UNIVERSITÀ DEGLI STUDI DI PADOVA

Department of Land, Environment Agriculture and Forestry

Second Cycle Degree (MSc)

in Food and Health

Kefir: a powerful fermented milk drink for the prevention of non-communicable diseases

Supervisor

Prof. Alessio Giacomini

Submitted by

Alessia Pegoraro

Student n.

2106663

ACADEMIC YEAR 2024/2025

Index

1. ABSTRACT

Kefir is a fermented milk beverage rich in probiotics. This product with ancient origins is becoming increasingly popular nowadays due to its beneficial effects on human health. The gut microbiota has recently been shown to play an important role in regulating many different biological aspects such as nutrients absorption, immune regulation, and neuronal transmission. It follows that by regulating the composition of the gut microbiota, individual health can be significantly influenced. This study aims to show how kefir, as part of a healthy diet and lifestyle, can help prevent non-communicable diseases. It has been shown that regular consumption of kefir can increase insulin sensitivity in individuals at high risk of developing type 2 diabetes mellitus. Kefir also helps reduce inflammation and regulates the accumulation of cholesterol in the arteries, preventing the risk of atherosclerosis. Recently, promising results have been found on the effect of kefir in limiting the growth and development of cancer cells.

2. INTRODUCTION

This thesis investigates the role of kefir as a powerful probiotic used as a preventive measure for non-communicable diseases. In recent years, there has been growing interest in alternative and natural methods of disease prevention, especially probiotics and functional foods. This is partly due to the advanced scientific understanding of the role of microbiota and the interaction between bacteria and host, made possible thanks to the advent of metagenomics, and partly due to the increasing awareness of the population of the interrelationship between the environment and human health. This philosophy has led people to rediscover some ancient methods of disease prevention. In addition, the spread of diseases due to the global syndemic (obesity, undernutrition, and climate change) has contributed to the urgent need for governments to take action to raise awareness among the population about the importance of a healthy diet and lifestyle in order to prevent the internalization in the healthcare systems.

Subject	n. paper published 2014-2024
Kefir	1996
Kefir + human health	273
Kefir + inflammation	113
Kefir + obesity	78
Kefir + diabetes	65
Kefir + cholesterol	104
Kefir + cancer	86

Table 2: Statistical research on the studies published in Scopus database on the topics treated in this thesis published in the last 10 years

2.1 What is kefir and where it comes from

Kefir is a fermented milk product, made by fermenting cow's, goat's, or sheep's milk with "kefir grains" as a starter culture. The origin of the practice of fermenting milk can be traced back to around 10000 B.C. **(Dhewa et al., 2015)** and was used as a method of preservation. During the fermentation process, microorganisms use milk sugars (mainly lactose) to produce lactic acid. Lactic acid lowers the pH value of milk and thus prevents the growth of spoiling or pathogenic microorganisms that normally cannot survive at an acidity level of less than 4.5. In addition to preventing milk spoilage, fermentation also gives the food some additional organoleptic properties and improves its digestibility **(Sunil et al., 2017)**.

The origins of kefir lie in the Caucasian mountains and in Tibet. People in these Asian regions used to produce it in their households using kefir grains, which they had inherited from their ancestors and passed down from generation to generation **(Sunil et al., 2017)**. The kefir grains have a yellowish colour and an irregularly lobed shape reminiscent of small cauliflower florets. They are composed of a mixture of lactic acid bacteria (LAB), yeasts and acetic acid bacteria that coexist in a symbiotic environment and are held together by a natural matrix of exopolysaccharides (the most common is kefiran), proteins and fats that form the gelatinous and slimy structure **(Apalowo et al., 2024)**. The biological origin of kefir grains has not been scientifically proven yet.

Figure 1.1. Map of the Caucasus region. Two blue arrows indicate the Grater and the Lesser Caucasus Mountains in the picture.

The health benefits of kefir have been first reported by Russian doctors working in the Caucasus region in the second half of the nineteenth century **(Bellamy et al., 2005)**. The consumption of this product helped in the effective treatment of tuberculosis, chronic diseases and intestinal problems of the population. Further studies have identified additional properties of kefir in improving the composition of the microbiota and the inflammatory profile, which contribute to its anti-carcinogenic, anti-hypertensive, and anti-diabetic effects **(Caricilli et al., 2013)**.

2.2 Kefir production

The traditional process for making kefir involves incubating milk with kefir grains at room temperature for 24 h, and at the end of the incubation period, the kefir grains are separated from the product by filtration, while the remaining fermented milk is kept for further consumption **(Otles et al., 2003)**. Nowadays, the production process has also been industrialized. The milk is first sterilized and then incubated with selected microorganisms at 20°C for 24 h. At the end, it is filtered and stored in the refrigerator **(Assadi et al., 2000)**. Many people believe that kefir is the same as yogurt, but in reality these two products have different properties: Yogurt is milk fermented by certain bacteria of the species *Lactobacillus delbrueckii* subsp*. bulgaricus* and *Streptococcus thermophilus,* while kefir contains many other genera of lactic acid bacteria and also yeasts, which give it a more acidic taste (due to lactic acid fermentation) and a little effervescence due to carbon dioxide and a small amount of alcohol (due to alcoholic fermentation) **(Sunil et al., 2017)**. The reason why milk kefir can be frequently confused for a yogurt, is that with the industrial manufacture of the product, the microorganisms are selected to guarantee a more favourable taste to sell it. People, especially in western countries are not very used to fermented foods, so they might not appreciate the sour taste given by yeasts fermentation. To make kefir more popular and saleable, many manufacturers don't include yeasts and select some species of bacteria, so the final product resembles yogurt, also thanks to the addition of fruit syrups and some gelling agents that make it tastier and more palatable, see **Figure 2.2** as an example.

Figure 2.2: Two labels of Kefir products in the Italian market. Image A) is lactose free milk with pomegranate and chia seeds, additives, LAB but without yeasts. Image B) Skimmed milk with starch, additives, LAB but without yeasts.

There is also the water version of kefir. Similarly to milk kefir, the grains in this case also consist mainly of LAB, acetic acid bacteria and yeasts, although the last group is more represented compared to milk kefir grains, which is why water kefir has a higher alcohol content (it should be a maximum of 1% w/v). Water kefir is traditionally prepared by adding 5-10% (w/v) water kefir grains to 5-10% (w/v) sucrose solution **(Cai et al., 2020)**. The most common way to add sucrose to water is to use dried figs, which add some minerals and nutritional properties to the finished food. If the fermentation process, which is carried out at room temperature for 24-72 hours, goes properly, the sucrose in the base solution should be completely converted into ethanol, acetic acid and various other products including volatile flavours. The preparation of water kefir is more complicated than that of milk kefir due to the variability of the fermentation process: the composition of the grains varies due to the available substrates; the use of tap water varies the content of chlorine and fluoride, which can damage the microorganisms in the grains. These factors can worsen the efficiency of the fermentation process, and the final product may contain some ingredients such as higher levels of sugar or trace minerals that are not suitable for some consumers **(Laureys et al., 2017)**. For these reasons, this product needs more attention in terms of safety practices. With the proper manufacturing practices, water kefir appears to have an effective mix of probiotic and beneficial components, like milk kefir, although studies are still ongoing because it is a newer product. It may be a valid alternative for people who cannot consume dairy products or who follow a special diet such as the vegan diet **(Bozkir et al., 2024)**.

It is precisely the biodiversity of the organisms involved in the fermentation process that gives kefir its popularity as a powerful drink full of probiotics. In the following chapters milk kefir microbial and nutritional composition is characterized, since the studies reported in the discussion part are related to it.

2.3 Microbial composition of kefir

Kefir grains consist of lactic acid bacteria, acetic acid bacteria, and yeasts. The microorganisms contained in milk kefir grains can be divided into four groups according to the type of fermentation they carry out: homofermentative and heterofermentative lactic acid bacteria and lactose-assimilating and non-lactose-assimilating yeasts **(Cheirsilp et al., 2011).** The most prevalent bacterial species are *Lacticaseibacillus paracasei* (*Lactobacillus paracasei)*, *Lactiplantibacillus plantarum* (*Lactobacillus plantarum)*, *Lactobacillus acidophilus*, and *Lactobacillus delbrueckii* subsp. *bulgaricus,* which make up the 20% of the lactobacilli in the final product, while *Lactobacillus kefiranofaciens* represents the 80% **(Zanirati et al., 2015)**. For the yeasts, instead, the most represented species are *Saccharomyces cerevisiae*, *Monosporozyma unispora (S. unisporus)*, *Kluyveromyces marxianus (Candida kefyr)*, and *Kluyveromyces marxianus* subsp. *marxianus* **(Diosma et al., 2014).**

Kefir grains are a real example of symbiosis between bacteria and yeasts. Among the yeast species found in kefir grains, there are a few lactose-positive, while the most are able to utilize galactose, lactate, and citrate for alcoholic fermentation. While LAB prefer to metabolize glucose, yeasts can change their metabolism according to the different carbohydrate concentrations and available oxygen, rather by metabolizing lactose into glucose, galactose, and ethanol, or by fermenting galactose left by galactose-exporting LAB **(Lopitz-Otsoa et al., 2006)**. Lactate-positive yeasts can slightly increase the pH, allowing for further growth of LAB, leading to increased lactate production in a cycle which maintains the acidity level stable and favourable for the consumption of the final product **(Cheirsilp et al., 2003)**. Moreover, some yeasts are also lipolytic and proteolytic, and they make important components for cell growth available, contributing to the synergism with LAB.

Lactic acid bacteria are Gram positive, usually non-motile and non-spore-forming, catalase and oxidase negative. They are usually used as starter cultures since they survive well at temperatures between 25-44°C and are strong acidifiers. They ferment carbohydrates into lactic acid, if homofermentative, or into a mixture of lactic acid, acetic acid, ethanol, and CO_2, if heterofermentative **(Mokoena et al., 2017).** The most prevalent species of lactobacilli found in milk kefir is *L. kefiranofaciens*, homofermentative, facultatively anaerobic, and rod-shaped. It is responsible for the production of kefiran, an exopolysaccharide composed of the same amount of glucose and galactose, which forms the matrix of the kefir granules. It is used for technological purposes such as

stabilizer, emulsifier, gelling agent, and thickener, but it is also known for its multiple health benefits in modulating the immune system and the gut microbiota **(Georgalaki et al., 2021).**

2.4 Nutritional characteristics of kefir

As a result of the synergistic fermentation of microorganisms, milk kefir is a source of readily digestible macro- and micronutrients. More than 20% of total concentration are vitamins such as the ones belonging to the B group (B1, B2, B5, B9), essential growth factors, but also vitamin C as a powerful antioxidant, vitamin A and K especially important for their immune-modulatory action **(Hamida et al., 2021)**. Due to the proteolytic activity of bacteria and yeasts especially, kefir also contains many essential aminoacids such as serine, threonine, alanine, lysine, valine, isoleucine, methionine, phenylalanine, and tryptophan, which can also be rapidly absorbed by the brain to control energy balance, support metabolic activities, and be used as precursors for neurotransmitters **(Oladayo et al., 2024)**. There are also different macro-elements in kefir, such as potassium, calcium, phosphorus, and magnesium, essential for bone health and the efficacy of the nervous signal, together with microelements like zinc, copper, and manganese. Recently, traces of alkaloids, phenols, and esters have been found, which contribute to making kefir a powerful antioxidant beverage **(Al-Mohammadi et al., 2021)**. Moreover, there is the important exopolysaccharide kefiran, produced by *L. kefiranofaciens,* with its immunomodulatory action, antimicrobial activity, and health benefits in metabolic disorders. Indeed, all these nutrients, which should also be part of a healthy diet, contribute to modulating the intestinal microbiota with a direct impact on the gut-brain axis **(Peluzio et al., 2021)**. A well-balanced intestinal microbiota is fundamental to guarantee the integrity of the intestinal wall and to regulate the absorption of nutrients and toxins, besides preventing the development of cancer due to chronic inflammation, which can result from dysbiosis. This is the reason why the introduction of kefir in a balanced diet can be crucial for the prevention of non-communicable diseases such as type 2 diabetes, atherosclerosis, and cancer.

2.5 Epidemiology and pathophysiology of type 2 diabetes

Diabetes is one of the top 10 leading causes of death **(WHO 2021)** and is considered a global burden on healthcare systems, as it measurably impairs individual quality of life in DALYs (Disability Adjusted Life per Year). Type 2 diabetes mellitus (T2DM) is particularly prevalent in the Pacific Islands and USA, and its prevalence is increasing in Western Europe. It is characterized by a genetic component, but unlike type 1 diabetes, type 2 diabetes does not manifest itself in childhood, but later in life, as modifiable factors are key to the development of this disease. Urbanization, consumption of unhealthy diets and sedentary lifestyles are considered to be the main risk factors for the spread of the disease in recent years. **(Moien et al., 2019)**.

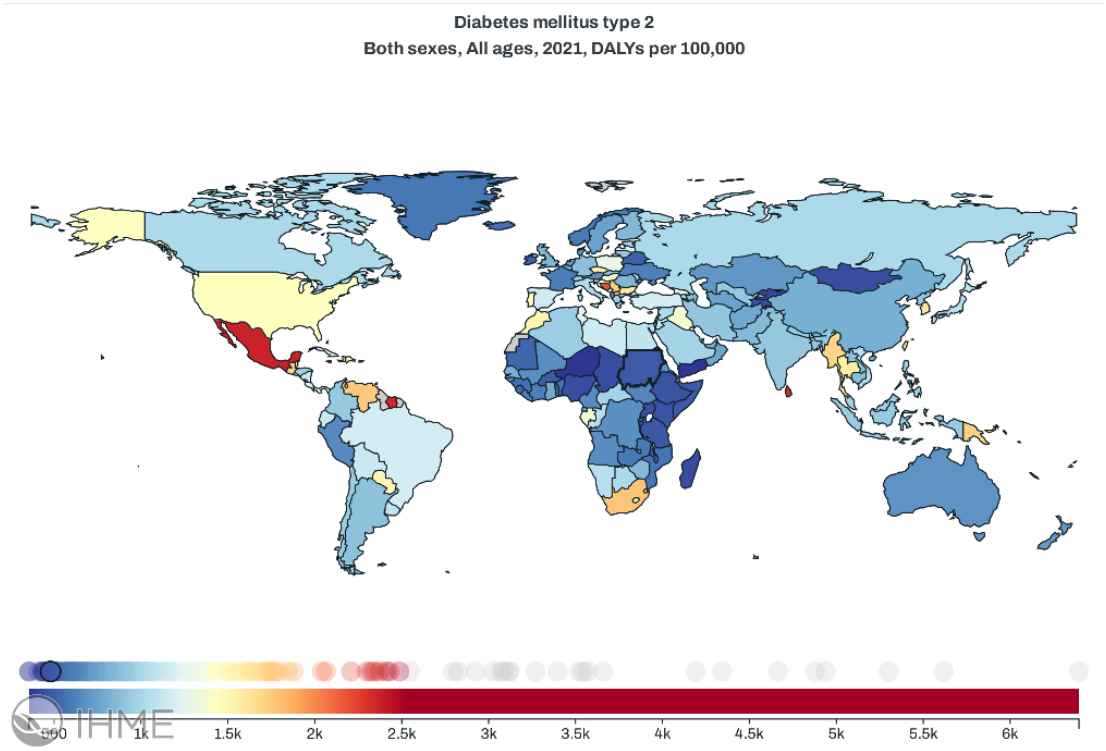

Figure 2.5: DALYs per 100,000 due to type 2 diabetes mellitus over the world in 2021. https://vizhub.healthdata.org/gbd-compare/#0

Over 90% of diabetes mellitus cases are T2DM, a heterogeneous disease linked to a contribution of genetic and environmental factors. It is characterized by both insulin resistance and deficient insulin secretion by pancreatic β-cells **(Stumvoll et al., 2005)**. Genetic predisposition plays an important role in T2DM development. Different genome-wide association studies have been conducted, and the genetic variants contributing to insulin resistance have been grouped: two reduce insulin secretion with fasting hyperglycaemia, nine lower insulin secretion with normal fasting glycaemia, and one alters insulin processing **(Dimas et al., 2014)**. T2DM is a polygenic disease, but environmental factors play a strong impact in disease manifestation and progression.

