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Metabolic effects of dietary approaches: ketone bodies & ketogenic diet

[Scientific Field: BIO/09]

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1 INTRODUCTION

Overweight is one of the major health problems related to the lifestyle of today's society, and recent scientific evidence indicates that the spread of overweight and obesity continues to increase dramatically throughout the world (Chopra *et al*., 2002 - King *et al*., 2009 - WHO, 2007). For the first time in history, there are more obese children than malnourished, an alarming fact considering that obesity is a high risk factor for the increased incidence of cardiovascular diseases, diabetes and tumors (Malvezzi *et al*., 2013 - Tamosiunas *et al*., 2014 - Wild *et al*., 2004). Thus, the prevention of weight gain has become increasingly important not only for everyday consumer health but also for those national or international institutions responsible for the health of their subjects. Today, people are becoming more and more interested in following a diet that allows them to reach, not only an adequate weight, but above all a good state of health.

One of the dietary approach that has attracted particular attention in recent years is the "ketogenic diet" (KD). This is a dietary program designed in the 1920s as a therapy for drug-resistant epilepsy (Freeman *et al*., 1998 – Hartman & Vining, 2007), which then gained popularity in the 1970s as a weight-loss diet (Atkins) (Astrup *et al*., 2004 – Caminhotto *et al*., 2015). Indeed, many current diets are based on the KD model, and, recently, this model was proposed for a number of neurological disorders like Alzheimer's and Parkinson's diseases (Kashiwaya *et al*., 2015 – Vanitallie *et al*., 2005).

But what actually is a "ketogenic" diet? The word "ketogenic" derives from the Greek and literally means ketones (*chetos*) origin (*genesis*), and the basis of the diet is to induce the synthesis of ketone bodies (KB), which take place of sugars for producing energy. This KB synthesis occurs essentially in two situations, fasting and diabetes. The fasting condition produces KB by forcing the organism to catabolize fats for energy, which through β-oxidation leads to the production of large acetyl-CoA amounts that remain in excess compared to the available oxaloacetate.

The KD is a high-fat, low-carbohydrates diet planned to achieve ketosis, characterized by increased levels of circulating KB. Under normal conditions and with a balanced diet, KB are produced in small quantities, but, under a KD, their synthesis is induced as a result of a very low carbohydrate intake and high fat intake. The low glucose supply stimulates the catabolism of fats to obtain energy, leading to the accumulation of acetyl-CoA and synthesis of KB by the liver, which are sent to the peripheral tissues where they are oxidized to produce energy (Fukao *et al*., 2004 – Laffel, 1999 – Nehling, 2004).

This physiological process represents an evolutionary advantage for the human species, which was exposed at its origins to cycles of glut, prolonged fasting and frequent famine. The big question today is: are there any risks to taking advantage of this metabolic response in order to obtain weight loss? This is a subject of heated debate by the scientific community. Indeed, the usefulness of KB for therapeutic purposes is unquestionable, since its positive effects are now recognized in the treatment of epilepsy and other neurodegenerative diseases. However, as a solution to rapid weight loss the ketogenic dietary approach requires further attention.

1.1 SYNTHESIS OF KETONE BODIES

Three compounds are called "ketone bodies": acetoacetate (AA), β-hydroxybutyrate (βHB) and acetone (Fig. 1). AA is produced and used, during intermediate metabolism, and it generates the other two KB. Acetone, which derives from the spontaneous decarboxylation of AA, is of clinical interest because its particular smell in the exhaled air allows the identification of ketosis. Instead,

βHB is produced from the reduction of AA, and is conventionally grouped with the other two KB, although βHB's ketone part is reduced to hydroxyl group. βHB is not volatile and is chemically stable, one of its metabolic fates being interconversion with AA (Fukao *et al*., 2004).

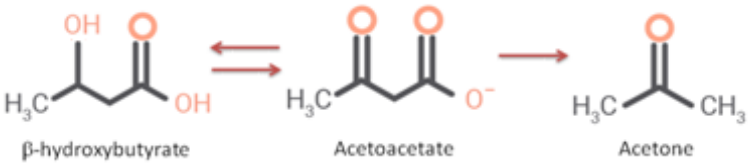

Fig. 1 Chemical structure of ketone bodies.

Under normal conditions and with a balanced diet, KB are produced in small quantities, because acetyl-CoA is mainly used in the citric acid cycle. The oxidative decarboxylation of pyruvic acid and β-oxidation are specifically regulated so that acetyl-CoA is not produced in excess. The main uses of acetyl-CoA (Fig. 2) shall include its complete oxidation to CO_2 and H_2O in the mitochondria, and its cytosolic utilisation for the biosynthesis of fatty acids and cholesterol.

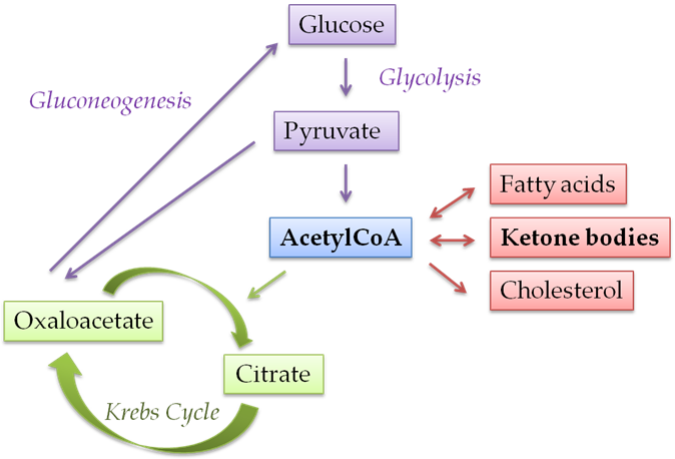

Fig. 2 Metabolic uses of acetyl-CoA.

One of the factors on which mitochondrial oxidation of acetyl-CoA depends is the mitochondrial levels of oxalacetate, generated mainly by the decarboxylation of pyruvic acid, the terminal product of glycolysis, and the metabolism of some aminoacids. Indeed, acetyl-CoA and oxaloacetate are condensed to form the citrate, which is used in the citric acid cycle of mitochondria, or exported to cytosol.

Under particular conditions, like when the intracellular glucose levels become too low, for example during prolonged starvation, fasting or as a result of low insulin levels in diabetes, oxaloacetate is depleted owing to its preferential utilization in the process of gluconeogenesis. This impedes the entry of acetyl-CoA into Krebs cycle. Therefore, in liver mitochondria, the excess of acetyl-CoA is converted into KB, which are sent through the bloodstream to the peripheral tissues where they are oxidized to produce energy, or excreted in the urine (Campbell & Farrel, 2012).

The mitochondrial synthesis of KB consists of three steps (Fig. 3):

- the first step is the enzymatic condensation of two molecules of acetyl-CoA, catalyzed by thiolase;
- the acetoacetyl-CoA condenses with acetyl-CoA to form β-hydroxy-β-methylglutaryl-CoA (HMG-CoA);

- HMG-CoA is cleaved to free AA, which is reversibly reduced by D-β-hydroxybutyrate dehydrogenase to βHB, or spontaneous decarboxylated to acetone.

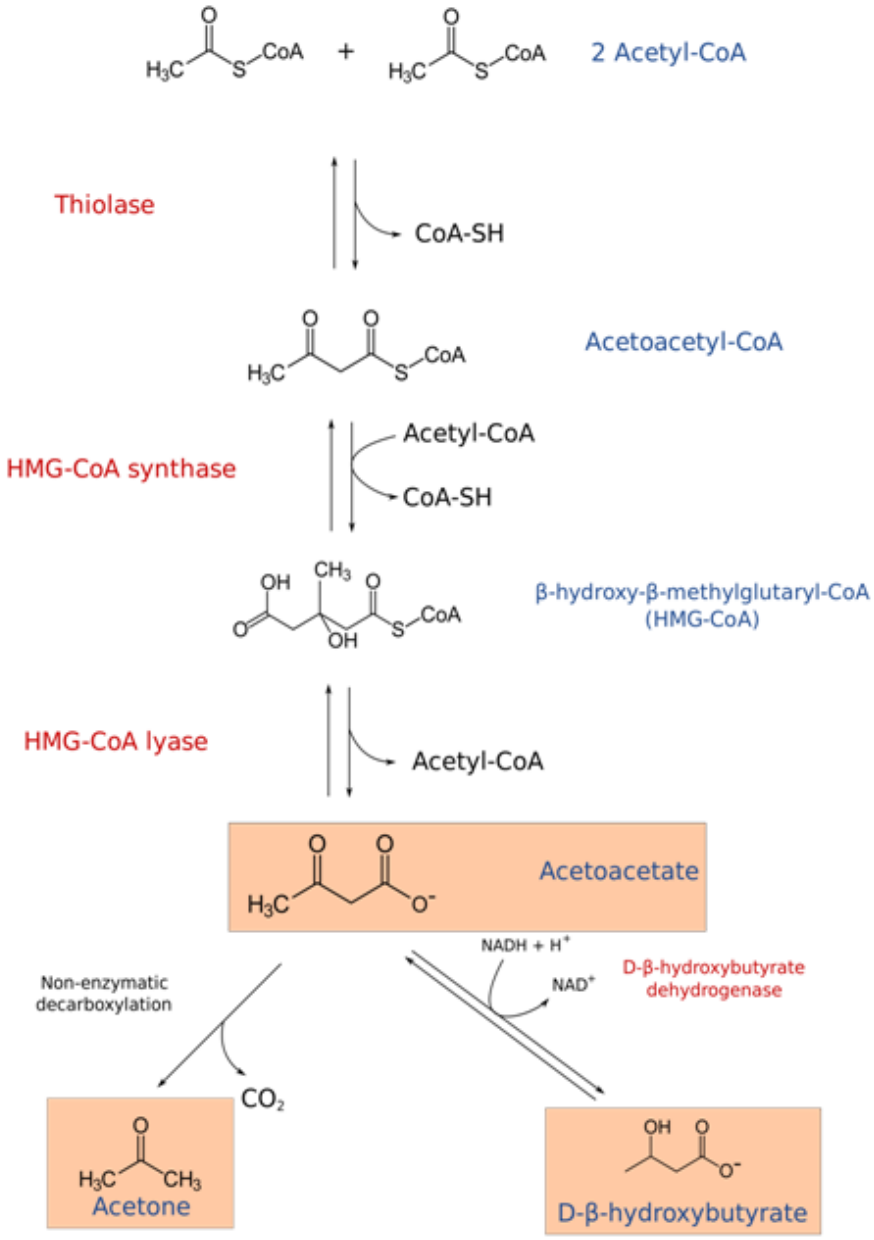

Fig. 3 Ketogenesis pathway.

The use of KB for energy purposes occurs in physiological conditions, such as morning ketosis, ketosis after effort or after eating a low-carbohydrates and high-protein meal (Pezzana *et al*., 2014). This physiological ketosis differs a lot from diabetic ketoacidosis, a severe status in which KB are over-produced by the liver. In fact, circulating levels of KB in normal conditions are generally 0,5 mM, while on a KD they are up to 5-7 mM, and in uncontrolled diabetes they can reach up to 25 mM. Moreover, the normal ratio between βHB and AA is 3:1, while on a KD it can reach 4:1, and in diabetic ketoacidosis 6:1 (Tab. 1) (Cahill, 2006 - Laffel, 1999).

	Physiological condition	*Ketogenic diet*	*Diabetic acidosis*
Circulating levels of KB	0,5 mM	5 - 7 mM	25 mM
βHB : AA ratio	3 : 1	4 : 1	6 : 1

Tab. 1 Different KB's circulating levels and βHB : AA ratio in the bloodstream.

When the amount of KB exceeds normal values in the blood (up to 3 mM) a metabolic decompensation takes place. KB are relatively strong organic acids that are potentially harmful because they lower blood pH below regular values, causing acidosis. It is important to note that prolonged acidosis can become a dangerous pathology, and is, in fact, the first cause of death of prolonged fasting, and can also be a severe consequence of uncontrolled diabetes. Buffer systems usually stabilize the body's pH, but if the fast conditions persist, they become exhausted and the pH reaches a non-optimal level for many enzymes. Moreover, the excessive amount of urine produced by kidneys to expel KB can produce a secondary imbalance of mineral salts (Campbell & Farrel, 2012).

1.1.1 Metabolic effects of ketone bodies

KB are emerging as crucial regulators of human health. For instance, βHB could be a valid energy support for peripheral tissues during fasting or intense physical exercise. Human βHB basal serum levels are normally low, about 0,1 mM, but after two days of fasting they can reach 1-2 mM, and under ketogenic treatment can even exceed 2 mM concentration (Kim & Rho, 2008). Intriguingly, βHB is more than just a metabolite as it also plays an important cellular signalling role. In fact, βHB is an endogenous inhibitor of histone-deacetylases (HDACs) and a ligand for at least two cell surface receptors that modulate lipolysis, sympathetic tone and metabolic rate (Fig. 4).

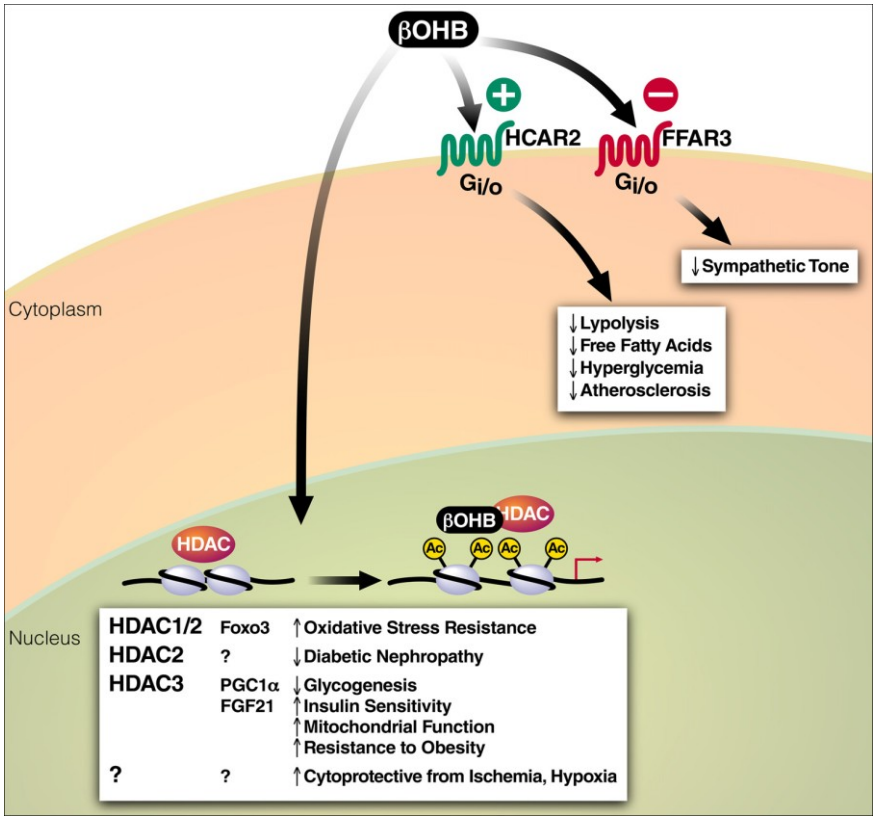

Fig. 4 Cellular signaling functions of the ketone body βOHB (βHB) (Newman & Verdin, 2014)

In addition, βHB alters protein acetylation through at least two mechanisms: i) by increasing the cellular pool of acetyl-CoA that is a substrate for histone acetyltransferases, and ii) by directly inhibiting the activity of class I HDACs (Newman & Verdin, 2014). HDACs are a family of proteins able to suppress gene expression through the deacylation of lysine residues of histone and non-histone proteins. The ability of βHB to affect HDAC activity, and thereby epigenetic regulation, can have notable implications on a wide variety of genes. For instance, βHB is able to modify the expression of genes involved in resistance to oxidative stress (acting in particular on HDAC-1) (Giacco & Brownlee, 2010) or in the development of metabolic diseases (Mihaylova *et al*., 2013). Thus, the ability of βHB to induce resistance to oxidative stress by inhibiting HDACs could explain the therapeutic benefit of a KD for neurological disorders (Newman & Verdin, 2014).
Antioxidant and oxidative stress-mitigating roles of KB have been widely described both *in vitro* and *in vivo*, particularly in the context of neuroprotection.
In this regard, Noh and colleagues showed (2016) how KB, particularly AA, offer protection from oxidative stress caused by glutamate. In fact, the results indicated that both AA and βHB significantly reduce glutamate-induced neuronal death in HT22 cells (a mouse hippocampal cell line) and in rat primary hippocampal neurons. Specifically, it was seen that pre-treatment with 5 mM of AA provided more effective HT22 protection (about 86%), than that offered by βHB (52%). Instead treatment with acetone provided no protection. Moreover, the effect of AA on ROS production was assessed; the data showed that a treatment for 8 hours with glutamate in HT22 leads to an increase in ROS levels, while the pre-treatment with AA has significantly decreased ROS production. Therefore, the results suggest that, in this *in vitro* model, the neuroprotective effect of AA is related to the decrease of mitochondrial ROS production (Noh *et al*., 2006).

1.2 KETOGENIC DIET FOR THERAPEUTIC PURPOSES

1.2.1 Epilepsy

To date, it has been demonstrated how the metabolic state of mild ketosis, which can be induced through KD administration, calorie restriction or fasting, represents an emerging tool for the metabolic management of epilepsy and a number of different neurological and metabolic diseases.
Epilepsy represents one of the most frequent neurological pathologies, concerning about 43 million people worldwide. The annual incidence of epilepsy is 40-70 cases per 100,000 people in industrialized countries and 100-190 per 100,000 in resource-poor countries (Fig. 5).

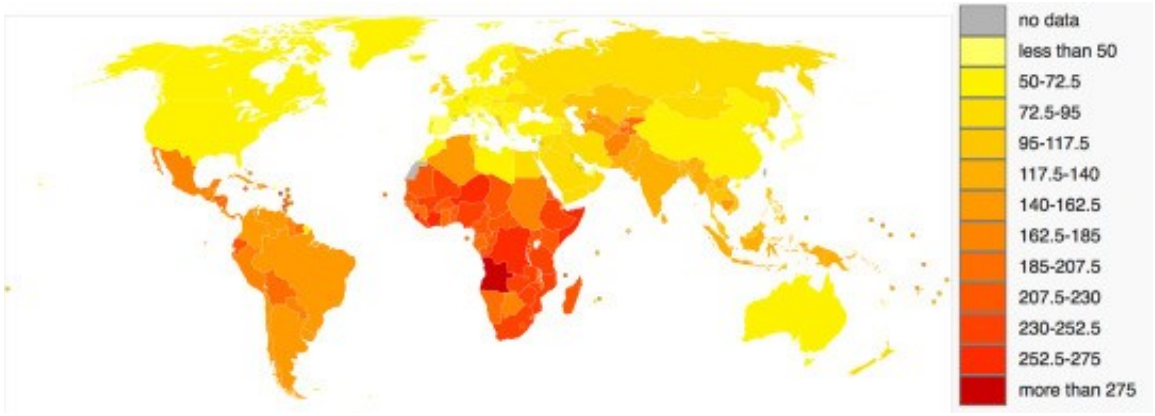

Fig. 5 Representation of the incidence of epilepsy (number of cases per 100,000 inhabitants) in the different countries of the world (Abramovici & Bragic, 2016).

In most cases, epilepsy is considered a benign condition: in fact, in 60% to 70% of diagnosed cases a remission is obtained after appropriate treatment with anti-epileptic drugs. However, 30-40% of patients with epilepsy do not respond to any antiepileptic treatment (Kwan & Brodie, 2000). Evidence suggests that the incidence of drug-resistant epilepsy is high: it has been reported that up to 525 individuals (9-93 years) presented with a new diagnosis of epilepsy, 37% having being shown to be drug resistant after 13 years of pharmacological treatment (French, 2007). Currently, KD is recognized as the best protocol to treat drug-resistant epilepsies.

It has been known for a long time that fasting can be used as a treatment for epilepsy; descriptions of the effectiveness of dietary restriction for this purpose date back to the time of Hippocrates and are mentioned in the New Testament. High-fat diets were considered about 150 years ago, but the first recognized therapeutic use of the diet seems to have been in the United States at the beginning of the last century, when a healer (Bernard Macfadden) and an osteopathic physician (Dr. Hugh Conklin) supported the use of fasting in a boy with epileptic seizures. As soon as human metabolism became better understood, it became clear that a high-fat diet could induce, like fasting, a state of ketosis. Simultaneously, Wilder introduced the concept of a diet composed of "ketogenic" and "anti-ketogenic" components for the treatment of epilepsy (Wilder, 1921). About 20 years ago, the treatment of a child at the Johns Hopkins Hospital raised worldwide scientific interest in KD (Freeman *et al*., 1998).

Therapeutic KD is high in fat content and low in carbohydrates. When carbohydrates are limited, ketones, responsible for the anticonvulsant action, are synthesized to provide an alternative fuel source to the brain. The typical ratio between fats and carbohydrates plus proteins (in terms of grams) is 4:1 (Kossoff & McGrogan, 2005). The efficacy of the KD against epilepsy has been demonstrated by taking into consideration a series of parameters, similar to those of patients in pharmacological treatment, such as seizure control and the absence of side effects. Currently, the classic KD and its variants are used in the treatment of drug-resistant epilepsy in 80 countries (2017) all over the world.

1.2.2.1 Dietary protocols

The diet is aimed at inducing and maintaining ketosis. The types of dietary protocols used up to now are distinguished i) by the way in which ketosis is induced and ii) based on the quality and quantity of the lipid and carbohydrate components. In summary, they can be grouped as follows (Tab. 2);

Protocol	
Classic	Long chain triglycerides, caloric and fluid restriction, induction of fasted ketosis
Classic updated	Long or medium-chain triglycerides (MCT), without fasting, without caloric and liquid restriction
Alternative	With increased carbohydrate content (MAD, LGIT)

Tab. 2 Main ketogenic protocols.

The classic KD is the original diet introduced into practice in the 1920s. It is typically composed of a 4:1 ratio of fat (in grams) to protein plus carbohydrates (in grams), thus shifting the predominant caloric source from carbohydrate to fat. Lower ratios of 3:1, 2:1, or 1:1 can be used depending on age, individual tolerability, levels of ketosis, and protein requirements (Zupec-Kania & Spellman, 2008).

In addition, different and more relaxed variant forms (Fig. 6) have been designed, including the KD combined with medium-chain triglycerides (MCT), the Modified Atkins Diet (MAD) and the Low Glycemic Index Treatment (LGIT).

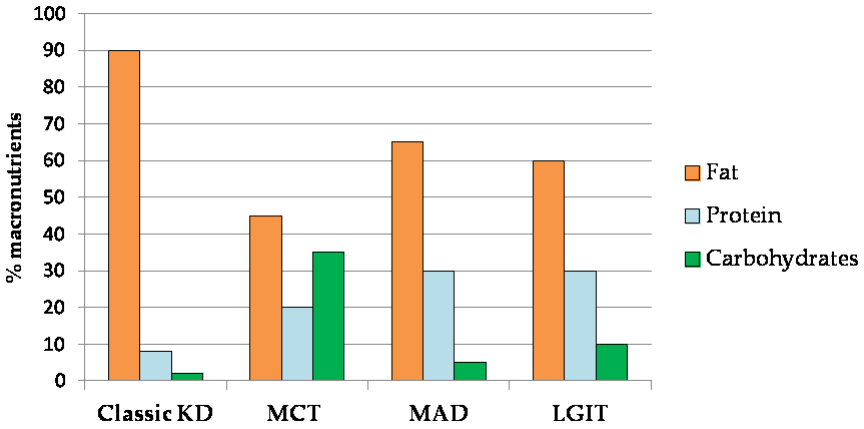

Fig. 6 Different diet compositions (%/kcal): classic KD and its variants.

The KD MCT variant uses medium-chain fatty acids provided by coconut and/or palm kernel oil as a diet supplement. Replacing part of the long-chain triglycerides (LCT) with MCT is a composition variant introduced in 1971 by Huttenlocher in Chicago. This variant increases the share of carbohydrates as MCT results in more ketones per kcal of energy than LCT, are absorbed more efficiently, and are carried directly to the liver in the portal blood. This increased ketogenic potential means less total fat is needed in the MCT diet, allowing the inclusion of more carbohydrate and proteins. The ketosis level achieved is similar to that of the classic protocol and, moreover, the diet with MCT has proved to be equally effective in the treatment of epilepsy (Neal *et al*., 2009).

The MAD variant, introduced in 2003, is based on a ratio of approximately 1:1, although this is not necessary in all meals, and includes 10-30 g of carbohydrate per day with no restriction of fluids, calories or protein. It allows users more flexibility and it does not require the calculating and weighing of food portions or an initial hospital stay for its implementation (Kossoff *et al*., 2008).

The LGIT is designed to prevent dramatic postprandial increases in blood glucose by limiting the quantity of carbohydrates consumed and restricting the carbohydrates sources to low glycemic index foods (< 50 GI). Compared to the classic KD, the LGIT variant allows for a less restricted total carbohydrate intake (Muzykewicz *et al*., 2009 - Pfeifer & Thiele, 2005).

The general protocol of the classic KD adopted at the Johns Hopkins Hospital (Tab. 3) has evolved over time, and many advances have been made regarding the mode of administration of the diet. Before beginning it, the patients are subjected to tests aimed at identifying any metabolic disorders that might interfere with the ability to generate adequate ketone amounts, like defective metabolic pathways of oxidation or fatty acids transport. However, there are some absolute contraindications to KD and these include pyruvate carboxylase deficiency, porphyria, lipid myopathies, and primary or acquired carnitine deficiency (Hartman & Vining, 2007 – Wheless, 2001).

Once admitted to hospital, the patients are fasted (fluids are administered) with glycemic controls every 6h. For glucose levels below 25-40 mg/dL, no treatment is needed unless the patient becomes symptomatic (for example with extreme lethargy and severe vomiting). On the second day 2/3 of the total calories programmed are administered, and on the third day, the total caloric intake calculated. The total amount of calories of the diet is based on anthropometric measures and dietary habits of the subject prior to the intervention.

Before diet
Nutrition history obtained
Minimize carbohydrate intake for 1 day
Fasting begins after dinner the evening prior to admission
Day 1
Admission to the hospital
Conversion to carbohydrate-free medications
Basic laboratory results obtained if not done previously (metabolic profile, urine calcium, urine creatinine, fasting lipid profile, antiepileptic drug levels)
Check fingerstick glucose every 6 hr; if <40 mg/dL, check every 2 hr
If symptomatic, or glucose <25 mg/dl, give 30 ml orange juice, measure blood glucose again
Parents begin classes
At dinner, one third of the calculated ketogenic meal given as "eggnog" (e.g., if the full meal is calculated as 150 ml, give 50 ml at this meal)
Blood glucose checks discontinued after dinner
Day 2
At breakfast and lunch, one-third of the calculated ketogenic meal given as "eggnog"
Symptomatic ketosis (e.g., nausea, vomiting) can be relieved with small quantities of orange juice
Parent classes continue
At dinner, two-thirds of the calculated ketogenic meal given as "eggnog"
Day 3
At breakfast and lunch, two-thirds of the calculated ketogenic meal given as "eggnog"
Parent classes conclude
At dinner, the first full ketogenic meal is given (not "eggnog")
Day 4
After breakfast (full ketogenic meal), the patient is discharged to home
Prescriptions written for carbohydrate-free medications, urine ketone test strips, a sugar-free, fat-soluble multivitamin and calcium supplements, citrate salts (if indicated)
Clinic follow-up appointment arranged

Tab. 3 Typical KD initiation regimen (Hartman & Vining, 2007)

The typical diet, in the clinic, includes 3 meals and 2 snacks during a period of 24h (Tab. 4).

