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Toward sustainable dietary patterns: new insights on plant-based diets and pulses consumption

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Abbreviations

AIC	Italian Association for Celiac Disease
Anti-tTG	Anti-Tissue Transglutaminase
BCM	Body Cell Mass
BCMI	Body Cell Mass Index
BDA	Banca Dati di composizione degli Alimenti per studi epidemiologici in Italia
BIA	Bioelectrical Impedance Analysis
BIVA	Bioelectrical Impedance Vector Analysis
BMI	Body Mass Index
BSA	Bovine Serum Albumin
BSG	British Society of Gastroenterology
CD	Celiac Disease
CDA	Celiac Disease Autoimmunity
CHO	Carbohydrates
CREA	Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria
CXCR3	Chemokine Receptor
DC	Dendritic Cell
EAA	Essential Amino Acids
ECW	Extra-Cellular Water
EFSA	European Food Safety Authority
EPIC	European Prospective Investigation into Cancer and Nutrition Study
ESPGHAN	European Society for Pediatric Gastroenterology, Hepatology and Nutrition
FA	Fatty Acids
FFM	Free Fat Mass
FM	Fat Mass
GFD	Gluten-Free Diet
GHG	Green House Gas
GI	Glycaemic Index
IBD	Inflammatory Bowel Disease
ICTs	Information and Communication Technologies
ICW	Intra-Cellular Water
IgA	Immunoglobulin A
IL-12	Interleukine 12
IL-15	Interleukine 15
INF-gamma	Interferone Gamma
IOM	Institute of Medicine
ISTAT	Italian Institute for Statistics
IU	International Unit
HLA	Human Leukocyte Antigen
HLA-DQ2	Human Leukocyte Antigen DQ2
HLA-DQ8	Human Leukocyte Antigen DQ8
LARN	Livelli di Assunzione di Riferimento di Nutrienti ed energia per la popolazione italiana
MD	Mediterranean Diet
MLC	Myosin Light-Chain
MLCK	Myosin Light-Chain Kinase
(MLCK)-P-MLC	Myosin Light-Chain Kinase signalling pathway
MyD88	Myeloid Differentiation Primary Response 88

NASPGHAN	North American Society for Pediatric Gastroenterology, Hepatology and Nutrition
NF-kb	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NICE	National Institute for Health and Care Excellence
(NK) T cells	Natural Killer T lymphocytes
PhA	Phase Angle
PREDIMED	Prevención con Dieta Mediterranea
PRI	Population Reference Intake
PT-G	Pepsin-Trypsin-resistant Gliadin
PTH	Parathyroid Hormone
RDA	Recommended Dietary Allowance
RER	Respiratory Exchange Ratio
RMR	Resting Metabolic Rate
Rz	Resistance
SDGs	Sustainable Development Goals
SDT	Suggested Dietary Target
TBW	Total Body Water
Th1	T helper lymphocyte 1
Th2	T helper lymphocyte 2
TNF- α	Tumor Necrosis Factor α
tTG	Tissue Transglutaminase
tTGA	Tissue Transglutaminase Autoantibody
TJ	Tight Junctions
UL	Upper Level
UVB	Ultraviolet B Light
VDR	Vitamin D Receptor
WHO	World Health Organization
Xc	Reactance
Z	Impedance
25(OH)D	25-hydroxyvitamin D or cholecalciferol
25(OH)D3	25-hydroxyvitamin D or calcidiol
1,25(OH)2D3	1,25-dihydroxyvitamin D3 or calcitriol

Abstract

Plant-based diets have been recognized to positively impact health, by contributing to the maintenance of a healthy status and to the prevention of non-communicable diseases (i.e. cardiovascular disease, diabetes). The positive health benefits of these diets are related to the intake of plant-foods they promote, such as vegetables, fruits, cereals, legumes and nuts and to the reduced intake of animal derived foods. Moreover, plant-based foods have a lower environmental impact compared to animal derived foods, making these diets environment-friendly.

Concerning plant foods, pulses emerge as a nutritionally valuable food. Indeed, pulse regular consumption is related to beneficial health outcomes. In addition, pulses represent an important sustainable protein source alternative to animal derived foods. Indeed, it must be underlined that pulses production is more sustainable than animal derived food production, contributing to make them a dietary protein source to be preferred.

In this perspective, this dissertation presents research studies aimed to support plant-based diets as healthy and sustainable dietary regimens starting from the promotion of pulse consumption. In this perspective, pulses intake and the causes of pulses disregard among consumers were initially investigated as a starting point to study strategies to foster and sensitize consumers on pulse intake, such as the designing of innovative pulse-based products, in collaboration with Fertitecnica Colfiorito s.r.l company, and the development of targeted nutrition education interventions. Furthermore, given the fundamental impact of a healthy diet on health, the effects of plant-based diets and dietary proteins quality on body composition parameters and resting metabolic rate were evaluated.

As pulses are gluten-free foods, also their role in gluten-free diets was underlined. Adhering to gluten-free diets is often complex for celiac patients causing nutritional imbalances. Therefore, this work intended to investigate the possible role of a specific micronutrient, vitamin D, in celiac disease onset. Moreover, the development of an on-line nutritional education course for the management of gluten-free diets was described.

Lastly, the attention was focused on the nutritional properties of rice, one of the most commonly consumed cereal among celiac patients, and its applicability in gluten-free diet.

Key-words: pulses, proteins, sustainability, Mediterranean Diet, body composition, health.

Chapter 1. Preface

Plant-based diets are becoming always more popular among consumers.

These diets promote the consumption of plant derived foods, such as vegetables, fruits, cereals and pulses. Among these foods, an important role is covered by pulses. Indeed, pulses exhibits positive health benefits for human health. At the same time, they represent an environment-friendly alternative to animal derived foods, like meat.

Therefore, more plant-based diets have a central role in the Agenda 2030 for a sustainable development.

The Agenda 2030 is an action plan, approved on 25th September 2015 by 193 countries that are part of the United Nations, targeting people, the planet and prosperity and intended to promote sustainability of all dimensions of development. A sustainable development considers current needs avoiding to compromise those of future generations and interests not only the environmental perspective but also the social and economic ones. As these contexts are connected, all of them must be taken into account to guarantee a better life for everyone.

Indeed, the Agenda 2030 defines 17 Sustainable Development Goals (SDGs) to be reached within 2030 that constitute a possible strategy to make the Earth more sustainable (Figure 1-1).

Sustainable Development Goals (SDGs)
1. No poverty
2. Zero hunger
3. Good health and well-being
4. Quality education
5. Gender equality
6. Clear water and sanitation
7. Affordable and clean energy
8. Decent work and economic growth
9. Industry innovation and infrastructure
10. Reduced inequalities
11. Sustainable cities and communities
12. Responsible consumption and production
13. Climate action
14. Life below water
15. Life on land
16. Peace, justice and strong institutions
17. Partnerships for the goals

Figure 1-1. Sustainable Development Goals (SDGs). Desa, U. Transforming our world: The 2030 agenda for sustainable development. 2016.

Considering the spreading of plant-based diets, the demand for plant-based foods is increasing and pulses are used as the primary ingredient. As plant-based foods consumption can support a reduction in animal derived foods intake, the interest in plant-based substitutes is increasing (i.e. meat substitutes). However, consumers usually tend to perceive nutritional quality of these products better than the one of animal products they are substituting but some plant-based foods can be found as processed or highly processed foods, therefore in some cases their nutritional composition may not be healthy. Accordingly, both nutritional education able to sensitize people and the study of new plant-based foods with improved nutritional characteristics is now of fundamental importance.

Giving the importance of a more plant-based diet and the fundamental impact that the diet has on human health and environment, this work aims to:

- study and develop new plant-based foods with optimized nutritional characteristics and improved palatability and acceptability;
- deeply exploit consumers food habits and willingness to improve plant-based and, in particular, legumes consumption;
- evaluate different diet approaches impact on body composition taking into account protein quality (i.e. different animal-based and plant-based proteins ratio);
- develop new educational tools in order to improve people awareness regarding healthy diet and sustainability.