The pathophysiology of the disease articulates in different phases: normoglycemia, with the development of insulin resistance; prediabetes, β-cells start to produce more insulin to contrast the loss of sensitivity and experience a progressive functional decline because they are not able to sustain the abnormal rhythm; diabetes, the final stage in which β-cells die due to overexposure to stress conditions so the patient presents high glucose levels in the blood that cannot be completely treated with the simple injection of recombinant insulin.

Insulin resistance contributes to increasing glucose production by the liver (glycogenolysis) and decreases glucose uptake by the cells. It is a mechanism that interests different organs; the most important are the muscles, adipose tissue, and the liver. T2DM usually develops with concomitant conditions that contribute to chronic inflammation **(Zheng et al., 2018)**. The prevalence of the

disease is higher in people affected by obesity. Obesity is a disease characterized by the accumulation of fat and dysfunction of the adipose tissue. Hypertrophied adipocytes experience a state of hypoxia, and they start dying. This brings to the accumulation of macrophages and inflammatory cytokines, which disrupt physiological cell signalling, including the one of insulin receptors. Adipose insulin resistance leads to impaired suppression of lipolysis, impaired glucose uptake, and enhanced free fatty acids release even with high insulin levels **(Czech, 2020)**. Moreover, when adipose tissue is no longer able to grow, it starts to accumulate as ectopic fat, affecting other organs, including the pancreas and the ability of β-cells to produce insulin.

Obesity and dysfunctional adipose tissue also affect the muscles. Insulin resistance on this site is the most important extra-pancreatic factor in the development of T2DM. Immune cells infiltration and secretion of proinflammatory molecules by ectopic fat accumulated inside skeletal muscles lead to myocyte inflammation, impaired metabolism, and insulin resistance by paracrine effects **(Petersen, et al., 2002)**. On this point, physical activity represents a very important factor to contrast the development of disease. The contraction of skeletal muscles increases the blood flow, enhancing glucose uptake from plasma; physical activity reduces intra-abdominal fat, which is the most inflammatory one and it has been demonstrated that moderate-intensity exercise improves glucose uptake by 40% **(Ross, 2003).**

Another important condition that can promote insulin resistance is gut dysbiosis. An imbalance in the microbial intestinal flora due to high-fat diets can lead to chronic inflammation. Moreover, intestinal dysbiosis reduces short-chain fatty acid synthesis, which promotes gut barrier integrity, pancreatic β-cells proliferation, and insulin biosynthesis **(Tan et al., 2014)**, contributing directly to the development of T2DM. This is where probiotics such as the ones contained in kefir, together with a healthy diet, can play an important role in the prevention of the disease, reverting the prediabetes stage to the normal physiological condition.

2.6 Epidemiology and pathophysiology of atherosclerosis

Atherosclerotic cardiovascular disease (ACD) is the leading cause of mortality worldwide **(WHO, 2021)**. The most common risk factors for developing the disease are a high body mass index (BMI), high blood pressure, high glucose and cholesterol levels, which is why cardiovascular pathologies are more prevalent in high-income countries where the obesity epidemic exacerbates the spread of cardiovascular diseases. However, rapid changes in the food system have made overprocessed foods available even in lower-income countries, where they have become even more affordable than healthy and nutritious foods **(Barquera et al., 2021)**. The mortality rate due to ACD also depends on the efficiency of healthcare systems, which are indeed more advanced in developed countries, where

the age-adjusted mortality rate tends to decrease, even if heart diseases remain the leading cause of death globally.

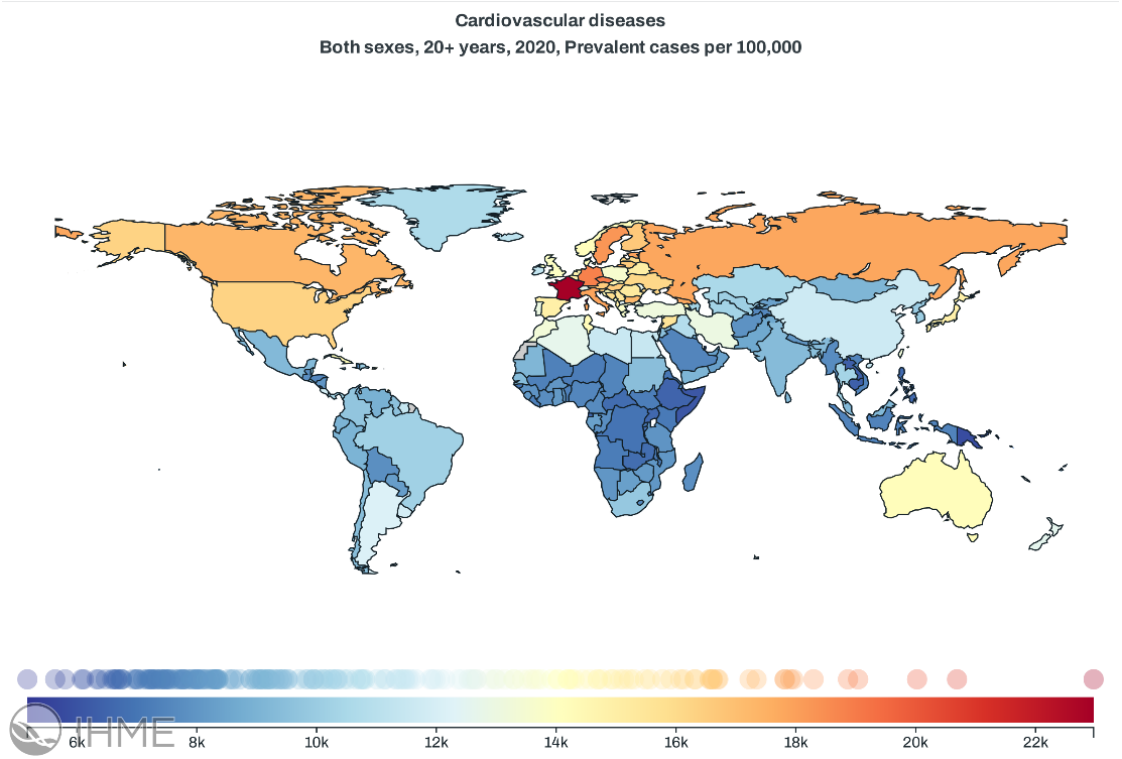

Figure 2.6: Prevalent cases per 100,000 of cardiovascular diseases in the world population over 20. https://vizhub.healthdata.org/gbd-compare/#0

Atherosclerosis is the main risk factor for cardiovascular diseases (CVDs), the main cause of mortality worldwide. Atherosclerosis is characterized by the accumulation of lipids, fibrous elements, and calcification, initiated by endothelial activation and an inflammatory cascade.

The vascular endothelium is formed by a single layer of endothelial cells that, together with collagen and elastic fibres, form the luminal layer. This layer is directly in contact with the bloodstream, and it is surrounded by the tunica media consisting of vascular smooth muscle cells (VSMC) and elastic collagenous tissue. Endothelium is the first defence barrier, which is able to sense mechanical stress (wall shear stress) and changes of metabolic factors in the blood to activate an eventual inflammatory response **(Jebari-Benslaiman et al., 2022)**.

The atherosclerosis condition is characterized by the accumulation of low-density lipoproteins (LDL) in the blood. This altered factor in the blood is sensed by the endothelium, which loses its ability to maintain homeostasis and promotes vasoconstriction, facilitating lipid infiltration. LDL accumulates in the intima, the space between endothelium and tunica media, crossing the endothelium by diffusion or transcytosis. Once in the intima, LDL particles are modified by oxidation (ox-LDL). The extracellular matrix of intima is full of reactive oxygen species (ROS), which easily modify

polyunsaturated fatty acids (PUFAs), but also the direct oxidation by the enzymatic activity of phospholipases and lipoxygenase contributes to LDL modification **(Steinbrecher et al., 1990)**. This process is amplified by the modification of apoB-100, a protein that couples LDL with its receptor, which increases the uptake of LDL particles through non-regulated receptors. Modified lipids and plaque formation contribute to exacerbating the shear stress sensed by the endothelium, which activates the inflammatory response through cytokines and macrophages **(Kraaijenhof et al., 2021)**.

The inflammatory response activated by the endothelium promotes the expression of adhesion molecules such as VCAM-1, ICAM-1 and chemokines, which attract monocytes. Monocytes migrate into the intima and differentiate in pro-inflammatory macrophages activated by the secreted inflammatory cytokines. There, they phagocytize ox-LDL particles, becoming foam cells and contributing to the development of the atherosclerotic plaque **(Peled et al., 2014).**

As the plaque grows, a sudden rupture of the endothelium may occur, creating a thrombus that travels through the bloodstream causing ischemia and tissue necrosis. To stabilize the plaque, VSMCs migrate from the tunica intima to the luminal side and form a fibrous cap with their extracellular matrix to avoid exposure to prothrombotic material **(Watson et al., 2018)**. This plaque can be further aggravated by the calcification process, which is a hallmark of advanced atherosclerosis, leading to the eventual complete occlusion of the vessel, a chronic manifestation of the disease **(Nakahara et al., 2017)**.

There are many modifiable risk factors that contribute to atherosclerosis development, first, an imbalanced high-fat diet and sedentariness. An interesting link is the one between atherosclerosis and the microbiota. Gut microbiota is responsible for many functions, such as energy storage, nutrient absorption, and regulation of the immune system **(Clemente et al., 2012)**. As illustrated above, atherosclerosis is mainly a process characterized by an uncontrolled and chronic inflammatory response. As known, inflammation can also be triggered by gut dysbiosis, which disrupts the permeability of nutrients and also allows LDL particles to pass easier to the blood flow. Another contribution of dysbiosis to atherosclerosis is the production of TMA by some bacterial species. TMA is a byproduct of bacterial metabolism of L-carnitine and choline, assumed through eggs, meat, and fish. This molecule is then absorbed by the gut, and it migrates to the liver by circulation. The liver oxidizes TMA into TMAO. TMAO is directly responsible for the progression of atherosclerosis since it increases the expression of scavengers, which reduce cholesterol efflux from the intima, and it promotes the recruitment of macrophages, increasing foam cells formation **(Eshghjoo et al., 2021)**.

A healthy diet is fundamental to regulating gut microbiota. As dysbiosis favours the production of inflammatory molecules by microbial synthesis, a balanced gut microflora is important to contrast them. For example, there are some bacteria such as E. coli, Bacteroides, and Clostridium, which

can regulate tryptophan metabolism, producing molecules with antioxidant properties such as indole, instead of toxic indoxyl sulphate **(Lee et al., 2010).** This is where kefir, can help in preventing the progression of atherosclerosis and regulate cholesterol levels in the blood.

2.7 Epidemiology and pathophysiology of colorectal cancer

Colorectal cancer (CRC) is a significant health problem as it represents the third most commonly diagnosed and the second most fatal cancer globally **(WHO, 2021).** The number of cases is increasing in the older population; this is why it is estimated that the global incidence of CRC will double in 2035 due to the population aging. Since this type of cancer can be treated by surgery, the most effective way to prevent CRC is screening average-risk individuals, a measure taken in the last years by European countries, Canada, specific regions of North and South America, Asia, and Oceania; in the United States, thanks to the implementation of screening programs, a downward trend in CRC morbidity has been registered **(Hossain et al., 2022)**. The screening campaign is also the reason why we have more data concerning the CRC diffusion in those countries, but it is estimated that it is spreading even across low-income countries where screening and prevention are less possible.

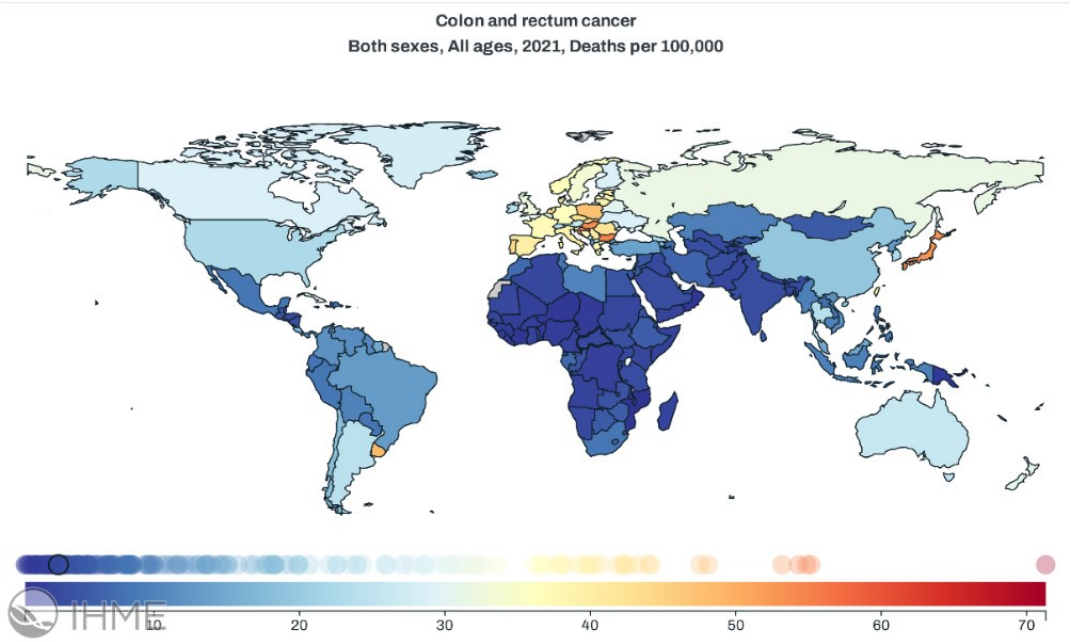

Figure 2.7: Deaths per 100,000 in the world population in 2021. https://vizhub.healthdata.org/gbd-compare/#0

Colorectal cancer is determined by both genetic and environmental factors. Like any cancer, it arises from DNA mutations that give rise to abnormal cells, in this case in the form of polyps and adenomas, which multiply uncontrollably until the metastatic phase begins. However, there are some modifiable risk factors that influence the development of the disease; these include diet, lifestyle, and chronic inflammation. In the early stages, symptoms are not as pronounced, while in advanced stages,

abdominal pain, changes in bowel habits, and unintentional weight loss are the most common symptoms. There are three main types of CRC: sporadic, hereditary, and colitis associated.