Breakfast
Breakfast quiche with bacon
Lunch
Belgian salad with avocados, pineapple, and pecans
Dinner
Sausage, potato, sauerkraut
Creamy milkshake
Snacks
Cheesecake with blueberries
(total: 1000 calories/ 3.8:1 ratio)
Total cost for the day: $2.94

Tab. 4 Example of daily menu of KD (Hartman & Vining, 2007)

The next phase of the KD consists of routine clinical visits (3, 6, 12, 18 and 24 months after beginning the diet) with laboratory tests and frequent meetings with the nutritionist. In general, once the efficacy of the diet has been established (usually within 3-6 months from the start of the diet), the drugs are reduced. However, if the patient has significant side effects from the drug therapy, the drugs can be eliminated earlier (Kossoff *et al*., 2004).

1.2.1.2 Side effects & complications

The KD is an effective medical therapy for epilepsy, but it must be carefully monitored because it is associated with a number of side effects. A recent review was aimed at retrieving prospective studies that monitored adverse effects in children receiving KD therapy for refractory epilepsy (Cai *et al*., 2017). It reported that more than 40 categories of side effects are associated with KD (Tab. 5). Some are predictable and potentially curable, such as constipation, dehydration, and hypoglycemia (Kang *et al*., 2004), others, such as cardiomyopathy and renal tubular acidosis, have been reported in isolated clinical cases, and their relationship with KD is unknown.

Adverse effects	Number of cases (%)	Adverse effects	Number of cases (%)
Constipation	175 (13.2)	Osteopenia	17 (1.2)
Gastrointestinal disturbances	132 (9.6)	Irritability	12 (0.9)
Vomiting	125 (9.1)	Tachycardia	12 (0.9)
Hyperlipidemia	63 (4.6)	Gastroesophageal reflux	10 (0.7)
Hyperuricemia	61 (4.4)	Flushed face	10 (0.7)
Lethargy	56 (4.1)	Hematuria	9 (0.7)
Hypercholesterolemia	53 (3.8)	Aspiration pneumonia	7 (0.5)
Infectious disease	53 (3.8)	Behavioral problems	7 (0.5)
Hypoproteinemia	52 (3.8)	Respiratory failure	6 (0.4)
Diarrhea	52 (3.8)	Reduced carnitine levels	6 (0.4)
Hypertriglyceridemia	44 (3.2)	Reduction of plasm zinc	5 (0.4)
Acidosis	42 (3.1)	Epistaxis and bruising	4 (0.3)
Hunger	33 (2.4)	Thrombocytopenic purpura	3 (0.2)
Lack of energy/fatigue	32 (2.3)	Pancreatitis	2 (0.1)
Pneumonia	29 (2.1)	Gallbladder stones	2 (0.1)
Dehydration	29 (2.1)	Fatty liver	2 (0.1)
Elevation of liver enzymes	28 (2.0)	Fatty diarrhea	2 (0.1)
Hypoglycemia	25 (1.8)	Hair thinning	2 (0.1)
Abdominal pain	23 (1.7)	Pica	1 (0.07)
Electrolyte disturbance	22 (1.6)	Pulmonary edema	1 (0.07)
Weight loss	20 (1.5)	Shock	1 (0.07)
Fever	19 (1.4)	Dysphagia	1 (0.07)
Urolithiasis	19 (1.4)	Urinary sediment	1 (0.07)
Taste problems	17 (1.2)		

Tab. 5 Reported adverse effects due to the KD in prospective studies (n=1376) (Cai *et al*., 2017)

One of the side effects that has been systematically detected is nephrolithiasis (observed in 6% of patients) (Furth *et al*., 2000); while other adverse effects of the diet are hypercalciuria, aciduria and hypocitruria which contribute to the formation of stones. For these reasons all the patients, before starting the diet, are screened for family history of nephrolithiasis and hypercalciuria. For those having high amount of creatinine, calcium in the urine and haematuria, or those taking carbonic anhydrase inhibitors, oral citrate salts were prescribed as prophylactic (Kossof *et al*., 2002). Finally, nephrolithiasis is treated by increasing fluid intake, alkalinisation of urine, and suspension of carbonic anhydrase inhibitors.

For very young children following the protocol, one consequence to be taken into consideration is the fact that growth (height and weight) may be compromised (Liu *et al*., 2003). Thus during the follow-up visits each child's growth is monitored and strictly controlled. During the admission phase of the diet, families are informed about the symptoms of possible side effects, though many such effects do not manifest themselves immediately but have long-term implications. In fact, there could be consequences for the health of the vascular system (atherosclerosis), bones (osteoporosis), and liver (Hartman & Vining, 2007).

In addition to a potential decrease in the children's growth, there can also be reduced bone mineral density due to the limited availability of calcium in the diet. Recently, an animal model study was conducted (Frommelt *et al*., 2014) to determine mineral absorption in a KD compared to a diet with a standard fat content. It was demonstrated that calcium absorption was considerably reduced in KD, suggesting a lower bioavailability of the mineral. Indeed, in 2008 a longitudinal intervention study addressed this issue by assessing the bone mineral content (BMC) in epileptic children. The subjects were 25 pre-adolescent children (1-14 years, 16 males and 9 females) undergoing a ketogenic regimen for 15 months. Along with BMC, other parameters were measured, such as anthropometric data, vitamin D, parathyroid hormone and electrolytes. The results were compared with a cohort of 847 healthy children. The results of this longitudinal study indicated that children with refractory epilepsy had, at baseline, compromised bone health and suboptimal growth status. During the observation period, a progressive loss of bone mineral content was detected, resulting

in osteopenia and osteoporosis despite the increased serum levels of vitamin D. Bone health in children with intractable epilepsy was poor, particularly for younger non-ambulatory children with low BMI status (Bergqvist *et al*., 2008).
Consideration must be given to the fact that the ketogenic therapeutic regimen is administered for a long time (>2-3 years). In this regard, Groesbeck and colleagues conducted a retrospective study of 28 children treated with the KD for 6-12 years; they reported that 21% had bone fractures. In particular, the first fracture occurred after only 1.5 years (median of the cases) from beginning the diet, and 14% of the subjects had, in their medical history, more fractures (Groesbeck *et al*., 2006) .

1.2.1.3 Anti-seizure effects of ketogenic diet

Despite the passing of a century since the first application of a KD, and the wide recognition of its efficacy, its mechanisms of action are only now being studied. In recent years, many hypotheses have been formulated to explain the anti-seizure effects of the KD. At present, it is becoming more apparent that the KD maybe works through multiple mechanisms that target fundamental biochemical pathways linked to cellular substrates and mediators responsible for neuronal hyperexcitability.
Fig. 7 shows a schematic summary of the principal mechanisms currently believed to be responsible for the anti-seizure activity of this dietary therapy. The most important effects, induced by ketosis, which have been accepted by the scientific community, are as follows (Rho, 2017):

- changes in neurotransmitter systems, including GABA, glutamate, and adenosine, as well as ion channel regulation. For instance, KB have been proposed to increase levels of GABA and enhances inhibitory neurotransmission by altering the metabolism of glutamate (Yudkoff *et al*., 2005). In addition, the activation of some ione-channles, such as ATP-sensitive potassium channels and two-pore domain channels, would hyperpolarize neurons and decrease neuronal excitability as well. In this respect, Ma and colleagues (Ma *et al*., 2007) demonstrated that, at physiological concentrations, KB reduce spontaneous discharges of GABAergic neurons in the rat substantia nigra, through ATP-sensitive potassium channels.
- enhancement of cellular bioenergetics and mitochondrial function; the mitochondrial biogenesis is predicted to increase ATP production and enhance energy reserves, leading to stabilized synaptic function and improved seizure control. Moreover, a remarkable increase in mitochondrial transcription proteins and enzymes was observed in rat hippocampus after the administration of a KD (Bough *et al*., 2006).
- glycolytic restriction or diversion; the KD also induces a small reduction in glycolysis, concomitant with an increase in non-glucose sources of fuel through the oxidation of fatty acids and KB. Thus, glycolytic restriction is thought to be an important mechanism mediating the anti-seizure properties of the KD.
- direct inhibitory action of fatty acids; intake of a high-fat diet increases the rate of fatty acid oxidation and this also changes the levels and types of polyunsaturated fatty acids (PUFAs) in the circulation, liver and brain. Since PUFAs are known to possess neuroprotective properties (Michael-Titus & Priestley, 2014), it has been assumed that this lipid species may help mediate the anti-seizure effects of the KD.

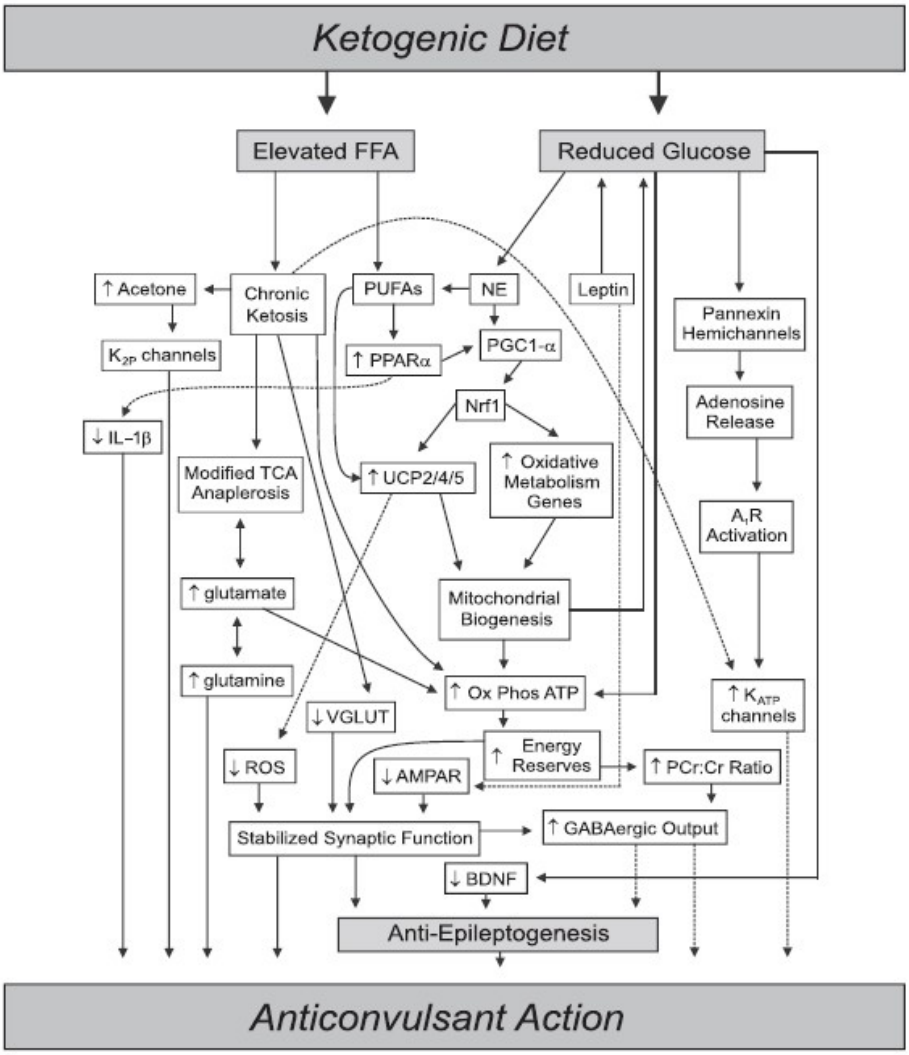

Fig. 7 Hypothetical pathways leading to the anticonvulsant effects of the KD (Rho, 2017).
[adenosine receptors: A1R - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor: AMPAR - brain-derived neurotrophic factor: BDNF - brain-specific uncoupling proteins: UCPs -–peroxisome proliferator-activated receptor-α: PPARα - peroxisome proliferator-activated receptor γ coactivator-1: PGC-1α - polyunsaturated fatty acids: PUFAs - vesicular glutamate transporters: VGLTs]

1.2.3 Specific epilepsy syndromes

Traditionally, the KD has been widely used in cases of drug-resistant epilepsy, but it has also become established as a therapy for a few specific epilepsy syndromes.
The glucose transporter type-1 deficiency syndrome (GLUT1-DS) was first described by De Vivo et al. quite recently (De Vivo *et al*., 1991), but no firm estimates of its incidence and prevalence can be made as the cases have been reported worldwide and are biased by physician awareness of the disorder. In Queensland, Australia, it was estimated that the incidence/prevalence is approximately 1:90,000, data are supported by a more recent Scandinavian study that reported a similar incidence/prevalence of 1:83,000 (Larsen *et al*., 2015). In Italy, there is no national registry of GLUT1-DS so the prevalence cannot be estimated (De Giorgis *et al*., 2015). This disease is caused by impaired glucose transport across the blood-brain barrier and into brain cells. The majority of patients carry heterozygous mutations in the SLC2A1 gene encoding the GLUT1 transporter (Awaad *et al*., 2015). If not identified and treated, children with GLUT1-DS develop microcephaly,

mental retardation, spasticity, and ataxia as a consequence of relative brain hypoglycemia. Currently, this epilepsy syndrome is effectively treated with KD, because the KB generated from dietary fatty acid oxidation are able to penetrate the blood-brain barrier and provide an alternative fuel for the central nervous system. As the developing brain of young children requires a lot of energy, the KD should be started as early possible, and should be maintained into puberty (Klepper, 2008). Even though KD is the first choice of treatment in GLUT1-DS patients, about 20% of such patients do not meet the compliance criteria or the diet loses its effectiveness over time, so for these reasons new therapeutic strategies need to be identified (Veggiotti & De Giorgis, 2014).

Similarly, children with pyruvate dehydrogenase complex (PDC) deficiency show improvements while on the KD. In PDC deficiency, pyruvate, the glycolytic end product, is not optimally metabolized through the tricarboxylic acid cycle, and this leads to an increased production of lactate and limited mitochondrial energy production (Patel *et al*., 2012). A recent Swedish publication reported a longitudinal cohort study of pediatric patients diagnosed with PDC deficiency and treated with KD. All the patients suffering baseline epileptic seizures improved during KD treatment.; half of them stopped having seizures within 1 year of starting the diet. Based on the results, the authors proposed that KD should be introduced as soon as possible after diagnosing PDC deficiency, as early initiation could prevent further metabolic injury to the brain. Moreover, they reported that the long-term effectiveness is highly dependent upon the condition of ketosis, which must be regularly assessed by monitoring the plasma ketone levels and adjusting dietary composition (Sofou *et al*., 2017).

One of the most malignant epileptic syndromes in which this specific diet may be particularly useful is the severe myoclonic epilepsy of infancy also known as Dravet syndrome (DS), which is associated in many cases with mutations in the gene SCN1A, a subunit of the sodium channel of muscle cells and neurons (Steel *et al*., 2017). DS is characterized by the onset of febrile hemiclonic or generalized status epilepticus in the first year of life. Febrile seizures recur, followed by the evolution of afebrile seizures including myoclonic, absence, and partial seizures between ages 1 and 4 years. The epilepsy is usually resistant to standard antiepileptic medication, and from the second year of life cognitive, behavioural, and motor impairments become apparent (Dravet, 1978 – Dravet, 2011). In 2006, a retrospective study evaluated the efficacy and tolerability of the KD in 13 patients with DS; ten of them had a significant reduction in the number of seizures. Considering the intractability of seizures in patients with DS, this result has shown that the KD is an attractive therapeutic alternative (Caraballo *et al*., 2006). More recently, another retrospective study evaluated both KD effectiveness and tolerability in comparison with multiple antiepileptic drugs. According to the results reported, such as good effect on seizures, good tolerability and few compliance problems due to formula treatment, the KD should be considered an early treatment option in infants with DS. In fact, data showed equal efficacy of the KD compared with various antiepileptic drugs currently available for the treatment of DS (Dressler *et al*., 2015).

1.2.4 New applications

Recently, the use of the KD has been proposed in a series of pathological conditions quite different from epilepsy syndromes.

For instance, in neurodegenerative disorders KD has been shown to have a positive effect, although it is not completely clear whether this is direclty due to the KD or wheter it is due to the metabolic changes the diet induces (Barañano & Hartman, 2008).

Parkinson's disease (PD), a condition in which the neurotoxine 1-methyl-4-phenyl-1,2,3,6-etrahydropyridine (MTPT) causes dopaminergic neurodegeneration, has been shown, through an animal model, to benefit from a protective βHB action, an effect that could be related to improved mitochondrial respiration and ATP production (Jabre & Bejjani, 2006 – Vanitallie *et al*., 2005). Additional evidence supporting the potential benefits of KB in PD is provided by another in vitro study demonstrating neuroprotective and anti-inflammatory effects of KD against MPTP-induced-neurotoxicity (Yang & Chen, 2010).

Also Alzheimer's disease has been found to benefit from the protective role of KB in that it can decrease amyloid β-peptide, which, in this progressive dementia, accumulates in brain regions critical for memory (Kashiwaya *et al*., 2013). Furthermore, a pilot clinical study involving 15 Alzheimer participants has shown that KD can actually improve memory in cognitively intact subjects, or subjects with mild cognitive impairment (Taylor *et al*., 2018).

Moreover, KD has emerged as a potential treatment for autism spectrum disorder (ASD). Since ASD is associated with metabolic dysfunction and autism is a common trait of epilepsy-associated diseases, the use of KD has been suggested to ameliorate some of the ASD-associated symptoms. In fact, one study and one case report indicated that children with ASD treated with a KD showed decreased seizure frequencies and exhibited behavioural improvements (Napoli *et al*., 2014). Indeed, a more recent clinical trial was done to test a modified KD for the improvement of core clinical impairments in 15 children with ASD (Lee *et al*., 2018), and it was found that components of the KD are possibly beneficial in improving social skills in children with ASD. However, additional studies are needed to understand how the KD improves behavior, because the mechanism of action is still unidentified.

Currently, there is the proposal that KD might prevent tumor progression and could be used as supportive therapy in patients with different type of cancer. The mechanism underlying this hypothesis is the fact that cancer cells require large amounts of glucose; in fact, cancer cell mitochondria are dysfunctional, so they cannot use fatty acids and KB, which, on the contrary, can be used by healthy cells. The goal of KD is to limit sources of energy for cancer cells by restricting carbohydrates, while providing fatty acids and KB to healthy cells (Seyfried *et al*., 2014). A systematic review of 13 studies evaluated the KD effects on survival time and tumor growth in animal models. From these studies, 9 articles indicated that KD significantly increased the mean survival time and there was a clear trend of slower tumor growth in pancreatic, prostate, gastric, brain, lung cancer (Khodadadi *et al*., 2017). However, few human data are available, and these provide little concrete evidence of the anti-tumor effects of KD, due to the lack of controlled trials and the limitation to individual cases (Klement, 2017).

1.3 KETOGENIC DIET FOR WEIGHT LOSS

Currently, the KD as a weight loss dietary protocol (e. g. Atkins) is one of the most discussed issues by the scientific community. Although this diet seems to promote a better control of hunger and a greater weight loss in the short time, it differs quite radically from the conventional guidelines for healthy diet (Fig. 8), and the long-term effects on health and disease risks need clarification.

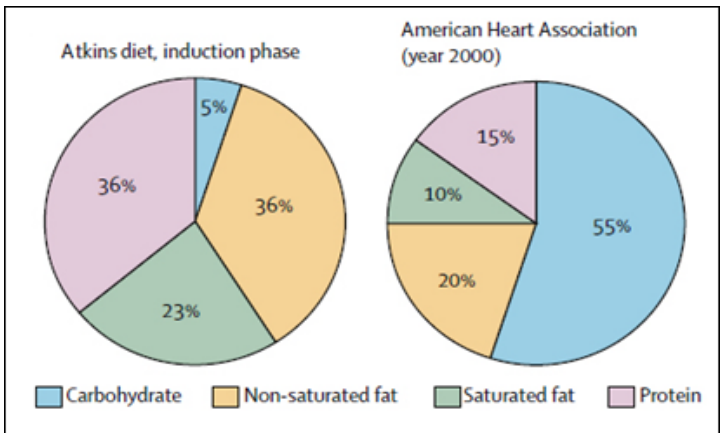

Fig. 8 Macronutrient's percentage contributions to daily caloric intake of Atkins diet compared to American recommendation (Astrup *et al.*, 2004).

The cardiologist Robert C. Atkins was the first to propose the KD with an aim different from the therapeutic one. He devised it in the '70s to prevent diabetes, however the diet became famous for its effectiveness in weight loss. The Atkins protocol applied with the purpose of weight control consists of 4 phases:

1 - the first is the induction phase, recommended for 2 weeks, where the aim is to bring the organism into ketosis. The intake of carbohydrates is limited to 20-25 grams daily, of which 12-15 grams must come from salad leaves, fruits and vegetables such as broccoli, spinach, pumpkin, cauliflower, tomato (legumes are not allowed at this stage because they are too rich in starche). Among the permitted foods, meat can be eaten ad libitum, as well as fish, shellfish and eggs.

2 - this second phase is still characterized by the strict control of carbohydrate intake, but gradually increasing it by 5 grams a week (the consumption of some fruits and legumes is granted).

3 - the carbohydrate intake is increased further, up to 10 grams a week. The goal is to find the amount of carbohydrates that allows to maintain the proper weight without getting fat.

4 - in the last phase the objective is to consolidate the eating habits adopted in the previous stages. Although an instinctive choice of food is encouraged, the regaining of weight means that the subject must return to the previous phases (Atkins, 2002).

The Atkins diet books have sold over 45 million copies in over 40 years and, given today's obesity epidemic, both the Atkins diet and its associated products are still very popular worldwide. This diet protocol promises to lose weight effectively despite the ad libitum consumption of meat, butter and other high-fat dairy products, while limiting carbohydrate intake to less than 30 grams/day. The apparent paradox that the unlimeted intake of fatty foods produces weight loss seems to be due to various reasons: the severe limitation of carbohydrates depleting glycogen stores, the suppressed appetite by the ketogenic nature of the diet, the satiating power of the high-protein content, the reduction of spontaneous food intake which leads to a reduction in energy intake, and finally, as the protocol is hypocaloric, the catabolism of fatty acids by β-oxidation (Astrup *et al.*, 2004).

1.3.1 Ketogenic diet vs low-fat diet

The differences in health benefits of carbohydrate-restricted diet and a calorie- and fat-restricted diet are of considerable public interest. There are concerns that a very-low-carbohydrate-diet (VLCD) leads to abnormal metabolic functions that could have a severe impact on human health. For this reason, many *in vivo* studies, meta-analyses and reviews have been published to understand the short- and long-term effects of a VLCD compared to other more conventional diets.

Is it really so beneficial to follow a restrictive diet like VLCD? What is the effectiveness of this dietary protocol in the long-term?

In 2003, three different randomized trials evaluated the effect of VLCD on several parameters, focusing on wight loss, compared to other types of diet. In the first study, 132 severely obese individuals (39% affected by type II diabetes, 43% by metabolic syndrome) were randomly divided into two groups and assigned to a low-carbohydrate diet (LCD) or a low-fat diet (LFD). After 6 months, subjects undergoing the LCD had lost 3.9 kg more than the other group (Samaha *et al.*, 2003). In the second study, which lasted 6 months as well, 53 obese women followed a VLCD or a low-calorie diet with 30% of fat energy. At the end of the treatment, the women who had followed the VLCD had lost more weight than those who followed the low-calorie diet (-8.5 kg vs. -3.9 kg, $p < 0.001$) (Brehm *et al.* , 2003). The third study was conducted for 12 months, on 63 non-diabetic obese subjects who followed Atkins diet or a conventional hypocaloric diet (25% of the energy from fats, 15% from proteins and 60% from carbohydrates). After 6 months the Atkins group showed better results, with a weight loss of 7% of body weight, compared to 3.2% of the other group ($p < 0.02$); however, after 1 year the difference between the groups was no longer significant ($p = 0.26$) (Foster *et al.*, 2003). Although this research showed that VLCD led to a greater weight loss during 3-6 months, the longer study conducted over 12 months indicated that, compared to the traditional hypocaloric protocols, the VLCD diet provided no additional long-term advantages.

Surprisingly, with regard to cardiovascular risk factors, according to the above studies, greater improvements were observed in people following the VLCD: triglycerides were significantly reduced, HDL cholesterol and sensitivity to insulin were increased, while the parameters of blood pressure and total lipids were unchanged. Apart from the specific composition of the diet, this result could have been due to the weight loss itself, which significantly improves the lipid profile and glucose tolerance. However, it must be remembered that these studies had some limitations, such as low adherence to the diet and high dropout rates.

A few years later, Foster and colleagues performed another *in vivo* study, once again to compare LCD vs LFD, but this time the time was prolonged to 2 years of follow-up. The study was a randomized, controlled trial conducted with outcome assessments at baseline, 3, 6, 12 and 24 months; participants were randomly assigned to LCD (153 subjects) or LFD (154 subjects). The results reported no differences in weight, body composition, or bone mineral density between the groups at any time point. As showed in previous studies, during the first 6 months, the LCD group had a greater reduction in diastolic blood pressure, triglyceride levels, and VLDL cholesterol levels, and a lower reduction in LDL cholesterol levels, than did the LFD group. However, in some variables already after 1 year, but especially at the end of the study (2 years) the differences in lipid profiles were no longer significant (Fig. 10). Therefore, the authors conclude that successful weight loss can be achieved with either LCD or LFD, without any particular cardiovascular disease risk factors at 2 years (Foster *et al.*, 2010).

