysis of the microbiota is focused on some of the most known bacteria responsible for being directly or indirectly involved in the production of SCFA, therefor, the presented results are also based upon the type of bacteria dominating in each enterotype (Bacteroides, Prevotella, and Ruminococcus), considered important SCFA producers [131]. Results indicate a predominance of Bacteroides and Prevotella in all groups suggesting that microbiota of the participants could be classified as enterotype 1 or enterotype 2. Bacteroides have been strongly related to high intake of protein and fat in long-term diet, while Prevotella genus have been associated with high levels of carbohydrate intake namely fibers, in long-term diet [65]. Our participants scored for a “medium adhesion” on PREDIMED assessment, meaning that they were not fully committed to the Mediterranean diet, which privileges the consumption of fruits, vegetables, and cereals. The findings are consistent with results from diet inquiry and it must be considered that disparities in Prevotella’s results at baseline compared with results after intervention are probably caused by single individuals in those groups, rather than for the intervention itself. The Ruminococcus genus, although not being predominant in any of the groups, showed a significant decrease in our statistical analysis for group B. These results suggest that Ruminococcus genus can be alcohol modulated. In fact, a study relating intestinal bacteria capable of ethanol oxidation with the pathogenesis of ethanol-related colorectal cancer, identified the

Ruminococcus genus as producers of acetaldehyde (AcH) from ethanol under aerobic conditions, and potential accumulators of AcH, along with Bifidobacteria [132]. Same authors demonstrate on another study, decreasing levels on Ruminococcus, associated with non-chronic alcohol consumers, consistent with our findings [133].

Findings indicate that 4wk beer intake was not sufficient to alter the overall community of gut microbiota, suggesting that the non-alcoholic fraction of beer may be insufficient to interfere in gut microbiota composition and favour the grow of selective bacteria.

Potential prebiotic effects of beer were assessed specially by evaluating the presence of Bifidobacterium and Lactobacillus pre- and post-intervention. Strains of Bifidobacterium and Lactobacillus can be enhanced by the use of prebiotics [134], furthermore, these bacteria *genus* are believed to exert positive health benefits on their host [135]. Results express a tendency for both Bifidobacteria and Lactobacillus to increase in groups B and C, while in group A observations were the opposite. Findings suggest that a small amount of alcohol may be necessary to trigger the prebiotic potential in beer, which could be attributed to the polyphenolic compounds or undigested carbohydrates enduring in beer.

Considering the pioneer characteristics of the study, taxonomy data obtained from the samples of the study participants' complex microbiota, must be consider preliminary. The percentage of genus not identified in each individual (around 30%) may have an impact on the present results. Future analysis must be performed in order to optimize the 16S ribosomal RNA gene sequencing method, allowing us to extract a more conclusive response about the influence of moderate beer consumption on human gut microbiota.

Concluding remarks

Beer is a fermented beverage widely consumed across generations. Giving a closer look to the current offer market, it's possible to understand that this trend is not yet to end, quite the opposite. Increasingly, supply is expanding and diversifying, seeking to satisfy consumer preferences. Despite the dozens of different beer types that malt can provide, nowadays it is possible to buy all kinds of beer, proving that is possible to reach every consumer. Another concept on the spot light these days is the production of artisanal beer. The claims made in respect of this product relate not only to the distinct character of the flavor, but also to the improvement that this type of beer may have on health. This means that consumers are increasingly being aware that beer consumption can actually be beneficial to health contradicting the misconception of "belly beer". It is up to the investigators to deepen these allegations and to clarify to what extent it can be affirmed that the consumption of beer is actually beneficial, or, at least not harmful to health.

Many studies have demonstrated that polyphenols can have a protective effect on human health. Polyphenols present in beer have been scrutinized and their beneficial effects have been described, however when a compound is present in a food matrix it must be taken on account the synergistic effects with the other existing compounds. Previous studies on the beer composition lead us to believe that the contents, both the polyphenols resulting from malt and hops, and products resulting from yeast activity, together with other compounds, such as the vitamins, can promote prebiotic properties into the beer.

In this clinical trial we were able to observe that indeed the beers, had effects on biochemical parameters and electrolytic markers, independently of their alcoholic content, and on gut microbiota, on an apparently level of alcohol dependence. This investigation will be further extended and future results on beer composition will allow a more conclusive description on the influence of beer intake in the gut microbiota. Further determination of LPS will also assist to observe if moderate beer intake can yield an anti-inflammatory effect on the intestine. In future beer related studies, deeper investigation to evaluate the bioavailability and activity of the phytoestrogen (8-PN) could also be interesting, considering the exposure to the IXN and his potential bioactivation by selective gut bacteria.

Commercial filtered beer has a negative impact on the availability of beer polyphenols, either if dealcoholized or not. If health claims are proven to be correctly associated with beer, efforts should be made to optimize the brewing process so that there is greater selectivity in the recovery of the compounds proven to be beneficial.

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