In particular, as a first step, this work intends to describe the general characteristics of pulses (chapter 2) and to investigate the state of art of pulse intake through nutrition surveillance (chapter 3).

Moreover, to promote pulse intake, this work means to present the pulse-based products developed in collaboration with the R&D office of Fertitecnica Colfiorito s.r.l company, with a specific reference to #Mordilanatura Colfiorito® Muesli. The design and development of this product was directed to a double aim. Firstly, it represents a strategy to offer consumer's a nutritionally balanced alternative pulse-based food, wishing to arouse consumer's curiosity and to improve pulse intake. Secondly, as the product is completely plant-based, it provides to consumers the possibility to choose nutritionally balanced alternatives to animal-based products increasing also the

sustainability of the diet (chapter 4). Furthermore, other several projects were defined and started with the company aiming to promote pulse intake in all consumers' categories by starting from the consideration of the needs of specific target of consumers (chapter 5).

In parallel, to sustain pulse consumption, the development and the administration of an e-learning nutrition education intervention meant for "Human Nutrition Biology" university students were carried out. The aim was focused on the improvement and the reinforcement of students knowledge on pulse nutritional properties to sensitize them on the positive effects of pulse consumption as they will be future nutrition trainers (chapter 6).

As a healthy and balanced diet is fundamental to guarantee a healthy status, firstly the effects of a Mediterranean diet on body composition in healthy and physically active subjects (chapter 7) was investigated and, secondly, the impact of plant-based dietary protocols with different animal/plant-proteins ratios on body composition and resting metabolic rate of healthy and physically active individuals was carried out (chapter 8).

Noteworthy, pulses are gluten-free foods and they can be widely used in gluten-free diets. Adhering to strictly gluten-free and nutritionally balanced diets is often difficult for celiac consumers, therefore possibly nutritional imbalances can occur. The last chapters (9, 10 and 11) are focused on pulses role in gluten-free diet as naturally gluten-free products.

Bibliographic references are reported at the end of each chapter.

List of publication, conference abstract and oral communications

Publications

Published

- G. Vici, D.R. Perinelli, D. Camilletti, F. Carotenuto, L. Belli, V. Polzonetti (2021). Nutritional properties of rice varieties commonly consumed in Italy and applicability in gluten free diet. *Foods*, 10, 1375.
- G. Vici, D. Camilletti, V. Polzonetti (2020). Possible Role of Vitamin D in Celiac Disease Onset. *Nutrients*, 12(4), 1051.
- L. Cesanelli, G. Vici, D. Camilletti, R. Ceci, L. Belli, V. Polzonetti (2020). Impact of 8 weeks of supplementation with *Withania somnifera* on strength in trained individuals. *Nutrafoods*, 1, 169-175.

In proceeding

- D. Camilletti, G. Vici, L. Malandrino, S. Zufolino, L. Belli, V. Polzonetti. E-learning nutritional education as innovative tool to sustain healthy food choices and practices. *In preparation*.

Conference abstract

- D. Camilletti, G. Vici, L. Cesanelli, L. Belli e V. Polzonetti. Legumes in the diet: consumption and perception in the consumer. XLI Congresso Nazionale della Società Italiana di Nutrizione Umana (SINU), on-line edition. 9th-10th April 2021.
- G. Vici, R. Carbone, D. Camilletti, L. Belli, V. Polzonetti. The importance of hydration in professional singers. XLI SINU on-line National Congress. XLI Congresso Nazionale della Società Italiana di Nutrizione Umana (SINU), on-line edition. 9th-10th April 2021.
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- D. Camilletti, G. Vici, L. Cesanelli, L. Belli, C. Annetti, A. Ciacci and V. Polzonetti. Alternative strategies and timing for legumes consumption in athletes, 4° Convegno Piattaforme Tematiche di Ateneo, Cibo e nutraceutici: parola chiave “caratterizzazione”, Camerino 9th July 2019.
- G. Vici, D. Camilletti, A. Mozzoni, L. Cesanelli, V. Polzonetti. Effects of specific re-hydration protocols after exercise in non-elite and elite athletes. 1st Annual ISSN Italy Conference and 3rd Sport Nutrition International Conference. Sport Nutrition from Science to Practice. Bologna 30th November - 1st December 2018.

Oral communications

- “Consumer targeted food packages to promote pulse consumption”, 5° Convegno on-line a cura delle Piattaforme Tematiche di Ateneo su “Alimenti e Nutrizione e Salute Umana e Animale”. Camerino (MC), 13th July 2021.
- “Promoting pulses consumption: the usefulness of innovative food formulations”. IX SINut National Congress. Bologna, 31th May - 1st June 2019.
- “The importance of legumes and new strategies to promote their consumption in the diet”. 18th European Young Cereal Scientists and Technologists Workshop. San Benedetto del Tronto (AP), 15th - 17th April 2019.

Chapter 2. Pulses

Introduction

A balanced diet is fundamental for maintaining a healthy status [1]. At present a healthy diet must be also a sustainable diet [2]. In the context of the current global socio-demographic changes, finding an alternative protein source to that of animal-based foods becomes more and more urgent [3]. Reducing meat consumption appears a strategy to hinder climate change and to preserve biodiversity [4]. The shift towards a more plant-based diet, even with the partial substitution of animal-proteins sources with plant-based ones, contributes to sustain environment and human health [5]. Therefore, protein plant foods consumption should be promoted.

Among protein plant foods, pulses cover an essential role as they contribute to both human and planet health [6]. Indeed, pulses exert a key aspect in the prevention of non-communicable diseases [7, 8]. Moreover, from the environmental side, pulses are involved in the achievement of the 17 Sustainable Development Goals (SDGs) described in the Agenda 2030, for example helping facing hunger and malnutrition and supporting sustainable agriculture productions [9].

As it is extensively evidenced in literature, pulses show interesting nutritional properties that will be described hereafter.

Carbohydrates and proteins are the main macronutrients present in pulses [10]. Carbohydrates include simple sugars, oligosaccharides and polysaccharides [11].

Among oligosaccharides, non-digestible components are worth of mentioning as they constitute dietary fiber [12], which is an important nutrient, acting for the prevention of chronic diseases such as cardiovascular disease, diabetes and cancer [13].

The preventive effect of fiber is due to its capacity to contribute to the reduction of glycaemic index of the food or plate [14].

Therefore, the intake of high fiber foods is essential to manage glycaemic response [15]. Pulses are well-known sources of dietary fiber and they have a low glycaemic index [14, 16]. According to Mediterranean diet, pulse consumption is recommended at least twice a week [17]. As a matter of fact, several studies pointed out that a regular intake of pulses contribute to improve blood lipid profile, glycaemic control and body composition [18-21]. Consequently, pulse consumption is useful for the prevention of cardiovascular disease, diabetes and for the management of body weight [22-24].

Pulses constitute a valuable source of plant-proteins for human consumption [25, 26]. In the context of the current global socio-demographic changes, finding an alternative protein source to that of

animal-based foods becomes more and more urgent [3]. Pulse production is more environment friendly and it is responsible for lower greenhouse gas emissions than meat production [27].

Despite the evident benefits of pulses, there is a general disregard for pulse intake by consumers [28]. Indeed, consumer choices are oriented by personal preferences, culinary education and innovative food products availability, all aspects that strongly influence pulse consumption [29].

From the other side consumers have become more sensitive concerning ethical issues related to animal welfare (ex. killing, suffering), leading to the avoidance of animal food products in their diet [30].

In this context, nutrition and environmental education is needed in order to exert or support a change in consumer's food behaviour [31].

The nutritional quality of food proteins is related to their amino acidic composition, protein digestibility and availability [27, 32]. More in details, plant-proteins are precisely deficient in the adequate content of essential amino acids for humans [27]. Pulses are characterized by a high content of lysine, leucine and arginine but lack of sulphur amino acids, such as methionine and cysteine, and tryptophan [32, 33]. Consequently, in order to improve the quality of pulse proteins by solving the matter of limiting amino acids for human nutrition, it is generally recommended to combine plant sources foods properly and to take into consideration the concept of amino acid complementarity [34, 35]. Specifically, the amino acidic composition of cereals proteins is complementary to those of pulse proteins [36]. Indeed, cereal proteins contain a higher amount of sulphur amino acids than pulses. Thus, pulses should be associated to cereals and cereals derivatives in each plate in order to guarantee the covering of the required daily intake of essential amino acids [37].