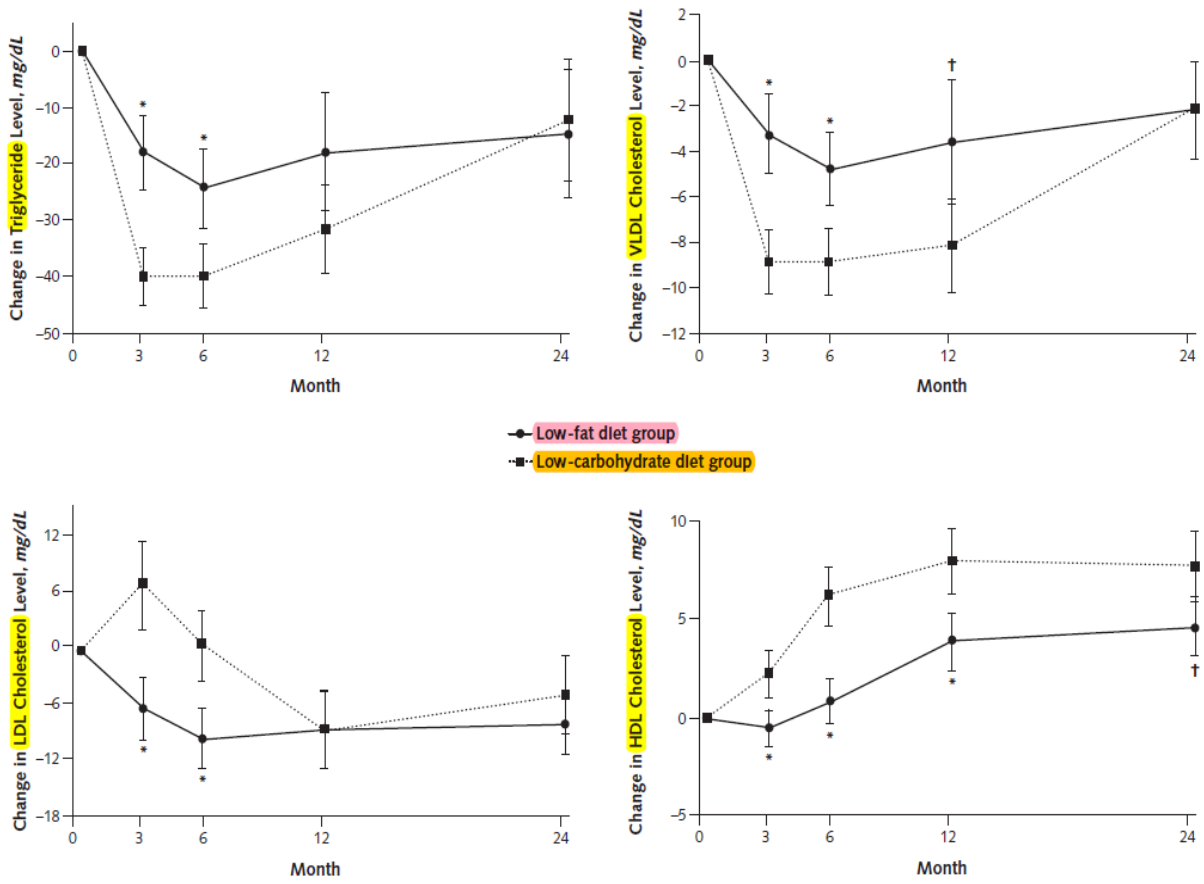

Fig. 10 Predicted absolute mean change in serum triglyceride, VLDL, LDL and HDL cholesterol concentrations in the low-fat and low-carbohydrate diet groups , * p <0.001, † p < 0.01 for between-group differences (Foster *et al.*, 2010).

There is an increasing necessity for longer studies, conducted both on overweight and large obese individuals, to evaluate weight loss effectiveness, with careful assessment of the energy balance and body composition, cardiovascular risk factors, diabetes, renal and skeletal health markers, without forgetting nutritional adequacy and quality of life. It is not known if moderately overweight people would have the same improvements in triglycerides and HDL-cholesterol, as demonstrated in obese participants of the studies, or if the levels of physical activity could change its effects. Thus, future studies should be undertaken for a sufficiently long time (at least 2 years) on different subjects to allow a careful monitoring of the above mentioned risk factors.

1.4 KETOGENIC DIET & MICROBIOTA

1.4.1 Role of microbiota

In recent decades several studies have highlighted the key role of the microbiota in maintaining a correct status of host's health (Rooks *et al.*, 2014). The "microbiota" is defined as the entire population of microorganisms residing in different districts of the human organism. The intestinal population consists mainly of over 1000 different bacterial species, reaching 10^{14} microbial cells. Instead, if we consider the microbiome, which is the number of genes present in all the genomes of these microbial cells, it is 200 times larger than the number of genes of the entire human genome. Microbial density reaches its maximum levels in the distal portion of the colon, with a

concentration of about 10^{11} bacteria/gram of enteric content, while a smaller portion is represented by viruses, fungi and *Archaea* (Ley *et al*., 2006).

Most of the microbiota in the gastrointestinal tract consist of anaerobic bacteria and, although more than 50 bacterial phyla have been described, human microbial communities are overwhelmingly dominated by: *Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria* and *Verrycomicrobia* (Fig. 11).

The first two phyla make up over 90% of the colon community (Dethlefsen *et al*., 2007), and *Firmicutes*, Gram positive bacteria, are very active from the fermentation point of view: the metabolism of undigested food components determines the formation of short-chain fatty acids (SCFA), particularly butyrate (Pryde *et al*., 2002). *Bacteroidetes*, Gram negative bacteria, are associated with numerous beneficial effects, due to their ability to digest polysaccharides and proteins, especially producing acetate and propionate (Macfarlane & Macfarlane, 2003).

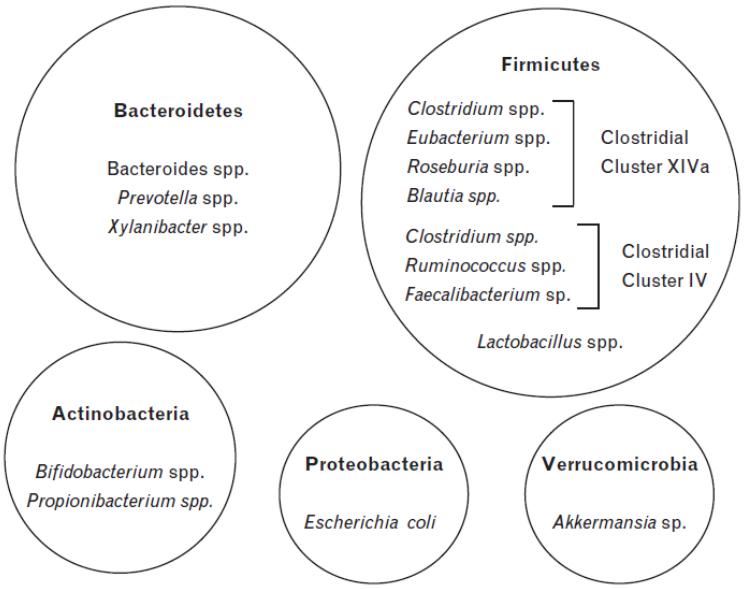

Fig. 11 Predominant species of the human colonic micrbiota (Chassard & Lacroix, 2013).

The microbiota performs various functions able to contribute in a concrete way to the state of health of the host, exerting a protective, structural and metabolic role (Fig. 12). The microbiota competes with pathogenic bacteria, producing substances capable of inactivating them, altering the intestinal pH, subtracting the nutrients and maintaining the integrity of the mucosal barrier. The mucosa is an important defence system against potentially immunogenic or pathogenic factors, food residues and organic secretions (Prakash *et al*., 2001). It also helps to preserve intestinal homeostasis, keeping cell junctions intact, and acts as a stimulus for the development and regulation of the immune system. The mucosa promotes the correct localization and response of Toll-Like Receptors (TLRs), key elements in the response to possible pathogens. The intracellular signal downstream of the TLRs induces the production of cytokines able to coordinate the inflammatory and immune response (Pessi *et al*., 2000). At metabolic level, the microbiota plays an important role in the synthesis of vitamins, such as vitamins K, B9 and B12. However, the main metabolic activity carried out by the intestinal microbiota is the fermentation of undigested substrates introduced with the diet, contributing to a large extent to energy recovery for the host, producing secondary metabolites, in particular SCFA (Wong *et al*., 2006).

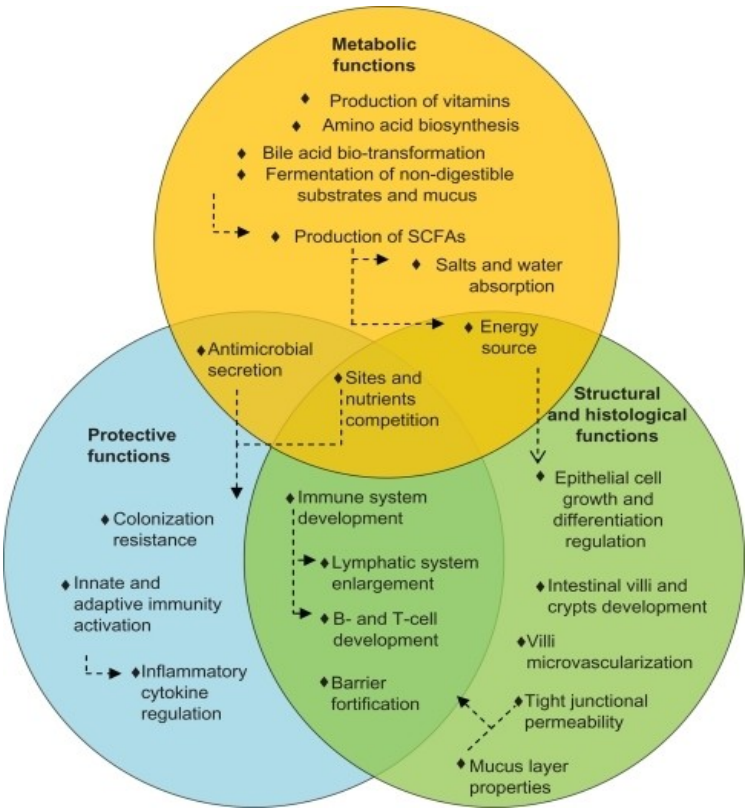

Fig. 12 Main functions of microbiota (Prakash *et al.*, 2011).

1.4.1.1 Short chain fatty acids

The colonic microbiota is able to ferment organic material that cannot be digested by the host in the upper gut, as humans lack the enzymes to degrade the bulk of dietary fiber. Fermentation results in multiple groups of metabolites of which SCFA are the major product. They are a class of saturated fatty acids characterized by an aliphatic chain length of less than 6 carbon atoms. Acetate (C2), propionate (C3) and butyrate (C4) are the most abundant, as they rapresent 90-95% of SCFA in the colon. Generally, the molar ratio acetate: propionate: butyrate is 60:20:20 and remains constant across the different regions of the colon, although absolute concentrations may vary (Macfarlane et al., 1992). Depending on the diet, the total concentration of SCFA ranges from 70 to 140 mM in the proximal colon and from 20 to 70 mM in the distal colon (Topping & Clifton, 2000). The formation of SCFA by intestinal bacteria is affected by numerous factors including the source and the amount of substrate available, the bacterial species composition of the microbiota, the intestinal transit time and ecological factors such as cooperative interactions between different groups of bacteria (Huazano-Garcìa & Lòpez, 2013). Absorption of SCFA in the colon is rapid and reaches 95% (Donohoe *et al.*, 2011), predominantly through apical carriers, such as the monocarboxylate-1 transporter and the sodium-coupled monocarboxylate-1 present in epithelial cells (Goncalves & Martel, 2013). This help promote the absorption of Na+, to maintain the base acid balance and energy homeostasis in the colonocytes, providing 5-10% of the total energy need (McNeil, 1984). Once absorbed by enterocytes, most SCFA are metabolized into lipids (Bergman, 1990 – Zambell *et al.*, 2003), resulting in a significant decrease in their concentration. SCFA that reach the liver are metabolized into lipids and small amounts are present in the peripheral blood (Bergman, 1990).

The main fermentable substrates in the intestine are the components of the dietary fiber, particularly the soluble fraction (Flint *et al*., 2008), such as "resistant" starch, non-starch polysaccharides, oligosaccharides and non-digestible sugars. Carbohydrate fermentation occurs through various biochemical reactions, among which the most important are glycolysis, for sugars with 6 carbon atoms, and the pentose-phosphate pathway, for sugars with 5 carbon atoms. The former is mainly used by *Lactobacilli* and *Bacteroides*, so that, after glucose phosphorylation, sugar is converted to pyruvate, a molecule that acts as an intermediate key for subsequent metabolic interactions with SCFA production. The second is the pentose-phosphate pathway used by bacteria belonging to the family *Bacillaceae*, *Enterobacteriaceae* and *Enterococcaceae*, in which the monosaccharides are converted into phosphoenolpyruvate (PEP). Subsequently the PEP is converted into fermentation products such as organic acids or alcohols (Miller & Wolin, 1996).
Furthermore, the anaerobic metabolism of peptides and proteins at the colon level, by the bacteria, leads to the production of branched SCFAs (Branched Chain Fatty Acids, BCFA): isobutyrate, isovalerate and 2-methyl-butyrate, deriving specifically from the catabolism of the valine amino acids, leucine and isoleucine (Cummings & Englyst, 1987).
SCFA are the main source of energy for enterocytes, thus any deficiency can determine the atrophy of the colonic mucosa. The colonic epithelium gets 60-70% of its energy from bacterial fermentation products, especially from butyrate (Cummings & Macfarlane, 1997), preferring it to acetate and propionate, but also to glucose. Several studies have shown how propionate and acetate can modulate the glyco-lipid metabolism, inhibiting the hepatic synthesis of cholesterol (propionate) (Demignè *et al*., 1995) and improving insulin sensitivity (acetate) (Brighenti *et al*., 1995). Furthermore, acetate also acts as a substrate for the synthesis of long chain fatty acids and as a co-substrate for the synthesis of glutamine and glutamate, while propionate acts as a precursor of gluconeogenesis in the liver (Roy *et al*., 2006). Butyrate is also able to modulate the gene expression by reducing the risk of formation of epithelial neoplastic cells in the colon. In vitro studies have shown the ability of butyrate to inhibit growth or promote the differentiation of human cells and induce apoptosis in tumor cells (Hague *et al*., 1995). It also seems to prolong the time of cell division and slow down the growth rate of colon-rectal tumor cell lines. Csordas (1996) associates this property of butyrate to the inhibition of histone deacetylase activity (HDACs) in enterocytes and immune cells.
The production of SCFA affects the gut environment, primarily by decreasing pH, as large amounts of acids are produced. The initial pH drop prevents the growth of pH-sensitive pathogenic bacteria, such as some belonging to Enterobacteriaceae and Clostridia (Duncan *et al*., 2009). At pH 5.5, butyrate-producing bacteria, for example *Roseburia* and *Faecalibacterium prausnitzii*, account for 20% of the total population, while in the most distal parts, where there is a lack of fermentable food fibers, the pH increases to 6.5. The butyrate-producing bacteria disappear almost completely and the bacteria associated with the *Bacteroides*, producers of acetate and propionate, become dominant (Walker *et al*., 2005). In addition, thanks to the intestinal pH reduction, SCFA contribute to the absorption of sodium, magnesium, vitamins and water at the colon, where they exert an anti-inflammatory action (Roberfroid, 2005). Butyrate inhibits the activation of the transcription factor NF-kB and consequently the production of pro-inflammatory cytokines (Segain *et al*., 2000; Luhrs *et al*., 2001). Overall, the various SCFA and the drop in pH induce colonic and systemic health effects (Fig. 13) (Chassard & Lacroix, 2013).

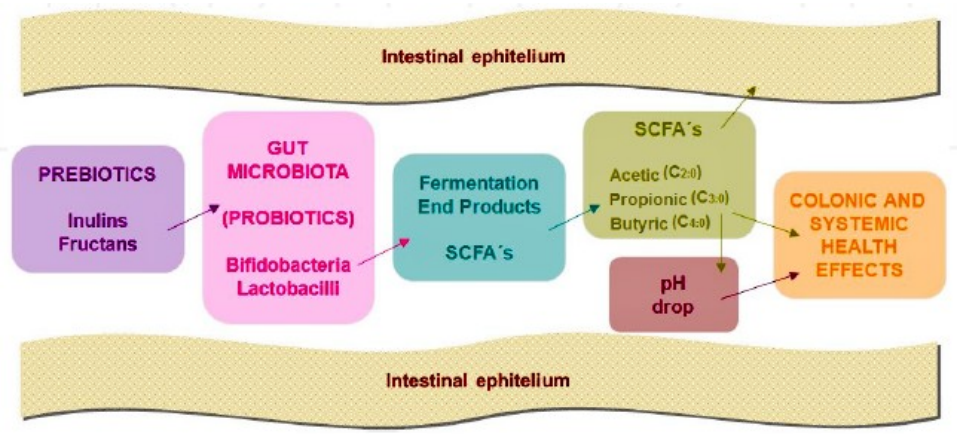

Fig. 13 General events taking place in the large intestine; prebiotics are fermented by bacteria to produce SCFA to improve host health (Huazano-Garcìa & Lopez, 2013).

1.4.2 Microbiota & diet

The association between microbiota and host is the result of numerous evolutionary processes, which have favoured the generation of a mutual beneficial condition for both the bacteria and for the host. When there is an altered composition of the intestinal ecosystem, a state called gut dysbiosis, physiological changes in the intestinal environment occur, disrupting the functions of the gut microbiota. Changes at the level of the microbial ecosystem have been indicated as crucial for the development of many diseases such as diabetes, obesity, inflammatory diseases, metabolic syndrome and also for some neurodegenerative and neurological diseases (Prakash *et al*., 2011).
It has been clearly demonstrated that the human intestinal microbiota can be modulated by multiple elements, both genetic and environmental factors which can influence the inter-individual diversity of gut microbiota. Among environmental ones, the role of diet on the composition of gut microbiota is definitely one of the most important (Albenberg & Wu, 2014- Bibbò *et al*., 2016 - David *et al*., 2014 - Gong & Yang, 2012). Indeed, diet can impact not only the host directly, but can also have an indirect effect through the intestinal microbiota. The composition and duration of diet can affect bacteria but also viruses, *Archaea*, and fungi. In addition to the composition of the intestinal microbiota, diet affects its production of metabolites, which can influence host physiology (Fig. 14) (Albenberg & Wu, 2014).
Alterations in the intestinal microbial population have been associated primarily with the consumption of dietary fiber from fruit and vegetables. In controlled trials in humans, variations in the intake of resistant starch and non-starch polysaccharides have shown the ability to modulate specific bacterial species such as *Rominococcus bromii* and *Eubacterium rectale*. It has been observed, by in vitro analysis on human faecal samples, that these taxa are able to metabolize specific substrates derived from soluble carbohydrate (Walker *et al*., 2011). Furthermore, animal models have shown the important capacity of the intestinal microbiota to metabolize glycans, deriving not only from the diet, but also from the mucus produced by the host. In fact, some microorganisms are able to modify their metabolism in relation to the availability of the substrates (Sonnenburg *et al*., 2005).

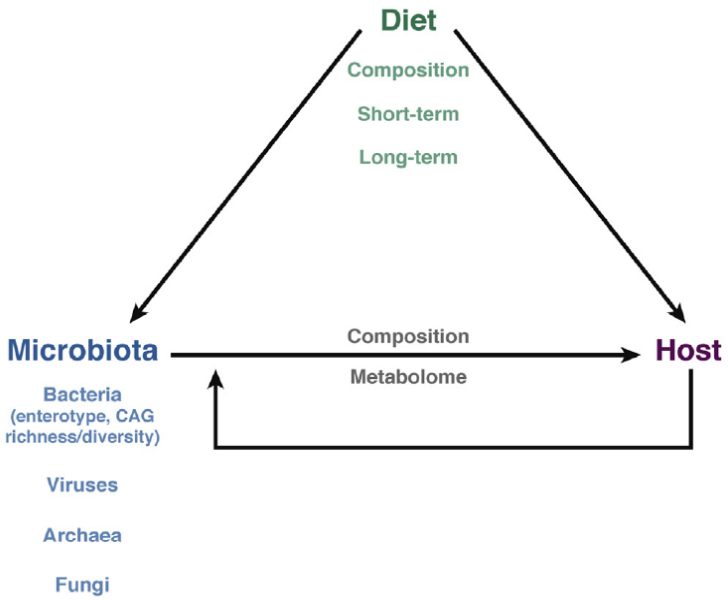

Fig. 14 Interactions among diet, the intestinal microbiota, and the host (Albenberg & Wu, 2014)

Moreover, a diet low in carbohydrates and rich in proteins alters the colon microbiota, favoring a potentially pathogenic and pro-inflammatory microbial profile, a decreased production of SCFA and an increased concentration of ammonia, phenols and sulfuric acid (Guarner & Malagelada, 2003). These metabolites largely compromise the structure of the colonic epithelium, causing inflammation of the mucosa, and can also interfere with the modulation of the enteric nervous system and intestinal motility. These factors can be of clinical importance in the approach to problems in which protein fermentation could be involved such as irritable bowel syndrome, inflammatory bowel disease and colorectal cancer prevention (Hugenholtza *et al*., 2013).

The diet seems to influence the intestinal microbiota by interacting at various levels on human physiopathology. For instance, a diet rich in fiber stimulates the production of SCFA; instead, diets rich in red meat have been associated with an increased risk of colon cancer development as they could promote the production of sulfate-reducing bacteria producing hydrogen sulfide, a genotoxic agent (Albenberg & Wu, 2014).

One of the most relevant studies in this field, which demonstrated the impact of diet in shaping gut microbiota, was published by De Filippo and collegues. They analyzed the gut microbiota of children aged 1-6 years old living in a rural village of Africa, specifically in Burkina Faso, and of Italian children living in Florence, in order to compare the environment typical of industrialized Europe with an environment close to that of Neolithic farmers. African children showed a significant enrichment in Bacteroidetes and depletion in Firmicutes with a unique abundance of Prevotella and Xylanibacter, completely lacking in Italian children. This result was consistent with a high abundance of SCFA and a lower level of Enterobacteriaceae in children from Burkina Faso compared to the Europeans. These results suggest that diet has a dominant role over other possible variables such as ethnicity, sanitation, hygiene, geography, and climate, in shaping the gut microbiota. The authors hypothesized that the reduction in microbial richness observed in Europeans compared with Burkina Faso children could indicate how the consumption of sugar, animal fat, and calorie-dense foods in industrialized countries is rapidly limiting the adaptive potential of the microbiota (De Filippo *et al*., 2010).

Diets greatly different from conventional ones, such as those with a low carbohydrate intake (e.g. KD, VLCD), are assumed to exhibit a very pronounced effect on the microbiota (David *et al*., 2014). Indeed, it has been shown, in animal model, that the consumption of a high fat diet is associated with large alterations in microbiota including a decrease in Bacteroidetes and an increase in both Firmicutes and Proteobacteria (Devkota *et al*., 2012 - Hildebrandt *et al*., 2009). However, very few studies have investigated the specific effect of a KD on the microbiota and they have been published only just recently. One of these is a pilot study perfomed on six patients affected by GLUT1-DS treated with KD. Fecal samples were collected and analysed before and after three months on the diet to evaluate any changes in the microbiota. Although at the phylum level there were no statistically significant differences at 3 months compared to baseline, *Desulfovibrio spp.* increased significantly. This bacterial group is supposed to be involved in the exacerbation of the inflammatory condition of the gut mucosa associated with the consumption of fats of animal origin. The authors concluded, considering the dysbiosis demonstrated by fecal samples, that a trial of pre- or pro-biotics could be suggested to potentially restore the correct balance of intestinal microbiota (Tagliabue *et al*., 2017). Another study, performed by Swidsinski and collegues, investigate once again the effect of KD on gut microbiota, but this time considering 10 patients affected by auto-immune multiple sclerosis (MS) using the diet for 6 months. They found that the total concentrations and diversity of substantial bacterial groups were reduced in MS patients. In particular, the results showed that some bacterial groups including *Roseburia*, *Bacteroides* and *Faecalibacterium prausnitzii* were diminished the most. Morevoer, the effects of a KD were biphasic. In fact, in the short term, the bacterial concentrations and diversity were further reduced, then at week 12 started to recover, and finally after 23-24 weeks significantly exceeded the baseline values. In summary, it seems that the KD can normalized concentrations of the colonic microbiome after 6 months in MS patients (Swidsinski *et al*., 2017). Nevertheless, both studies were performed with a very small number of subjects, therefore more research is needed on this topic to better understand the role of KD on human gut in different contexts.

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2 AIM OF THE STUDY

The overall aim of the present Ph.D. thesis was to investigate the metabolic effects of KB and KD, through both *in vitro* and *in vivo* approaches. Thus, the research project has been subdivided in two part, as described below:

PART I

In the first part we focused our attention on the vascular risk represented by KD-induced oxidative stress. We evaluated the effects of physiological levels of KB by measuring markers of oxidative stress using an *in vitro* model of endothelium. With this aim, we decided to investigate: a) the DNA oxidative damage by comet assay in order to study the genotoxicity due to KB; b) the ability of KB to modulate DNA susceptibility toward a secondary oxidative insult and c) the cell adaptive metabolic response to KB exposure, by activation of the Nrf2, which is a transcriptional factor involved in the cellular response to a stress, by western blot, immunofluorescence and real time PCR.

PART II

The second part was devoted to verify the impact of the KD on human intestinal environment, by an *in vivo* study. This project was in collaboration with the Human Nutrition and Eating Disorder Research Center of the University of Pavia, where the subjects were recruited. After the collection of the samples, three different activities were scheduled: a) analysis of the composition of gut bacteria (NGS, qPCR) performed in collaboration with the Department of Health Sciences of Università degli Studi di Milano; b) assessment of markers of bacterial metabolism (SCFA) and c) evaluation of fecal water citotoxicity and genotoxicity (Trypan blue, Comet assay).

3 PART I: *IN VITRO* STUDY

3.1 MATERIALS & METHODS

3.1.1 Cell culture

For this in vitro study, we chose to use a cell line of human microvascular endothelial cells (HMEC-1) (Fig. 15). HMEC-1 are a long-term cell line immortalized by simian-virus-40 large T antigen, provided by the Center for Disease Control and Prevention, Atlanta, GA, USA. These cells have been reported to retain the morphologic, phenotypic and functional characteristics of normal human microvascular endothelial cells (Ades *et al*., 1992). Moreover, comparing different endothelial cell lines, HMEC-1 is considered the most suitable model to investigate microvascular endothelium (Bouïs *et al*., 2001).

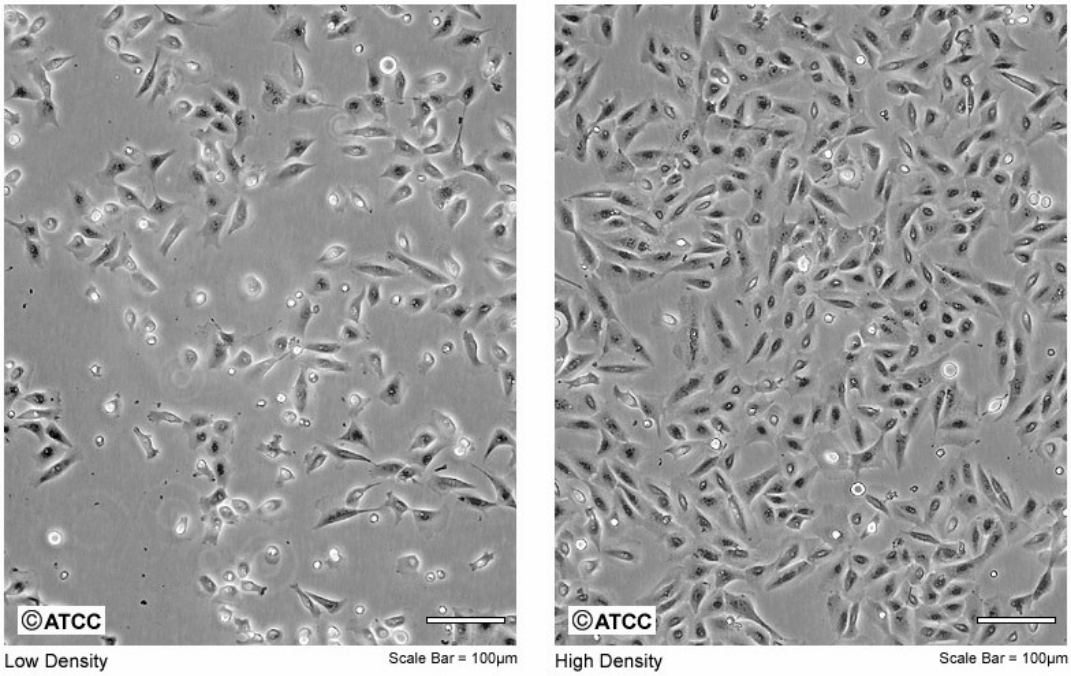

Fig. 15 HMEC-1 cells at low and high density (ATCC).