Sporadic CRC originates from multiple spontaneous somatic genetic modifications, resulting in the transition from normal mucosa to benign adenoma to dysplasia and in the end carcinoma. The most common mutations are malfunction of the mismatch repair genes, which may account for 15% of sporadic colon cancer, and the APC gene mutation which is believed to be responsible for at least 80% of sporadic colon cancer cases **(Cappell, 2008)**. The mismatch repair genes physiologically recognize errors in nucleotide matching during DNA replication and initiate excision of the erroneous strand, so when modified the errors due to base pairs in DNA replication accumulates and mutations arise. The APC gene instead, is responsible for regulating cell growth and apoptosis, so when modified it promotes uncontrolled aberrant cells replication, a hallmark of cancer **(Jen et al., 1994)**.

CRC predisposition can be hereditary. Familial adenomatous polyposis (FAP) is a disease caused by a single autosomal dominant mutation of the APC gene. Patients affected by FAP develop multiple adenomatous polyps throughout the colon during puberty, which develop into colon cancer in adulthood. Another disease is hereditary non-polyposis colorectal cancer (HNPCC), which has been shown to be due to mutations in one of the mismatch repair genes that increase the risk of carriers developing cancer **(Robbins et al., 2002)**.

Colitis-associated colorectal cancer (CAC) has been demonstrated to be linked to colonic inflammation through both epidemiological and clinicopathological studies. An example of chronic inflammation that interests the colon is inflammatory bowel disease (IBD). Inflammation accelerates the mutational processes, and it is the main cause of the development of polyps that can degenerate into CRC **(Zhou et al., 2023)**. Colon inflammation can be driven by imbalances in microbiota, in fact, differences in the microbiota composition between CRC patients and healthy individuals have been proven by different metagenomic studies.

Microbiota can alter the progression of CRC through the modulation of different mechanisms such as inflammation and DNA damage, or by the production of regulative molecules. CRC patients have shown a reduction in bacterial biodiversity in their intestines compared to healthy individuals, and this can alter nutrient absorption and the intestinal environment **(Shah et al., 2018).** Among bacteria with a proven role in tumour progression, we find *Bacteroides fragilis*, which produces a toxin that causes diarrhoea and IBD; *Fusobacterium nucleatum,* which contributes to the conversion of adenoma to cancer; *Enterococcus faecalis*, which produces superoxide, a powerful ROS that can damage the DNA of epithelial cells; *Helicobacter pylori,* which damage the colon epithelium through inflammation driven by the blockage of the proton pumping system; some strains of *E. coli* containing pathogenic islets can contribute to the development of CRC **(Dokht et al., 2022)**.

From this last evidence of the involvement of microbiota in the susceptibility and progression of CRC, a healthy diet and the introduction of probiotics have been suggested as a preventive measure for chronic inflammation. Kefir contains many good bacterial species with key functions like competition with damaging bacteria for the site of adhesion, production of bacteriocin, improvement of intestinal permeability, and regulation of immune response **(Bermudez-Brito et al., 2012)**.

3. DISCUSSION

To sustain the thesis that kefir can be a powerful fermented milk drink for the prevention of non-communicable diseases, here are reported one representative in-vivo study on animal models and one representative study on human patients or cells for each of the three pathologies under investigation: type 2 diabetes mellitus, atherosclerosis and colorectal cancer. To conclude, there is also reported one personal exploratory research study about kefir knowledge and consumer's habits conducted by a survey on a small sample of the population of Veneto region.

3.1 Kefir and type 2 diabetes prevention, an in vivo study

The first reference is an in-vivo study on rats by AL-Shemmari et al., titled «Evaluation of Antidiabetic and Antihyperlipidemic Activity of Kefir in Alloxan Induced Diabetes Mellitus Rat». Published in the *Scientific Journal of Medical Research* in 2018.

This study was conducted to investigate the antihyperglycemic effect of skimmed milk kefir in alloxan-induced diabetic male Wister rats. Hyperglycaemia is the characteristic sign of diabetes mellitus, so to investigate kefir effect on the induced pathology, blood glucose level has been selected as the clue parameter. After 40 days of kefir-treatment diabetic induced rats reported lower levels of blood glucose compared to the positive control. The results obtained by kefir-treatment were found to be comparable to the ones obtained by a hypoglycaemic drug.

3.1.1 Design of experiment

Kefir

Skimmed milk was sterilized at 121°C for 15 min and then, incubated with 10% w/v of kefir grains for 20h at 20°C. The grains utilized were Tibetan kefir grains, containing *Lactobacilli*, *Lactococci*, and yeasts together with a small amount of acetic acid bacteria. The grains were then removed by using cheesecloth layers and the remained fermented milk was kept at 4°C until used.

Test groups

This experiment was conducted on 48 male rats weighing 200-250 g and aged 12-14 weeks. The rats were all fed standard pellet and water *ad libitum* and kept under standard conditions (temperature and light). The rats were divided into four groups (12 rats each): the first group was the negative control group (Control), which was drenched with normal saline; the second group (DM) was the positive control group, the rats were injected with alloxan (150 mg/kg, single dose), a toxic agent that induces apoptosis of β-cells and causes diabetes mellitus, and orally drenched with normal saline; the third group (DM + Kefir) was the diabetic intervention group, injected with alloxan (150 mg/kg, single dose) and orally drenched with skimmed milk kefir (3.6ml/200g); the fourth group (DM + Gliben clamide) was the diabetic drug-treated test group, which was injected with alloxan (150 mg/kg, single dose) and drenched orally with Gliben clamide (600µg/kg), a hypoglycaemia-inducing drug.

Analysis

Blood samples were collected via heart puncture from six animals of each group at day 20 and at day 40 of experiment. Spectrophotometric analysis was used to collect measurements of blood glucose levels. I elaborated the data reported in the study (Means ± Standard error) performing statistical analysis using GraphPad Prism software. Difference between groups has been calculated by using One-way ANOVA test and reported in the form of histograms.

3.1.2 Results

The significant increase in glucose levels confirms the induction of diabetes mellitus in all groups injected with alloxan compared to the negative control group (Control v.s DM $p < 0.0001$; Control vs DM + kefir and DM + Gliben clamide $p < 0.01$).

After 20 days of experiment, no difference was detected between rats treated with kefir and rats treated with Gliben clamide. Blood glucose levels of the two treated groups were significantly reduced compared to the positive control (DM) with a p-value < 0.05.

After 40 days of experiment, no difference was detected between DM + Kefir and DM + Gliben clamide groups still. On the other hand, the decrease in blood glucose levels of the two treated groups compared to the positive control was more significant: p-value < 0.0001 even if the levels were higher than the negative control ($p < 0.01$).

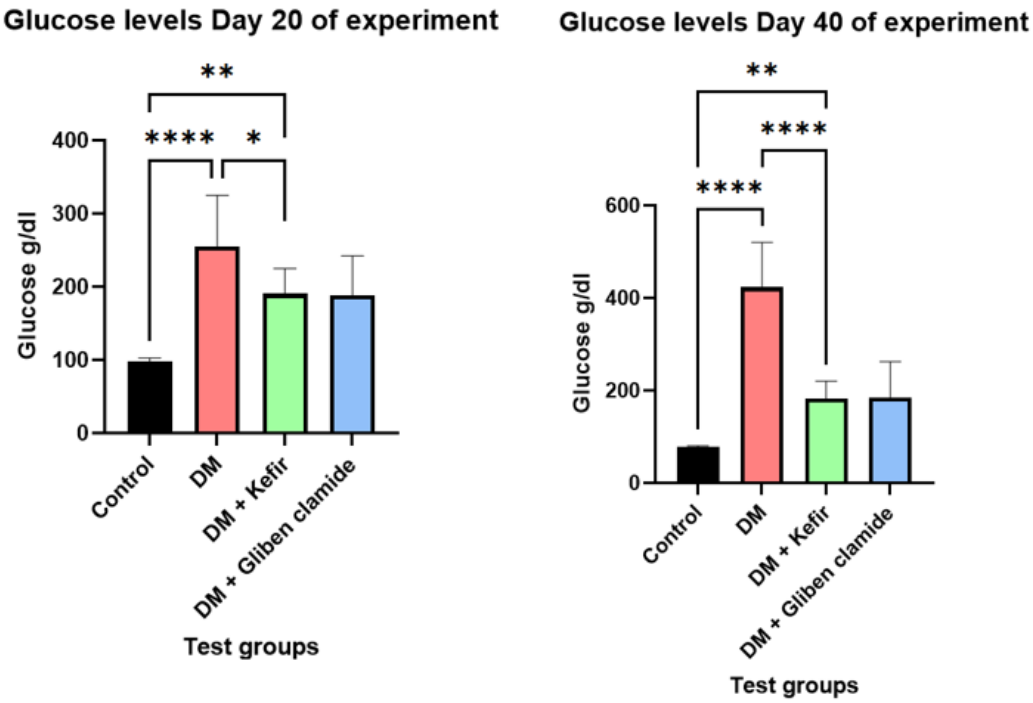

Figure 3.1.2: Effects of Kefir in alloxan induced diabetic mice on (A) blood glucose levels at 20 days of experiments, and (B) blood glucose levels at 40 days of experiments. The groups in comparisons are negative control (black), positive control (red), kefir-treated group (green), drug-treated group (blue). Statistical analysis: One-way ANOVA performed with GraphPad Prism software. Statistical signs: *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001. [modified]

3.1.3 Conclusions

From the results obtained by this study it is possible to conclude that kefir has a lowering effect on glycemia in diabetic induced rats. Since alloxan, the chemical agent used to induce the pathology, reduces insulin production, it is possible to assume that the anti-diabetic effect of kefir results in an increase in insulin sensitivity. Insulin sensitivity reduction is the first step of type 2 diabetes, so it can be a promising frontier for the prevention of the pathology if the same effect is transposed in human beings.

3.2 Kefir and type 2 diabetes prevention, clinical trials

The second reference is a systematic review and meta-analysis on randomized controlled clinical trials by Salari et al., titled «Effect of Kefir Beverage Consumption on Glycaemic Control: A Systematic Review and Meta-analysis of Randomized Controlled Clinical Trials». Published in *Complementary Therapies in Clinical Practice* in 2021.

This systematic review and meta-analysis of randomized controlled trials aimed to demonstrate the efficacy of milk kefir administration on the glycaemic control. Due to its anti-inflammatory and regulating properties on the gut microbiota, milk kefir is thought to have an anti-diabetic effect, particularly in the regulation of blood glucose levels. This property was investigated here by examining the change in fasting blood glucose levels, glycated haemoglobin and insulin in patients at high risk of T2DM comparing those who assumed a regular quantity of milk kefir to a control pool. After the analysis of six randomized control trials, data suggested that kefir consumption can decrease both blood glucose and insulin levels.

3.2.1 Design of experiment

Kefir

The kefir utilized in the selected studies was made by fermenting milk with kefir grains containing mainly lactic acid bacteria such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus* and yeasts such as *Kluyveromyces*, *Candida*, *Saccharomyces* and *Pichia.* The final fermented beverage contained bioactive peptides, bacteriocins, antioxidants, vitamins and calcium.

Test groups

Databases such as PubMed, Scopus, ISI Web of Science and Google Scholar were searched up to July 2020. Six randomized controlled trials, conducted on 323 subjects in total, were selected. All the trials report the effects of kefir beverage consumption on fasting blood sugar (FBS), 4 on glycated haemoglobin (HbA1c), and 3 on insulin levels.

Characteristics of included randomized clinical trials in the Meta-analysis.

First author, year	Country	Participants N	Age (range or mean)	Duration (day)	Study design	Intervention Group	Comparison Group	Reported data	Notes about participants
Judiono et al. (2014)	Indonesia	F/M: 106	NR	30	RCT, Parallel	A standard diet supplemented with 200 ml/day kefir	A standard diet	FBG HbA1c Insulin	Diabetes mellitus outpatients
Ostadrahimi et al.(2015)	Iran	F/M: 60	35–65	56	RCT, Parallel	600 ml kefir containing probiotics twice a day	600 ml conventional fermented milk(dough) twice a day	FBS HbA1c	Diabetic patients
Golunuk et al. (2017)	Turkey	M: 36	18–25	15	RCT, Parallel	300 ml/day kefir + 1 hour aerobic exercise	only 1 hour aerobic exercise	FBS	Male individuals
El-Bashiti et al. (2019)	Palestine	M: 21	37–65	70	RCT, Parallel	250 ml/day kefir + metformin	only metformin	FBS HbA1c	Diabetic male patients
Bellikci-Koyu et al.(2019)	Turkey	F/M:22	18–65	84	RCT, Parallel	180 ml/day kefir	180 ml/day unfermented milk	FBS HbA1c Insulin	Subjects with Metabolic syndrome
Jenko et al. (2020)	Slovenia	F/M: 27	30–60	56	RCT, Cross-over	300 ml/day kefir	300 ml/day milk	FBS	Healthy overweight participants

Table 3.2.1: This table reports the characterization of the six randomized control trials selected for this study

The duration of trials varied in the studies between 15 and 84 days, and the age of the participants ranged from 18 to 65 years. All studies examined patients who already had T2DM or who were at high risk of developing the disease. The intervention groups were given a certain amount of milk

kefir, ranging from 180 ml to 300 ml per day. Three studies included an additional intervention such as lifestyle modification (2 studies) or metformin (1 study). The control groups received a placebo in form of conventional or standard fermented milk in 3 studies or only the additional intervention in the 3 studies where this was implied.

Analysis

The comparison of the levels of FBS, HbA1c, and insulin between kefir and control groups of each clinical study were estimated using weight mean difference. P-values less than 0.05 were considered as statistically significant.

3.2.2 Results

Analysis of the studies showed that FBS was significantly improved among the intervention groups administered with kefir beverage in comparison to the control groups. FBS resulted in a reduction in the mean difference of -10.28 with a p-value of 0.001.

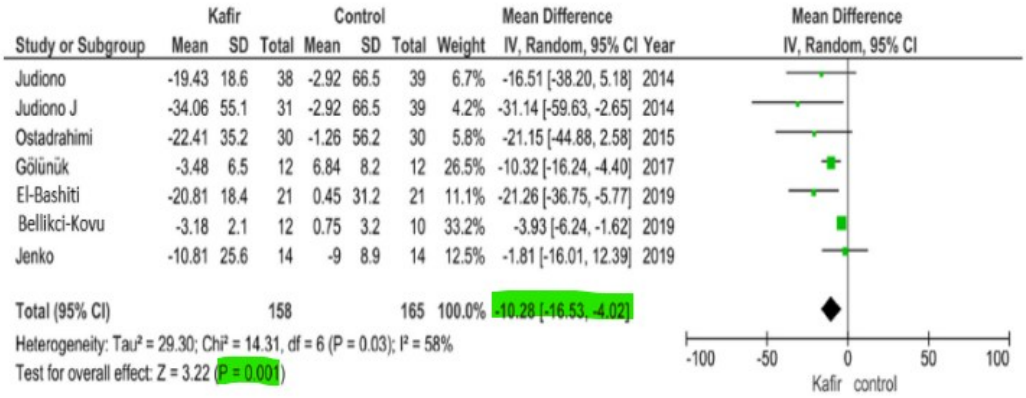

Table 3.2.2 (1): Effect of kefir consumption on FBS. Forest plot depicting the meta-analysis of randomized controlled clinical trials (RCTs) investigated for the effect of kefir in glycaemic control on FBS compared with the control in pooled analysis.