Pulses naturally have a low content of fats [38]. They contain mono- and polyunsaturated fats, they are low in saturated fats and contain plant sterols [39-41]. Therefore, increasing pulse consumption can contribute to ameliorate diet quality not only by promoting plant-based proteins and fiber intake but also by promoting unsaturated fat intake. Western diets are characterized by a high content of saturated fats, negatively impacting on health [42]. Consequently, shifting to a more plant-based diet can exert numerous health benefits, for both humans and planet health [43, 44].

Pulses contain group B vitamins (thiamine, niacin, riboflavin, pyridoxine and folate), vitamin A and vitamin E [31, 45] and several minerals, such as iron, potassium, phosphorus, calcium, magnesium and zinc [10, 31]. Although the mineral content described in pulses is remarkable, it is necessary to take into account the low mineral bioavailability due to the presence of anti-nutritional factors. Anti-

nutritional factors, like phytic acids and phenolic compounds, exert a chelating effect on numerous minerals (i.e. zinc, iron, calcium, magnesium), inhibiting their absorption [46, 47]. As previously mentioned, pulse processing (i.e. soaking, thermal treatments, germination) can aid reducing the concentration or the activity of anti-nutritional compounds, thus improving mineral bioavailability and utilization in pulses and optimizing pulses nutritional profile [48, 49].

Anti-nutritional factors are secondary metabolites produced by plants in specific conditions (i.e. biological stress). At the same time, when ingested, these molecules are able to reduce or compromise the absorption of nutrients of the foods they are contained in [50].

Anti-nutritional factors include different types of molecules such as lectins, phytohaemagglutinins, tannins, phytic acid, oxalates, phenolic compounds, saponins, trypsin inhibitors [51] and specific oligosaccharides [52]. Each one of them has a particular action, for example trypsin inhibitors hinder the activity of trypsin enzyme while tannins are responsible for the production of insoluble complexes with proteins, thus compromising protein digestion and absorption [51].

It was found that some anti-nutritional factors behave as bioactive compounds exerting positive health benefits (such as phytates, saponins, tannins) [53, 54]. Accordingly, these molecules appear to display a dual role and the dose introduced entails to discriminate if the molecule behaves as a bioactive compound (lower doses) or as an anti-nutritional factor (higher doses) [51, 55]. Anyway, it should be taken into account that the concentration of these compounds varies according to the type of pulses considered and that it can be reduced by soaking, domestic cooking methods (i.e. boiling) and by applying specific treatment methods (i.e. hulling, germination) or a combination of them [56].

Soaking is the slow process of water imbibition of the seed and it is a fundamental part in pulses preparation [57]. It favours protein denaturation and starch gelatinization, which contribute to seed softening when pulses are cooked. Soaking is characterized by a rapid initial uptake of water which gradually gets slower until saturation [57].

This process differently impacts on the anti-nutrients content in pulses by inducing its reduction [58]. More in details, Shi et al. reported that the concentration of lectins and oxalates is effectively decreased by soaking in Canadian pulses [56]. Moreover, according to Shi et al., soaking combined with cooking is more effective in reducing the activity of enzyme inhibitors than soaking itself [59]. In particular, soaking and cooking appears to exert a significant effect on α -amylase inhibitors, promoting α -amylase activity and carbohydrates bioavailability [59].

Other common cooking methods are boiling and pressure cooking.

Boiling is one of the most common domestic cooking methods used for pulses preparation. It consists in diving pulses in boiling water (100°C, the temperature is maintained stable during cooking).

Une et al. found that boiling results incisive in significantly reducing the concentration and the activity of anti-nutritional compounds, in particular of polyphenols, tannins and trypsin inhibitors, in both whole and hulled Japanese red sword beans after soaking [60]. Simultaneously, boiling increases nutrient availability, for example proteins and starch [61]. Indeed, thanks to the partial reduction of anti-nutritional compounds, more space in the matrix becomes available for the enzymatic degradation of starch aiding to raise its digestibility [62].

Pressure cooking is a rapid method for pulses homemade cooking. It requires the use of a pressure cooker, a specific pot able to halt the leak of water and steam, leading to the establishment of a specific pressure (2 bar) and temperature (120°C) and the consequent fast cooking of foods. Indeed, pressure cooking allows to halve cooking time respect to other cooking methods (i.e. boiling). As well as boiling, pressure cooking increasing protein digestibility by inducing protein denaturation and the structural destruction of anti-nutritional factors [63]. Moreover pressure cooking appears to be useful in preserving the concentration of antioxidant compounds [64].

The choice of the best cooking method to use for domestic pulse preparation and nutritional quality preservation is not therefore univocal.

Processing methods such as hulling and germination are other optimal alternatives to allow pulses edibility by avoiding cooking [65].

Hulling is a mechanical process used to remove the seed coat of pulses. It is also an effective method to reduce the concentration of anti-nutritional compounds because it is found that these molecules are mainly present in the cotyledon of the seed [56].

Moreover, it has been reported that the decrease of the activity and of the concentration of anti-nutritional compounds after soaking and hulling promotes an increased activity of α -amylase, raising starch digestibility, and, simultaneously, fosters an improved protein digestibility in moth bean cultivars [63]. At the same time protein availability and digestibility is improved [63, 66].

Germination is the maturation process of pulse seed. Seeds are induced to grow under controlled conditions (temperature, humidity, light...) until developing sprouts.

Germination is a low-cost, effective and environmental-friendly process leading to the production of germinated edible seeds and sprouts for human consumption [36, 67].

It begins when dry seeds start to absorb water during soaking and it is concluded when the embryonic axis elongates [68].

This process is responsible for the optimization of the nutritional quality of pulses [36, 69]. Indeed, it causes the decrease of anti-nutritional compounds content [67] and in parallel the degradation of macronutrients (carbohydrates, proteins and fatty acids) [70].

Accordingly, germination leads to an improvement of nutrient bioavailability and digestibility, for example in terms of proteins and carbohydrates [71-73]. Starch digestibility appears to be increased for the intensified activity of α -amylase but primarily for the reduction of anti-nutritional compounds content [72]. Indeed, the removal of anti-nutritional compounds during germination clears space in the matrix, allowing for the facilitated action of enzymes and thus contributing to ameliorate starch digestibility [71].

Furthermore, germination is characterized by the release of amino acids and simple sugars [36], mainly due to the decrease of anti-nutritional compounds and for the biosynthetic activity of seedlings, that require proteins for plant development [74]. In addition, storage proteins are subjected to hydrolysis in order to provide free amino acids for plant growth [74].

Germination appears to modify the composition of pulses, improving their nutritional profile in particular considering specific nutrients, like amino acids. Variation in amino acid composition of sprouted pulses may be of great interest, especially starting from the consideration that pulse proteins are deficient of some essential amino acids, in particular sulphur amino acids [27].

In this context, germination appears an easy technology to be used in order to implement the quality of plant-based diets [75].

Regarding preservation methods, canning is one of the most commonly used. It is a long-life preservation method frequently used for pulses. Canning provides for the pre-cooking of foods and the subsequent packaging. During the packaging a partial vacuum is established because the products are canned when they are still hot. Finally, the product is sterilized [65].

Canned legumes show a reduced content of proteins and fiber compared to pulses prepared with the domestic method (soaking and boiling). This result is due to the greater matrix disruption occurring in canning than those in boiling. Consequently, proteins and fiber are lost in the preserving liquid at a higher concentration. For the same reason, canning generally induces a greater leaching of micronutrients (such as vitamins and minerals) than boiling [10]. Margier et al. observed that canning and domestic cooking (boiling) do not affect iron and zinc content specifically in white beans and chickpeas but canning causes a loss of iron and magnesium in kidney beans compared to boiling.

Authors confirm a higher reduction of mineral content with canning than with domestic cooking [10].