HMEC-1 cells were cultivated in MCDB131 medium supplemented with 10% fetal bovine serum, 10 ng/mL epidermal growth factor, 1 µg/mL hydrocortisone, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 20 mM HEPES buffer.

The cells were cultured in a 75 cm^2 flask and, before the cells reached the complete confluence (about 80%), a dilution of 1 to 4 was carried out every 3-4 days. In particular, when cells reached the sub-culturing density of 80% the medium was removed and cells were washed with PBS. Then the cells were supplemented with trypsin-EDTA solution and left in the incubator for few minutes, until their detachment. Trypsin action was arrested by addition of complete medium, cell suspension was centrifuged for 5 min at 350xg. After removing supernatant, cell pellet was re-suspended in complete medium and seeded at 4.5×10^3 cells/cm^2.

For the analysis, cells were seeded at a density of 5×10^4 cells/cm^2 in 60 mm Petri dishes and allowed to reach confluence in 24 h; then, cells were supplemented with KB dispersed in complete medium, and incubated at 37 °C in an atmosphere containing 5% of CO_2, according to the experimental design.

3.1.2 Treatment with ketone bodies

Stock solutions for AA (acetoacetic acid lithium - A8509; Sigma Chemical Co.) and βHB (β-hydroxybutyric acid – 298360; Sigma Chemical Co.) were prepared in sterilized water. HMEC-1 cells were supplemented with different concentrations of KB: from 2 to 20 mM for βHB and from 0.5 to 5 mM for AA. To simulate the physiological condition both βHB and AA were incorporated simultaneously in cell treatments in a ratio 4:1, which occurs in vivo after the induction of ketosis (Fukao *et al*., 2004).

3.1.3 Cell viability

Cell viability was determined by the MTT reduction assay (Fotakis & Timbrell, 2006). The test is based on the ability of the MTT compound (tetrazolium salt, 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl-tetrazolium bromide) to be metabolized by a mitochondrial enzyme, succinate dehydrogenase. The reduction of MTT leads to the formation of blue crystals of formazan, which is insoluble in water. Vital cells, unlike non-viable cells, reduce MTT, so the amount of formazan produced is proportional to the number of live cells present. The procedure used in this study has been already described (Meroni *et al*., 2018).

3.1.4 Comet assay

The comet assay (Single Cell Gel Electrophoresis) is an in vitro assay of increasing use in toxicology, not only for testing the genotoxic potential of substances in vitro, but also for human bio-monitoring in vivo studies (Sasaki *et al*., 2000). It is used to quantify the breaking of single strands of DNA (corresponding to oxidized bases) and the formation of alkali labile sites, at the single cell level. This technique has been demonstrated in several studies to have excellent sensitivity in the determination even minimal levels of oxidative damage; moreover, it does not require an excessive number of cells and is simple to perform (Tice *et al*., 2000 – Erba *et al*., 2014). The procedure involves the incorporation of cells in agarose, their lysis, the passage of the lysate in alkaline buffer, which determines the complete unfolding of the nuclear DNA, the electrophoresis, the colouring phase and the processing of images acquired under a microscope (Fig. 16).
DNA strands without breaks migrate poorly and tend to remain concentrated in a nucleoid, while fragments generated by strand breaks migrate in inverse proportion to their length and molecular weight. At the end of the electrophoretic migration, the cell lysate is coloured with ethidium bromide and visualized by fluorescence microscope: the sample assumes the appearance of a comet, with a tail that is longer and brighter the more DNA is fragmented (Tice *et al*., 2000).
In this study, the Comet assay was used to evaluate the genotoxicity of KB exposure on HMEC-1. The experimental procedure was designed according to the criteria reported in literature (Erba *et al*., 2014), applying appropriate modifications (Meroni *et al*., 2018). Particularly, based on the results of the viability test, cells were exposed to KB (βHB 4 mM, AA 1 mM) for 2, 24 and 48 h at 37 °C with 95% humidity and 5% CO_2. Every treatment was performed in triplicate; both negative (cells without KB) and positive (cells treated with H_2O_2: 50 μM for 5 min) controls were included in each batch. Among the stimuli used to produce quantifiable oxidative damage to DNA and modulated by the presence of antioxidants, hydrogen peroxide has proved to be the most effective and reproducible (Erba *et al*., 2003).

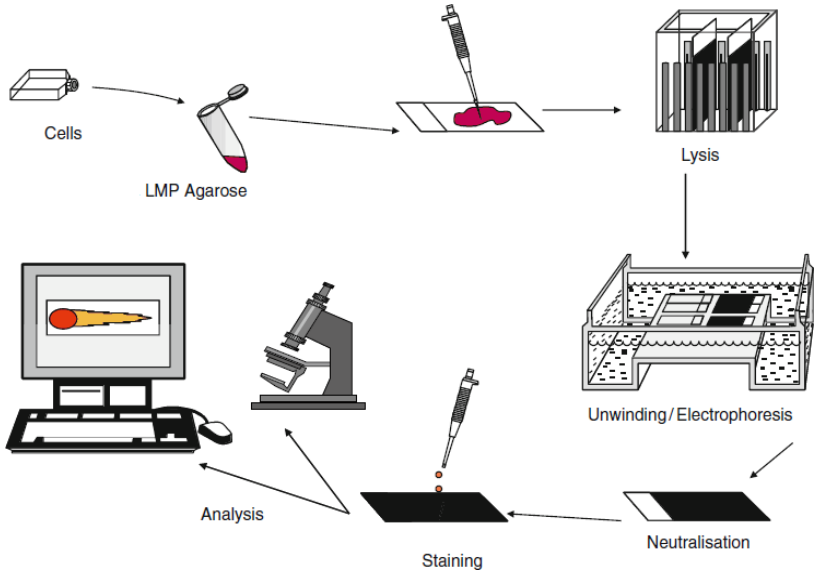

Fig. 16 Different steps of Comet assay.

After incubation, an aliquot of cells was used for the trypan blue exclusion test in order to verify cell viability (Strober, 2001). Another aliquot of cells was centrifuged (11,000 g, for 15 s; SL16R Thermo Fisher Scientific), re-suspended in 1.5% low melting point agarose, and spread on a microscope slide previously covered with 1% normal melting point agarose. Embedded cells were lysed and electrophoresis was performed at 25 V and 300 mA for 20 min. The slides were then immersed in neutralization buffer, stained with ethidium bromide and analyzed using a fluorescence microscope (BX60 Olympus, Tokyo, Japan) equipped with Image-Pro Plus software (Immagini & Computer, Bareggio; Milano, Italy).

In order to achieve realistic pictures of DNA damage it is necessary to randomly acquire from 50 to 60 images per slide. The different luminescence represents the DNA of the individual cells. When the DNA is not damaged, it does not migrate during electrophoresis and forms rounded images (Fig. 17-A). Instead, the DNA fragments of a damaged cell migrate to the positive pole during electrophoresis. The image displayed in this case will be similar to a comet (Fig. 17-B), with a head (the brightest part) that represents the undamaged DNA and a tail formed by the fragments of the migrated DNA. The extent of DNA damage, in terms of breakage, is estimated by the acquisition of two parameters: percentage of DNA in the tail (measurable as fluorescence intensity after staining) and tail length. It is possible to calculate the "tail moment", defined as the product of the relative fluorescence intensity of the tail due to its length, which is assumed as an index of DNA damage (Collins *et al*., 2001). For these reasons, in this study 50 images were analyzed for each slide and the tail moment was registered.

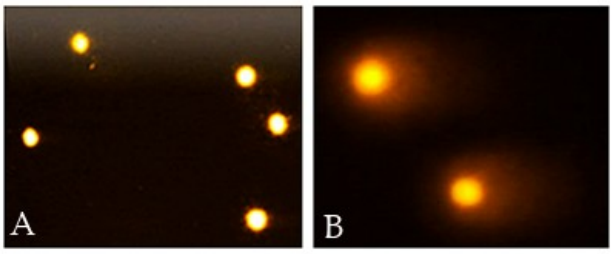

Fig. 17 Example of less (A) and more (B) damaged DNA.

3.1.5 RNA extraction and gene expression

Real-time reverse transcription quantitative PCR (RT-PCR) is the most versatile method for single cell mRNA analysis. It offers quantitative information about transcript levels, and it can be used for fast, accurate, sensitive and cost-effective gene expression analysis (Derveaux *et al*., 2010 - Kubista *et al*., 2006). In this study, total RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany), following the protocol suggested by the manufacturer. Then, 0.8 μg of RNA was reverse-transcribed employing the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Real-Time PCR was performed using an iCycler thermal cycler (Bio-Rad Laboratories) with cDNA corresponding to 10 ng of total RNA as the template. The PCR mixture included 0.2 μM primers (sequences in Tab. 6) and 1X SYBR Green PCR Master Mix (Bio-Rad Laboratories) in a final volume of 20 μL.

Genes	Primer Sequences	
Nrf2	F: 5′-AGCACATCCAGTCAGAAACC-3′	R: 5′-TGAAACGTAGCCGAAGAAAC-3′
HO-1	F: 5′-CAACATCCAGCTCTTTGAGG-3′	R: 5′-AGAAAGCTGAGTGTAAGGAC-3′
GAPDH	F: 5′-AGGGCTGCTTTTAACTCTGG-′	R: 5′-CATGGGTGGAATCATATTGG-3′

Tab. 6 List of primer used for real-time reverse transcription-polymerase chain reaction (RT-PCR).

Amplification and real-time data acquisition were performed using the followed cycle conditions: initial denaturation at 95 °C for 3 min, followed by 45 cycles of 10 s at 95 °C and 30 s at 58 °C. The fold change in expression of the different genes in treated HMEC-1 cells compared with control cells was normalized to the expression of GAPDH and was calculated by the equation $2^{-\Delta\Delta Ct}$ using iQ5 software version 2.0 (Bio-Rad Laboratories, Hercules, CA, USA). All reactions were performed in triplicate, and the accuracy was monitored by analysis of the PCR product melting curve.

3.1.6 Western blot analysis

Western blotting is a technique that allows the identification of a protein within a sample by recognition by specific antibodies, after electrophoresis. The method is based on an electrophoretic separation such as SDS-PAGE, (Sodium dodecylsulphate-polyacrylamide gel electrophoresis, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate), the transfer of the separated proteins on a support consisting of a nitrocellulose membrane or of PVDF and the detection of the bands of interest by incubation with specific antibodies. The technique allows not only to detect the presence of a target protein, but also an estimation of its relative level of expression.

After treatment with KB, cell pellets were lysed with a buffer containing 10 mM HEPES pH 8.0, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.05% Nonidet P-40 (NP-40), 1 mM Na3VO4 and protease inhibitors. In order to isolate nuclear compartments, lysates were centrifuged at 2500 g at 4 °C for 10 min and the supernatants (cytoplasm extracts) were collected. Then, pellets were re-suspended in a lysis buffer with protease inhibitors, and nuclear extracts were clarified by centrifugation (10,000 g at 4 °C for 5 min) and collected in new tubes. The concentration of protein in the samples was measured by Bradford's method (Bradford, 1976).

Proteins were denatured by boiling for 5 min in sodium dodecylsulfate (SDS) sample buffer, loaded into 10% SDS-PAGE gels and subsequently transferred onto PVDF membranes by electroblotting. Then, the membranes were incubated in Tris-buffered Saline for 1 h. Blots were incubated with

primary antibodies in the appropriate blocking solution at 4 °C overnight. The following primary antibodies were used: Nrf2 (C-20, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) dilution 1:400 in TBS-T+5% (w/v) non-fat dried milk; GAPDH (FL-335, Santa Cruz Biotechnology, Inc.) dilution 1:500 in TBS-T+5% (w/v) non-fat dried milk; lamin A/C (N-18, Santa Cruz Biotechnology, Inc.) dilution 1:500 in TBS-T+5% BSA. Membranes were washed three times for 10 min and then incubated with the appropriate secondary antibody conjugated with horseradish peroxidase for 1 h. For the immunological detection of proteins, the enhanced chemiluminescence system (Pierce Biotechnology, Waltham, MA, USA) was used. The acquisition of PVDF membrane images and the densitometric analysis of blots was performed using an Alliance MINI HD9 (UVItec) apparatus (Cleaver Scientific, Warwickshire, United Kingdom) and related software.

3.1.7 Immunofluorescence staining

Cells were preliminarily washed twice with PBS and fixed using a solution of 4% (w/v) paraformaldehyde in PBS for 10 min at room temperature. For permeabilization and blocking, cells were incubated with PBS + 1% (w/v) bovine serum albumin solution (BSA) and 0,2% (v/v) Triton X-100 for 30 min at room temperature. Cells were then incubated with anti-Nrf2 (C-20) rabbit polyclonal antibody (Santa Cruz Biotechnology) at dilution of 1:200 in blocking solution overnight at 4°C. After removing the primary antibody, cells where washed three times in blocking solution and incubated with the anti-rabbit FITC-conjugated secondary antibody at dilution of 1:100 in blocking solution for 1 h at room temperature in the dark. Cells were analyzed using a fluorescence microscope (IX50 Olympus) equipped with VarioCam acquisition camera and Image-Pro Plus software (Media Cybernetics).

3.1.8 Statistical analysis

Experimental results are expressed as mean ± S.D. of three (four for western blot) independent measurements. The data for various parameters were analyzed by one-way analysis of variance (ANOVA) with SPSS Statistics 22 (IBM). Tukey test was used to detect significant differences ($p < 0.001$ for comet assay analysis, $p < 0.05$ for cell viability, western blot and PCR analysis).

3.2 RESULTS

The results reported in this section have been recently published (Meroni *et al*. 2018).

3.2.1 Cell viability

Cells were treated with various concentrations of βHB and AA for different times (24, 48 and 72 h). Concentrations up to 4 mM βHB and 1 mM AA showed no significant effects on cell viability up to 48 h (Fig. 18). Higher concentrations were harmful at 48 h, and lower concentrations were damaging at 72 h. Considering this outcome, and assuming that 48 h is an appropriate time to detect a metabolic cellular response to an external stimulus, all further investigations were performed on HMEC-1 cells exposed to 4 mM βHB and 1 mM AA.

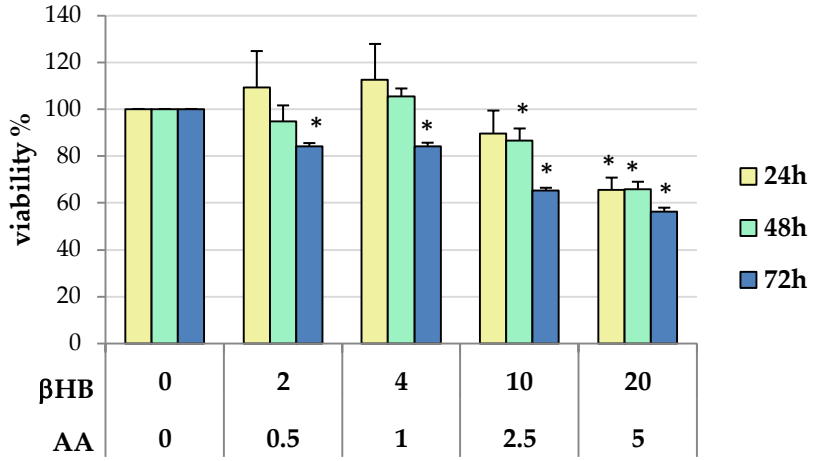

Fig. 18 Viability of HMEC-1 treated with KB (βHB 2–20 mM and AA 0.5–5 mM). Data are expressed as mean ± SD. * $p < 0.05$ compared with control (βHB = 0 and AA = 0).

3.2.2 Genotoxicity of ketone bodies

Figure 19 shows the results relating to DNA damage, both in cells supplemented with KB (βHB 4 mM and AA 1 mM) for 2, 24 and 48 h and control cells. KB induced moderate (<30%) (Klinder *et al*., 2007) stress to cells at every incubation time point; in particular, it was found that DNA damage, expressed as the percentage of DNA in the tail, was about to 21% in KB-treated cells vs. 2% in control cells (C = 2.03 ± 0.21, KB2h = 19.25 ± 2.04, KB24h = 21.09 ± 4.20, KB48h = 23.75 ± 2.02% of DNA in the tail, $p < 0.001$). The results show that the DNA damage induction was not related to the duration of incubation, but it occurred rapidly.

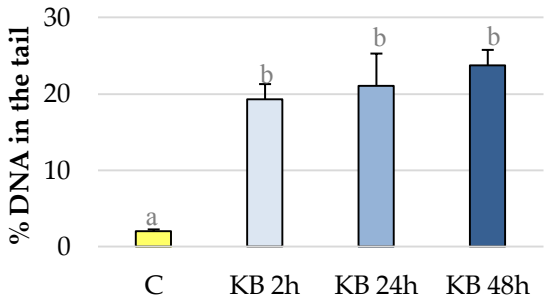

Fig. 19 Analysis of DNA damage in cells treated with KB (4 mM βHB, 1 mM AA) for 2, 24, 48 h; results are expressed as the % of DNA in the tail (mean ± SD). Data not sharing a common letter are significantly different, $p < 0.001$.

Then, we investigated the ability of KB to modulate DNA susceptibility toward a secondary oxidative insult. To this aim, cells were supplemented with KB (again for 2, 24 or 48 h) or not (control), and subsequently subjected to H_2O_2 50 μmol/L for 5 min. Firstly, there was a net increase in oxidative DNA damage in control cells exposed to H_2O_2 (C = 2.03 ± 0.21 vs. C + OX = 60.92 ± 2.73, % DNA in the tail, $p < 0.001$); cells previously treated with KB and exposed to H_2O_2 showed DNA damage as well. However, DNA damage decreased when the duration of exposure to KB increase. In particular, there was a significant decrease (−36%, $p < 0.001$), in H_2O_2-induced oxidative DNA damage in the cells treated with KB for 48 h (KB48 + OX = 39 ± 2.42% DNA in the tail) compared to the oxidized control cells (C + OX = 60.92 ± 2.73% DNA in the tail) (Fig. 20).

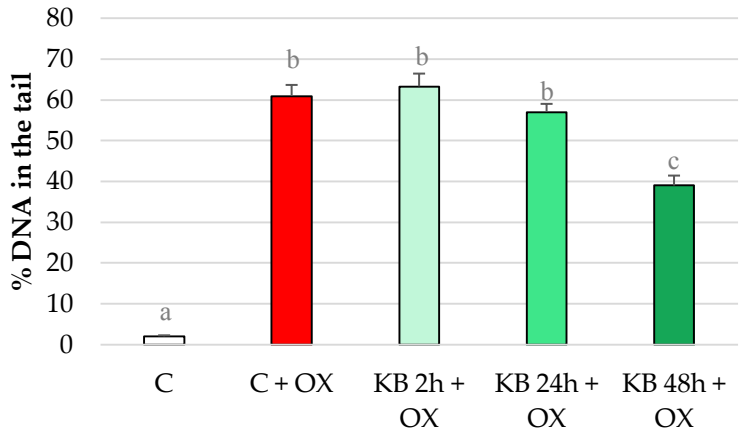

Fig. 20 Analysis of DNA damage in HMEC-1 cells treated with KB (4 mM βHB, 1 mM AA) for 2, 24 or 48 h and subsequently oxidized with 50 μM H_2O_2 for 5 min; results are expressed as the % of DNA in the tail (mean ± SD). Data not sharing a common letter are significantly different, $p < 0.001$.

3.2.3 Gene and protein expression of Nrf2

Since we hypothesized a rapid activation of Nrf2 pathway, HMEC-1 cells were exposed to KB for shorter times (2, 6, 14 and 24 h). Firstly, regarding the gene expression of Nrf2, a significant increase was observed at 2 h in KB-treated cells compared to control cells (variation about 13%, $p < 0.05$). At later time points, instead, there was a decrease in Nrf2 mRNA levels (Tab. 7).

Control	KB 2h	KB 6h	KB 14h	KB 24h
0.89 ± 0.02^{a}	1.01 ± 0.01^{b}	0.82 ± 0.08^{a}	0.66 ± 0.03^{c}	0.58 ± 0.01^{c}

Tab. 7 Nrf2 mRNA expression by real-time PCR in control HMEC-1 cells and cells treated with KB for 2, 6, 14 or 24 h. mRNA expression was normalized to the level of the housekeeping gene GAPDH. Data are the means ± SD of three independent experiments. Data not sharing a common letter are significantly different, $p < 0.05$.

The western blot analysis (Fig. 21) showed an increase in the total amount of Nrf2 at 2 h (by about 30%) in cells treated with KB compared to the control cells. Furthermore, considering only the nuclear extracts, the amount of Nrf2 was greater in KB-treated cells than in the control. Particularly, as reported in Fig. 21C, the level of Nrf2 in the nucleus was significantly different at 2 h in cells treated with KB compared to control cells (KB2h = 50.30 ± 2.2; Control = 34.95 ± 2.9, % vs. total, $p < 0.05$).

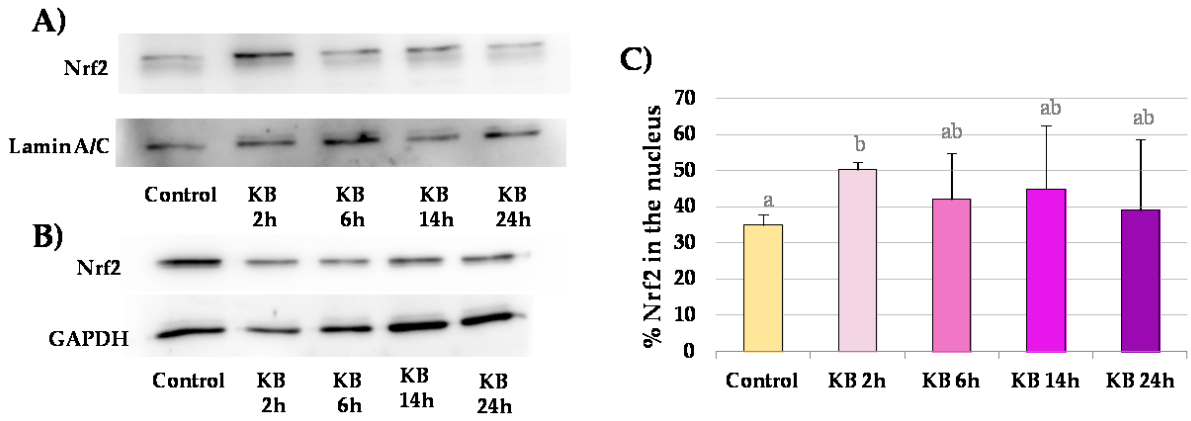

Fig. 21 Western blot analysis. Nrf2 protein expression in control cells or cells treated with KB for 2, 6, 14 or 24 h in the nuclear (A) and cytoplasmic (B) fractions. Lamin A/C was used as a nuclear marker. GAPDH was used as a cytoplasmic marker. The western blot image is representative of four independent experiments (A,B). Densitometric analysis of Nrf2 protein expression was performed using lamin A/C as the loading control and Nrf2 nuclear translocation are expressed as the % of Nrf2 in the nuclear fraction compared to the total amount of Nrf2 in whole cells. Data are the mean ± SD of four independent experiments. Data not sharing a common letter are significantly different, $p < 0.05$ (C).

Moreover, the western-blot results were further confirm by immunofluorescence analysis. In fact, as we can see from figure 22, the nuclei of KB cells are brighter than the control after 2 h of treatment, indicating Nrf2 translocation in the nucleus and, therefore, its activation.

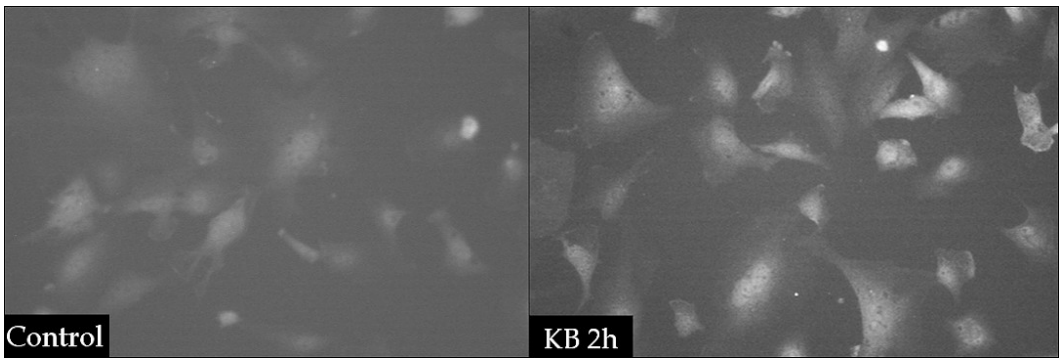

Fig. 22 Immunofluorescence analysis with anti-Nrf2 antibody in control and KB-treated cells for 2 hours (original magnification 20x).

3.2.4 HO-1 gene expression

Activation of the Nrf2 pathway was investigated by assessing the gene expression of HO-1. The results show that HO-1 mRNA levels were significantly higher in cells treated with KB compared to control cells, at all time points measured (Tab. 8). Particularly, after 2 and 6 h there were the greatest increases in mRNA levels (C = 0.40 ± 0.05, KB2h = 1.00, KB6h = 0.91± 0.01, $p < 0.05$), while at later time points, although there was a decrease, the values were still higher than in control cells.

Control	KB 2h	KB 6h	KB 14h	KB 24h
0.40 ± 0.05^{a}	1.00^{b}	0.91 ± 0.01^{b}	0.53 ± 0.09^{c}	0.65 ± 0.03^{c}

Tab. 8 Real time PCR analysis of HO-1 mRNA expression in control HMEC-1 cells and cells treated with KB for 2, 6, 14 or 24 h. mRNA expression was normalized to the level of the housekeeping gene GAPDH. Data are the means ± SD of three independent experiments. Data not sharing a common letter are significantly different, $p < 0.05$.