The analytical results of 4 studies including parameters on glycated haemoglobin indicated that kefir consumption results in reduced HbA1c levels with a mean difference of -0.64. However, this result was not statistically significant.

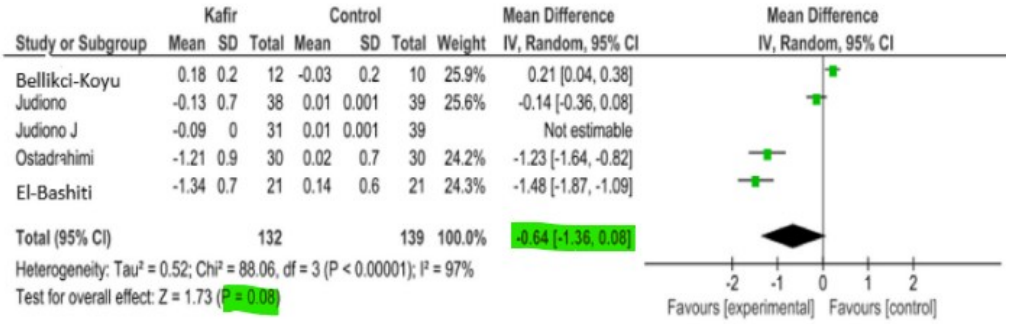

Table 3.2.2 (2): Effect of kefir consumption on HbA1c. Forest plot depicting the meta-analysis of randomized controlled clinical trials (RCTs) investigated for the effect of kefir in glycemic control on HbA1c compared with the control in pooled analysis.

After the analysis of 3 studies containing data about insulin levels, it has been found a significant reduction of blood insulin in the intervention groups compared to the controls, with a mean difference of -2.87 and a p-value of less than 0.00001.

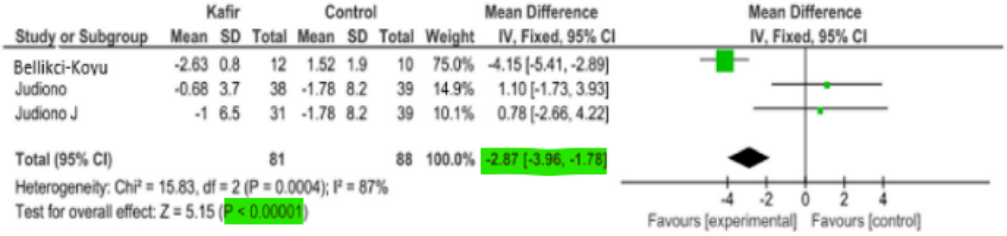

Figure 3.2.2 (3): Effect of kefir consumption on insulin levels. Forest plots of randomized controlled clinical trials illustrating the pooled effects of kefir consumption on insulin levels.

3.2.3 Conclusions

Data comparison of the randomized control trials selected show an overall decrease in both insulin levels and fasting blood sugar levels in intervention groups who assumed kefir regularly with respect to control groups. If the improvement of fasting blood sugar levels does not correspond to a higher level of insulin but the contrary instead, this means that the assumption of kefir improve insulin sensitivity. By improving insulin sensitivity, it is possible to prevent the development of T2DM from the initial stage.

3.3 Kefir and atherosclerosis prevention, an in vivo study

The third reference is an in-vivo study on mouse model conducted by Chang et al., titled «Kefir Peptides Attenuate Atherosclerotic Vascular Calcification and Osteoporosis in Atherogenic Diet-Fed ApoE−/− Knockout Mice». Published in *Frontiers in Cell and Developmental Biology* 11 on 2023

Atherosclerosis and vascular calcification are consequences of chronic inflammation due to the accumulation of low-density lipoproteins (LDL) in the intima of the arteries. The intervention strategy to treat this pathology involves reducing the inflammatory status and improving the lipid profile. Kefir is known for its anti-inflammatory properties, but this study aims to show that it can also contribute to improving lipid profile, in particular thanks to the bioactive peptides it contains. The effects of kefir peptides were demonstrated using a mouse model. LDL levels, inflammation levels and body weight of atherosclerosis induced mice treated with kefir peptides were compared with those of the control groups. At the end of the study an improvement of all the factors examined was find in the kefir-treated groups.

3.3.1 Design of experiment

Kefir

Kefir was obtained by fermenting milk with kefir grains containing lactic acid bacteria, acetic acid bacteria, and yeasts. Kefir peptides (KP) were extracted by Phermpep Biotech. Co. to synthetize KP powder (KEFPEP®) implied in this study. The KP powder counted 23.2 g of peptides and 487 kcal in 100g. The solution for oral administration was freshly prepared in phosphate-buffered saline (PBS).

Test groups

This study included only male mice. To induce dysfunctional lipid profile condition, a knock-out of Apolipoprotein E (ApoE) was used. ApoE mediates the binding of lipoproteins circulating in the bloodstream to specific receptors, allowing their distribution and redistribution in different tissues of the body. It has been demonstrated that lack of ApoE results in hyperlipidaemia and atherosclerosis-like cardiovascular diseases. To induce the disease, mice were fed with atherogenic diet, high in fat and cholesterol content. The first negative control group was the wild type, male C57BL/6 (B6) fed with standard pellet (WT + CD). The second negative control group with the genetic modification was the knockout ApoE−/− mice (B6.129P2Apoetm1Unc/J) fed with standard pellet (mock + CD). The third group was the positive control: ApoE−/− mice fed with atherogenic diet (mock + AD). The third and fourth groups represented the intervention groups: ApoE−/− mice fed with atherogenic diet,

treated with 328 mg/kg low-dose KPs (KPs-L + AD) and 656 mg/kg high-dose KPs (KPs-H + AD) respectively. The duration of treatment was 13 weeks in total.

Analysis

Blood samples were collected by retro-orbital bleeding under isofurane anesthesia for serological examination. Total cholesterol and triglycerides were measured using IDEXX dry-slide technology and the VetTest chemistry analyser. Malondialdehyde, a marker of oxidative stress, was measured by TBARS assay kit, while the tumour necrosis factor alpha (TNF-α) and (ox-LDL) were determined by using mouse-specific ELISA kits. Data were represented in box-plot (median + first and third quartiles) using GraphPad Prism software. One-way ANOVA was used to analyse statistically significant differences represented by the symbols: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to the wild type control group; and #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ compared to the genetically modified negative control group.

3.3.2 Results

The differences of final weight gain between groups were not statistically significant at the end of the study, since all the subjects were fed by the same caloric intake. Total cholesterol and oxidative status were both higher in ApoE knockout mice fed with atherogenic diet (AD) then in the controls, indicating that the pathology was well induced. Total serum cholesterol levels slightly increased in mock+CD mice compared to WT but remained close to normal serum levels. It increased significantly in AD-fed knockout mice ($p < 0.001$) compared to WT. Oral administration of KPs decreased total cholesterol levels to a significant extent ($p < 0.01$), even though not sufficiently to reach the normal status [**Fig. 3.3.2A**].

Ox-LDL was significantly higher in the group without intervention (mock+AD) with respect to the controls (WT+CD, mock+CD) with a p-value lower than 0.001. The kefir intervention groups (KPs-L+AD and KPs-H+AD) presented ox-LDL levels significantly lower than the positive control group (mock+AD), with a statistical significance $p<0.001$, enough to reach the normal status with no statistical difference in respect to the negative controls [**Fig.3.3.2C**].

MDA levels were higher in all the AD-fed knockout mice ($p<0.001$) than in the controls, but kefir intervention (KPs-L+AD and KPs-H+AD) lowered it significantly compared to the positive control group (mock+AD) with a p-value lower than 0.01 [**Fig. 3.3.2D**].

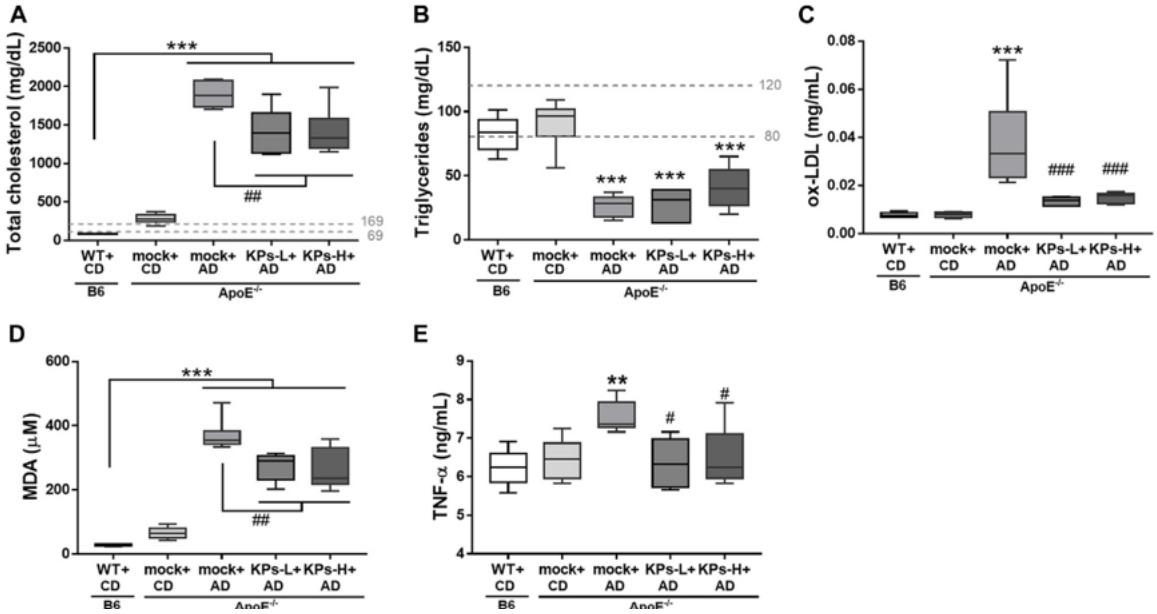

*Figure 3.3.2: Effects of KPs on serum (A) cholesterol, (B) triglyceride, (C) ox-LDL, (D) MDA, (E) TNF-α. The dashed lines indicate the normal ranges of target serum markers in mice. Statistical signs (n = 6): *p < 0.05, **p < 0.01, ***p < 0.001 vs. WT + CD/B6, and #p < 0.05, ##p < 0.01, ###p < 0.001 vs. mock + AD/ApoE−/−.*

TNF-α, inflammatory cytokine, was found with higher levels in the untreated group (mock+AD) compared to the negative controls (WT+CD and mock+CD). Both the intervention groups treated with kefir (KPs-L+AD and KPs-H+AD) presented TNF-α levels closed to the negative controls, significantly lower ($p<0.05$) than the positive control group(mock+AD) [**Fig, 3.3.2E**].

Triglyceride levels in the blood were found significantly lower ($p<0.001$) in the AD-fed knockout mice compared to the controls (WT+CD and mock+CD) [**Fig, 3.3.2B**].

3.3.3 Conclusions

Kefir consumption improves lipid profile in ApoE knockout mice fed with atherogenic diet by lowering total cholesterol, lipid oxidation and inflammatory cytokines.

The possible controversial data can be the one regarding triglycerides where AD-fed mice present lower values in respect to negative controls. This data can be explained by the fact that the loss of ApoE function in the process of hepatic LDL vesicles secretion leads to the accumulation of triglycerides in the liver and lower levels detectable in the bloodstream. This brings to subsequent hepatic dysfunction proven by the measurement of increased serum hepatic enzyme activity.

The most promising effect regards ox-LDL levels that kefir consumption in pathologic subjects can restore until reaching the values of healthy individuals.

3.4 Kefir and atherosclerosis, a clinical trial study

The fourth reference is a randomized controlled clinical trial by Bellikci-Koyu et al., titled «Probiotic Kefir Consumption Improves Serum Apolipoprotein A1 Levels in Metabolic Syndrome Patients: A Randomized Controlled Clinical Trial». Published in *Nutrition Research* 102 in 2022.

This randomized controlled clinical trial aims at demonstrating the capacity of kefir in improving glycaemic control, lipid profile, blood pressure, and inflammatory status in patients with metabolic syndrome, with a specific focus on dyslipidaemia. Dyslipidaemia is a condition that consists in an altered lipid profile with an increase in LDL cholesterol and high risk of developing atherosclerosis. In this study patients where accurately selected, and the parameters of the ones treated with kefir were compared to a placebo group. Significant improvements were found in the kefir-treated group for LDL-C and Apo A1, principal protein component of human HDL, fundamental for the correct lipid metabolism.

3.4.1 Design of experiment

Kefir

Kefir was produced from a bulk culture. To prepare the bulk culture 12% of skimmed milk powder was dissolved in 500 ml of water and sterilized in autoclave at 115°C for 10 min and cooled to 25°C. 1 g of DC1500I culture obtained from Danisco (Olsztyn, Poland) was added to 500 ml of milk. The milk was then mixed and incubated at 25°C until it reached a pH of 4.6. For kefir preparation, raw milk was heat-treated at 80°C for 10 minutes and brought to 25°C, then inoculated with 3.25 % bulk culture. When the milk reached a pH of 4.7, the milk was filtered and transferred to a cold storage. The microbial composition of the kefir included *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *diacetylactis*, *Leuconostoc mesenteroides* subsp. *cremoris*, *Lactobacillus kefyr*, *Kluyveromyces marxianus*, and *Saccharomyces unisporus.*

Test groups

Patients with metabolic syndrome were recruited from the outpatient clinics in the Department of Internal Medicine, Endocrinology Division at Ege University, Izmir, Turkey. The age of participants ranged between 18 and 65 years. In additional of abdominal obesity, to be eligible for the study, patients must have presented 2 of the following components: fasting plasma glucose concentration higher than 100 mg/dL or previously diagnosed type 2 diabetes; raised concentration of triglycerides, more than 150 mg/dL; reduced concentration of high-density lipoprotein cholesterol, HDL-C lower than 40 mg/dL in men and lower than 50 mg/dL in women; raised blood pressure: systolic blood pressure higher than 130 mm Hg or diastolic blood pressure higher than 85 mm Hg, or treatment of

previously diagnosed hypertension. The exclusion criteria were having dairy allergy, already consuming probiotics regularly, having type 1 diabetes, assuming antibiotics, having severe hypothyroidism, or being in a pregnant or lactating status. The subjects were divided in two groups, 31 each for a total of 62 patients, using stratified randomization based on sex. The intervention group assumed 180 ml/day of kefir, while the control group assumed 180 ml/d of unfermented milk as placebo. The duration of the study was 12 weeks, dietary intake was monitored by dietary diaries and interviews.

Analysis

Blood samples were collected following the 10h fasting overnight. Serum glucose levels, insulin levels, total cholesterol, HDL-C, LDL-C, triglycerides and Apo A1 measurements were collected at weeks 4, 8 and 12 of experiment. Data were presented as mean ± standard deviation and differences between groups were calculated by paired t-test. Here data are represented in histograms made by using GraphPad Prism software and the significance of the difference between groups is reported by the symbols: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

3.4.2 Results

At week 0 all the parameters were measured to prove there were no initial significant differences between groups. At the end of the study there was no significant change in fat mass and waist circumference in either group. Among all the parameters measured the only significant difference between milk group and kefir intervention group was found in the levels of ApoA1. Apolipoprotein 1 was significantly increased in kefir group compared to milk control group (*$p < 0.05$) [**Fig 3.4.2(1)**].