An aspect to take into consideration when consuming canned products in general is the salt content. As it is widely known salt intake should be taken under control in the diet [76-78]. Consequently, after the decision to consume canned legumes, it is recommended to wash them using running water [65].

Given the valuable nutrient content of pulses, their role for health is essential [16]. Moreover, pulse production is environmental sustainable, contributing to attribute them a higher consideration in the diet [6]. In this perspective pulses should be part of the diet and a more plant-based diet should be supported. Therefore, studies aiming to deepen the role of pulses in the diet and innovative pulse-based products development are of fundamental importance.

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Chapter 3. Nutrition surveillance: the state of art of pulse consumption

Introduction

Pulses could represent an alternative protein source to that of animal-based foods [1]. Pulse should be proposed to reduce meat consumption, thus constituting a strategy to hinder climate change and to preserve biodiversity [2]. Therefore, a shift towards a more plant-based diet is desirable, even with the partial substitution of animal-proteins sources with plant-based ones, to sustain environment and human health [3]. Pulses have an essential role as they contribute to both human and planet health [4]. Moreover, pulses constitute a pillar of Mediterranean diet, which exerts positive effects on human health, in particular, by contributing to the prevention of chronic diseases, such as cardiovascular disease and diabetes [5-9]. According to Mediterranean diet, pulses should be consumed at least 2 times per week [10].

Although the motivations for pulse consumption are numerous, consumers do not have the propensity in choosing pulses in their weekly diet [11, 12]. The long time required for pulse preparation (due to soaking and cooking) is generally advanced as an apparent justified excuse to avoid pulses, especially in the modern schedule of daily activities [11]. Another barrier for pulse intake is the lack of information about the health benefits derived from their consumption among consumers [13].

Starting from these considerations, it comes into light the necessity to deeply examine pulse consumption habits of consumers. In particular, it could be useful to determine which are pulses preferred by consumers and which is their knowledge on pulse nutrient composition and recommended weekly intake aiming to overcome the barriers for their reduced consumption, to study strategies to promote pulse intake and to design new projects in order to develop pulse-based products able to satisfy consumers' requests.

Aim of the study

The aim of the study was to investigate pulse consumption habits, preferences, possible reasons for reduced consumption and knowledge of pulse nutrients content through the administration of an on-line questionnaire to a group of Italian consumers to put the basis for projects focused on both the development of new healthy pulse-based products and of new educational activities.

Materials and methods

The population sample

789 adult subjects were involved in the study. The inclusion criteria for the participation to the study were living in Italy and be aged at least 18 years old. If these criteria were satisfied, participants were asked to confirm their adhesion to the study by providing a written consent.

Survey development

The study was based on the administration of an on-line questionnaire with multiple choice questions and open answer questions, entitled “Pulses, yes or not? That is the problem”, Appendix I) to investigate aspects related to pulse consumption.

The estimated time for the completion of the questionnaire was 15 minutes.

As the study was carried out during COVID-19 first lock-down, it was decided to distribute the questionnaire using technological tools. Indeed, the questionnaire was structured using Google forms® and the respective link was distributed over a period of 20 days in March 2020. Answers were registered in anonymous form.

The questionnaire was divided into 2 sections. The first part contained questions related to subjects’ personal data. The second section regarded pulse intake and pulse consumption habits (Table 3-1).

Table 3-1. Sections and questions of the questionnaire.

Sections	Questions
1. Demographic data	Age
	Gender
2. Pulses	Weekly frequency of pulse intake
	Favourite pulses
	Pulse forms generally consumed
	Consumption of pulse flours
	Knowledge on the recommended weekly frequency of pulse intake
	Nutritional content of pulses
	Willingness to increase pulse consumption in the diet
	Reasons for reduced pulses intake in the population
	Foods consumed with pulses in a meal

For each questions participants could choose among different options. For the question “Favourite pulses” subjects were asked to express their degree of appreciation, giving a score from 0 to 5 (0 = not appreciated, 5 = highly appreciated). If participants had never tasted a specific pulse type, they had the possibility to check “never tasted”) for each type of pulse proposed (lentils, chickpeas, peas, beans, broad beans, wild peas, soybeans and lupin beans). For the questions “Pulse forms”,

“Nutritional properties of pulses”, “Foods consumed with pulses in a meal”, subjects could select simultaneously more options, consequently, percentages are calculated on the total answer obtained for each answer (Appendix I).

Data analysis

Statistical analysis was performed using GraphPad Prism version 9.4.0 for Windows, GraphPad Software, San Diego, California USA. The normality of data distribution was tested through the Shapiro-Wilk test. Descriptive statistics were run and data were expressed as number and percentage (%) when appropriate, as mean±standard deviation (SD), if data distribution was normal, otherwise as median and min–max range. Differences between groups were estimated using the unpaired *Student’s t-test* and non-parametric mean comparison test, as appropriate. *Chi-square* test or *Fisher’s exact* test was used to compare differences in categorical variables. $p < 0.05$ was considered statistically significant.

Results

Sample characteristics

789 adults subjects between 18 and 89 years of age took part to the study (39.7 ± 16.6). Of these, 548 were females (69.5%) between 18 and 85 years of age (38.8 ± 15.8) and 249 were males (30.5%) between 18 and 89 years of age (41.8 ± 18.1).

Pulse weekly intake

Pulses are consumed once a week by the 32.1% of participants, twice a week by the 37.4% and three times a week by the 15.6%. Only the 5.4% of the subjects declared a frequency of intake of more than three times a week (Figure 3-1).

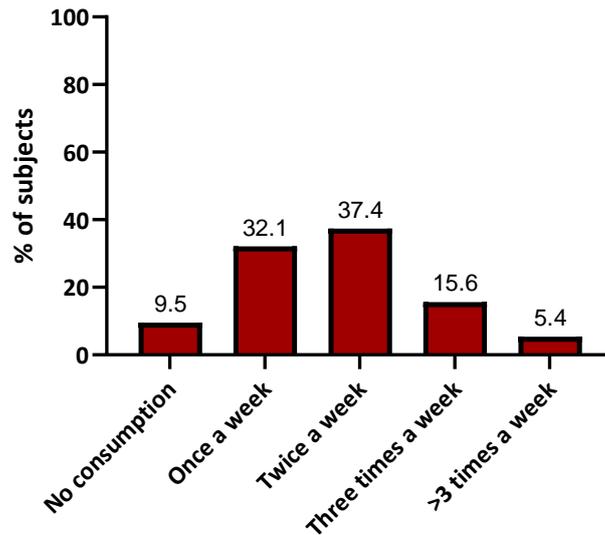


Figure 3-1. Frequency of pulse consumption (% of subjects).

Preferred pulses and never tasted pulses

Among pulses, lentils and peas are the most preferred ones (mean score = 3.5 ± 1.6 for both), followed by chickpeas (mean score = 3.3 ± 1.7), beans (mean score = 3.1 ± 1.6), broad beans (mean score = 2.6 ± 1.8) and lupin beans (mean score = 2.2 ± 1.8). Wild peas and soybeans obtained the lowest mean scores (1.8 ± 1.5 and 1.5 ± 1.7 , respectively).

It has been possible to observe that some typology of pulses (i.e. wild peas) have been never tasted by almost a quarter of the participants while other have been tasted by almost all the population (i.e. lentils) (Figure 3-2).

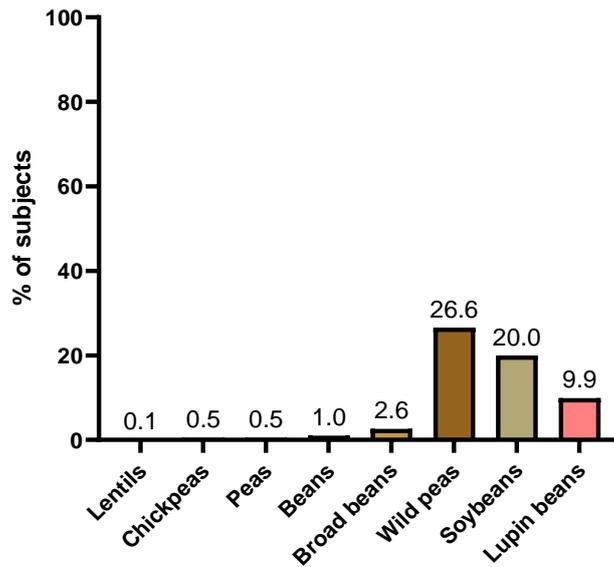


Figure 3-2. Never tasted pulses (% of subjects).