3.3 DISCUSSION

Although KD is used for multiple applications, less is known about its global impact on human body. In fact, the mechanisms by which KD achieves neuroprotection have been investigated by several studies (Noh *et al*., 2006 - Jarrett *et al*., 2008 - Milder & Patel, 2012), whereas the systemic effects of KB circulating in the bloodstream are not completely understood. Moreover, the adverse effects of KD on the cardiovascular system are still controversial. In fact, in epileptic patients following a KD treatment, has been observed an increase of serum levels of cholesterol, triglycerides and LDL, that are risk factors for cardiovascular diseases (CVD) (Kang *et al*., 2004 – Coppola *et al*., 2014). In addition, changes in vascular function and structure have been reported (Kapetanakis *et al*., 2014). Instead, in non-epileptic subjects, the consumption of a KD has been associated with an improvement in some cardiovascular risk factors, even though the effect of long term use of KD and its lipid composition should be better assessed (Kosinski & Jornayvaz, 2017). Another important risk factor for CVD is endothelial dysfunction, often resulting from cellular oxidative unbalance (Drexler 1997 – Anderson *et al*., 1995). In this context, considering that KB exposure has been shown to alter red-ox cellular state, it appears critical to investigate the KB effects on endothelial model. For these reasons, we decided to investigate the effects of exposure to KB in HMEC-1 endothelial cells by measuring markers of oxidative stress and metabolic responses.

Conflicting data are reported in literature concerning the production of ROS by KB and KD in different models. Some studies (Stafford *et al*., 2010 – Shukla *et al*., 2014) found ROS reduction owing to KB metabolism, while other studies reported increased oxidative stress (Allen *et al*., 2014), or no change (Poff *et al*., 2015) in cancer models. In primary cells, Veech reported that the metabolism of KB should reduce the amount of Q semiquinone, thereby decreasing ROS production (Veech, 2001). Conversely, other investigations in primary cells models showed different results. For instance, Shi et al. investigated the pro-oxidant activities of βHB in primary calf liver cells; they demonstrated that βHB decreased antioxidant defences and increased the oxidizing species, causing stress at least within 24 h (Shi *et al*., 2014). Another study seems to confirm this thesis, because it showed that, in mitochondria from the hippocampus of rats fed a KD, there was an increase both of H_2O_2 and 4-HNE levels, markers of oxidative stress. These results suggest the induction of mild oxidative stress immediately after the beginning of a KD (Milder *et al*., 2010). More specifically of our interest, it has been demonstrated that ketones can generate oxygen radical and overall contribute to increased oxidative stress also in endothelial cells. For instance, the study of Kanikarla-Marie et al., showed that KB cause increased oxidative stress by up-regulating NADPH oxidase 4 in HUVEC cells (Kanikarla-Marie & Jain, 2015). Accordingly, another research demonstrated that elevated levels of KB can result in lipid peroxidation, which is supposed to be increased by oxygen radicals generated by AA (Jain *et al*., 1998). Thus, our results are in agreement with these recent data, as they demonstrate that KB, at concentrations equal to 4 mM for βHB and 1 mM for AA, induced moderate oxidative stress. Although they significantly increased oxidative DNA damage after 2 h, we detected that this stress was not related to the duration of exposure. In fact, we did not find any significant differences in DNA damage among the three time points of exposure (2, 24 and 48 h). Therefore, we hypothesized that, during 48 h of treatment, the oxidative stress generated at 2 h may induce some cellular metabolic responses. One of the protective mechanisms promoted by the cell as a consequence of KB-induced stress could be the activation of antioxidant enzymes.

With the aim to investigate this hypothesis, we exposed HMEC-1 cells to KB and afterwards to a secondary oxidative insult, in our study represented by H_2O_2. Surprisingly, oxidized cells

previously treated with KB for 48 h showed significantly less DNA damage compared to control oxidized cells. This intriguing result suggests that KB cause moderate oxidative stress and, at a following time point, they can activate a cellular response that results in protection against a secondary insult.

Lately, several studies have been carried out to understand the positive role of KB in some pathologies, not only epilepsy, but also other neurological diseases and cancer. For instance, a study published by Noh et al. proved that AA protects neuronal cells from oxidative glutamate toxicity. In particular, cells treated with AA showed a significant decrease in glutamate-induced ROS production compared with cells treated with glutamate alone (Noh *et al*., 2006). Moreover, Shimatzu and collegues implanted mice with subcutaneous pump delivering PBS or βHB for 24 h, in order to test the potential protective role of βHB against oxidative stress. Then mice received an intravenous injection of paraquat, which produces superoxide anions. The authors found that paraquat treatment of βHB-treated mice led to a significant prevention of carbonylated proteins, while in control mice there was an increase of their production. Equally, the increase of 4-HNE was suppressed in mice receiving βHB, compared to PBS-treated mice (Shimazu *et al*., 2013). Likewise, Jarrett et al., reported that KD increased mitochondria reduced glutathione (GSH) levels and improved red-ox status, thereby resulting in decreased mitochondrial production of ROS in rats fed a KD for three weeks (Jarrett *et al*., 2008). Consistently with the papers above, our data suggest the capacity of KB to promote cellular responses that result in the prevention of oxidative damage induced by a secondary insult, maybe by implementing the antioxidant defences.

The activation of the Nrf2 pathway may be a potential mechanism by which this protection occurs (Milder & Patel, 2012 – Liśkiewicz *et al*., 2016). Nrf2 is a transcription factor normally sequestered in the cytoplasm by binding to the protein Keap1 (kelch ECH associating protein 1), which continually ubiquitinates Nrf2, targeting it for the proteasome where is degraded. When stress occurs, Keap1 is inactivated and Nrf2 is stabilized, therefore, de novo synthesized Nrf2 translocates into the nucleus. There, it forms a heterodimer with small Maf (sMaf) proteins and activates the transcription of target genes by binding to antioxidant response elements (ARE) (Lee & Johnson, 2004 - Kobayashi & Yamamoto, 2005 - Kensler *et al*., 2007 - Bryan *et al*., 2013 - Kim & Keum, 2016). Some studies have reported that enhanced nuclear translocation of Nrf2 protects against oxidative stress injury (Gu *et al*., 2015 – Liao *et al*., 2016). For these reasons, firstly we decided to evaluate Nrf2 gene expression, then its translocation into the nucleus. Since we hypothesized that the activation of a cellular response would happen fast, as we saw protection at 48 h, we exposed HMEC-1 cells for 2, 6, 14 or 24 h to KB. Primarily, the gene expression results showed higher mRNA Nrf2 levels at 2 h; thus, it seems that there was very rapid stabilization of Nrf2, which implies its fast de novo synthesis. However, the levels of Nrf2 mRNA expression from 6 to 24 h were lower, but we cannot say that Nrf2 was non-active. Indeed, it has been indicated by several papers that some agents can increase the nuclear translocation of Nrf2, but not alter its gene expression level (Ishii *et al*., 2000 - Nguyen *et al*., 2003 - Liao *et al*., 2016). Accordingly, the findings obtained from the western blot analysis showed an increase in the total amount of Nrf2 in KB-treated cells compared to control as well as nuclear accumulation already at 2 h. This result was further confirmed with the immunoflorescence analysis. The trend of increased Nrf2 nuclear accumulation means that the association between Keap1 and Nrf2 has changed; the transcription factor was stabilized and was not degraded by the proteasome anymore, therefore its translocation into the nucleus was promoted. In view of these data, we hypothesized that KB, at first, cause the production of low levels of ROS, which may serve as a redox signaling stimulus and activate the transcription factor Nrf2. Milder and colleagues were

the first to propose that the consumption of KD could activate the Nrf2 pathway. In fact, they observed Nrf2 accumulation in nuclear fractions from the hippocampus and liver of rats fed a KD for up to three weeks, suggesting chronic Nrf2 nuclear translocation and activation (Milder & Patel, 2012). Newly, the impact of KD on tumor growth in an animal model was investigated; the authors reported that, among the several factors protecting against cancer, one was the stimulation of Nrf2 (Liśkiewicz *et al*., 2016). Both of these studies clearly showed higher Nrf2 nuclear levels, even the different dietary intervention with KD.

In order to confirm the effective involvement of the Nrf2 pathway, we decided to analyze the gene expression of one of its target genes: HO-1. It is one of the most important enzymes up-regulated by Nrf2, its function is to catalyze the conversion of heme into biliverdin, carbon monoxide and free iron, and it has both anti-inflammatory and antioxidant properties (Immenschuh & Ramadori, 2000 - Paine *et al*., 2010 - Loboda *et al*., 2016). Moreover, among the antioxidant proteins regulated by Nrf2, we chose HO-1 because its induction is strongly related to the red-ox-dependent Keap1/Nrf2 system when stress occurs (Paine *et al*., 2010 – Liao *et al*., 2016). Our results confirm the activation of Nrf2/HO-1 pathway, because they showed, at all time points, an increase in HO-1 gene expression, particularly after 2 and 6 h of KB exposure, accordingly with other papers (Yao *et al*., 2007 - Chen *et al*., 2011 - Liao *et al*., 2016). It has been shown by several studies that activation of the Nrf2/HO-1 pathway protects different types of cells (Nguyen *et al*., 2003 - Gu *et al*., 2015 - Liao *et al*., 2016) and, particularly, this pathway has been specifically correlated to the effects of KD. In the study mentioned above by Milder et al., a new mechanism of action of KD has been suggested, which include Nrf2 activation. The authors proposed that the Nrf2 pathway was systemically activated by KD via redox signalling, resulting in the induction of protective proteins and cellular adaptation. In fact, their results showed that, after the consumption of a KD, both Nrf2 and HO-1 protein expression were higher compared to control (Milder & Patel, 2012). This hypothesis has been confirmed further, by Liśkiewicz and collegues, with the investigation of the long-term effects of KD on tumor growth. Indeed, they demonstrated that Nrf2 levels increased in rats fed a KD compared to control (Liśkiewicz *et al*., 2016). Hence, our results, accordingly to the studies reported, overall demonstrate rapid activation of Nrf2 pathway after KB-induced stress.

More detailed analysis should be performed in upcoming studies, planned to evaluate the effects of AA and βHB individually supplemented, with the aim to clarify their specific influence on DNA oxidative damage and Nrf2 activation. Additionally, the expression of other Nrf2 target genes, different from HO-1, should be assessed, to confirm KB activation of Nrf2 pathway once again.

3.4 CONCLUSIONS

The results obtained in this *in vitro* study demonstrate the activation of a cellular metabolic response caused by the effect of KB exposure. The conclusions of this project are graphically summarised in Fig. 23. Ketones, by inducing moderate oxidative stress, activate the transcription factor Nrf2, which translocates into the nucleus. By binding to the ARE, Nrf2 activates the transcription of target genes, among which HO-1. As a consequence, the metabolic response caused by KB exposure makes cells more protected against a secondary insult, such as H_2O_2, leading to a reduction in DNA oxidative damage.

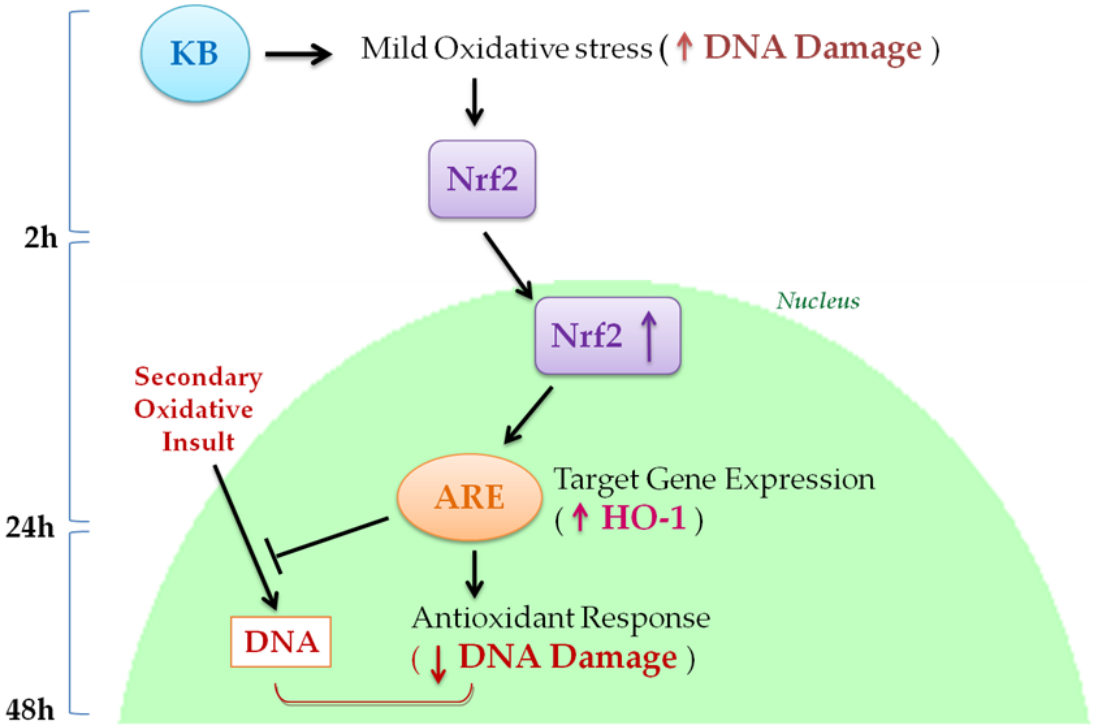

Fig. 22 Proposed mechanism of action of KB in HMEC-1 cells.

The choice to investigate the effects of KB in endothelial cells is a novelty in this field of research, since it is something different from the neurological context. Although the positive effects of the ketogenic therapy have been clearly demonstrated (Freeman *et al.*, 1998 – Tieu *et al.*, 2003 - Hartman & Vining, 2007 – Kashiwaya *et al.*, 2013), the use of this diet as a weight loss method is still controversial. Every time a KD is applied it should be considered that this diet could have an impact on cell metabolism in different tissue functions. The mechanism hypothesized in our *in vitro* study is that KB could activate the transcription factor Nrf2, which improves the cellular ability to detoxify and remove harmful substances by the activation of cellular defence processes (Lee & Johnson, 2004 – Uruno & Motohashi, 2011 – Bryan et al., 2013). However, the up-regulation of Nrf2 does not always leads to protection; for instance, it has been reported that it may promote carcinogenesis in various tissues. This is due to different activities of Nrf2: improvement of mitochondrial function, suppression of apoptosis, and redirection of glucose metabolism toward NADPH generation and anabolic pathways, which overall generate cell proliferation (Holmström *et al.*, 2013 - Suzuki & Yamamoto, 2015 - Tebay *et al.*, 2015). Moreover, considering the activation of Nrf2 as a result of impaired oxidative cell status, it could be harmful promote this function in the long term. In conclusion, when a KD is applied, both for therapeutic or weigh loss purposes, a careful examination of the risk-benefit ratio should be done.

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4 PART II: *IN VIVO* STUDY

4.1 SUBJECTS AND METHODS

4.1.1 Study design

In order to study the effects of the KD on human intestinal environment, and verify if there are any differences in microbiota composition respect to a standard diet, a case-control observational multi-center study was designed. We enrolled 12 patients (6 females and 6 males, age range 2-46 years) affected by GLUT1-DS or refractory epilepsy, both pathologies for which KD is mandatory, at the Department of Child Neurology and Psychiatry of C. Mondino National Neurological Institute in Pavia. They were matched to a respective control group represented by a healthy population, comparable by gender, age and body mass index; these subjects were recruited at the Pediatric Clinic of the San Paolo Hospital in Milan.

Exclusion criteria were: fecal incontinence; gastrointestinal disorders or use of antibiotic or probiotic/prebiotic supplements during the month prior to the study; treatment with antiepileptic drugs at the time of the study, age < 2 years old.

The recruitment (Fig. 23) began in September 2016 and lasted untill June 2018. In this period, 33 patients were diagnosed of refractory epilepsy or GLUT1-DS and undergoing a ketogenic diet, implemented as a therapy at the Human Nutrition Research Center in Pavia. We faced numerous recruitment challenges, mostly because the majority of subjects (21) did not have fecal continence, therefore they were unsuitable for the study.

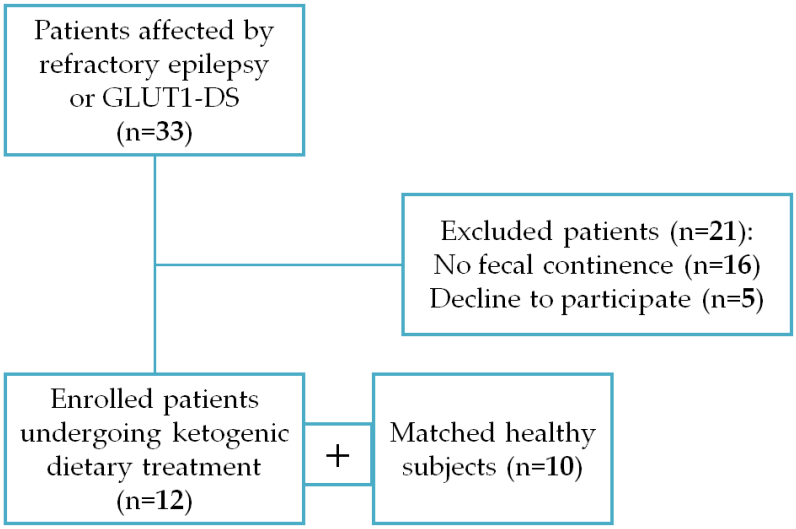

Fig. 23 Patients selection and enrollment

Partecipants enrolled to the study, both under KD treatment (KD) and controls (CTR), received specific instructions for the total collection of a fecal sample.

In addition, a 1-month, prospective, single-center, single-arm study of the effects of the KD on gut microbiota was designed (Fig. 24), with the aim to investigate the specifc effect of KD beside the pathological condition. To this aim, we enrolled 7 patients (4 females and 3 males, age range 2-46 years) affected by GLUT1-DS or refractory epilepsy, at the Department of Child Neurology and Psychiatry of C. Mondino National Neurological Institute in Pavia; they were asked to collect a fecal sample before (t0) and after one month of treatment with a classic KD (t1).

Fig. 24 Schematic diagram indicating experimental protocol

The study protocol complied with the principles of the Declaration of Helsinki, and was approved by the Institution Review Board. All patients (or parents if the patient was <18 years of age) provided written informed consent before the beginning of the study. The study purpose and protocol were exhaustively explained to all participants and their parents, who signed an informed consent before study enrollment.

4.1.2 Ketogenic diet treatment

The treatment with KD was implemented at the Human Nutrition Research Center outpatient clinic according to a standardized protocol (Tagliabue *et al.*, 2012). The patient's protocol has provided for a first visit during which the information relating to the physiological and pathological history were gathered, anthropometric measurements (weight, height, BMI, bicipital, tricipital and subscapular folds, waist circumference) were recorded, biochemical tests (lipid profile, glycaemia, uric acid, creatinine) were performed, body composition assessment parameters (bioimpedentiometry, percentage fat mass) were predicted, and measured by indirect calorimetry, basal metabolic rate were measured. Prior to start the diet, blood tests were performed to check for contraindications, dyslipidemia, hyperuricemia or elevated levels of creatinine. Subsequently, surveys were carried out on the eating habits of patients through a very detailed 7-day food diary, both in terms of quantity and quality of food and drinks consumed during the day.
After this evaluation the dietician worked out, for each patient, several individual and personalized KD plans. Firstly, the caloric needs were established considering an average between the measured basal metabolic rate, the energy intake obtained from the food diary and the level of physical activity of the patients (usually low). Proteins were established on the basis of population recommended intake levels based on sex and age of patients, while glucose/protein and lipid intake were calculated in relation to the ketogenic ratio of the diet followed by the patient (2:1 or 3:1 or 4:1). All patients were instructed to start a 1:1 ketogenic diet at home and gradually proceed to 2:1, 3:1 or 4:1 ketogenic ratios in order to obtain blood values 2.0 mml/l of βHB. Parallel to the reduction of the carbohydrate quantity and therefore to the limitation of the fruit and vegetable intake, it was evaluated the intake of a vitamin-mineral supplement to avoid any deficiencies. The integrator, strictly sugar free, must take into account age, sex of the patient, and established ketogenic relationship. Among the supplementations it was also recommend an adequate intake of liquids, favoring in consumption of water, preferably strongly mineralized, or alternatively drinks without sugar. No probiotic or prebiotic supplementation was provided in the first month of treatment. Families were instructed to check blood (for glucose and KB control) daily during the induction phase and twice per week thereafter, and to report the values by e-mail.

4.1.3 Collection and preparation of samples

Patients were instructed to collect the total stool of one day in disposable bedpans, keep the sample refrigerated and deliver it to the laboratory within 24-48 h from the recovery. Aliquots of stool were immediately divided for different biological assessment and for preparation of fecal water, then were stored at -80°C. The humidity of fecal samples was determined, after drying at 105°C overnight, by weight difference of a sample aliquot according to the official AOAC method n. 923.03 (AOAC 1995).

4.1.3.1 Preparation of fecal water

Fecal water (FW) was obtained, from frozen samples, after defrosting them for 2 hours at room temperature; then samples were diluted 1:1 (w/v) in sterilized PBS and homogenised manually until a uniform consistency was achieved. The samples, transferred into ultracentrifuge tubes (Beckman Ultra-clear tubes; Beckman Limited, High Wycombe, UK) centrifuged at 24000 rpm (35000g) for 2 h at 20°C (Beckman L7-55 Ultracentrifuge; Beckman Limited, High Wycombe, UK), and supernatants were carefully decanted and stored at -80°C. For citotoxicity and genotoxicity evaluation, FW samples were rapidly defrosted, centrifuged at 11.000 rpm for 2 min at 20°C to remove any residual, and filtered through 0,45μm filter (VWR International, USA); filtered solutions were used to assess FW toxicity.

4.1.4 Fecal water toxicity

4.1.4.1 Cell culture

Human Caco-2 cells were obtained from the European Collection of Animal Cell Cultures (United Kingdom). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma–Aldrich) supplemented with 10% heat inactivated (30 min at 56°C) fetal bovin serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 0.1 mMnon-essential amino acids, in an incubator with an atmosphere of 95% air and 5% carbon dioxide. The culture medium was routinely changed every 2 days, and always the day before exposure to fecal water. All cell culture reagents were purchased from Sigma–Aldrich (St. Louis, MO, United States) and chemicals from Merck (Darmstadt, Germany).
When cells reached a subculturing density of 70% confluence, they were detached by means of trypsinization: the medium was removed from the flask (75 cm^2), and the cells were washed with PBS and treated with 2.5 mL of fresh trypsin–EDTA solution in the incubator. The trypsin action, lasting 5 min, was arrested by the addition of 4 mL of complete medium. The cell suspension was then transferred to a 15 mL tube and centrifuged 5 min at 1000g. After removing the supernatant, the cell pellet was resuspended in complete medium and seeded at 10^4 cells/cm^2 (Natoli *et al*., 2012).

4.1.4.1.1 Cell differentiation

When Caco2 cells differentiated, an in vitro system is obtained that simulates both the morphology and the functionality of the human intestinal epithelium. In fact, the differentiated Caco2 cells express narrow junctions, microvilli and a series of enzymes typical of enterocytes (Fig. 25) (Sambuy *et al*., 2005).

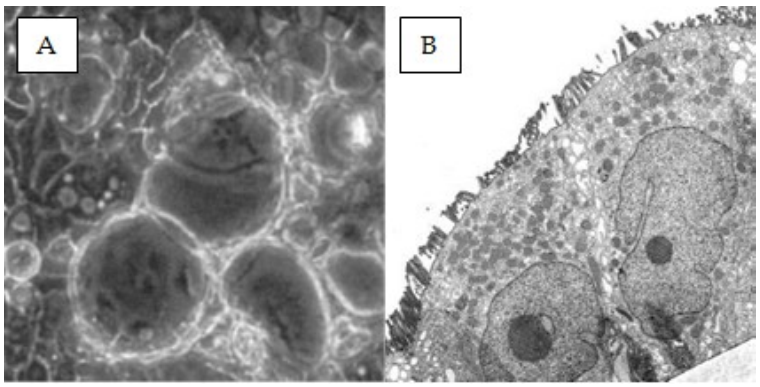

Fig. 25 Image of Caco-2 cells obtained by optical microscope (A) and electron microscope (B). The cell is polarized, that is with the microvilli, intended for the absorption of nutrients, exposed upwards, in the direction of the culture medium, which corresponds to the intestinal lumen. The adjacent cells are joined by tight junctions.

For toxicity experiments, considering the permeability of the tight junctions determined by measurements of trans epithelial electric resistance of cell monolayers, cells were seeded on 60mm plates (Cellstar, Greiner, Germany) at a density of 10^5 cells/cm^2, maintained for 10 days in complete medium; the medium was changed three times a week (Sambuy *et al*., 2005).

4.1.4.2 Citotoxicity and genotoxicity

A suspension of differentiated Caco-2 cells (3.5 x 10^5 cells/ml in 380 µL) was incubated with FW (120 µL) or medium (120 µL negative control) or H_2O_2 500 µM (120 µL positive control) for 30 min at 37°C on a shaking platform (Erba *et al*., 2014). Every FW recovered from stool samples was analyzed in quadruple and controls (negative and positive) were included in each batch. After the incubation, an aliquot of this cell suspension was used to assess citotoxicity of FW, by measuring cell viability, with Trypan Blue exclusion test (expressed as percentage of viable cells). Another aliquot of cell was used to assess genotoxicity of FW with Comet Assay, according to the procedure previously described (3.1.4).

4.1.5 SCFA measurement

SCFA concentrations were assessed in accordance with the method proposed by Weaver et al. (1997), modified as follows. Stool (200 mg) were suspended in 1 mL of double distilled water, homogenized on a vortex mixer, and, after 30 min, centrifuged (15000 rpm) for 15 min at 10°C (Beckman Coulter Centrifuge). Aliquots (0.5 µL) of supernatant were added with 200 µL 85% orthophosphoric acid, and 300 µL of 2-ethyl-butyric acid (109959 Sigma-Aldrich,Milan, Italy) 3.3 mM in H_2SO_4 2% as internal standard. SCFA were gently extracted for 1 min with 1 mL ethyl-ether/heptan (1:1 v/v) and centrifuged for 10 min at 3000 rpm. The aqueous phase was frozen and the organic layer was removed for analysis by a Varian 3400 CX (Conquer Scientific, San Diego, CA, USA) gas liquid chromatograph equipped with a Varian 8200 CX autosampler and an HP-FFAP fused-silica capillary column (30 m, 0.53 mm i.d. with a 1-mm film). Injector and detector temperatures were 90°C and 260°C, respectively. The initial oven temperature was 60°C and was increased by 5°C/min to 130°C and then by 20°C/min and held at 200°C. Quantification of the SCFA was obtained through calibration curves of acetic, propionic, iso-butyric, butyric, and iso-valeric

acid in concentrations between 0.5 and 10 mM (10 mM 2-ethyl-butyric acid as internal standard) (Weaver *et al*., 1997). Results are expressed as mg/g of dry weight of feces.