A subgroup analysis was then performed in participants with dyslipidemia (LDL-C >130 mg/dL). Here, together with the significant difference (*$p < 0.05$) in ApoA1 between the intervention and control group [**Fig. 3.4.2(2)B**], also differences in LDL-C were detected.

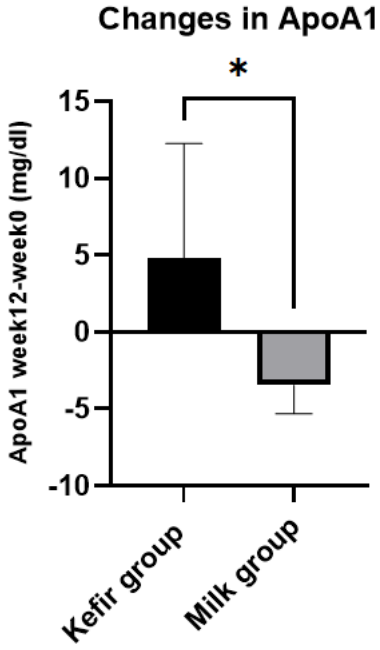

*Figure 2.4.2 (1): changes in levels of ApoA1 between week 12 and week 0, comparison between kefir and milk groups. Statistical signs: *p < 0.05, **p < 0.01, ***p < 0.001. [modified]*

Levels of LDL-C within the group treated with kefir lowered after 12 weeks of treatment with a statistical significance of *p < 0.05, while there was no variation within the placebo group [**Fig. 3.4.2(2)A**].

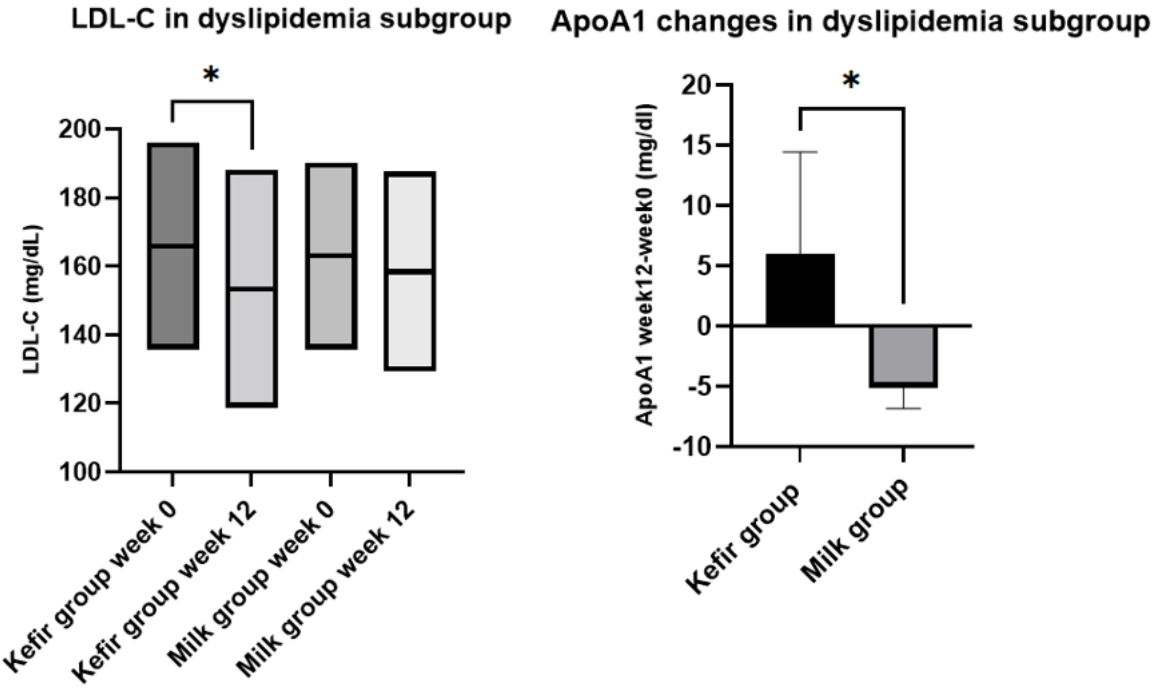

*Figure 3.4.2 (2): (A) LDL-C differences within kefir group and milk group at week 0 and week 12 represented by boxplots; (B) changes in levels of ApoA1 between week12 and week 0, comparison between kefir and milk groups. Statistical signs: *p < 0.05, **p < 0.01, ***p < 0.001. [modified]*

3.4.3 Conclusions

Here kefir acts by increasing the production or secretion of ApoA1. This lipoprotein helps in clearing arteries and making the passage of LDL particles more fluid.

The impact of kefir consumption in improving lipid profile is significantly detectable in patients with dyslipidaemia who in standard conditions present abnormally higher LDL concentrations. Lipid-lowering property of kefir is probably due the kefir peptides capacity of blocking lipogenesis and decreasing inflammatory responses by reducing fatty acid synthase and increasing p-acetyl-CoA carboxylase expression. Thus, kefir consumption can prevent the development of atherosclerosis in people with concomitant metabolic pathologies such as dyslipidaemia.

3.5 Kefir and colorectal cancer, an in vivo study

The fifth reference is an in vivo study on rats conducted by Guiomar De Almeida et al., titled «Kefir Modulates Gut Microbiota and Reduces DMH-Associated Colorectal Cancer via Regulation of Intestinal Inflammation in Adulthood Offsprings Programmed by Neonatal Overfeeding». Published in *Food Research International* 152 in 2022.

In this study, Wistar rat is used as an animal model to demonstrate the positive effects of kefir on preventing the development of colorectal cancer (CRC). The effect of kefir was investigated both in a group of subjects under standard condition and in a group of subjects at high risk of developing colorectal cancer. Obesity was considered here as a risk factor for the development of CRC, as it is associated with chronic inflammation and dysbiosis. The study groups, consisting of normal and obese rats, were further divided into two subgroups, one of which was treated with milk and the other with kefir for 60 days. On day 67, a carcinogen was administrated to all subjects. On day 240, the study was completed, and it was found that kefir-treated subjects in both test groups had a lower number of tumours and lower levels of inflammatory cytokines.

3.5.1 Design of experiment

Kefir

The kefir grains used in this study were obtained from the Department of Nutrition and Health of the Federal University of Viçosa, Minas Gerais, Brazil. Whole milk was fermented with the grains at 20°C for 24h, then filtered and kept refrigerated until use. The process was repeated daily to obtain fresh kefir. The pH was maintained at 3.9-4.1; the microbial composition corresponded to 10^8 CFU/mL LAB with a prevalence of *Lactococcus* and *Lactobacillus* and 10^6 CFU/mL yeasts with a prevalence

of *Aspergillus* and *Cordyceps*. It has been shown to fulfil the standards proposed by the Codex Alimentarius.

Test groups

Wistar rats were obtained from Center of Reproduction Biology of the Federal University of Juiz de Fora, Minas Gerais, Brazil. After the day of birth, male pups and their dams were distributed either in a small litter (SL), three male pups per dam, or in a normal litter (NL), ten male pups per dam. At birth, no difference in body weight was observed between the pups included in the study. During lactation, the dams of the NL and SL control groups received 1 ml of filtered water by gavage once daily, while the dams of the test groups, the kefir-treated normal litter (KNL) and the kefir-treated small litter (KSL) received 1 mL of kefir milk by gavage once daily. After day 21 of lactation, the pups of the four groups (NL, SL, KNL, KSL) continued to receive the same treatment with water or kefir and free access to standard rodent chow. On day 67, all animals were injected with 40 mg/kg of the carcinogen 1,2-dimethylhydrazine (DMH). On day 240, the experiment was terminated, and the measurements were undertaken.

Analysis

Body weight was monitored once every 4 days until day 240 of experiment when even fat mass measurements were undertaken (epididymal fat + retroperitoneal fat + mesenteric fat). To measure the number of tumours, necropsy of vital organs was performed and the incidence was represented by the percentage of animals with tumours over the total. The intestine was opened and fixed for evaluating the number of tumours in the proximal, medial and distal segments. To measure the concentration of inflammatory cytokines such as interleukin 1 (IL-1β), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α), colonic tissue cells were centrifugated and the supernatant was analysed with ELISA kit. Data were statistically evaluated using the mean ± standard error of the mean (SEM) and data representation was obtained by the statistical program GraphPad Prism. One-way ANOVA and Newman-Keuls post-test were used as parametric analysis to determine the difference between groups.

3.5.2 Results

Body weight trend during lactation showed an increase in both small litter groups (SL, KSL) compared to both normal litter groups (NL, KNL) [**Fig. 3.5.2(1)A**]. At the end of the study SL rats weighed significantly more than all the other groups: NL (+21.78%; $p < 0.01$), KNL (+15.43%; $p < 0.05$) and KSL (+10.98%; $p < 0.05$) [**Fig. 3.5.2(1)C**]. At day 240, SL rats presented the highest fat mass among the test groups NL (+53.83%; $p < 0.001$), KNL (+48.85%; $p < 0.001$) and KSL groups (+20.04%; $p < 0.01$). Also, kefir treated small litter rats (KSL) presented significantly higher fat mass

than KNL and NL groups (p<0.01), while no difference in adipose tissue weight was detected between NL and KNL rats [**Fig. 3.5.2(1)E**].

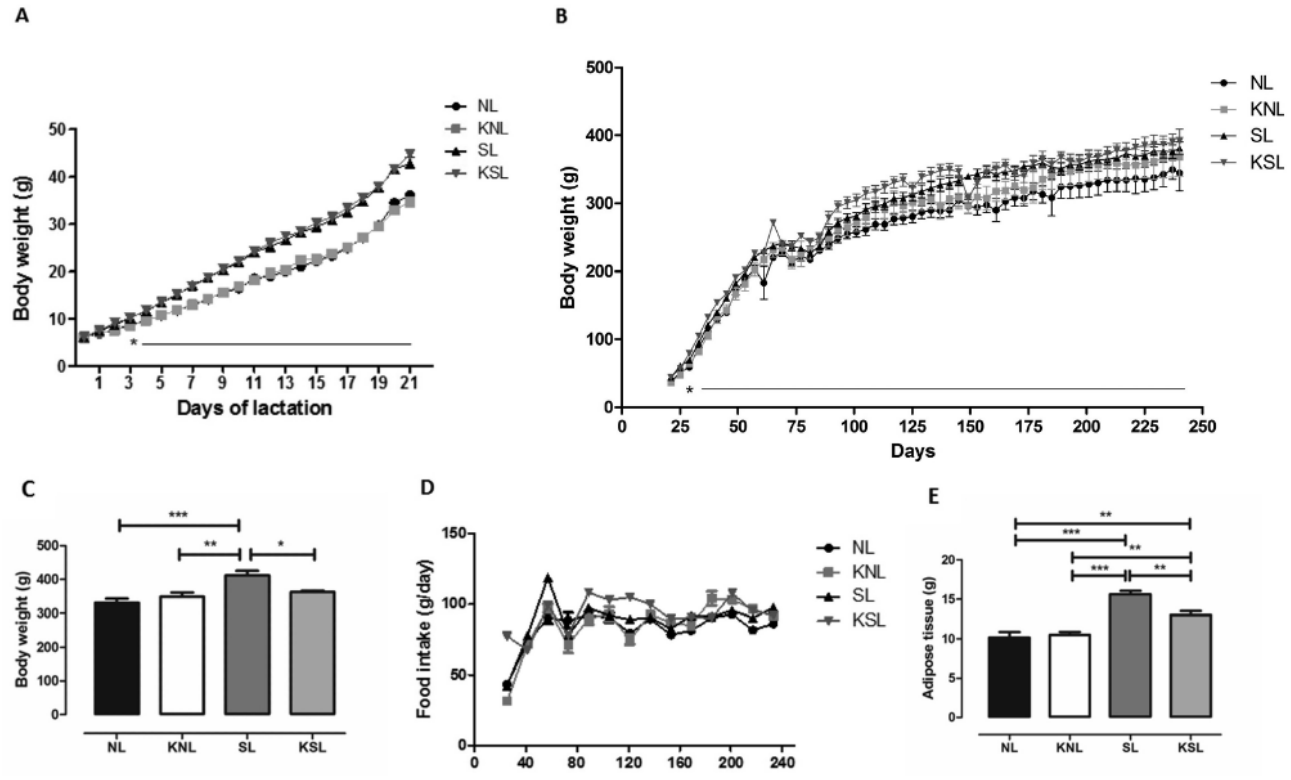

Figure 3.5.2 (1): Body weight of Wistar rats during lactation (A); Body weight of the groups that received 1,2-dimethylhydrazine (DMH) twice a week for two weeks until 240 days old (B); Body weight at 240 days old (C); Food intake at weaning until 240 days old (D); Sum of visceral adipose tissue (E). Results are expressed as mean ± SEM (n = 6–8) and were analyzed by one-way ANOVA, followed by Newman-Keuls test (p < 0.05, ** p < 0.01, *** p < 0.001).*

The quantity of the inflammatory cytokines IL-1β, IL-6, TNF-α was significantly higher in non-treated groups, both NL and SL, than in kefir treated groups. Even though both kefir groups showed promising results in lowering the levels of inflammatory cytokines, there was still a significant difference between them with higher value in KSL group with a p-value < 0,001 [**Fig. 3.5.2(2)**].

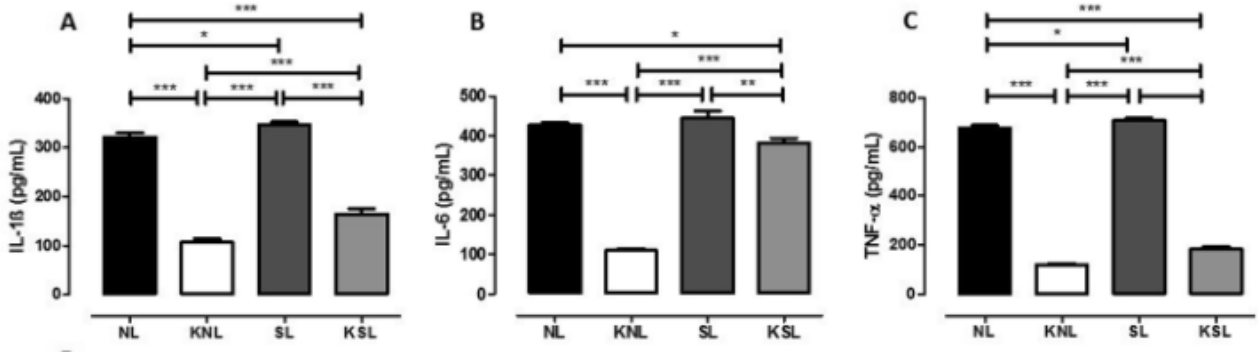

Figure 3.5.2 (2): Concentration of inflammatory cytokines, IL-1β (A), IL-6 (B), TNF-α (C), IFN-γ (D), nitric oxide (NO) (E), and zonulin (F). Results are expressed as mean ± SEM (n = 6) and were analyzed by one-way ANOVA, followed by Newman-Keuls test (p < 0.05, ** p < 0.01, *** p < 0.001).*

All animals belonging to SL group contracted tumours. The incidence of tumours was reduced in KSL group (-71.43%; $p < 0.004$) and equal to NL group, while the KNL group reported absence of developing tumours after the experimental period [**Fig. 3.5.2(3)A**]. Tumour inhibition by kefir-treatment was more efficient in the medial part of the colon where animals from other groups tended to develop the highest number of tumours [**Fig. 3.5.2(3)B**].