Pulse forms

It emerged that participant usually consume canned legumes (30.0%), followed by pulses (28.6%), frozen legumes (28.4%) and fresh legumes (26.8%). It also emerged that prepared frozen legumes and ready to eat legume-based dishes are not commonly chosen, indeed, the 38.5% and the 54.4% of participants, respectively, answered “Never” (Table 3-2).

Table 3-2. Pulse forms consumption (% of subjects).

Pulse forms	Never	Rarely	Sometimes	Usually	Always
Fresh legumes	11.8%	15.6%	33.8%	26.8%	12.1%
Pulses	10.3%	11.8%	28.6%	28.6%	20.6%
Canned legumes	16.7%	11.5%	26.7%	30.0%	15.1%
Frozen legumes	12.9%	12.3%	35.5%	28.4%	10.9%
Prepared frozen legumes	38.5%	14.9%	27.5%	12.6%	6.5%
Ready to eat legume-based dishes	54.4%	14.6%	20.1%	8.8%	2.0%

Use of pulse flours

The 48.1% of the subjects never uses pulse flours, while the 26.7% consumes them rarely, the 19.7% sometimes and the 5.5% often as depicted in Figure 3-3.

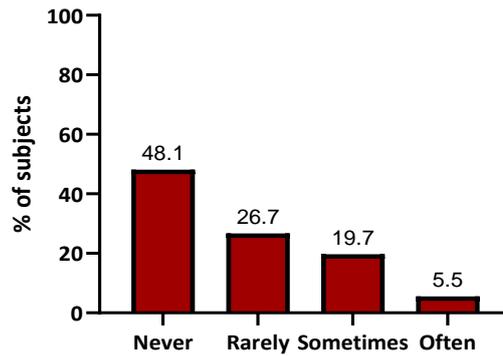


Figure 3-3. Frequency of pulse flours consumption (% of subjects).

Knowledge on pulse recommended frequency of consumption and nutritional composition

Participants were asked to answer to questions related to their general knowledge on pulses. It was found that almost half of the population (52.0%) knows that pulses should be consumed at least two times a week, as recommended by Mediterranean diet [10]. At the same time, 30.0% of the population stated that legumes should be consumed either 3 times a week or more than 3 times a week. Only the 10.0% of the subjects thought that legumes should be consumed either once a week or twice a week, while 8.0% of participant declader not to know the recommendations of Mediterranean diet about legumes consumption (Figure 3-4).

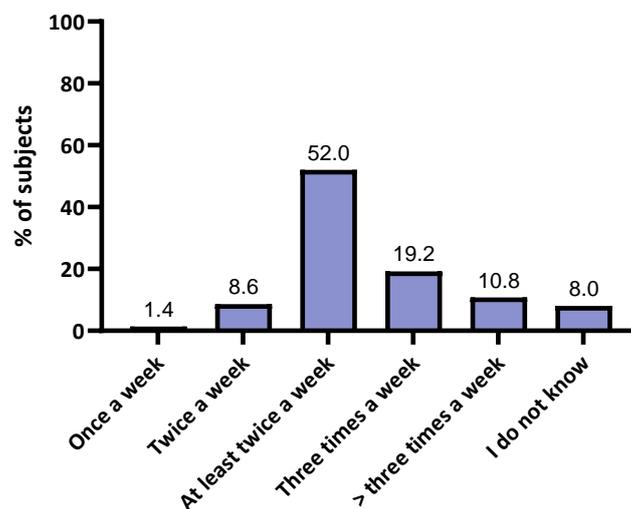


Figure 3-4. Knowledge on pulse frequency of consumption (% of subjects).

Regarding pulse nutrient content, participants were asked which nutrients are present in pulses among carbohydrates, fiber, proteins, fats, vitamins and minerals. They could answer: “Yes”

(meaning for presence of that specific nutrient) or “No” (meaning for lack of that specific nutrient) or “I don’t know” (meaning to declare to have no idea about the nutrient presence in pulses). Concerning carbohydrates and fiber content, the 53.7% of participants asserted pulses contain carbohydrates and the 83.7% declared they contain fiber. The 91.5% of participants affirmed pulses are characterized by the presence of proteins. Regarding fats, the 14.8% of participants answered “Yes”. For vitamins and minerals, the 68.1% and the 68.7% of participants, respectively answered “Yes” (Table 3-3).

Table 3-3. Knowledge on pulse nutrient content (% of subjects).

Nutritents in pulses	Yes	No	I don't know
Carbohydrates	53.7%	22.3%	24.0%
Fiber	83.7%	2.9%	13.4%
Proteins	91.5%	1.1%	7.4%
Fats	14.8%	51.8%	33.4%
Vitamins	68.1%	7.4%	24.5%
Minerals	68.7%	5.1%	26.2%

Willingness to increase pulse consumption

Participants had to express their willingness to increase pulse intake in their diet. It was found that the 53.4% showed the availability in increasing pulse consumption if they were better informed on the health benefits of pulse intake. The 30.7% appeared inclined in consuming pulses only with the condition to consume them as they prefer, the 11.3% stated not to be available and the 4.6% declared to agree with increasing pulse intake exclusively if forced (Figure 3-5).

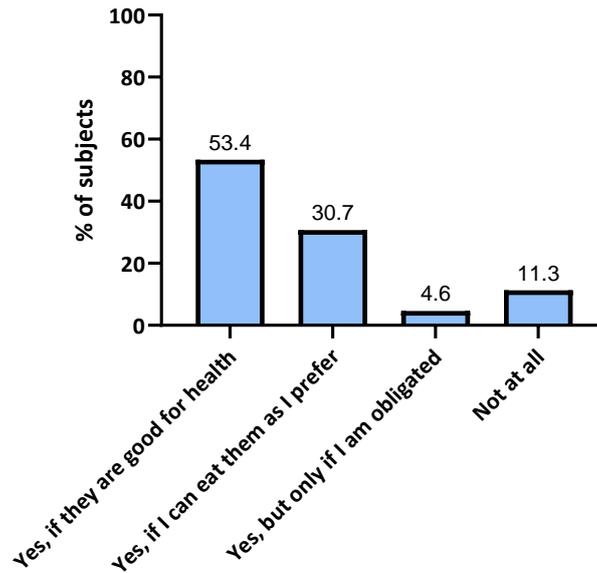


Figure 3-5. Willingness to increase dietary pulse intake (% of subjects).

Reasons for reduced pulse intake in the population

Furthermore, it was requested to participants to select which are the reasons why people do not consume pulses according to Mediterranean diet recommendations [10]. The 40.1% of participants stated that consumers do not consume pulses because they forget to buy or to cook them. The 18.3% of the subjects affirmed pulses are not consumed because they are not appreciated by family members. The 15.3% declared consumers are not used to consume pulses while the 15.3% of participants stated that the reason is related to pulses dislike (Figure 3-6).

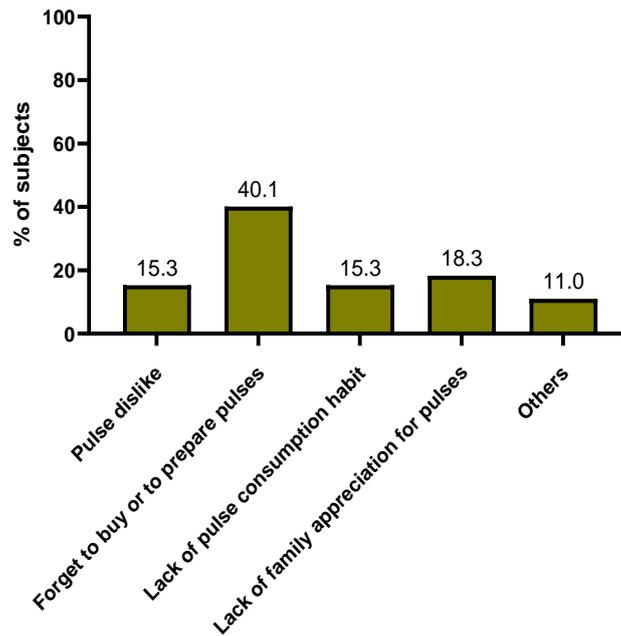


Figure 3-6. Reasons for the reduced pulses intake (% of subjects).