4.1.6 Gut microbiota analysis

Stool total bacterial DNA extraction was performed using the Spin stool DNA kit (Stratec Molecular, Berlin, Germany), according to the manufacturer's instructions. Twenty-five ng of DNA extracted from each stool sample was utilized to construct a sequencing library. 16S rRNA gene amplicon libraries were performed with a two-step barcoding approach according to Illumina 16S Metagenomic Sequencing Library Preparation (www.illumina.com). In the first-step PCR, 16S rRNA genes (V3-V4 region) of all bacteria were amplified as described by Klindworth *et al*. (2013). For library preparation, DNA samples coming from first PCR step were amplified with dual-index primers using Nextera DNA Library Preparation Kit (Illumina, San Diego, CA, USA).
Each sample possessed specific barcode sequences at the 5'- and 3'-end of the PCR amplicon to discriminate among each other in the pooled library. Library concentration and exact product size were measured using a KAPA Library Quantification Kit (Kapa Biosystems, Woburn, MA, USA) and an Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA, USA), respectively.
A pooled library (20 nM) and a PhiX control v3 (20 nM) (Illumina) were mixed with 0.2 N fresh NaOH and hybridization buffer HT1 (Illumina) to produce the final concentration at 12 pM each. The resulting library was mixed with the PhiX control v3 (5%, v/v) (Illumina) and 600 μL loaded on a MiSeq® v2 (500 cycle) Reagent cartridge for obtaining a paired-end 2 × 250 bp sequencing. All sequencing procedures were monitored through the Illumina BaseSpace® application. FASTQ files were demultiplexed by Illumina MiSeq Reporter and a total of 2.5 Gbases raw reads were obtained. Sequencing reads are available in NCBI Short Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA447916.

4.1.6.1 Microbiota profiling

The 16S rRNA sequences obtained were analyzed using Pandaseq (Masella *et al*., 2012). Low quality reads were filtered and discarded, then the reads were processed using the QIIME pipeline (release 1.8.0; Caporaso JG et al., 2011) and clustered into Operational Taxonomic Unit (OTUs) at 97% identity level. Taxonomic assignment was performed via RDP classifier (Wang *et al*., 2007) against the Greengenes database (release 13_8 http://greengenes.secondgenome.com).
Alpha-diversity was computed through the QIIME pipeline using Chao1, observed species, Shannon diversity and Faith's Phylogenetic diversity metrics; statistical evaluation of differences in alpha-diversity indices was performed by a non-parametric Monte Carlo-based test, using 9999 random permutations. Beta-diversity's principal coordinates analysis (PCoA) was performed using weighted and unweighted UniFrac distances, while "adonis" and "anosim" functions were employed to determine statistical separation of the microbiota profiles.

4.1.6.2 Real-time PCR quantification

Absolute quantification by real-time PCR was performed before and after KD in 7 patients. After extraction with the Prepman Ultra kit (Applied Biosystem, USA), the DNA of the following control strains was used to determine the standard curves: *Roseburia intestinalis* DSM 14610 and *Faecalibacterium prausntzii* DSM 17677 from the international DSMZ (Leibniz-Institut

DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). The other microorganisms used as a control are part of the collection of the Laboratory of Clinical Microbiology of the Department of Health Sciences of the University of Milan: *Bifidobacterium animalis* and *Enterobacteriaceae*.

Real Time PCR was carried out using the StepOne Plus instrument (Applied Biosystems) and the SYBR1Green chemistry (ThermoScientific, USA). The analysis was performed in a total volume of 15 µl, and each sample analyzed in triplicate. Standard curve was carried out using five serial dilutions of control DNA and specific 16S rRNA primers reported in Tab 9.

	PRIMER 5′ -> 3′
Enterobacteriaceae	fw: CAT TGA CGT TAC CCG CAG AAG AAG rev: CTC TAC GAG ACT CAA GTC TGC
Bifidobacterium spp.	fw: CGC GTC YGG TGT GAA AG rev: CCC CAC ATC CAG CAT CCA
Roseburia spp.	fw: TAC TGC ATT GGA AAC TGT CG rev: CGG CAC CGA AGA GCA AT
Faecalibacterium prausnitzii	fw: GGA GGA AGA AGG TCT TCG rev: AAT TCC GCC TAC CTC TGC ACT

Tab. 9 Forward and reverse primers of the species in analysis.

The following thermal cycling parameters were used for amplification of DNA: 95°C for 10 minutes followed by 40 cycles of 20 seconds at 95°C, 3 seconds at 95°C, and 30 seconds at 60°C. A melting curve analysis was also performed to verify amplicon specificity.

4.1.7 Statistical analysis

Variables are expressed as means and standard deviations. Because of the paired data, comparisons of continuous variables before and after 1 month of KD were performed by the paired t-test. All calculations were performed using SPSS version 17.0 for Windows (SPSS, Inc., Chicago, IL, USA). A value of p-values < 0.05 (two sided) was considered statistically significant.

For microbial profiling, statistical comparisons were performed using MATLAB software (Natick, MA, USA). For evaluating differences in relative abundances of bacterial groups at each taxonomic level, a Mann-Whitney U-test was performed, excluding a normal distribution of data at every level (Shapiro-Wilk test at 0.99 confidence). Unless otherwise stated, p-values < 0.05 were considered as significant.

4.2 RESULTS

4.2.1 KD vs CTR

4.2.1.1 Gut microbiota analysis

The gut microbiota was characterized by next-generation sequencing using V3–V4 hyper-variable 16S rRNA genomic region. On average, 43492.000 high-quality reads were considered for gut microbiota analysis of KD (n = 11; one subject was excluded because he was too young compared to the others and showed completely different results) and healthy controls (n = 10); reads were then grouped in a total of 47917 operational taxonomic units (OTUs), which could be assigned to specific taxonomies down to the genus level.
Firstly, bacterial composition within each sample (α-diversity) was measured using OTU-based methods (Chao1, Fig. 27A; observed species, Fig. 27B; and Shannon indexes, Fig. 27C).

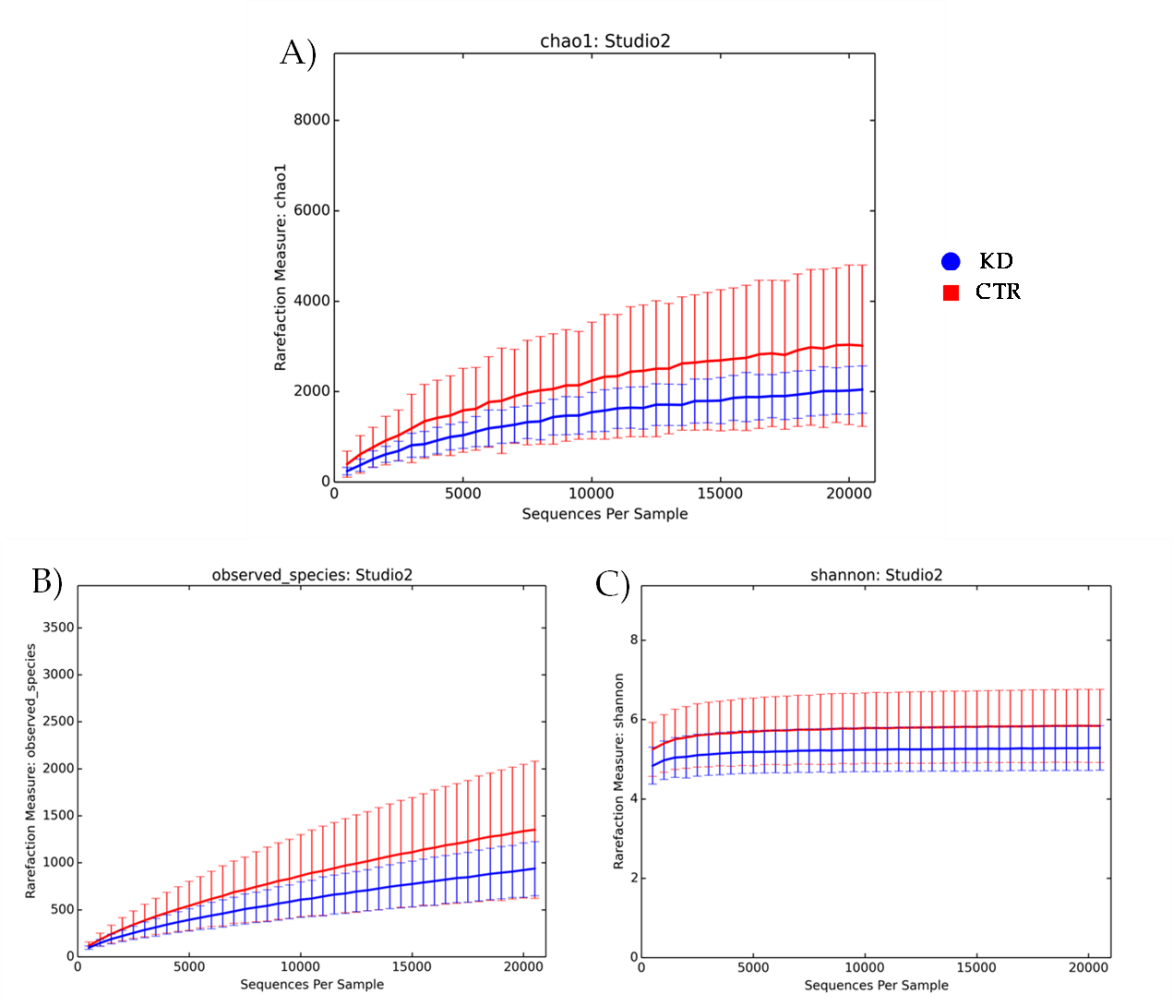

Fig. 26 α rarefaction curves according to Faith's phylogenetic diversity index. α-diversity plot of KD (blue) versus control (red) samples. KD patients show a reduced biodiversity compared to healthy controls. Differences are statistically significant ($p < 0.05$) for Chao1 (A), not for observed species (B) or Shannon indexes (C).

Even though we could observe a reduction in α-diversity in KD group, the number of subjects analyzed was not sufficient to reach statistical significance ($p < 0.05$, permutation-based non-parametric *t*-test) for all metrics, but only for Chao1 index.
Furthermore, to investigate differences in the two studied groups, we assesed β-diversity using weighted (Fig. 27A) and unweighted (27B) UniFrac distances.

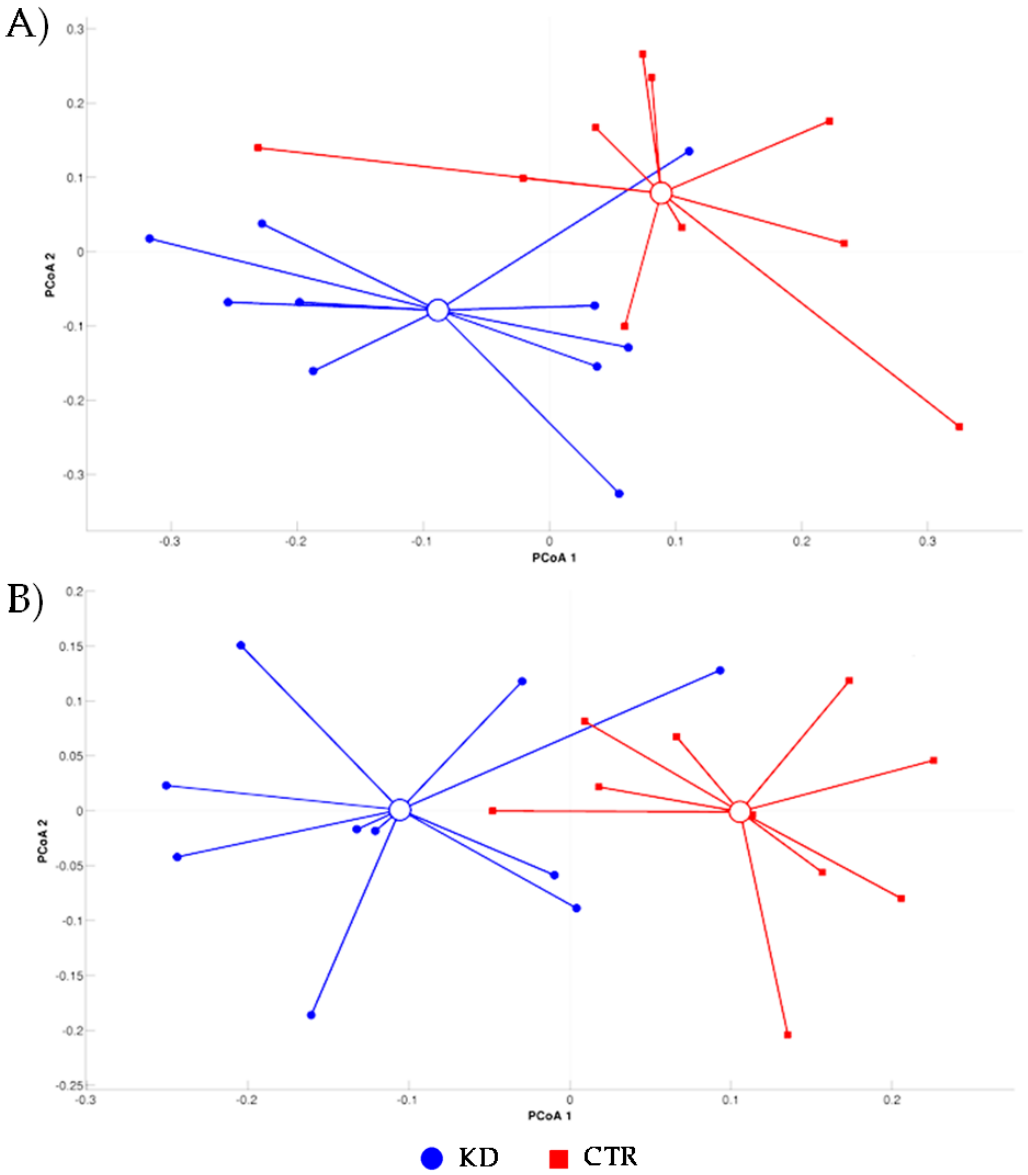

Fig. 27 Principal Coordinate Analysis (PCoA) according to unweighted (A) and weighted (B) Unifrac distances. The first two components of the variance are represented. PCoA of KD (blue) versus CTR (red) samples. Differences are statistically significant ($p < 0.05$).

β-diversity analysis highlighted a statistically significant separation between the centroids of KD and CTR groups according to the weighted and unweighted Unifrac distance ($p = 0.001$; $p = 0.002$, respectively). This important difference may be due to principal components of the gut microbiota, as the separation for both Unifrac metric was significant.
To evaluate possible differences in taxa distribution among KD and control subjects we analyzed the relative microbial abundance at different taxonomic levels. At the phylum level (28A) *Bacteriodetes* and *Firmicutes* were the predominant bacteria taxa in feces of both KD and CTR; in

particular, these two taxa rapresent 96.4% (CTR) and 92.8% (KD) of total microbiota. However, data showed a different ratio *Firmicutes*/*Bacteriodetes*, as *Firmicutes* were significantly increased in KD compared to CTR (KD: 81.9 ± 10.8, CTR: 59.3 ± 12.4; mean ± sd, $p < 0.001$), on the contrary *Bacteroidetes* were reduced (KD: 10.9 ± 10.1, CTR: 37.1 ± 10.7, $p < 0.001$).
Accordingly, this difference was reflected also in the results of the family level (Fig. 28B), as we detected a significant decrease in *Bacteroidaceae* (KD: 7.8 ± 6.9, CTR 30.3 ± 10.7, $p < 0.001$) and an increase, in *Ruminococcaceae*, although not statistically significant (KD: 39.0 ± 14.6, CTR: 31.3 ± 13.1, $p = 0.42$). Moreover, KD showed significant differences in other smaller families, particularly in *Lachnospiraceae*, *Rikenellaceae* and *Erysipelotrichaceae* (KD: 24.1 ± 10.9, 0.7 ± 0.8, 1.1 ± 0.8; CTR: 13.7 ± 4.4, 3.1 ± 2.2, 0.4 ± 0.3; respectively, $p < 0.05$).

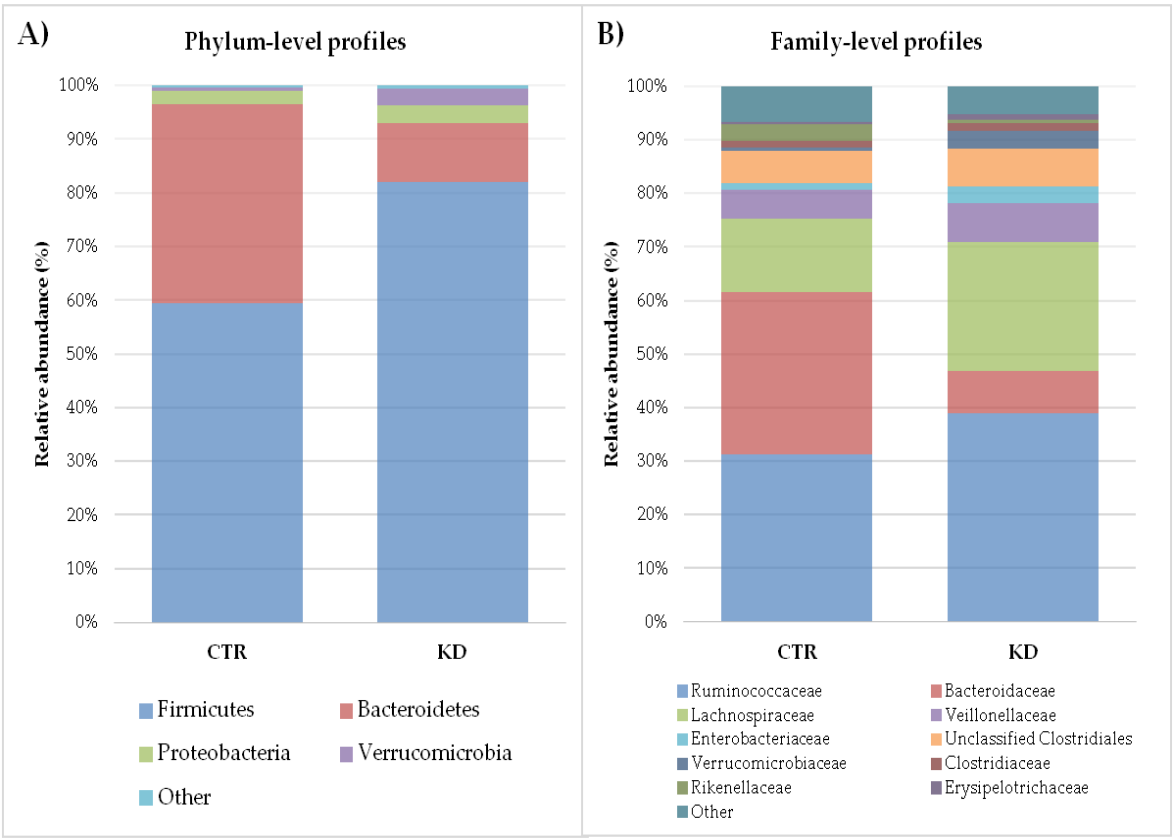

Fig. 28 Bar charts representing the average relative abundance of subjects on a ketogenic diet (KD) and healthy control (CTR) microbiota, classified using the 16S rRNA gene. A) Mean relative abundances of fecal bacterial phyla; B) Mean relative abundances of fecal bacterial families.

At the genus level (Tab. 10), the most important results, statistically significant, were the reduction in *Bacteroides* (KD: 7.8 ± 6.9, CTR: 30.3 ± 10.7, $p < 0.001$) and the increase in unclassified *Ruminococcaceae* (KD: 15.5 ± 13.2, CTR: 6.7 ± 7.2, $p < 0.05$). Moreover, in KD group we found a significant decrease of the genera *Unclassified Rikillenaceae* (KD: 0.63 ± 0.75, CTR: 2.83 ± 2.11, $p < 0.05$) and *Veillonella* (KD: 0.36 ± 0.33, CTR: 1.60 ± 2.34, $p < 0.05$); on the contrary, a statistical significant increase was observed for *Blautia* (KD: 2.83 ± 3.17, CTR: 0.90 ± 0.72, $p < 0.05$), *Unclassified Erysipelotrichaceae* (KD: 1.11 ± 0.68, CTR: 0.45 ± 0.33, $p < 0.05$), *Prevotella* (KD: 0.85 ± 0.78, CTR: 0.08 ± 0.006, $p < 0.05$).

Genus	Relative abundance				
	KD		CTR		
	Mean	SD	Mean	SD	p-value
Bacteroides	**7.78**	6.97	**30.29**	10.74	***0.00***
Unclassified Ruminococcaceae	**15.46**	13.16	**6.71**	7.27	***0.05***
Faecalibacterium	**9.00**	10.46	**10.78**	6.30	0.34
Dialister	**4.55**	7.17	**4.04**	5.48	0.68
Unclassified Clostridiales	**7.24**	9.32	**6.00**	6.58	0.97
Unclassified Lachnospiraceae	**5.68**	3.01	**3.76**	2.58	0.24
Roseburia	**6.97**	6.36	**2.99**	1.59	0.16
Escherichia	**3.00**	6.11	**0.86**	2.09	0.88
Ruminococcaceae (other)	**2.98**	2.89	**3.65**	3.12	0.97
Oscillospira	**6.91**	8.82	**3.79**	2.36	0.91
Ruminococcus	**3.92**	2.54	**5.77**	5.19	0.43
Akkermansia	**3.14**	5.05	**0.64**	1.07	0.09
Blautia	**2.89**	3.17	**0.90**	0.72	***0.05***
Coprococcus	**2.83**	2.19	**1.74**	1.75	0.16
Unclassified Rikenellaceae	**0.63**	0.75	**2.83**	2.11	***0.00***
Lachnospira	**0.50**	0.54	**1.08**	1,33	0.68
Clostridium	**1.98**	4.20	**0.54**	0.84	0.32
Unclassified Erysipelotrichaceae	**1.11**	0.68	**0.45**	0.33	***0.01***
Veillonella	**0.36**	0.33	**1.60**	2.34	***0.03***
Prevotella	**0.85**	0.78	**0.08**	0.06	***0.00***
Desulfovibrio	**0.01**	0.02	**0.19**	0.32	*0.35*

Tab. 10 Relative abundances of fecal bacterial genera of subjects on a ketogenic diet (KD) and healthy control (CTR); data are expressed as means ± standard deviations.

4.2.1.2 SCFA levels

Changes in microbial species may alter the amounts of microbial metabolites, in particular SCFA, produced as fermentation products from food components that are undigested in the upper intestine. Concentrations of SCFA, expressed as mg/g dry weight feces, in the two groups of analysis, are reported in Tab. 11. We found a significant statistical difference between CTR and KD for all the SCFA measured ($p < 0.05$), except for butyrate ($p = 0.065$). SCFA were strongly reduced in patients on a KD compared to healthy subjects. This result is consistent with the gut microbiota analysis, which showed a depletion of *Bacteroidetes*, able to ferment indigestible polysaccharides and produce SCFA.

Fecal SCFA (mg/g dry feces)					
	KD		CTR		
	Mean	SD	Mean	SD	p-value
total SCFA	**10.56**	5.9	**23.1**	8.5	*0.005*
Acetate	**4.31**	2.9	**11.13**	4.4	*0.003*
Butyrate	**3.18**	1.9	**5.1**	1.7	0.065
Propionate	**2.14**	1.2	**4.52**	2	*0.008*
Iso-valerate	**0.59**	0.3	**1.43**	0.6	*0.002*
Iso-butyrate	**0.35**	0.2	**0.96**	0.5	*0.002*

Tab. 11 Fecal SCFA concentrations in patients on a ketogenic diet (KD) and healthy controls (CTR). Data are expressed as means ± standard deviation.

4.2.2 Specific effect of KD on human gut

4.2.2.1 Ketogenic dietary treatment

Since we observed differences in microbiota composition and SCFA concentrations in patients undergoing a KD respect to healthy subjects followed a conventional diet, we wanted to further investigate the specific effect of this diet on human gut health. We selected 7 patients, who collected fecal samples before starting the dietary therapy and after 1 month of KD. Adherence to the KD protocol was documented by constant ketonuria in all subjects. All participants completed the protocol, tolerated the diet well, and there were no adverse effects.

Table 12 shows the daily dietary intake before and after the beginning of the KD. The total energy intake did not differ significantly before and after the treatment with KD. In terms of nutritional composition, on the classical KD all macronutrients changed significantly; in particular fat (expressed as percentage of total dietary energy) increased from 44.7 ± 17.9% to 85.3 ± 4.0% kcal ($p < 0.05$), while protein and carbohydrates (expressed as percentage of total dietary energy) were reduced from 14.5 ± 4.6% to 9.0 ± 2.9% ($p < 0.05$) kcal and from 38.1 ± 15.2% to 5.1 ± 3.4% ($p < 0.05$), respectively.

	Pre intervention (t0)		Post Intervention (t1)		
	Mean	SD	Mean	SD	p-value
Energy Intake (kcal/24h)	**1432.3**	424.6	**1512.1**	320.7	0.499
Energy Intake (kcal/kg)	**57.1**	33.3	**57.3**	28.6	0.932
Protein (g/kg)	**1.8**	0.8	**1.2**	0.3	*0.030*
Protein (% energy)	**14.5**	4.6	**9.0**	2.9	*0.005*
Carbohydrates (g/kg)	**2.1**	1.2	**0.9**	1.1	0.150
Carbohydrates (% energy)	**38.1**	15.2	**5.1**	3.4	*0.001*
Fat (g/kg)	**2.8**	2.0	**5.4**	2.7	*0.015*
Fat (% energy)	**44.7**	17.9	**85.3**	4.0	*0.001*
Saturated Fat (% energy)	**14.3**	8.0	**22.3**	8.6	0.142
Monounsaturated Fat (% energy)	**12.5**	6.2	**29.2**	6.6	*0.000*
Polyunsaturated Fat (% energy)	**4.3**	2.9	**12.0**	4.6	*0.000*
Cholesterol (mg/day)	**171.7**	101.1	**200.8**	107.8	0.623

Tab. 12 Daily dietary intake before and after the treatment with KD.

4.2.2.2. Humidity of fecal samples

We decided to evaluate the humidity of fecal samples in order to assess differences intra-subject; results are reported in Tab. 13. Although this parameter was quite different among the 7 patients, only for one subject (4) we found differences before and after the diet. Moreover, the values obtained were used to report SCFA concentrations as mg/g dry weight feces.

	Humidity (%)	
subjects	**t0**	**t1**
1	77.9	78.8
2	65.9	61.4
3	74.3	76.9
4	81.9	64.8
5	83.1	77.4
6	55.5	55.0
7	61.5	61.4

Tab. 13 Humidity of fecal samples of 7 subjects before (t0) and after (t1) the diet.

4.2.2.3 Changes in gut microbiota after KD

To evaluate possible differences in taxa distribution before and after the diet we analyzed the relative microbial abundance at diverse taxonomic levels for each subject; particularly, Fig. 29 shows the results regarding the phylum level. We found no significant statistical difference at every level analyzed (phylum, family, genus), due to the huge heterogeneity of the these patients.