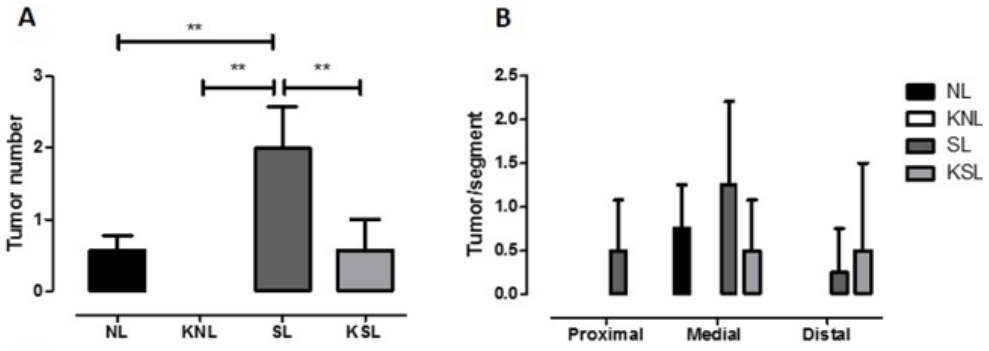

*Figure 3.5.2 (3): Tumor evaluation. In each colon, the tumors were assessed by sum total of the number of tumors (A), and the number of tumors in each segments of colon (B). Results are expressed as mean ± SEM (n = 7–8) and were analyzed by one-way ANOVA, followed by Newman-Keuls test (** p < 0.01).*

3.5.3 Conclusions

The results obtained from this in-vivo study are really promising. It is demonstrated that in mice at high risk of developing CRC kefir-treatment from birth can decrease the risk-levels to the once registered in healthy subjects. In healthy subjects instead, kefir-treatment from birth seems to totally eliminate the risk of developing tumours.

3.6 Kefir and colorectal cancer, a study on human cells

The sixth reference study was conducted on human cancer cells by Khoury et al., titled «Kefir Exhibits Anti-Proliferative and pro-Apoptotic Effects on Colon Adenocarcinoma Cells with No Significant Effects on Cell Migration and Invasion». Published in the *International Journal of Oncology* 45, in 2014.

This study aims to demonstrate the anti-cancer properties of kefir. Kefir is rich in probiotics that produce anti-inflammatory and anti-carcer compounds. These molecules can interfere with cell signalling and can regulate pathways, such as those leading to cell apoptosis and cell transformation. To further investigate how kefir affects the development of cancer cells *in vitro*, this study shows tests performed on the CRC cell lines Caco-2 and HT-29 for cytotoxicity, proliferation, and migration. The results show that kefir inhibits proliferation and induces apoptosis in colorectal cancer cells, but it has no significant effects on the motility and invasion of the same cells *in vitro*.

3.6.1 Design of experiment

Kefir

150 ml of skimmed milk was inoculated with kefir grains (50g, composed of lactobacilli and yeasts) at 20°C for 24h. The fermented milk was then strained to remove the grains and the filtrate was centrifugated (35,000 rpm for 10 min at 4℃) to remove the cell-component. The supernatant was stored at -20°C until use.

Test groups

Human colorectal adenocarcinoma cell lines Caco-2 and HT-29 were obtained from ATCC (American Type Culture Collection). Cells were culture in DMEM with 10% of FBS and 100 U penicillin/streptomycin at 37°C and 5% CO_2. The cells of both cell lines were divided into two groups: a test group treated with kefir and a control group treated with filtered (0.45-µm then 0.22-µm filter, Millipore) skimmed milk. The treatment was given at different concentrations (v/v): 0, 5, 10, 15, and 20% and the measurements were undertaken after 24, 48, and 72 hours.

Analysis

To determine cytotoxicity, cells were grown in a 24-well plates at $2x10^6$ cells/ml. The cells were treated with different concentrations of milk or kefir and then washed, trypsinised and collected in a tube with PBS. In each collection tube trypan blue was added and after microscope counting the percentage of viable cells was determined.

For the proliferation test, cells were grown in 96-well plates at $1x10^6$ cells/ml. Cells were treated with different concentrations of milk or kefir. After 24, 48, and 72 h, 10 µl of cell proliferation reagent was added in each well. Absorbance was then read at 450 nm and subtracted to the corresponding sample well.

The impact of kefir on cell cycle was determined by flow cytometry. Cells were seeded in 6-well plates and the 10% of milk or kefir treatment was added. Cells were then washed with PBS and fixed with ethanol 70%. After incubation overnight at 40°C cells were resuspended in binding buffer and propidium iodide. Cells were then analysed with Accuri C6 flow cytometer determining the respective cell cycle phases based on DNA content.

To determine the expression of genes regulating apoptosis reverse-transcription PCR was used. Cells were grown in 6-well plate at density of $1x10^6$ cells/ml and treated with 0,5 and 10% of kefir or milk for 24 hours. After the treatment all RNA was extracted and reverse transcriptase chain reaction (RT-PCR) was used to amplify the RNA of transforming growth factor α and β1 (TGF-α, TGF-β1),

then the fragments were run on 0.8% agarose gel stained with ethidium bromide at 100 V for 30 min and visualized under UV light.

For motility assay images of cells moving randomly were collected with the microscope every 60 seconds for 2 hours at a cell temperature of 37°C.

Statistical analysis was performed using VassarStats: Website for Statistical computation. Data were represented with mean ± SEM and the significance of difference between groups was evaluated by t-tests or χ2 tests.

3.6.2 Results

Kefir reduces the viability of both cell lines in a time- and dose-dependent manner. The inhibitory concentration of kefir ranges between 10, 12, and 18% (v/v) at 72, 48, and 24 h, respectively [**Fig. 3.6.2(1)**] while for HT-29 cells the kefir inhibitory action was detected at 48 and 72 h with a concentration of 12 and 10% respectively.

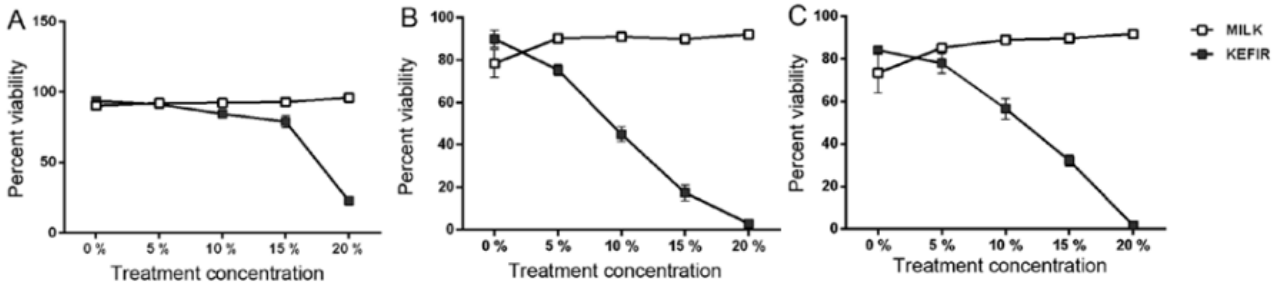

Figure 3.6.2 (1): Effect of kefir on viability of colorectal cancer (CRC) cell lines using trypan blue assay. Viability of Caco-2 cells after (A) 24 h, (B) 48 h, and (C) 72 h of treatment with different concentrations of milk and kefir cell-free fractions. Results are reported as the percent of viable cells out of the total number of cells (dead and alive). Data represent mean ± SeM from three independent experiments.

Kefir inhibits proliferation of cancer cell lines in a dose-dependent manner. All the cells belonging to control groups treated with milk showed a significant increase ($p<0.05$) in proliferation compared to kefir-treated cells. The decrease in proliferation of Caco-2 cells line was 95% at 24 h, 94% at 48 h, and 97% at 72 h compared to untreated cells [**Fig. 3.6.2(2)**]. The effect on HT-29 cells was slightly less evident but still significant: 60% at 48 h, and 38% at 72 h.

To verify if the inhibition of proliferation was due to an induction of cell cycle arrest, flow cytometry test was used. Cells of both lines were assigned to their respective phases based on DNA content: ub-g0/G1 cells were <2n, G0/G1 cells were 2n, and S/M phase cells were >2n. It was detected that Caco-2 cells belonging to sub-G0/G1 increased from 14 to 5.2% with 10% kefir treatment while the S/M phase cells decreased from 15.5 to 3.6%. The same trend was observed in HT-29 cells.

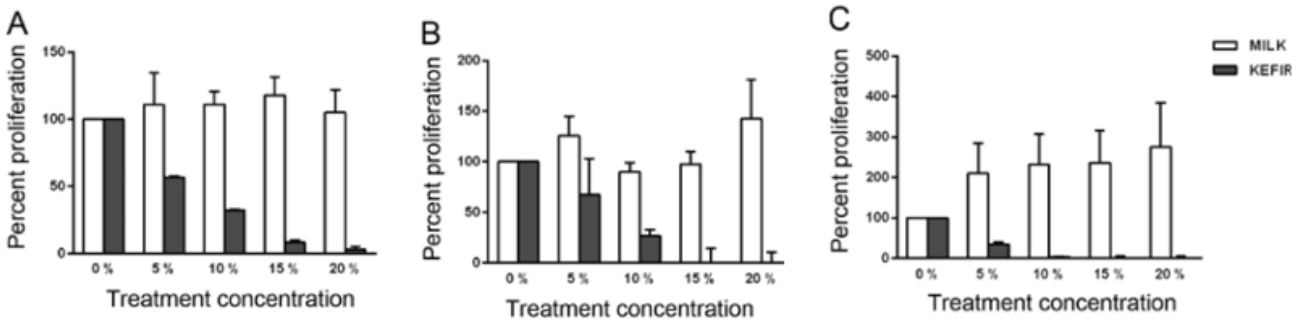

Figure 3.6.2 (2): Effect of kefir on the proliferation of colorectal cancer (CRC) cell lines. Proliferation of Caco-2 cells after (A) 24 h, (B) 48 h, and (C) 72 h of treatment with different concentrations of milk and kefir cell-free fractions. Results were normalized to the untreated cells. Data represent mean ± SEM from three independent experiments.

To determine possible mechanism for the anti-proliferative effect of kefir the expression of TGF-α and TGF-β1 was assessed at the mRNA level using RT-PCR. TGFs are growing factor cytokines that play an important role in cell proliferation pathways. In HT-29 cell line the expression of TGF-α and TGF-β1 was observed to decrease in a dose-dependent manner in kefir-treated cells while no change was detected in the control milk-treated group [**Fig. 3.6.2(3)**].

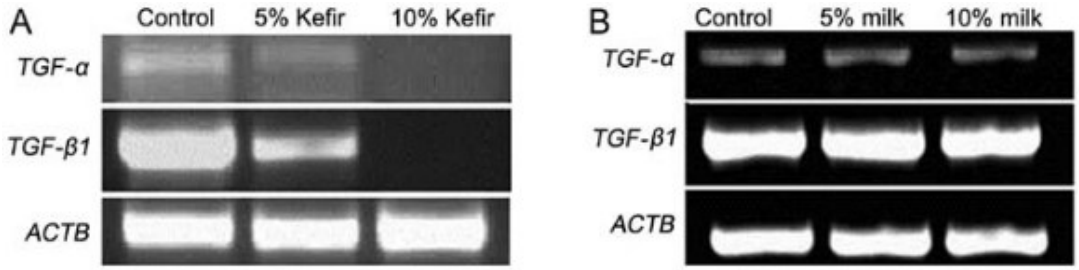

Figure 3.6.2 (3): Expression of genes involved in proliferation and apoptosis in kefir-treated HT-29 cells. The expression of transforming growth factor α (tgf-α) and transforming growth factor-β1 (tgf-β1) in 0, 5, and 10% (v/v) (A) kefir- and (B) milk-treated HT-29 cells.

To verify if kefir has an effect at the metastatic phase, the difference in migratory ability of treated cells was compared to the control ones. No difference was detected in neither Caco-2 nor HT-29 cell lines [**Fig. 3.6.2(4)**].

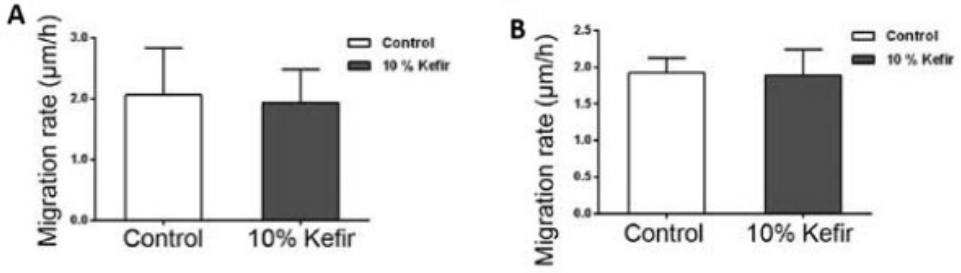

Figure 3.6.2 (4): Effect of kefir on the migration ability of treated cells in vitro. (A) Quantitation of the migration rate of HT-29 cells. (B) Quantitation of the migration rate of Caco-2 cells.

3.6.3 Conclusions

Kefir treatment seems to reduce the proliferative potential of cancer cells by promoting cell cycle arrest, target point of many anti-cancer drugs. Kefir also seems to promote apoptosis of cancer cells, boosting the immune system protective function. However, kefir has no effect in cancer cells migration capacity. These findings suggest that kefir could be a powerful ally to prevent cancer development in the initial stages but when metastases occur it is no more effective.

3.7 Investigation on kefir knowledge and consumption in Veneto region

Having seen from a scientific and experimental point of view how kefir can help in the prevention of non-communicable diseases, it is interesting to investigate how this product is perceived by the population. I conducted an exploratory marketing research study on a sample of the Veneto region population. The objective of the study was to investigate whether the knowledge of kefir is linked to some constitutive factors, and if the awareness of what kefir is, translates in the consumption of the product. Then, by investigating consumer's habits it would be possible to evaluate the topics for further research with the purpose of elaborating a potentially functional marketing strategy to drive consumer choosing kefir for maintaining a healthy lifestyle.

3.7.1 Design of experiment

Exploratory research is by definition qualitative and based on a small sample. In this case, 58 respondents took part in the study. The sampling method was simple random probability sampling for the first set of respondents and a snowball effect for which the respondents selected first attract other, secondary respondents was used. The survey was conducted through the administration of an online questionnaire.

3.7.2 Results

The results show that 51 people, representing 89% of the total sample, were aware of kefir as a fermented milk drink.