Foods associated with pulses in a meal

Subjects were asked about foods they generally consume with pulses. They could select simultaneously more options, consequently, results are reported as percentages calculated on the total answer obtained for each answer (Table 3-4).

From the obtained data, it was found that pulses are correctly associated with cereals and derivatives by the 60.3% of the participants.

In addition, legumes are usually either associated with vegetables by the 62.3% of the population or consumed alone by 53.4% of participants.

Regarding the association of pulses with animal-derived foods, it emerged that the 34.2% of participants consume pulses with meat while less common are associations with fish, cheese and eggs (16.5%, 15.5% and 17.1%, respectively).

Table 3-4. Foods associated with pulses (% of subjects).

Foods commonly associated with pulses	Yes	Sometimes	No
Cereals, pasta, bread	60.3%	27.1%	12.6%
Meat	34.2%	28.2%	37.6%
Fish	16.5%	22.1%	61.3%
Cheese	15.5%	20.1%	64.4%
Eggs	17.1%	19.0%	63.9%
Vegetables	62.3%	23.0%	14.7%
Alone	53.4%	32.7%	13.9%

Discussion

This study investigated pulse consumption habits, preferences, possible reasons for reduced consumption and knowledge of pulse nutrients content in 789 adult subjects.

It is worth underlying that pulse intake is associated to several health benefits, for example to the prevention of non-communicable diseases [14]. Although the positive outcomes derived from a regular pulse intake, pulses are not consumed respecting the adequate weekly frequency [11, 15, 16].

In this study, it emerged that around 40% of the participants do not consume pulses following the adequate weekly frequency of intake with data showing a consumption of either 0 or 1 times per week of pulses instead of at least 2 time per week as described by Mediterranean diet guidelines [10]. These results were in line with another study conducted in Italy by Fiore and colleagues in which the “legume consumers” were less than 50% in a group of 561 young and adult residents in Sicily [17].

In order to better understand the reason why people do not consume legumes, knowledge about recommended weekly frequency of pulse intake was investigated. From collected data, it emerged that only the 10% of the population has no idea regarding legume consumption, however, another 40% do not know the correct frequency. Indeed, of this 40%, the 30% assessed legumes should be consumed either 3 times a week or more than 3 times a week while the 10% declared legumes should be consumed either once a week or twice a week.

More in deep, participants were asked about their knowledge on pulse nutritional composition and foods combination.

Almost all participants agree pulses contain proteins. Similar results were found by Szczybyło et al. who reported that the 72% of sample participants confirms pulses are a good source of proteins [11]. According to the subjects, pulses contain also other nutrients such as fiber, carbohydrates, vitamins and minerals.

As far as, the shift towards a more plant-based diets is becoming of fundamental importance [18], it must be underlined that a plant-based diet can be defined as a healthy diet only if it is nutritionally balanced. In the perspective to support nutritional education of consumers, it is necessary to consider the importance to combine pulses with cereals. Indeed, pulses and cereals can be considered complementary foods in terms of adequate intake of essential amino acids for humans [19, 20].

In this regard, participants were asked which are the foods they usually associate pulses with. It was pointed out that almost half of participants correctly combine pulses with cereals and cereals derivatives. However, it was also found that pulses are also associated with animal-protein sources, with vegetables or even consumed alone. Although fish, cheese and eggs combination are not so common, combination with meat is consumed from the 35% of participants.

These results underlines that most of the participants are conscious regarding pulses consumption and nutritional properties. However, in practice, participants appears to face difficulties to follow legumes consumption guidelines and to consume them combined in the right way to obtain a balanced meal.

These results underlines the necessity to intervene with nutrition education to instruct consumers both on the importance of legumes consumption and adequate food combinations in order to increase their consumption in the context of a well-balanced diet.

So, in order to better understand how to increase legume consumption by study and develop both nutrition education programs and new plant-based foods with characteristics in line with people needs, it results of great importance to study factors that can be linked to pulses consumption above all in terms of applicability and palatability and by investigating preferences and needs.

Factors to take into account are taste and preferences of pulses [21]. By the analysis of preferred legumes, lentils and peas got the highest appreciation scores, followed by chickpeas and beans. Low appreciation scores were found for wild peas and soybeans. Similar results were pointed out by Śmiglak-Krajewska, showing that peas, beans, soybeans and lentils are the most commonly used by Polish people [22] and Szczybyło and colleagues that reported beans as the most consumed pulses [11]. The same was pointed out by Duarte and colleagues that showed Portuguese population

preference exclusively represented by common beans. Authors explained this result as probably linked to the presence of numerous Portuguese traditional dishes containing beans [13]. The same situation may be found in Italy. Indeed, lentils and peas are the basic ingredients of various Italian traditional meals such as lentils with cotechino or peas with cuttlefish.

This concept could also be linked to the lowest appreciation score obtained by wild peas and soybeans. Soybeans is not a food of Italian culinary tradition and this may lead to a less common consumption by Italian population. Although wild pea is a typical pulse of some Italian regions (i.e. Apulia, Marche region, Umbria, Abruzzo, Tuscany, Lazio and Molise), it is an ancient pulse, whose use and consumption were common for previous generations but have been lost during time. To support this, looking at never tasted legumes, wild peas and soybeans obtained the highest percentage (26% and 20% of the population, respectively) meaning that almost a quarter of the participants either do not know these kind of legumes or never have the chance to try them.

Referring to legume taste, it emerged that the percentage of respondents disliking pulses is actually low, around 15%. However, other reasons were reported as forget to buy or to cook them and family dietary habits. In more cases, family habits appear to have an important role in pulse consumption since family context can influence children dietary habits, playing an important role in promoting healthy dietary behaviours since childhood [23]. For this reason, families should be involved in nutrition education interventions to acquire competences in cooking and preparing pulses aiming to get tasty plates and to sustain their consumption.

Linked to legumes preparation, high cooking time has been reported as a barrier for pulse consumption [13].

In this regard, the pulse form chosen for consumption was investigated.

Data pointed out that canned legumes are usually selected by participants in line with what reported also by Smiglak-Krajewska and colleagues [22]. It may be argued that this choice is due to advantage in preparation, indeed, they do not require long time to be prepared.

Interestingly, from obtained data, it is possible to note that ready to eat legume-based dishes and prepared frozen legumes are not frequently chosen by participants. It may be hypothesized that, although the lack of time for cooking, consumers are inclined to prefer unprocessed products. Actually, processed and ultra-processed products contain a higher salt and fat content than unprocessed ones [24, 25] and maybe participants could be sensitive to this.

Another important aspect related to legume consumption improvement and legumes preparation is related to pulse flours utilization. Indeed, pulses flour can be used to prepared food products that

usually are made from wheat flour (i.e. pasta, cookies, crackers) [26]. This make pulses flour an interesting and promising ingredients in order to improve legumes consumption. The use of pulse flour should be promoted, especially for baked goods preparation. Indeed, pulse-flours based products allow to reduce or eliminate the unpleasant taste of pulse flours and to benefit from pulse nutritional properties [27, 28]. However, pulse flours have been never used by almost half of the subjects according to the results obtained on the studied population. This underline the importance to focus the attention on pulses flour knowledge and utilization also trough specific nutritional education activities and programs.

About willingness to increase pulse intake, it emerged that almost half of participants declared to be available to raise pulse consumption if they are beneficial for health. From this, it could be argued that, consumers should be better informed on the health benefits of pulses. Although, Melendrez-Ruiz et al. stated that the knowledge of pulse consumption benefits may not be enough to push consumers to use pulses in their diet [29], this should be considered one of the factors that should be deeply explained to consumers to improve their awareness about legume consumption.