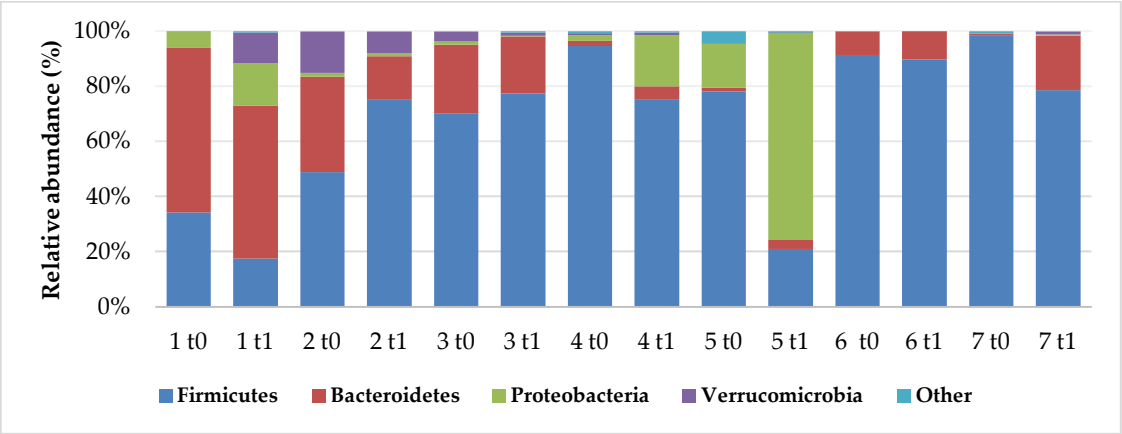

Fig. 29 Bar charts representing the average relative abundance of fecal bacterial phyla of subjects before (t0) and after (t1) the ketogenic treatment.

Then, we performed quantitative RT-PCR in order to validate the NGS data and evaluate possible changes in specific gut bacteria after one month of KD. We detected the microbial abundance of four bacterial groups, which are important for a good state of gut health: *Enterobacteriaceae*, as a marker of inflammatory status, *Bifidobacterium* spp., *Roseburia intestinalis* and *Faecalibacterium prausnitzii*, able to produce SCFA by fermentation of non-digestible carbohydrates. Results, expressed as genomes/g feces, are reported in Fig. 30. Data showed a decrease in *Faecalibacterium prausnitzii* after one month of KD, that was statistically significant ($p = 0.0170$). For the others bacteria analyzed we did not find any significant difference, however we saw a reduction-trend for *Bifidobacterium spp.*

Once again, the small number of subjects (7) and their heterogeneity constituted a limitation, as it was more difficult to detect significant differences before and after the ketogenic therapy.

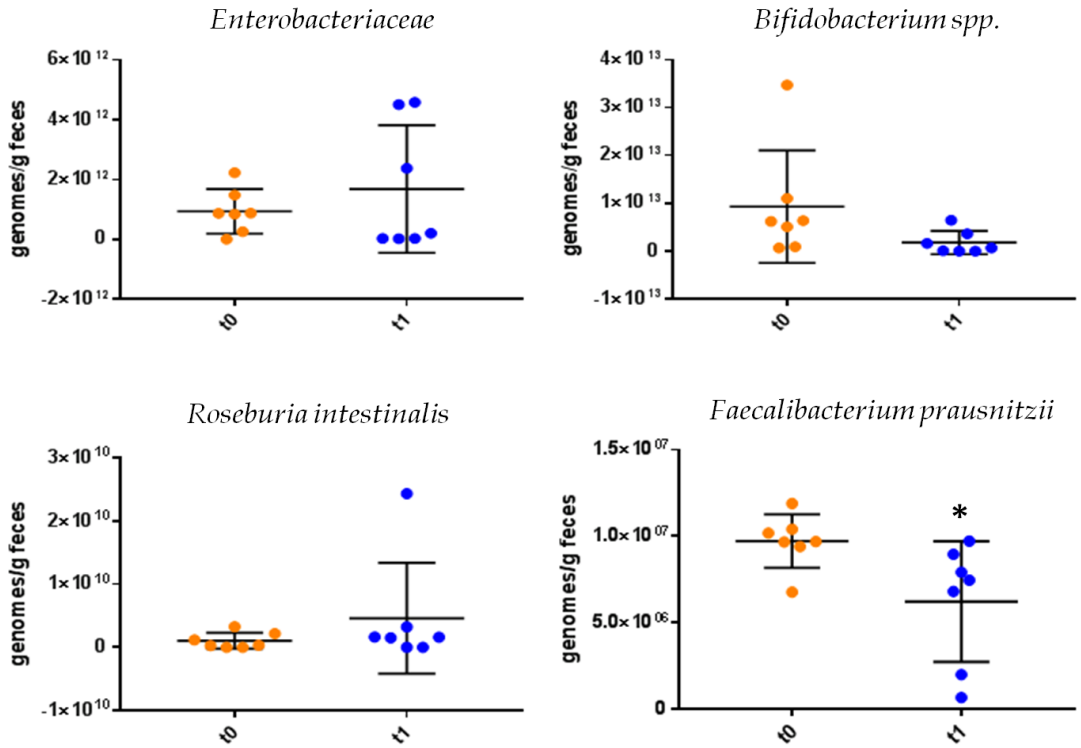

Fig. 30 Microbial abundance detected with RT-PCR of *Enterobacteriaceae*, *Bifidobacterium* spp., *Roseburia intesinalis*, and *Faecalibacterium prausnitzii*, before (t0 : orange) and after (t1: blue) the KD in 7 patients.

4.2.2.4 SCFA decrease with KD

We measured SCFA concentrations before and after KD; results are reported in Tab. 14. We found a significant statistical difference before (t0) and after (t1) the KD for total SCFA, acetate, butyrate and propionate ($p < 0.05$); on the contrary, iso-valerate and iso-butyrate were not statistically significant. Overall, the results showed that the KD strongly decrease the amount of SCFA in these patients, indicanting an alterated status of the gut microbiota, that is independed of the disease state. Furthermore, comparing these data with the results obtained from healthy controls samples, it appeared that SCFA concentrations of CTR and t0 were quite similar, instead a strong reduction of SCFA occurred in t1 samples, as Fig. 31 shows.

Fecal SCFA (mg/g dry feces)					
	t0		t1		
	Mean	SD	Mean	SD	p-value
total SCFA	**18.76**	8.2	**10.11**	5.45	*0.004*
Acetate	**8.29**	4.47	**4.07**	2.73	*0.005*
Butyrate	**4.77**	2.33	**2.84**	1.5	*0.008*
Propionate	**4.09**	1.72	**2.27**	1.24	*0.028*
Iso-valerate	**0.98**	0.54	**0.59**	0.22	0.105
Iso-butyrate	**0.62**	0.32	**0.34**	0.16	0.066

Tab. 14 Fecal SCFA concentrations before (t0) and after one month (t1) of the beginning of the KD. Data are expressed as means ± standard deviation.

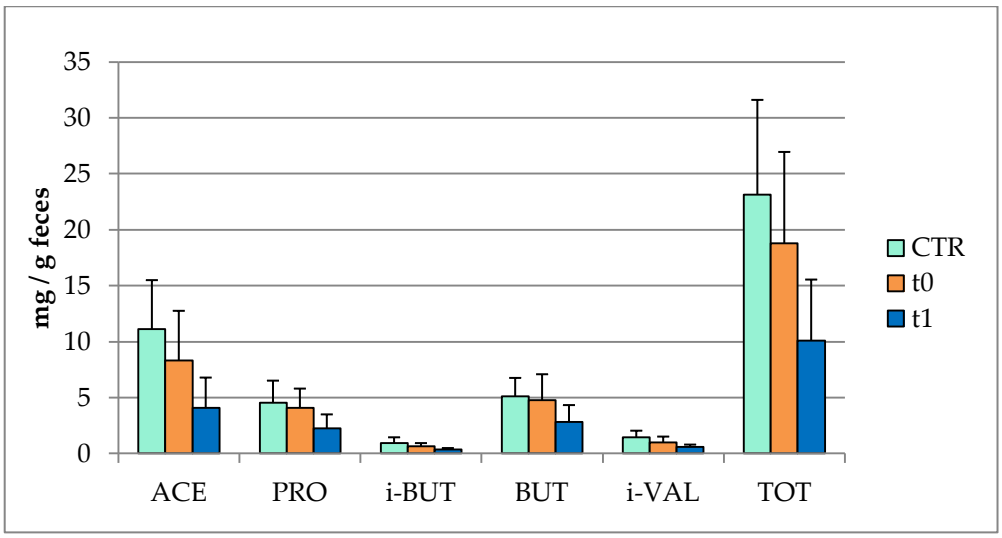

Fig. 31 Comparison between fecal SCFA concentrations of healthy controls (CTR), patients before (t0) and after one month (t1) of KD. Data are expressed as means ± standard deviation.

4.2.2.5 Fecal water toxicity

The toxicity of fecal water, an additional parameter of gut human health, was analyzed in our group of 7 patients. Firstly, we measured the citotoxicity by Trypan Blue assay (Fig. 32). Only one fecal water sample (3 t1) resulted citotoxic, as the percentage of viability was below 50% (43.1 ± 1), however all the other samples were not citotoxic. We decided to go further with the analysis and take into account also this sample, to confirm its level of toxicity. Additionally, we did not find any statistical significant difference before and after the KD for each subject.

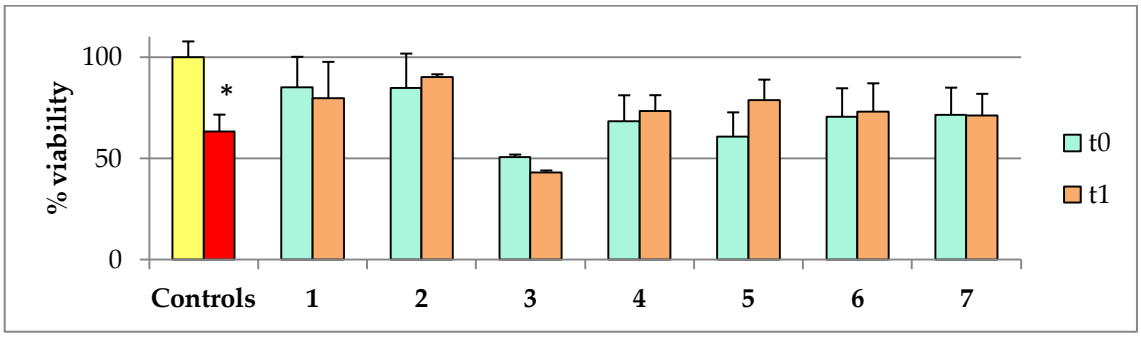

Fig. 32 Citotoxicity of fecal water before (t0, green) and after (t1, orange) the KD in 7 patients and in positive (red, H_2O_2) and negative (yellow, medium) controls. Data are expressed as mean ± standard deviations; * means $p < 0.05$.

Afterwards, we measured fecal water genotoxicity with Comet assay; results are reported in Fig. 33 as percentage of DNA in the tail. As expected, the subject with a high citotoxicity (sample 3) showed the highest genotoxicity, both before (t0) and after (t1) the KD; this result might be due to the particular clinical condition of the patient. The other subjects showed a medium level of genotoxicity, following the classification of Venturi et al. (1997) (range between 25.3 and 41.8%). Moreover, we detected significant statistically differences in 4 patients between t0 an t1 (samples 2,4,5,6); the level of genotoxiciy decrease after the ketogenic therapy (2t0: 40.5 ± 2.8, 2t1: 28.6 ± 2.5; 4t0: 31.9 ± 0.8, 4t1: 29.3 ± 1; 5t0: 41.8 ± 3.4, 5t1: 30.2 ± 0.8; 6t0 33.4 ± 0.3, 6t1 25.3 ± 2.9; $p < 0.05$) therefore KD could have an impact on human intestinal environment, at least in these patients.

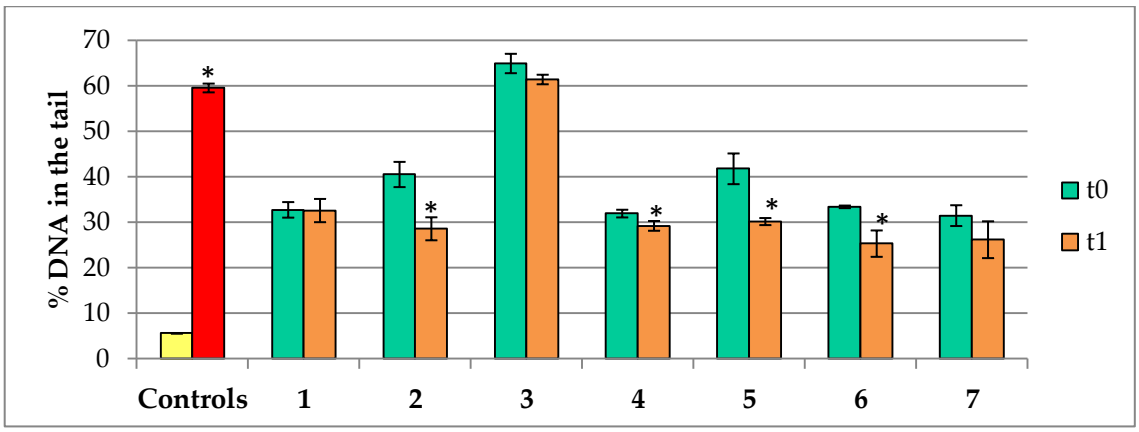

Fig. 33 Genotoxicity of fecal water before (t0, green) and after (t1, orange) the KD in 7 patients, expressed as % of DNA in the tail (mean ± standard deviation); * means $p < 0.05$.

4.3 DISCUSSION

The classic KD is a high-fat very low-carbohydrate diet, used worldwide for the treatment of refractory epilepsy, and increasingly applicated in different diseases such as GLUT1-DS and other metabolic or neurological disorders (Barañano & Hartman, 2008 – Veggiotti & De Giorgis, 2014). Patients stay on this diet for months or lifelong, but very few *in vivo* studies have been published in literature about KD global effects on human health. Moreover, it is well known that the diet is one of the most important external factor that strongly influence the composition of gut microbiota (Bibbò *et al*., 2016 – David *et al*., 2014 - De Filippo *et al*., 2010). It has been demonstrated that diet and derived microbial metabolites have strong implications with the development of food associated diseases including obesity, malnutrition, eating disorders, but also intestinal inflammatory diseases and colorectal cancer (Requena *et al*., 2018 – Yadav et al., 2018). Considering the unbalanced macronutrient composition of the KD, which is completely different from a conventional diet, it is possible that it might induce some changes in gut microbiota, which have been raraly studied in patients on ketogenic treatment for various pathological conditions. In addition to the fact that the number of *in vivo* studies about this argument is few, the results reported are not always comparable, both for type of pathologies considered and methodologies used. For these grounds, our study firstly focused on differed intestinal microbiota between healthy and epileptic or affected by GLUT1-DS patients; besides, the specific effect of KD, regardless of the disease, was investigated deeply through further analysis of gut microbiota before and after one month of KD treatment.

The primary important outcome of the first part of our research is that the gut microbiota of patients differs significantly from that of healthy controls; this difference is supported by several analysis perfomed and described above. Our data suggest that the KD microbial population is reduced in richness and uniformity; indeed, both α- and β-diversity of KD and healthy microbiome differed significantly. The differences indicated shifts in composition of KD microbiome; in particular, α-diversity expresses the mean species diversity in sites at a local scale, while β-diversity quantifies the number of different communities in a sample dataset. A rich microbial ecology promotes the fundamental cross-talk and cross-feeding between species that guarantees the resilience of the gut ecosystem; conversely, one of the features of intestinal dysbiosis is the loss of diversity (Hubbel S.P., 2001 - Whittaker R.H., 1972). Despite the small cohort enrolled, owing to the difficulties occurred during the recruitment (most of the patients affected by drug-resistant epilepsy and GLUT1-DS do not have fecal continence), our results are consistent with what was observed in other papers studying the microbiota alteration in patients undergoing a ketogenic treatment. For instance, a recent study by Swidsinski and collegues showed a reduced mass and diversity of the colonic microbiome in patients with multiple sclerosis on a KD (Swidsinski *et al*., 2017). An additional research reported lower gut microbiota diversity in infants affected by refractory epilepsy in comparison with healthy infants, as indicated by the Shannon index, a composite index that takes into account both richness and evenness of the bacterial (Xie *et al*., 2017). The same outcome has been observed also in a murine model of autism spectrum disorder, used in a study (Newell *et al*., 2016) that showed an anti-microbial-like effect of KD by significantly decreasing total host bacterial abundance.

At microbial composition level, in our KD cohort we found a strong reduction in *Bacteroidetes* and an increase in *Firmicutes*, compared to the healthy controls, indicating an altered status of gut microbiota. The phyla *Bacteroidetes* and *Firmicutes* include the most abundant bacterial division of the human gut microbiota (Qin *et al*., 2010). Dysbiosis between *Firmicutes* and *Bacteroidetes* in the

human gut has been described in previous studies in association with some disorders. For instance, an altered ratio *Firmicutes*/*Bacteriodetes* due to a lower level of *Bacteriodetes* was reported in obesity (Ismail et al., 2010 – Ley et al., 2006), but also inflammatory bowel disease (IBD) (Zhou & Zhi, 2016), and type 2 diabetes (Remely *et al*., 2013). This type of dysbiosis is associated to an inflammatory status which could have negative implications for host health. More important, our taxonomic analysis indicated that the altered ratio between *Firmicutes*/*Bacteroidetes* was due to a significant decrease in *Bacteroidetes*, and consequently in *Bacteroidaceae* and *Bacteroides*, in KD group compared to CTR. Although the effects of high-fat diets on gut have been investigated in different papers, the results are conflicting. A recent review reported that, in human intervention studies, the high fat intake could increase genera such as *Bacteroides* (Requena *et al*., 2018). On the other hand, in an animal study using mice fed with a standard carbohydrates diet and then switching to a high-fat diet, showed a decrease in *Bacteroidetes* and an increase in *Firmicutes*. This was seen for both genotypes (in the presence or abscence of obesity), indicating that this alteration was due to the high-fat diet itself, not to the obese state of the mice (Hildebrandt *et al*., 2009). Despite in literature there are not *in vivo* studies totally comparable to our research, the decrease in *Bacteroidetes* has been reported in other papers regarding the effect of KD on gut (Swidinski *et al*., 2017 - Xie *et al*., 2017), accordingly to our findings.

Alterations in the abundance of microbial communities lead to changes in the quantity and quality of some microbial metabolites, such as SCFA. For this reason, in order to further investigate the dysbiosis highlighted in KD group, we determined the amount of SCFA with gas chromatography. To our knowledge, this is the first study that measures SCFA concentrations during a ketogenic diet treatment, as the papers published to date are focused exclusively on microbiota profiling. However, we believe that additional analyzes are appropriate to better assess the global effect of the KD on human gut. Our results showed a statistically significant decrease in total SCFA concentrations, and also for each SCFA except for butyrate, that showed a decreased-trend too, although not statistically significant ($p = 0.08$), in KD group compared to CTR. It is worthy of note that SCFA exert multiple beneficial effect on human energy metabolism (den Besten *et al*., 2013), thus, this trend implies an impaired metabolic activity which might be detrimental for human health. The reduction of SCFA has been shown in studies investigating the gut microbiota in different pathologies, such as anorexia nervosa (Borgo *et al*., 2017), Rett syndrome (Borghi *et al*., 2017) and systemic inflammatory response syndrome (Yamada *et al*., 2015). The decrease in SCFA concentrations observed in our study, can be explained, firstly, as a consequence of the reduction in carbohydrates content, and consequently of the insoluble fiber, in the KD. In fact, it is recognised that non-digestible carbohydrate availability in the large intestine can shape the whole microbial community, and the reduced intake of fermentable dietary carbohydrate might impact on both the activity and the abundance of different bacterial group able to produce SCFA (Chassard & Lacroix, 2013 - Kleesen *et al*, 2001 – Koropatkin *et al*., 2012). Therefore, the difference between KD and conventional diet with standard amount of carbohydrates (accordingly to the Italian Food-based Guidelines about 45-60% of total energy intake), and the low amount of fiber (about 8-9g/day in a KD, instead that 25g/day accordingly to the Italian Food-based Guidelines), could explain these results. Moreover, we found a decrease in *Bacteroidetes* phylum, which is mainly able to produce acetate and propionate (den Besten *et al*., 2013 – Rios Covian *et al*., 2017). Nevertheless, in this first step of the *in vivo* research, we compare patients undergoing a KD as a required therapy for their disease, with healthy subjects. Therefore it should be considered that the dysbiosis observed in KD

group, both for microbial populations and SCFA production, may be due to their pathological condition.

In order to overcome this issue, the second step of our research has included the evaluation of the specific KD effect on the gut, regardless the pathology, by collecting samples before the diet and after one month. We selected 7 patients who have to begin the ketogenic treatment as a therapy and asked them to collect fecal samples in these two different time points, with the aim to assess variations before and after the diet in the same subject.

Considering the data obtained from the microbiota profiling (α-diversity, β-diversity, taxonomy analysis), which showed differences between KD and healthy CTR group, but no variations within the same patient by comparing t0 versus t1, we investigated, in our cohort of 7 subjects, the microbial abundance of particular bacterial groups with quantitative RT-PCR. We choose four different bacteria in order to validate the NGS analysis and evaluate changes before and after the dietary treatment in this specific cohort of subjects; moreover, the bacterial groups selected were markers of inflammatory status (*Enterobacteriacea*) and fermentation activity (*Bifidobacterium sp., Roseburia intestinalis, Faecalibacterium prausnitzii*), to better investigate the status of gut health. After performing the analysis, we did not find any statistical significant difference in *Enterobacteriaceae*, *Roseburia intestinalis* and *Bifidobacterium spp*., although for the last one a decrease-trend was observed. Instead, we detected a significant reduction in *Faecalibaterium prausnitzii*, which is important for the fermentation of non-digestible carbohydrates and the production of SCFA, particularly of butyrate. These findings suggest that the low amount of fiber (8,7 ± 4,4 g/day during the diet) might alter the different fibrolytic species responsible for fiber fermentation, even if the reduction of these species was not uniform.

We further measured the concentrations of SCFA before and after the KD, and we found that they were reduced after the dietary intervention. Moreover, we compared the results obtained from healthy subjects analysis to the diseased group not on a KD (t0 group), and we did not find differences ($p = 0.51$), as the SCFA concentatrions were quite similar. Instead, a strong reduction of SCFA was observed after one month of dietary therapy, as significant statistical differences were detected for acetate, proprionate and butyrate comparing the groups t0 versus t1. The significant decrease in butyrate, not showed in KD versus CTR, is consistent with the reduction in *Faecalibacterium prausntizii* in the 7 subjects analyzed. These important results mean that the change in microbiota composition and in markers of bacterial metabolism may be caused by a specific impact of the ketogenic protocol, beside the pathological condition of people required to follow this diet as a therapy.

Additionally, we evaluated the toxicity of fecal water to better understand the global impact of the KD on human gut. We decided to analyze this aspect because diet could affect the composition of human feces thus determining intestinal environment and exposition of colon mucosa to risk factors (de Kok & van Maanen, 2000 – Mai *et al*., 2009). Therefore, Trypan blue test and Comet assay were performed with the aim to evaluate if the consumption of a KD, regardless to the pathological condition, might result in differences in fecal water cytotoxicity/genotoxicity. Since there is a high inter-individual variability of fecal water activity in the population (Erba *et al*., 2014 - Osswald *et al*., 2000), we choose to perform these experiments only for the cohort of 7 subjects to assess the effect of the diet, not the level of toxicity in a healthy group. Actually, previous studies evaluated the toxicity using the same subject before the dietary treatment as the control, since it is the most accurate way to assess the specific effect of the diet itself (Erba *et al*. 2012 – Woods *et al*., 2002). Results showed that the majority of samples were included in the medium genotoxicity category, except for one subject

(number 3) who displayed the highest citotoxicity and consequently genotoxicity. This apparently "outlier" data is due to the particular clinical condition of the patient, since, in addition to be epileptic, he was affected by celiac disease. Although the patient followed a gluten-free diet before the dygnosis of epilepsy, it could not be enough to restore its inflammatory status, and this might be the reason explaining the more damaged colon mucosa and the higher value of fecal water toxicity. These findings confirmed the quality of the genotoxicity marker, as the % of DNA damage was consistent with the level of citotoxicity. Moreover, we found a decrease-trend of genotoxicity level after one month of ketogenic diet; actually, data showed a significant statistical reduction in four subjects. We hypothesized that this effect may be due to the effectiveness of the ketogenic therapy, which improves the overall health condition of these patients. However, the findings obtained in this study are comparable with others detected in healthy Italian people (Erba *et al*., 2014), which showed similar level of genotoxicity of fecal water (medium level of genotoxicity). Thus, accordingly to our results, it seems that neither neurological or metabolic pathologies not the KD could adversely affect the level of fecal water toxicity in our cohort.

4.4 CONCLUSIONS

In conclusion, we found that, first of all, the microbiota gut composition of patients affected by refractory epilepsy or GLUT1-DS undergoing a KD as a therapy, was significantly different than healthy control subjects, thus it is possible that KD may influence human gut. Moreover, KD group showed an intestinal dysbiosis, highlighted by reduced biodiversity and richness in microbial populations. In addition, we found an altered ratio *Firmicutes*/*Bacteroidetes*, due to a significant decrease of *Bacteroidetes* phylum in KD group, consistent with the status of dysbiosis.

This study was the first to evaluate the SCFA concentrations in relation with the consumption of a ketogenic dietary protocol; we found lower levels of SCFA in KD group compared to CTR, but also analyzing the same subjects before and after the diet. These data underline how KD is able to affect gut health by altering microbiota composition and its metabolic activity. This can mainly be due to reduction in carbohydrates content, and in bacterial genera able to do fermentation, like *Fecalibaterum prausnitzii* and *Bacteroides*.

Both citotoxicity and genototixicy of fecal water analyzes have shown that ketogenic treatment does not adversely affect human colon mucosa, as the results indicated medium level of toxicity, comparable with levels found in other studies of healthy population.

There are some limitations that need to be clarified. Firstly, the small size of the group of the partecipants due to the difficulties encountered during the recruitment of the subjects. Beside the rarity of the pathologies considered, most of the patients affected by drug-resistant epilepsy or GLUT1-DS do not have fecal continence, thus it was not possible to ask them to collect biological samples. Secondly, it would be appropriate to evaluate the effect of KD on human gut for longer time of follow-up, as other studies (Newell *et al*., 2016 - Swidinski *et al*., 2017) reported a re-estabilishment of the dysbiosis condition after several weeks of ketogenic dietary treatment.

Although the limits described, this study allow us to conclude that KD has an impact on the human gut, highlighting the need for further research to avoid long-term effects and optimize the therapy. Considering our findings, it may be reasonable to suggest a supplementation of probiotics/prebiotics to potentially restore the microbiota stability.

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5 CONCLUSIONS

In conclusion, through this Ph.D. thesis it was possible to provide novel results about the effects of KB and ketogenic dietary treatment. These findigs are particular important if we consider the increasing use of this diet for different purposes, not only as a therapy for refractory epilepsy.

In the first part of the research, we provided data on the vascular risk represented by KD-induced oxidative stress using an *in vitro* model of endothelium. We demonstrated that KB might have an impact on cell metabolism in tissue functions different from the neurological one. The mechanism hypothesized in our *in vitro* study is that KB could activate the transcription factor Nrf2, which improves the cellular ability to remove harmful substances by the activation of cellular defence processes. However, the activation of Nrf2 is the result of impaired oxidative cell status, therefore it could be harmful to promote this metabolic response in the long term.