The purpose of this study was to determine whether knowledge of kefir as a fermented milk product is determined by age or education and what consumer's habits are in relation to the product. First, I divided the sample demographically, to overcome the differences in sample representativeness of the three categories: 18-30 years old (34 respondents); 30-50 years old (16 respondents); over 50 years old (8 respondents). Then I analysed the subgroups separately, comparing kefir knowledge with other parameters.

In the subgroup of 18-30 years of age, 30 out of 34 people know what kefir is. 30% of people knowing kefir had a secondary school degree, 23% had the master's degree; and 47% had a bachelor's degree. Out of the 30 people who know what kefir is, 13 people do not use it, 16 people buy it at the supermarket and 1 prepare it at home.

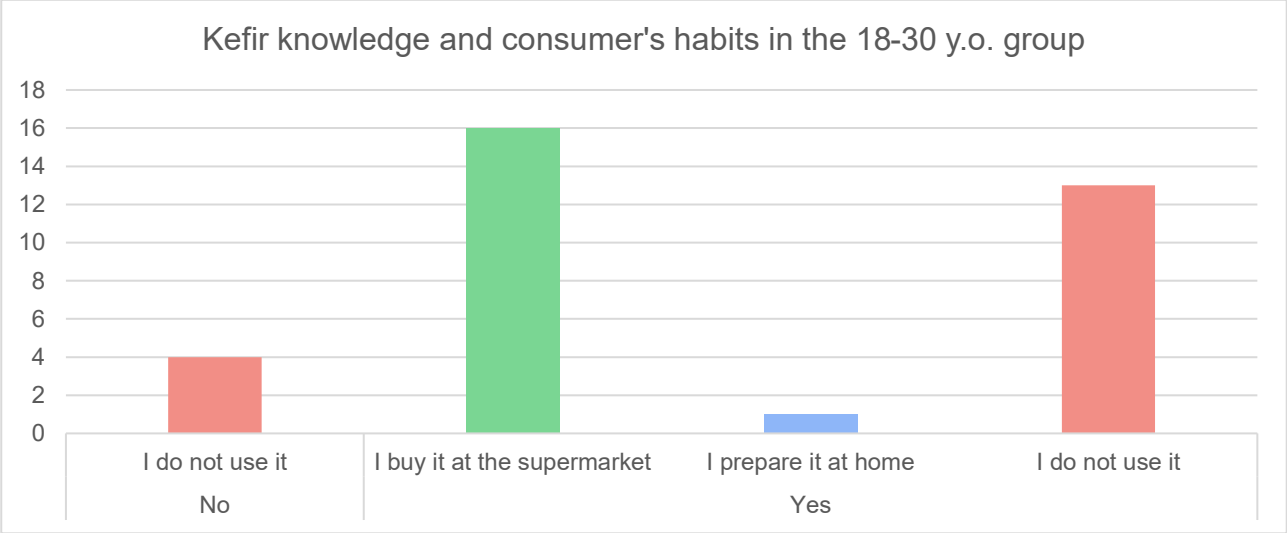

Figure 3.7.2 (1): Histogram reporting data of the subgroup 18-30 years of age. The graph reports the number of respondents on y axis and consumer habits in relation to kefir knowledge on the X axis.

In the subgroup of 30-50 years of age, 13 out of 16 people know what kefir is. 70% of the people knowing kefir had a secondary school degree, 15% had the master's degree; and 15% had a bachelor's degree. Out of the 13 people who know what kefir is, 6 people do not use it, 4 people buy it at the supermarket and 3 prepare it at home.

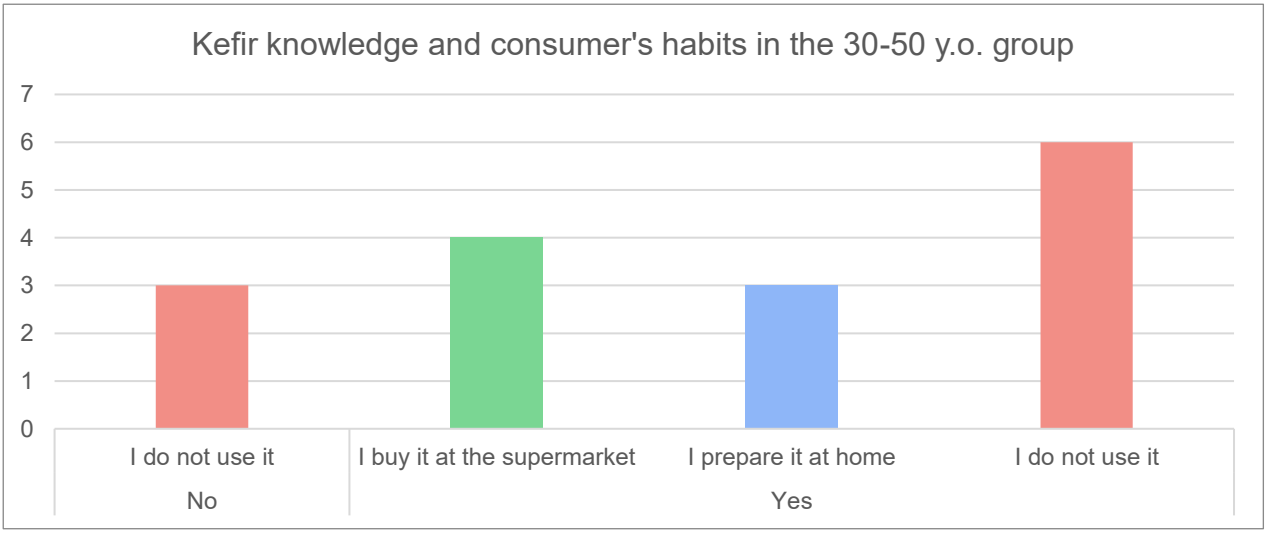

Figure 3.7.2 (2): Histogram reporting data of the subgroup 30-50 years of age. The graph reports the number of respondents on y axis and consumer habits in relation to kefir knowledge on the X axis

In the subgroup of over 50 years of age, all 8 respondents know what kefir is. 75% of the people in this group had a secondary school degree while 25%. Out of the 8 respondents in this group, 1 do not consume kefir, 2 people buy it at the supermarket and 5 prepare it at home.

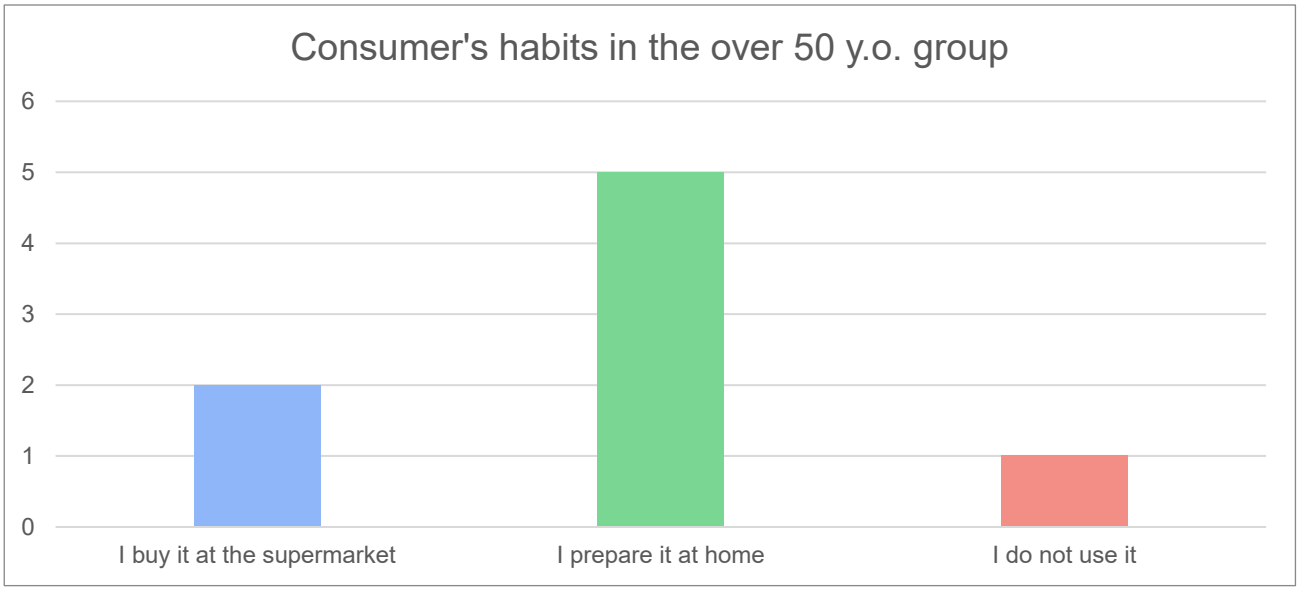

Figure 3.7.2 (3): Histogram reporting data of the subgroup over 50 years of age. The graph reports the number of respondents on y axis and consumer habits.

After assessing the differences between the stratifications of the testing population I investigated for which purpose people tend to consume kefir. 14% of the population consumes kefir because they like its taste, 7% because someone suggested to them to consume this product, 26% because it helps them with digestion. The remaining 53% of the respondents do not consume kefir.

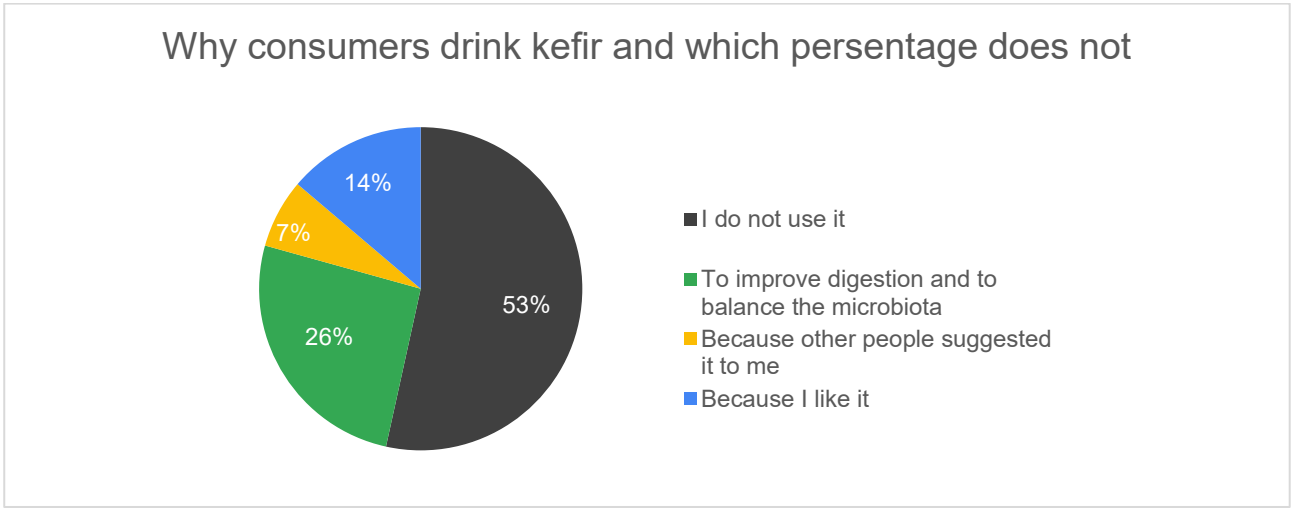

Figure 3.7.2 (4): Pie chart illustrating the percentage of testing population which do not consume kefir (black); consumes kefir because they like it (blue); consumes kefir because someone introduced them to the product (red); consumes kefir to ameliorate digestion (green).

3.7.4 Conclusions

By observing the gathered data, that should be used as preliminary knowledge for further quantitative researches on the same topic, it is possible to drive the following conclusions.

Overall, the population seems aware of kefir as a fermented milk drink, however the majority do not consume it still. Kefir knowledge and degree of study do not follow a direct trend, so there is not a proportional relationship between these two variables. This assumption is especially confirmed by the third group, where most people knowing kefir only achieved middle school education. From these initial data it is possible to observe the phenomenon of marketing penetration, which reaches people of all ages and education without the necessity of any action from their side.

Among people under 50 most of kefir consumers buy the product at the supermarket, this can probably be due to less free time availability and growing culture differences compared to older people. The group over 50 is an exception in different fields: 100% knows kefir, just 1 respondent do not consume it and most of consumers prepare it at home. The subgroup over 50 is also the least numerous so it is difficult to drive generalizable estimates, but these findings are an interesting incipit for further examination.

Ameliorating digestion seems to be the reason which attracts more kefir consumers. There is also a great part of kefir consumers that sustain kefir has a good taste and they enjoy consuming it. This are both important factors to consider when evaluating decision making to promote healthy choices such as kefir consumption. A suggested marketing intervention should be stressing more on kefir taste and freshness along with its beneficial properties in reinforcing immune system, improving digestion, preventing metabolic diseases.

4. CONCLUSIONS

Kefir has an anti-inflammatory property due to the high quantity of probiotics and prebiotics it contains. Lactic acid bacteria and acetic acid bacteria pass through the intestine and contribute to create a good environment for the growth of beneficial flora, and this decreases the risk of dysbiosis. Dysbiosis is the condition by which nutrients are not absorbed correctly, and the metabolic imbalance activates our immune system which leads to an inflammatory condition. Inflammation is the first step of many non-communicable diseases such as the ones investigated in this study: diabetes, atherosclerosis and cancer.

From in vivo studies we can observe that kefir can help in the regulation of blood glucose levels in diabetic induced mice. This effect can be observed also in humans, where both fasting blood glucose levels and circulating insulin levels decrease. Kefir consumption seems to increase insulin sensitivity in people at the pre-diabetes stage and that is indeed a good preventive measure. The mechanism by which kefir increases insulin sensitivity is probably due to its anti-inflammatory properties. By reducing inflammation, cell signalling improves thus it restores the regulatory system of glucose absorption in individuals at high risk of developing the pathology.

For the prevention of atherosclerosis, it is proven by both in vivo studies and in clinical trials that kefir consumption improves lipid profile. Atherosclerosis is due to the accumulation in the intima of the arteries of LDL particles followed by chronic inflammation and oxidative stress. Kefir consumption improves nutrient absorption, reducing the circulating LDL in the blood. Moreover, kefir contains many antioxidant molecules that decrease the level of ox-LDL if accumulation in the intima occurs and lowers the concentrations of inflammatory cytokines. The cholesterol lowering effect is mostly due to the increased production of apolipoproteins belonging to the class of HDL both in rats and in humans assuming kefir regularly. HDL molecules help in clearing the arteries, favouring the correct circulation of LDL particles. This is the prove that kefir can be a good preventing measure even for atherosclerosis.

For cancer prevention there are very promising results in animal models. Kefir consumption seems to reduce the risk of developing cancer in obese rats, restoring the risk condition to the one of healthy subjects. On healthy subjects instead, the kefir consumption seems to totally prevent the development of tumours. The results however cannot be generalized to humans yet. The conditions of experiments in animal models are perfectly controlled, from light and temperature to food consumption and rats in this study assumed kefir from gestation to adulthood. There are not significant studies in humans yet but the effect of kefir in CRC cells has been investigated instead. Kefir-treatment seems to reduce the proliferative capacity of cancer cells by promoting cell cycle arrest and apoptosis. The production of inflammatory cytokines by cancer cells is reduced. However, kefir-treatment has no significant impact on cancer cells migration capacity, so when the tumours enter the metastatic phase kefir has no more effect. Even if results seem to be very promising, we need to count the fact that even in experiments with cells the conditions are totally under control, so it is premature to generalise these findings to humans where both pathology and treatments are present in a systemic uncontrolled context.