Conclusions

Legumes have been a basic part of Italian population diet since the shift to a western diet occurred with the advent of modern times and the sudden changes in lifestyle and eating habits of last years. Legumes consumption drastically decrease despite their nutritional properties.

In this context, this study highlighted that to get a better scenario nutritional interventions are required. Consumers must be adequately informed on pulse nutritional and health benefits in order to make healthy dietary choices and to become aware of the importance of regular pulse intake. In addition, focus should be address on the study and development of new and innovative legume-based products feasible to use in order to improve their consumption by the population.

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Chapter 4. Fertitecnica Colfiorito s.r.l. collaboration: #Mordilanatura
Colfiorito® Muesli

Nutritionist role in the company

Today the importance of nutrition for health has been widely evidenced by scientific literature [1-5] and a growing interest for healthy diet and foods has spread among consumers [6, 7]. In this context, companies invest considerable internal resources to provide to consumers products with high nutritional value [8]. Industrial PhD can contribute to foster universities and companies collaboration to respond to consumers needs and the nutritionist inserts as a figure aiding in the optimization of the nutritional quality of company foods proposals and in giving nutritional support to consumers.

A nutritionist is firstly a healthcare expert, who is able to define individual's nutritional requirements starting from the evaluation of his food habits and body composition. By this knowledge, the nutritionist provides nutritional support in several conditions, from healthy to pathologic ones and in specific physiologic conditions (such as pregnancy and breastfeeding), to both the individual, at different ages, and the community.

Moreover, the nutritionist background on food nutritional properties and on the positive and negative consequences derived from specific food consumption makes the nutritionist cover a fundamental role in the nutrition education of consumers. Actually, the nutritionist accompanies them towards healthy and more environment sustainable dietary choices, for example by reading food labels. Furthermore, the nutritionist can be involved in the development of food products with short and clear food labels and in the reformulation of existing ones, supporting companies in selecting healthy ingredients and reducing the content of salt, sugar, saturated and trans fats [9]. In this context, a multidisciplinary approach, involving different company sectors (i.e. marketing) and the competences of a nutrition expert, represents a successful strategy for high quality product development and to respond to national policies and consumers' requests [9].

In addition, a nutritionist can give a contribution to the design of innovative food products. In particular today, considering the growing interest for plant-based products, the necessity to develop high nutritional quality plant-based products is fundamental [10]. In this scenario, the food sector has to respond to consumers' needs participating to the shift towards more plant-based and sustainable diets, facing the challenges and opportunities came into light [11].

Therefore, the responsibility food industries and companies have on consumers' nutrition and health appears undeniable. Thus, in order to make food industries actions positively impact on consumers' wellbeing, the role of nutritionist seems pivotal.

Starting from these considerations, my role in the company covered different tasks:

- research and development in collaboration with company R&D department,
- support to the personal branding of the company,
- provide nutritional information to consumers through the website and the social media of the company (monthly “Question&Answer” session, short communications...),
- supply and collect scientific evidences related to nutrition (bibliographic search),
- promote nutrition education and the fundamental role of pulses in the diet through product’s company.

Considering the above mentioned activities, the figure of nutrition experts intends to appear as an added value for the company.

This chapter and the following one describe the products resulted from the collaboration with Fertitecnica Colfiorito s.r.l. company. This chapter illustrates the steps of the main project that lead to the development of a pulse-based muesli, the #Mordilana Colfiorito® muesli. Chapter 5 explains secondary projects expanded upon the three years of PhD: “Bottega Colfiorito®” project, Piatto fatto® regional recipes elaboration and a project aimed to develop a specific pulse-based product line for mothers and infant weaning.

Introduction

An increasing interest for plant-based diets is spreading worldwide [12]. This finding reflects the attention of consumers not only for healthy diets but also for diets taking care of the environment [13]. In this context, plant derived foods have a primary role. In particular, among plant foods, pulses emerge as sustainable foods because their production is responsible for a low environment impact [14]. Reducing meat consumption appears a strategy to hinder climate change and to preserve biodiversity [15]. The shift towards a more plant-based diet, even with the partial substitution of animal-proteins sources with plant-based ones, contributes to sustain environment and human health [16]. Starting from these considerations, pulse consumption should be promoted in the diet. Although the reasons for pulse consumption are numerous, the long time required for pulse preparation (due to soaking and cooking) is generally linked to pulses avoidance, especially in the modern schedule of daily activities [17]. Indeed, in the modern lifestyle a lack of time and care for preparing meals has emerged, causing the progressive reduction of diet quality [18, 19]. In addition, it must be considered that meals are often consumed outside home inducing consumers to choose ready-to-eat-dish or processed foods, which are characterized by an unfavourable nutritional content [20].

Beydoun et al. pointed out that consuming meals away from home is associated to reduced diet quality [21]. Contrarily, Kramer et al. highlighted that preparing meal at home is responsible for positive health outcomes favouring obesity prevention [22]. Therefore, it appears necessary to study usable food products with a valuable nutritional profile. At the same, it is fundamental to promote nutrition education among consumers to sensitize them on the strategies to use to prepare a nutritionally balanced meal in a short time or to prepare meals in advance [23, 24]. Furthermore, it must be underlined that pulses are often consumed for lunch or dinner, for example in common preparation like soups, resulting sometimes unpleasant to consumers [25]. In this perspective, it could be advantageous to study strategies to suggest consumers to use pulses in non-traditional preparations or in unconventional meal consumption contexts, such as for breakfast or snacks. Breakfast could represent a possible meal to target to introduce pulses on a daily basis. In addition, given the poor nutritional quality of breakfast [26, 27] and the valuable nutritional profile of pulses [28], the introduction of pulses at breakfast could represent a strategy to improve it.

Breakfast is often described as the most important meal of the day [29, 30], but several are the definitions that can be used according to the country considered [31]. Betts et al. proposed a general

explanation of breakfast, suggesting it is the first meal consumed within 2 hours after a long sleeping in any 24-hour period [32].

First of all, breakfast has a fundamental role in regulating energy balance and diet [33]. Indeed, a regular breakfast consumption has been associated with higher intakes of micronutrients, a fruit and vegetables rich diet and reduced intake of soft drinks; therefore, to an overall high quality dietary improvement [34, 35]. Not only, breakfast consumption has been connected to several positive health benefits, such as a favourable dietary profile, the maintenance of a normal body mass index (BMI), increased physical activity behaviour, appetite regulation and improved metabolic profiles, in both children and adults [36].

Moreover, breakfast consumption also enhances cognitive performance and academic achievement [37].

On the contrary, skipping breakfast is linked to lower quality diets, reduced cognitive performance, negative health outcomes and irregular sleeping behaviour, often causing impaired food intake regulation [35, 38]. Consequently, breakfast skipping is responsible for appetite changes and decreased satiety, which could induce overeating and impaired insulin sensitivity [31]. In addition, breakfast skipping is related to an increased risk of developing type 2 diabetes [39].

Furthermore, subjects who are used to skip breakfast generally tend to have unhealthy lifestyle behaviours, such as smoking, being sedentary and drinking [40]. A study conducted on Malaysian primary and secondary school children found similar results, as a matter of fact an inverse association emerged between breakfast skipping and increased risk of overweight and obesity [41]. Generally, lack of time and of hungry in the morning is the reasons for skipping breakfast [42, 43]. All the above mentioned evidences demonstrate that skipping breakfast should be hindered, especially in the young population, as it has been described that inadequate nutritional habits acquired in childhood and adolescence may continue during adulthood, increasing the risk of overweight and obesity [44]. Therefore, nutrition education intervention must be promoted since an early age in order to support healthy dietary behaviours [45].

Another aspect to take into account is the type of breakfast to consume. Indeed, children eating a healthy breakfast entail a lower risk to be overweight or obese as described by “OKkio alla SALUTE” Italian survey (2007) [46]. Another Italian survey “OKkio alla SALUTE” (2019) underlines that on the day of data collection only the 8.7% of children skipped breakfast but the 35.7% did not consume a nutritionally balanced breakfast [47]. Consequently, breakfast nutritional quality is a key factor for a healthy diet [48, 49].