In the second part of the research, an *in vivo* study was performed to verify the impact of the KD on human gut. The primary important outcome was that, comparing patients following a KD with healthy subjects, the microbiota composition was signficanlty different, characterized by a status of dysbiosis. For the first time in this field of research the SCFA concentrations in relation with the consumption of a KD were evaluated. We found lower levels of SCFA in KD group compared to controls, but also analyzing the same subjects before and after the diet. Thus, KD is able to affect gut health by altering microbiota composition and its metabolic activity. This can mainly be due to the reduction of carbohydrates and fiber content, and of bacterial genera able to do fermentation. Finally, the analysis of fecal water toxicity showed that ketogenic treatment did not adversely affect human colon mucosa.

In the end, everytime a KD is applied, both for therapeutic or weight loss purposes, a careful examination of the risk-benefit ratio should be done. There is a need for further research in order to avoid long-term effects of this diet and optimize the therapy in different pathologies.

6 SCIENTIFIC PRODUCTS

6.1 Copies of papers published

Meroni E.; Papini N.; Criscuoli F.; Casiraghi M.C.; Massaccesi L.; Basilico N.; Erba D. (2018) Metabolic responses in endothelial cells following exposure to ketone bodies. *Nutrients*, 22, 10(2), E250, DOI:10.3390/nu10020250.

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Article

Metabolic Responses in Endothelial Cells Following Exposure to Ketone Bodies

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Abstract: The ketogenic diet (KD) is a high-fat, low-carbohydrate diet based on the induction of the synthesis of ketone bodies (KB). Despite its widespread use, the systemic impact of KD is not completely understood. The purpose of this study was to evaluate the effects of physiological levels of KB on HMEC-1 endothelial cells. To this aim, DNA oxidative damage and the activation of Nrf2, a known transcriptional factor involved in cell responses to oxidative stress, were assessed. The exposure of cells to KB exerted a moderate genotoxic effect, measured by a significant increase in DNA oxidative damage. However, cells pre-treated with KB for 48 h and subjected to a secondary oxidative insult (H_2O_2), significantly decreased DNA damage compared to control oxidized cells. This protection occurred by the activation of Nrf2 pathway. In KB-treated cells, we found increased levels of Nrf2 in nuclear extracts and higher gene expression of HO-1, a target gene of Nrf2, compared to control cells. These results suggest that KB, by inducing moderate oxidative stress, activate the transcription factor Nrf2, which induces the transcription of target genes involved in the cellular antioxidant defense system.

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ORIGINAL RESEARCH
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Effects of Sub-lethal Concentrations of Silver Nanoparticles on a Simulated Intestinal Prokaryotic–Eukaryotic Interface

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Nanotechnology applications are expected to bring a range of benefits to the food sector, aiming to provide better quality and conservation. In this research, the physiological response of both an *Escherichia coli* mono-species biofilm and Caco-2 intestinal cells to sub-lethal concentrations of silver nanoparticles (AgNPs) has been investigated. In order to simulate the anaerobic and aerobic compartments required for bacteria and intestinal cells growth, a simplified semi-batch model based on a transwell permeable support was developed. Interaction between the two compartments was obtained by exposing Caco-2 intestinal cells to the metabolites secreted by *E. coli* biofilm after its exposure to AgNPs. To the best of the authors' knowledge, this study is the first to investigate the effect of AgNPs on Caco-2 cells that takes into consideration previous AgNP-intestinal biofilm interactions, and at concentrations mimicking real human exposure. Our data show that 1 μg/mL AgNPs in anaerobic conditions (i) promote biofilm formation up to 2.3 ± 0.3 fold in the first 72 h of treatment; (ii) increase reactive oxygen species (ROS) production to 84 ± 21% and change the physiological status of microbial cells after 96 h of treatment; (iii) seriously affect a 72-h old established biofilm, increasing the level of oxidative stress to 86 ± 21%. Moreover, the results indicate that oxygen renders the biofilm more adequate to counteract AgNP effects. Comet assays on Caco-2 cells demonstrated a protective role of biofilm against the genotoxic effect of 1 μg/mL AgNPs on intestinal epithelial cells.

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Phytate/calcium molar ratio does not predict accessibility of calcium in ready-to-eat dishes

Daniela Erba,* Federica Manini, Erika Meroni and Maria C Casiraghi

Abstract

BACKGROUND: Phytic acid (PA), a naturally occurring compound of plant food, is generally considered to affect mineral bioavailability. The aim of this study was to investigate the reliability of the PA/calcium molar ratio as a predictive factor of calcium accessibility in composed dishes and their ingredients.

RESULTS: Dishes were chosen whose ingredients were rich in Ca (milk or cheese) or in PA (whole-wheat cereals) in order to consider a range of PA/Ca ratios (from 0 to 2.4) and measure Ca solubility using an *in vitro* approach. The amounts of soluble Ca in composed dishes were consistent with the sum of soluble Ca from ingredients (three out of five meals) or higher. Among whole-wheat products, bread showed higher Ca accessibility (71%, PA/Ca = 1.1) than biscuits (23%, PA/Ca = 0.9) and pasta (15%, PA/Ca = 1.5), and among Ca-rich ingredients, semi-skimmed milk displayed higher Ca accessibility (64%) than sliced cheese (50%) and Parmesan (38%). No significant correlation between the PA/Ca ratio and Ca accessibility was found ($P = 0.077$).

CONCLUSION: The reliability of the PA/Ca ratio for predicting the availability of calcium in composed dishes is unsatisfactory; data emphasized the importance of the overall food matrix influence on mineral accessibility.

Macromolecular and Micronutrient Profiles of Sprouted Chickpeas to Be Used for Integrating Cereal-Based Food

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ABSTRACT

Pulse flour may be used to improve nutritional traits of gluten and gluten-free formulations in traditional food such as bread or pasta. However, owing to some intrinsic nutritional, textural, and sensory properties, the use of pulses as ingredients for production of enriched food remains limited. In this study, we investigated the modification in macromolecules and micronutrients in industrial-scale flour from partially sprouted chickpeas to define its possible use as an ingredient in cereal-based foods. Controlled sprouting resulted in significant decrease of antinutritional compounds (e.g., phytic acid and serine protease inhibitors) and in an increase of free minerals and vitamins. Sprouting also affected the overall structural organization of proteins (such as aggregate formation) and their thiol/disulfide balance, and it promoted release of peptides. All of these had a positive effect on dough mixing properties, in particular for dough development. Formulations with enrichment in sprouted chickpea flour (wheat/chickpea ratio = 100:20) were tested also as for their dough leavening properties, which improved with respect to flour from nonsprouted chickpeas. Taking into account the modifications induced by partial sprouting on an industrial scale, we can conclude that sprouted chickpea flour represents an interesting ingredient for production of enriched cereal-based food with better nutritional and rheological characteristics.

6.2 Copies of abstract of oral presentation and posters

Meroni E. (2018) Metabolic effects of dietary approaches: ketogenic diet & ketone bodies. 23th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 19-21 Septmber, Oristano, Italy. *Oral Presentation*.

Meroni E.; Papini N.; Criscuoli F.; Casiraghi M.C.; Massaccesi L.; Basilico N.; Erba D. (2018) Metabolic responses in endothelial cells following exposure to ketone bodies. 15th International Conference on Clinical Nutrition, 24-26 May, Vienna, Austria. *Award for the Best Oral Presentation*.

Meroni E.; Raikos V. (2018) Formulating orange oil-in-water beverage emulsions for effective delivery of bioactives: influence of carrier oil type on chemical stability, antioxidant activity and in vitro bioaccessibility of lycopene. 15th International Conference on Clinical Nutrition, 24-26 May, Vienna, Austria. *Award for the Best Poster Presentation*.

Meroni E.; Domingo G.; Erba D.; Cattò C.; Cappitelli F.; Vannini C.; Bracale M. (2018) Effects of sub-lethal concentrations of silver nanoparticles on Caco-2 cells. 5th International Conference on Foodomics: from Data to Knowledge, 10-12 January, Cesena, Italy. ISBN 978-88-902152-8-5. *Poster Presentation*.

Meroni E.; Raikos V. (2017) Stabilità fisico-chimica, proprietà antiossidanti di bevande addizionate con ß-carotene, bioaccessibilità del carotenoide: influenza della tipologia di olio carrier. 38th National Congress SINU (Italian Society of Human Nutrition), 20-22 November, Torino, Italy. *Poster Presentation*.

Meroni E. (2017) Metabolic effects of dietary approaches: ketogenic diet & ketone bodies. 22nd Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 20-22 September, Bolzano, Italy. ISBN 978-88-98416-97-4. *Poster Presentation*.

Meroni E.; Papini N.; Erba D. (2016) Effects of exposure to ketone bodies in endothelial cells. 37th National Congress SINU (Italian Society of Human Nutrition), 30 November-2 December, Bologna, Italy. *Award for the Best Poster Presentation*.

Meroni E. (2016) Metabolic effects of dietary approaches: ketogenic diet & ketone bodies. 21th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 14 -16 September, Portici, Italy. ISBN 978-88-99648-06-0. *Poster Presentation*.

23th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 19-21 Septmber, Oristano, Italy.

Metabolic effects of dietary approaches: ketone bodies & ketogenic diet

Meroni Erika

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This PhD thesis dealt with the investigation of the metabolic effects of ketone bodies (KB), produced during a ketogenic diet (KD). The work was structured in two main projects. Firstly, we assessed, with an in vitro model of endothelium, the vascular risk represented by KD-induced oxidative stress, by studying the cito- and geno-toxicity of KB and the activation of a cellular response after KB exposure. Afterwards, the in vivo impact of therapeutic ketogenic diet on human intestinal environment has been verified, by the evaluation of several parameters: toxicity of fecal water, markers of bacterial metabolism and composition of gut microbiota composition. The results of this thesis contribute to improve knowledge about the mechanisms of action of the KD and its global effect on human health.

15th International Conference on Clinical Nutrition, 24-26 May, Vienna, Austria.

Metabolic responses in endothelial cells following exposure to ketone bodies

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The ketogenic diet (KD) is a high-fat, low-carbohydrate diet based on the induction of the synthesis of ketone bodies (KB). Despite its multiple applications, the impact of KD on the human body is not completely understood The purpose of this study was to evaluate in vitro the biological effects of KB on HMEC-1 endothelial cells. The exposure of cells to KB exerted a moderate genotoxic effect, measured by a significant increase in DNA oxidative damage ($p<0.001$) that was not related to the duration of exposure (2-48 h). To investigate the ability of KB to modulate DNA susceptibility to oxidative stress, we exposed cells to a secondary oxidative insult (H2O2) after KB supplementation. Surprisingly, cells treated with KB for 48 h showed significantly less DNA damage compared to control oxidized cells. One possible mechanism by which this protection might occur is through the activation of the Nrf2 pathway. In KB-treated cells, we found increased levels of Nrf2 in nuclear extracts and higher gene expression of HO-1, a target gene of Nrf2, compared to control cells. These results suggest that KB induce moderate oxidative stress, which activates the transcription factor Nrf2 and induces the transcription of target genes involved in the cellular antioxidant defense system. Consequently, in endothelial cells, the metabolic responses to KB-induced stress via Nrf2 pathway activation makes cells more resistant to a secondary insult, in this case H2O2, leading to a reduction in DNA oxidative damage.

15th International Conference on Clinical Nutrition, 24-26 May, Vienna, Austria.

Formulating orange oil-in-water beverage emulsions for effective delivery of bioactives: influence of carrier oil type on chemical stability, antioxidant activity and in vitro bioaccessibility of lycopene

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The carotenoids inclusion in diet is considered to contribute to human health, as their antioxidant properties are associated with a reduced risk for the development of chronic diseases. Thus, the consumption of lycopene, which is the most predominant carotenoid in human plasma, is important for various biological functions. The addition of lycopene in food formulations is problematic because of its lipophilic nature and high susceptibility to oxidation. However, its bioavailability can increase when co-ingested with other lipids. A popular method for increasing carotenoid bioavailability is emulsification. The purpose of the present work was to develop an edible orange oil-in-water beverage emulsion containing lycopene as a bioactive ingredient. Particularly, the influence of carrier oil type on the chemical stability, antioxidant properties and bioaccessibility of lycopene in orange oil-in-water beverage emulsions was investigated. The emulsions were formulated with orange oil (A), which was partially (50%) replaced with tributyrin (B) or corn oil (C). The addition of corn oil enhanced the physical stability of the beverage during chilled storage by inhibiting Ostwald ripening. The formation of oxidation products was insignificant during storage for 28 days at 4 °C, regardless the type of added oil. Lycopene was more susceptible to chemical degradation in the presence of unsaturated, long chain triglycerides and the retention followed the order: A (87.94%), B (64.41%) and C (57.39%). Interestingly, bioaccessibility of lycopene was significantly lower for emulsions formulated with 50% corn oil as opposed to 100% orange oil as indicated by the simulated in vitro gastric digestion model.

5th International Conference on Foodomics: from Data to Knowledge, 10-12 January, Cesena, Italy.

Effects of sub-lethal concentrations of silver nanoparticles on Caco-2 cells

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Nanoparticles (NPs) are widely used in the agri-food industry as additives, for example for functionalizing food processing surfaces and for packaging, to improve mechanical and antimicrobial properties. Given these widespread applications, NPs exposure represents a potential toxicological risk for human health. Of special interest is the effect of NPs on human gut microbiota, considering the range of consumer goods that can be intentionally, or accidentally ingested.
In this research, the metabolic response of Caco-2 intestinal cells exposed to sub-lethal concentrations of silver nanoparticles (AgNPs) has been investigated. In order to understand the complex interplay among AgNPs, gut biofilm and its host, an experimental model which reproduces an interactive gut ecosystem was developed. This simplified system was composed by Escherichia coli mono-species biofilm and Caco-2 monolayer. Interaction between the two compartments was obtained by exposing Caco-2 intestinal cells to the metabolites produced by E. coli biofilm after its exposure to AgNPs.
A label-free quantitative proteomic analysis, performed on Caco-2 monolayers treated for 24 hours with AgNPs (1mg/L) or metabolites produced by biofilm after its exposure to AgNPs, showed several differentially expressed proteins (DEPs). Respect to the control, we found 40 up-regulated and 260 down-regulated proteins after AgNPs treatments whereas, in Caco-2 cells treated with biofilm metabolites, we detected 175 up-regulated and 226 down-regulated proteins. Taking advantage of bioinformatics tools and over-represented Gene Ontology (GO) categories, DEPs were annotated into functional categories and metabolic pathway involved. Proteomic analysis showed that AgNPs and biofilm metabolites have different mechanism of action, highlighting the importance of the biofilm on the fate and toxicity of silver nanoparticles.

38th National Congress SINU (Italian Society of Human Nutrition), 20-22 November, Torino, Italy.

Stabilità fisico-chimica, proprietà antiossidanti di bevande addizionate con β-carotene e bioaccessibilità del carotenoide: influenza della tipologia di olio carrier.

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Premesse Il consumo di β-carotene è importante per varie funzioni biologiche e non solo per essere un precursore della vitamina A. L'aggiunta di β-carotene nelle formulazioni alimentari è problematica a causa della sua lipofilia e della sua alta suscettibilità a luce, ossigeno, temperatura e pH. Tuttavia, la sua biodisponibilità può aumentare se co-ingerito con altri lipidi.
Obiettivo Indagare l'effetto di differenti oli carrier del β-carotene, in bevande contenenti: 92% acqua, 3% proteine di siero di latte, 4% olio, 0,7% acido citrico e 0,5% β-carotene.
Metodi Sono state preparate tre bevande con diverse fasi olio: A) 100% olio di arancio B) 50% olio di arancio + 50% tributirina (trigliceridi a catena corta = SCT) C) 50% olio di arancio + 50% olio di mais (trigliceridi a catena lunga = LCT) e sono state valutate: 1) stabilità fisica (Turbiscan) e 2) shelf life (HPLC) dopo quattro settimane di stoccaggio a 4°C; 3) proprietà antiossidanti (FRAP, TBARS, dieni coniugati) e 4) bioaccessibilità di β-carotene (digestione gastro-intestinale in vitro).
Risultati Le bevande formulate con 50% di LCT sono fisicamente più stabili rispetto a quelle con 50% di SCT. Non è stata osservata alcuna degradazione del β-carotene durante lo stoccaggio, indipendentemente dalla composizione della fase olio. La formazione di prodotti di ossidazione primaria e secondaria alla fine dello stoccaggio è risultata non significativa. La bioaccessibilità del β-carotene è sensibilmente influenzata dal tipo di olio carrier e diminuisce nell'ordine C > B > A.
Conclusioni La tipologia di olio carrier ha un impatto significativo sulle proprietà fisico-chimiche delle bevande addizionate con β-carotene. Si ipotizza che l'aumento della bioaccessibilità del carotenoide (~ 30%) nelle bevande formulate con LCT sia dovuto alla maggiore capacità di solubilizzazione delle micelle dopo il protocollo di digestione in vitro.

22nd Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 20-22 September, Bolzano, Italy.

Metabolic effects of dietary approaches: ketone bodies & ketogenic diet

Meroni Erika

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The first part of the PhD thesis project is an in vitro study. Its aim was to investigate the effects of exposure to ketone bodies (KB) in HMEC-1 endothelial cells by measuring markers of oxidative stress. In particular, it was evaluated: 1) the DNA oxidative damage by Comet assay in order to study the genotoxicity due to KB; 2) the effect of a secondary oxidative insult after exposure to KB; 3) the activation of Nrf2 pathway, which is a transcriptional factor involved in the cellular response to a stress, by western blot and real time PCR.

37th National Congress SINU (Italian Society of Human Nutrition), 30 November-2 December, Bologna, Italy.

Effetti dell'esposizione ai corpi chetonici in cellule endoteliali

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[2]Dipartimento di Biotecnologie Mediche e Medicina Traslazionale, Università degli Studi di Milano, Segrate (MI)

Premesse La "dieta chetogenica" (KD) è un programma alimentare progettato negli anni '20 come terapia per l'epilessia farmaco-resistente, e che si è diffuso, a partire dagli anni '70, come una dieta dimagrante (Atkins). Gli effetti "in vivo" delle KD sono argomento di discussione, non tanto per le finalità terapeutiche, quanto per la salubrità di questi approcci dietetici formulati per la rapida perdita di peso.
Obiettivi Indagare, in cellule endoteliali HMEC-1, gli effetti dell'esposizione a concentrazioni fisiologiche di corpi chetonici (KB).
Metodi Dopo aver esposto le cellule ai ◎◎, è stato valutato il danno ossidativo al DNA (Comet Assay) conseguente a differenti tempi di esposizione (2h, 24h e 48h), ed il danno al DNA determinato da uno stimolo ossidativo (H2O2) applicato successivamente tali esposizioni.
Risultati L'esposizione ai KB esercita un moderato stress rilevabile da un significativo aumento del danno ossidativo al DNA a partire dalle 2 ore (20% DNA nella coda) senza mostrare un ulteriore aumento significativo ai tempi successivi. L'insulto ossidativo applicato dopo 2 h di esposizione, aumenta significativamente il danno al DNA (60% di DNA nella coda), mentre le cellule trattate con KB per 48h mostrano, dopo stress ossidativo con H2O2, un danno significativamente inferiore (40% di DNA nella coda, $p<0.001$).
Conclusioni I risultati ottenuti suggeriscono che l'esposizione delle HMEC-1 ai KB determini una condizione di lieve stress ossidativo che, stimolando le difese antiossidanti della cellula, la renderebbe più protetta verso stress successivi. Si ipotizza l'attivazione di qualche fattore di trascrizione coinvolto nella risposta cellulare allo stress ossidativo.

21th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 14 -16 September, Portici, Italy.

Metabolic effects of dietary approaches: ketone bodies & ketogenic diet

Meroni Erika

Department of Food, Enviromental and Nutritional Sciences, University of Milan, Milan, Italy

The overall aim of this PhD project is to investigate the metabolic effects of ketone bodies, in particular β-hydroxybutyrate and acetoacetate, by "in vitro" and "in vivo" studies. The main objectives will be: 1) to verify the effects exerted by ketone bodies on cellular responses, by studying the activation of transcriptional factors (like Nrf2) and post-translation changes of proteins; 2) to verify the impact of this dietary approach on human intestinal environment, by evaluation of composition of gut bacteria, marker of bacterial metabolism and toxicity of fecal water.

6.3 Awards

Best Oral Presentation – Young Researchers Forum at the 15th International Conference on Clinical Nutrition, Vienna, Austria, 24 – 26 May 2018.

Best Poster Award at the 15th International Conference on Clinical Nutrition, Vienna, Austria, 24 – 26 May 2018.

Young Researchers Award for the best poster presentation at the National Meeting of Italian Society of Human Nutrition (SINU), Bologna, Italy, 30 November -2 December 2016.

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Clinical Nutrition 2018

Young Researchers Forum

Prof/Dr/Mr/Ms. **Erika Meroni**

University of Milan, Italy

for presenting the oral entitled

Metabolic responses in endothelial cells following exposure to ketone bodies

at the "15th International Conference on Clinical Nutrition"
held during May 24-26, 2018 in Vienna, Austria

The award has been attributed in recognition of research paper quality, novelty and significance.

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Prof/Dr/Mr/Ms. Erika Meroni

University of Milan, Italy

for her Poster Presentation on

"Formulating orange oil-in-water beverage emulsions for effective delivery of bioactives: Influence of carrier oil type on chemical stability, antioxidant activity and in vitro bioaccessibility of lycopene"

at the "15th International Conference on Clinical Nutrition"

held during May 24-26, 2018 in Vienna, Austria

Royce P Vincent

King's College Hospital NHS Foundation Trust, UK

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In occasione del 55° Congresso Nazionale SINU - Bologna, 30 novembre - 2 dicembre 2016

si ringrazia

ERIKA MERONI

vincitore del premio Giovani Ricercatori per il poster dal titolo:

EFFETTI DELL'ESPOSIZIONE AI CORPI CHETONICI IN CELLULE ENDOTELIALI

Prof. Pasquale Strazzullo

Presidente SINU

6.4 Ph.D Erasmus Traineeship

Erasmus Traineeship: The Rowett Institute of Nutrition and Health, University of Aberdeen, Aberdeen, UK. January – May 2017.

Traineeship title: Physicochemical stability, antioxidant properties and bioaccessibility of beta-carotene and lycopene in beverage emulsions: influence of carrier oil type

Detailed programme of the traineeship: Carotenoids are a group of more than 600 lipophilic compounds that contribute to the yellow, orange and red colours of fruits and vegetables, and their inclusion in the diet is considered to contribute to human health and well-being. The main objective of this study is to develop an edible orange oil-in-water beverage emulsion containing beta-carotene or lycopene, in which the oil phase is partially replaced by long chain triglycerides or small chain triglycerides in order to investigate the influence of carrier lipid on several parameters affecting the bioaccessibility of the lipophilic bioactive compound. In particular, the specific objectives are: 1) to verify the physicochemical stability of the emulsions under four weeks of chilled storage; 2) to evaluate their antioxidant properties and 3) to assess the bioaccessibility of beta-carotene by using an in vitro gastro-intestinal digestion model.

COPIES OF PAPER PUBLISHED:

Meroni E., Raikos V. Lycopene in beverage emulsions: optimizing formulation design and processing effects for enhanced delivery. *Beverages*, 2018, 4(1), 14; DOI:10.3390/beverages4010014.

Meroni E., Raikos V. Formulating orange oil-in-water beverage emulsions for effective delivery of bioactives: improvements in chemical stability, antioxidant activity and gastrointestinal fate of lycopene using carrier oils. *Food Res Int*, 2018, 106, 439-445, DOI: 10.1016/j.foodres.2018.01.013.

Meroni E., Raikos V. Physicochemical stability, antioxidant properties and bioaccessibility of β-carotene in orange oil-in-water beverage emulsions: influence of carrier oil type. *Food Funct*, 2018, 9(1), 320-330, DOI:10.1039/C7FO01170A.

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Review

Lycopene in Beverage Emulsions: Optimizing Formulation Design and Processing Effects for Enhanced Delivery

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Abstract: Lycopene is a desired ingredient in food formulations, yet its beneficial effects on human health remain largely underexploited due to its poor chemical stability and bioavailability. Oil-in-water emulsions may offer multiple advantages for the incorporation and delivery of this carotenoid species. Engineering and processing aspects for the development of emulsion-based delivery systems are of paramount importance for maintaining the structural integrity of lycopene. The selection of emulsifiers, pH, temperature, oil phase, particle size, homogenization conditions and presence of other antioxidants are major determinants for enhancing lycopene stability and delivery from a food emulsion. Process and formulation optimization of the delivery system is product-specific and should be tailored accordingly. Further research is required to better understand the underlying mechanisms of lycopene absorption by the human digestive system.

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Formulating orange oil-in-water beverage emulsions for effective delivery of bioactives: Improvements in chemical stability, antioxidant activity and gastrointestinal fate of lycopene using carrier oils

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ABSTRACT

The influence of carrier oil type on the chemical stability, antioxidant properties and bioaccessibility of lycopene in orange oil-in-water beverage emulsions was investigated. The emulsions were formulated with orange oil (A), which was partially (50%) replaced with tributyrin (B) or corn oil (C) because of their distinctively different fatty acid composition. The addition of corn oil enhanced the physical stability of the beverage during chilled storage by inhibiting Ostwald ripening. The formation of oxidation products was insignificant after storage for 28 days at 4 °C, regardless the type of added oil. Lycopene was more susceptible to chemical degradation in the presence of unsaturated, long chain triglycerides and the retention followed the order: A (87.94%), B (64.41%) and C (57.39%). Interestingly, bioaccessibility of lycopene was significantly lower for emulsions formulated with 50% corn oil as opposed to 100% orange oil as indicated by the simulated *in vitro* gastric digestion model.

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Physicochemical stability, antioxidant properties and bioaccessibility of β-carotene in orange oil-in-water beverage emulsions: influence of carrier oil types

Erika Meroni *[a] and Vassilios Raikos [b]

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This study investigated the effect of carrier oils (short-chain triglycerides (SCT) *vs.* long-chain triglycerides (LCT)) on the physical stability, chemical degradation, antioxidant properties and bioaccessibility of β-carotene-loaded orange oil-in-water beverage emulsions. Beverages formulated with 50% LCT (corn oil) were physically more stable compared to the ones with 50% SCT (tributyrin) as indicated by the Turbiscan 32 Stability Indices during chilled storage for 28 days. No chemical degradation of β-carotene was observed during storage regardless of the carrier oil composition. The formation of primary (conjugated dienes) or secondary (thiobarbituric acid reactive substances) oxidation products at the end of the storage period was insignificant. The bioaccessibility of β-carotene was significantly affected ($P < 0.05$) by the type of carrier oil and decreased in the following order: 50% LCT + 50% orange oil > 50% SCT + 50% orange oil > 100% orange oil. The high bioaccessibility of β-carotene (30.06%) in the beverages formulated with LCTs was attributed to the enhanced solubilisation capacity of the lipophilic carotenoid in mixed micelles after the *in vitro* digestion protocol.

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