From these findings it is possible to conclude that kefir is a powerful fermented milk drink that helps in the prevention of many diseases. This is why the consumption of this product should be promoted also using marketing interventions. The exploratory survey conducted in this study demonstrates that the majority of the population know kefir as a product, but it is still the minority of the population

that consumes it. This highlights that the message of the importance of kefir consumption to keep the body healthy is not stressed enough. Future marketing research can be conducted on how the awareness of beneficial properties of the product can change consumers habits and if the response is positive, maybe invest more on spreading the message.

5. BIBLIOGRAFY

Al-Mohammadi, A.-R.; Ibrahim, R.A.; Moustafa, A.H.; Ismaiel, A.A.; Abou Zeid, A.; Enan, G. Chemical Constitution and Antimicrobial Activity of Kefir Fermented Beverage. Molecules 2021, 26, 2635

Apalowo, Oladayo Emmanuel, Grace Adeola Adegoye, Teresia Mbogori, Jayanthi Kandiah, e Tolulope Mobolaji Obuotor. «Nutritional Characteristics, Health Impact, and Applications of Kefir». *Foods* 13, fasc. 7 (27 marzo 2024): 1026. https://doi.org/10.3390/foods13071026.

Assadi, M.M., Pourahmad, R. and Moazami, N. (2000). Use of isolated kefir starter cultures in kefir production. World Journal of Microbiology & Biotechnology 16: 541–543.

Barquera, Simon, Andrea Pedroza-Tobías, Catalina Medina, Lucía Hernández-Barrera, Kirsten Bibbins-Domingo, Rafael Lozano, e Andrew E. Moran. «Global Overview of the Epidemiology of Atherosclerotic Cardiovascular Disease». *Archives of Medical Research* 46, fasc. 5 (luglio 2015): 328–38. https://doi.org/10.1016/j.arcmed.2015.06.006.

Bellamy, I. and MacLean, D. (2005). Radiant Healing: The many paths to personal harmony and planetary wholeness: Joshua Books, Queensland, Australia, pp. 272

Bellikci-Koyu, Ezgi, Banu Pınar Sarer-Yurekli, Cem Karagozlu, Fadime Aydin-Kose, Ahmet Gokhan Ozgen, e Zehra Buyuktuncer. «Probiotic Kefir Consumption Improves Serum Apolipoprotein A1 Levels in Metabolic Syndrome Patients: A Randomized Controlled Clinical Trial». *Nutrition Research* 102 (giugno 2022): 59–70. https://doi.org/10.1016/j.nutres.2022.02.006.

Bermudez-Brito M, Plaza-Díaz J, Muñoz-Quezada S et al (2012) Probiotic mechanisms of action. Ann Nutr Metab 61:160–174

Bozkir, Eda, Birsen Yilmaz, Heena Sharma, Tuba Esatbeyoglu, e Fatih Ozogul. «Challenges in Water Kefir Production and Limitations in Human Consumption: A Comprehensive Review of Current Knowledge». *Heliyon* 10, fasc. 13 (luglio 2024): e33501. https://doi.org/10.1016/j.heliyon.2024.e33501.

Cappell, Mitchell S. «Pathophysiology, Clinical Presentation, and Management of Colon Cancer». *Gastroenterology Clinics of North America* 37, fasc. 1 (marzo 2008): 1–24. https://doi.org/10.1016/j.gtc.2007.12.002.

Caricilli, A.; Saad, M. The Role of Gut Microbiota on Insulin Resistance. Nutrients 2013, 5, 829–851.

Chang, Gary Ro-Lin, Wei-Yuan Cheng, Hueng-Chuen Fan, Hsiao-Ling Chen, Ying-Wei Lan, Ming-Shan Chen, Chih-Ching Yen, e Chuan-Mu Chen. «Kefir Peptides Attenuate Atherosclerotic Vascular

Calcification and Osteoporosis in Atherogenic Diet-Fed ApoE−/− Knockout Mice». *Frontiers in Cell and Developmental Biology* 11 (6 aprile 2023): 1158812. https://doi.org/10.3389/fcell.2023.1158812.

Cheirsilp B, Shimizu H, Shioya S. Enhanced kefiran production by mixed culture of Lactobacillus kefiranofaciens and Saccharomyces cerevisiae. J Biotechnol 2003; 100: 43-53.

Cheirsilp, B., and Radchabut, S. (2011). Use of whey lactose from dairy industry for economical kefiran production by Lactobacillus kefiranofaciens in mixed cultures with yeasts. N. Biotechnol. 28, 574–580. doi: 10.1016/j.nbt.2011.0 1.009

Clemente, J.C.; Ursell, L.K.; Parfrey, L.W.; Knight, R. The Impact of the Gut Microbiota on Human Health: An Integrative View. Cell 2012, 148, 1258–1270

Czech, M.P. Mechanisms of insulin resistance related to white, beige, and brown adipocytes. Mol. Metab. 2020, 34, 27–42.

D. Laureys, A. Van Jean, J. Dumont, L. De Vuyst, Investigation of the instability and low water kefir grain growth during an industrial water kefir fermentation process, Appl. Microbiol. Biotechnol. 101 (2017) 2811–2819.

Dhewa, T., Mishra, V., Kumar, N. and Sangu, K.P.S. (2015). Koumiss nutritional and therapeutic values. In: A.K. Puniya (ed.). Fermented Milk and Dairy Products. CRC Press, Boca Raton.

Dimas, A.S.; Lagou, V.; Barker, A.; Knowles, J.W.; Magi, R.; Hivert, M.F.; Benazzo, A.; Rybin, D.; Jackson, A.U.; Stringham, H.M.; et al. Impact of type 2 diabetes susceptibility variants on quantitative glycemic traits reveals mechanistic heterogeneity. Diabetes 2014, 63, 2158–2171.

Diosma, G., Romanin, D. E., Rey-Burusco, M. F., Londero, A., and Garrote, G. L. (2014). Yeasts from kefir grains: isolation, identification, and probiotic characterization. World J. Microbiol. Biotechnol. 30, 43–53. doi: 10.1007/s11274013-1419-9

Dokht Khosravi, Azar, Sakineh Seyed-Mohammadi, Ali Teimoori, e Aram Asarehzadegan Dezfuli. «The Role of Microbiota in Colorectal Cancer». *Folia Microbiologica* 67, fasc. 5 (ottobre 2022): 683–91. https://doi.org/10.1007/s12223-022-00978-1.

Eshghjoo, Sahar, Arul Jayaraman, Yuxiang Sun, e Robert C. Alaniz. «Microbiota-Mediated Immune Regulation in Atherosclerosis». *Molecules* 26, fasc. 1 (1 gennaio 2021): 179. https://doi.org/10.3390/molecules26010179.

Georgalaki, Marina, Georgia Zoumpopoulou, Rania Anastasiou, Maria Kazou, e Effie Tsakalidou. «Lactobacillus Kefiranofaciens: From Isolation and Taxonomy to Probiotic Properties and Applications». *Microorganisms* 9, fasc. 10 (16 ottobre 2021): 2158. https://doi.org/10.3390/microorganisms9102158.

Guiomar De Almeida Brasiel, Poliana, Sheila Cristina Potente Dutra Luquetti, Julliane Dutra Medeiros, José Otavio Do Amaral Corrêa, Alessandra Barbosa Ferreira Machado, Ana Paula Boroni Moreira, Vinícius Novaes Rocha, Claudio Teodoro De Souza, e Maria Do Carmo Gouveia Peluzio. «Kefir Modulates Gut Microbiota and Reduces DMH-Associated Colorectal Cancer via Regulation of Intestinal Inflammation in Adulthood Offsprings Programmed by Neonatal Overfeeding». *Food Research International* 152 (febbraio 2022): 110708. https://doi.org/10.1016/j.foodres.2021.110708.

Hamida, R.S.; Shami, A.; Ali, M.A.; Almohawes, Z.N.; Mohammed, A.E.; Bin-Meferij, M.M. Kefir: A Protective Dietary Supplementation against Viral Infection. Biomed. Pharmacother. 2021, 133, 110974.

Hossain, Md. Sanower, Hidayah Karuniawati, Ammar Abdulrahman Jairoun, Zannat Urbi, Der Jiun Ooi, Akbar John, Ya Chee Lim, et al. «Colorectal Cancer: A Review of Carcinogenesis, Global Epidemiology, Current Challenges, Risk Factors, Preventive and Treatment Strategies». *Cancers* 14, fasc. 7 (29 marzo 2022): 1732. https://doi.org/10.3390/cancers14071732.

Jebari-Benslaiman, Shifa, Unai Galicia-García, Asier Larrea-Sebal, Javier Rekondo Olaetxea, Iraide Alloza, Koen Vandenbroeck, Asier Benito-Vicente, e César Martín. «Pathophysiology of Atherosclerosis». *International Journal of Molecular Sciences* 23, fasc. 6 (20 marzo 2022): 3346. https://doi.org/10.3390/ijms23063346.

Jen J, Powell SM, Papadopoulos N, et al. Molecular determinants of dysplasia in colorectal lesions. Cancer Res 1994;54:5523–6.

Khan, Moien Abdul Basith, Muhammad Jawad Hashim, Jeffrey Kwan King, Romona Devi Govender, Halla Mustafa, e Juma Al Kaabi. «Epidemiology of Type 2 Diabetes – Global Burden of Disease and Forecasted Trends»: *Journal of Epidemiology and Global Health* 10, fasc. 1 (2019): 107. https://doi.org/10.2991/jegh.k.191028.001.

Khoury, Nathalie, Stephany El-Hayek, Omayr Tarras, Marwan El-Sabban, Mirvat El-Sibai, e Sandra Rizk. «Kefir Exhibits Anti-Proliferative and pro-Apoptotic Effects on Colon Adenocarcinoma Cells with No Significant Effects on Cell Migration and Invasion». *International Journal of Oncology* 45, fasc. 5 (novembre 2014): 2117–27. https://doi.org/10.3892/ijo.2014.2635.

Kraaijenhof, J.M.; Hovingh, G.K.; Stroes, E.S.; Kroon, J. The iterative lipid impact on inflammation in atherosclerosis. Curr. Opin. Lipidol. 2021, 32, 286–292.

Lee, J.-H.; Lee, J. Indole as an intercellular signal in microbial communities. FEMS Microbiol. Rev. 2010, 34, 426–444.

Lopitz-Otsoa, Fernando, Aitor Rementeria, Natalia Elguezabal, e Javier Garaizar. «Kefir: Una Comunidad Simbiótica de Bacterias y Levaduras Con Propiedades Saludables». *Revista Iberoamericana de Micología* 23, fasc. 2 (giugno 2006): 67–74. https://doi.org/10.1016/S1130-1406(06)70016-X.

Mokoena, Mduduzi Paul. «Lactic Acid Bacteria and Their Bacteriocins: Classification, Biosynthesis and Applications against Uropathogens: A Mini-Review». *Molecules* 22, fasc. 8 (26 luglio 2017): 1255. https://doi.org/10.3390/molecules22081255.

Otles, S. and Cagindi, O. (2003). Kefir: A Probiotic Dairy-Composition, Nutritional and Therapeutic Aspects. Pakistan Journal of Nutrition 2: 54–59.

Peled, M.; Fisher, E.A. Dynamic Aspects of Macrophage Polarization during Atherosclerosis Progression and Regression. Front. Immunol. 2014, 5, 579.

Peluzio, M.d.C.G.; Dias, M.d.M.E.; Martinez, J.A.; Milagro, F.I. Kefir and Intestinal Microbiota Modulation: Implications in Human Health. Front. Nutr. 2021, 8, 638740.

Petersen, K.F.; Shulman, G.I. Pathogenesis of skeletal muscle insulin resistance in type 2 diabetes mellitus. Am. J. Cardiol. 2002, 90, 11G–18G.

Ray, Ramesh C., e Didier Montet, a c. di. *Fermented Foods. Part II, Technological Interventions*. Boca Raton, FL: CRC Press, 2017.

AL-Shemmari, Ihab Ghazi Mahdi, Raeed Abdel Mahdi Kassim Altaee, e Ayyed Hameed Hassan. «Evaluation of Antidiabetic and Antihyperlipidimic Activity of Kefir in Alloxan Induced Diabetes Mellitus Rat». *Scientific Journal of Medical Research* 02, fasc. 06 (2018): 83–86. https://doi.org/10.37623/SJMR.2018.2606.

Robbins DH, Itzkowitz SH. The molecular and genetic basis of colon cancer. Med Clin North Am 2002;86:1467–95.

Ross, R. Does exercise without weight loss improve insulin sensitivity? Diabetes Care 2003, 26, 944–945.

Salari, Afsaneh, Sara Ghodrat, Alireza Gheflati, Lida Jarahi, Mohammad Hashemi, e Asma Afshari. «Effect of Kefir Beverage Consumption on Glycemic Control: A Systematic Review and Meta-analysis of Randomized Controlled Clinical Trials». *Complementary Therapies in Clinical Practice* 44 (agosto 2021): 101443. https://doi.org/10.1016/j.ctcp.2021.101443.

Shah MS, DeSantis TZ, Weinmaier T et al (2018) Leveraging sequence-based faecal microbial community survey data to identify a composite biomarker for colorectal cancer. Gut 67:882–891

Steinbrecher, U.P.; Zhang, H.; Lougheed, M. Role of oxidatively modified LDL in atherosclerosis. Free Radic. Biol. Med. 1990, 9, 155–168

Stumvoll, M.; Goldstein, B.J.; van Haeften, T.W. Type 2 diabetes: Principles of pathogenesis and therapy. Lancet 2005, 365, 1333–1346

Tan, J.; McKenzie, C.; Potamitis, M.; Thorburn, A.N.; Mackay, C.R.; Macia, L. The role of short-chain fatty acids in health and disease. Adv. Immunol. 2014, 121, 91–119.

Watson, M.G.; Byrne, H.M.; Macaskill, C.; Myerscough, M.R. A two-phase model of early fibrous cap formation in atherosclerosis. J. Theor. Biol. 2018, 456, 123–136.

Y. Cai, A. Sounderrajan, L. Serventi, Water kefir: a review of its microbiological profile, antioxidant potential and sensory quality, Acta Scientifci Nutritional Health 4 (2020) 10–17.

Zanirati, D. F., Abatemarco, M., Cicco Sandesb, S. H., Nicolia, J. R., Nunes, A. C., and Neumanna, E. (2015). Selection of lactic acid bacteria from Brazilian kefir grains for potential use as starter or probiotic cultures. Anarobe 32, 70–76. doi: 10.1016/j.anaerobe.2014.12.007

Zheng, Y.; Ley, S.H.; Hu, F.B. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. Nat. Rev. Endocrinol. 2018, 14, 88–98

Zhou, Royce W., Noam Harpaz, Steven H. Itzkowitz, e Ramon E. Parsons. «Molecular Mechanisms in Colitis-Associated Colorectal Cancer». *Oncogenesis* 12, fasc. 1 (26 ottobre 2023): 48. https://doi.org/10.1038/s41389-023-00492-0.