To guarantee breakfast nutritional quality, some foods should be preferred to others according to the ingredients they contain. The presence of fiber, proteins, vitamins and minerals are of fundamental importance while the amount of total sugars, both naturally or added, and salt, which are generally the most regarded ingredients, should be taken under control especially for the negative impact they have on health [50-54].

In the context of breakfast food choices, cereal and muesli are a common preference [55].

A study developed on a group of Australian children and adolescent uncovered that children consuming cereals at breakfast have a better total dietary intake profile [48]. It has been found that fortified breakfast cereal consumption is related to a higher dietary suitability, especially for vitamin D, vitamin B and iron. When breakfast cereals are regularly consumed at breakfast, the quality of the diet is improved and the risk of overweight, obesity and non-communicable disease is reduced [56]. Moreover, Smith et al. found that ready to eat cereal consumption contributes to the daily intake of folate, iron, whole grains, vitamin B₆, vitamin B₁₂, thiamine, niacin, vitamin A, zinc, riboflavin, vitamin D but also added sugars, fiber, total carbohydrates and vitamin C [57].

Considering the popularity of cereals and muesli, it is fundamental to regard their nutrient composition and their promotion through honest marketing techniques. Unfortunately, more often communication is unfair and suggests the consumption of unhealthy but tasty breakfast cereals, which are rich of sugars and nutritionally poor, thus inducing consumers, especially children, to choose them [58]. Indeed, many cereals are energy dense, with a high fat, sugar and sodium content but with a low fiber content [57]. Monique et al. found that breakfast cereals addressed to children contain significantly more sugar and sodium and less fiber and protein than breakfast cereal for other population categories [59]. Consequently, it becomes fundamental to read properly nutritional labels, ensuring cereals and muesli have few and healthy ingredients. At this regard, the choice of wholegrain breakfast cereals should be preferred compared to refined grain cereals as they are nutritionally more advisable [36]. Actually, wholegrain breakfast cereals consumption has been associated to a lower risk to develop cardiovascular diseases and hypertension [60] and to the prevention of chronic diseases [61, 62].

Moreover, whole cereals for breakfast play a pivotal role as breakfast fiber intake contributes to significantly increase the daily intake of dietary fiber [63]. Despite this, many of the breakfast and muesli-type products available in the market are distinguished by a lower fiber content and therefore a high glycaemic index (GI) [64].

Low GI breakfast have a beneficial effect on health. It has been discovered that lower GI breakfast may influence positively cognitive functions [65].

Moreover, it is necessary to adequately combine foods to have a nutritionally balanced breakfast. For example, the addition of milk to cereals or muesli aids to improve the nutritional quality of breakfast and the overall diet, indeed, favouring to meet the recommended macro- and micronutrient needs [66]. Considering all these aspects, breakfast composition requires great attention.

At this regard, to ameliorate breakfast from the nutritional point of view, it was hypothesized the possibility to introduce pulses.

Pulses are universally recognized fiber rich foods. They are high in both soluble and insoluble fiber and they have a very low glycaemic index [28, 67]. Pulses are also a source of proteins [68]; anyway, these proteins are considered of a low biological value low as they do not contain an adequate amount of essential amino acids for human organism [69]. In order to fulfil the complete pattern of essential amino acids, it is suggested to combine pulses with cereals, whose proteins display pulses complementary amino acids [70]. In this way the perfect amino acidic balanced is defined [71]. Commercial muesli and granola are generally characterized by a high content of added sugars and saturated fats and they are poor in fiber [72]. In addition, they contain salt, thus contributing to increase salt intake in the daily diet.

Therefore, the idea was to develop a breakfast product with a high nutritional quality, differently to the breakfast competitor products available in the market. Given all the nutritional benefits of pulses [28], it may be convenient to introduce them also in the breakfast meal. Indeed, as pulses are a source of proteins and fiber [28, 73], their addition contributed to increase protein and fiber content of the muesli. In addition starting from the consideration that muesli are cereal-based, the addition of pulses allowed to have a balance in terms of protein quality as pulses and cereals are complementary regarding essential amino acids [74], Moreover, a pulse-based muesli could be an opportunity to offer pulses in an alternative and more appealing way.

Aim of the project

The aim of the project “#Mordilana Colfiorito® Muesli” was to design innovative and optimized muesli formulations containing pulses suitable for both children and adults.

For the project, the contribution of the different competences of the quality assurance board and the marketing office from the company and those of the university was essential.

Materials

For the development of the recipes, ingredients nutritional values were taken into account. The proportion of each ingredient in the recipes was decided on the basis of a preliminary marketing analysis on ingredient content in competitor muesli. This analysis allowed to define the proportion of the ingredients in the formulations starting from consumers' preferences (data on marketing evaluation can not be disclosed since they are considered confidential by the company). Each recipe had to contain:

- cereals and pulses (cereal flakes around 30%, puffed cereals around 10%, pulses around 10%, percentages referred to the total ingredients of each mix),
- nuts (i.e. almonds, walnuts, hazelnuts),
- seeds (i.e. pumpkin seeds, sunflower seeds, flaxseeds, hemp seeds, chia seeds),
- dried fruits (i.e. strawberries, apple, cinnamon apple, orange, lemon, blueberries).

Starting from these food categories, ingredients were differently combined.

All raw materials (cereal flakes, pulses, nuts and seeds and dried fruits) were purchased by company suppliers.

Methods

Muesli recipes formulation

The company decided to provide to consumers at least 4 nutritionally balanced muesli formulations based on the presence of both pulses and cereals.

Muesli formulations were developed starting from the study of the nutritional profile of each ingredients of interest.

Each ingredient was selected taking into account the following aspects:

- nutritional properties,
- taste,
- cost,
- consumer acceptance.

In addition, all ingredients were selected to get a final product with no sugars added, additives and preservatives.

The work was divided in 5 steps.

Step 1. Ingredients of interest were chosen by analyzing their nutritional properties from info sheets, national food databases (CREA database - Consiglio per la Ricerca in agricoltura e l'analisi dell'Economia Agraria - www.crea.gov.it and BDA database - Banca Dati di composizione degli Alimenti per studi epidemiologici in Italia - www.bda-ieo.it) and scientific literature.

Step 2. After the nutritional analysis, each ingredient was tested in order to appreciate the flavour through the organization of panel sensory tests. Tests were performed by consuming each raw material with both milk and yogurt, foods that are commonly consumed for breakfast. Unflavoured yogurt was used for the panel test not to alter the taste of the single ingredients.

Step 3. After ingredients choice, several nutritionally balanced muesli combinations were proposed and examined elaborating different muesli recipes.

Step 4. An internal panel test in the quality department was organized to taste the proposed recipes and eventually modify them, by optimizing the combination of ingredients. Variations suggested were recorded in order to trace all the steps of recipe design. The final objective was to obtain the perfect balance in terms of taste by maintaining an optimal nutritional composition.

Step 5. Focus group were organized in order to get a trend of consumers' opinion, to provide modifications to the recipes, if needed, and to get the final versions. A focus group is a tool for qualitative research, based on an apparent informal discussion, guided by a moderator and an observer, involving a restricted number of participants, that is frequently used to investigate specific topics in social contexts [75-78].

The organization of three focus groups was assigned to an external agency, expert in focus group coordination and collaborating with the company.

Four designed formulation of the 5 formulations obtained after the step 4 were selected (muesli with emmer, apple and crunchy soybean, superfood muesli, muesli with whole cereal flakes and muesli with dried fruits) based on marketing analysis carried out by the external agency.

The discussion carried out during the focus group was conducted by the agency moderator (a psychologist) who proposed the items of interest (breakfast and muesli) to the target group of subjects selected by the agency and previously informed about the situation. Subjects were invited to participate to the discussion and to express their opinion spontaneously, in order to understand their preferences and dietary behaviour.

The focus group was divided into 4 phases:

- Warming up. In this first phase the discussion was directed at the focus item gradually.

- Relationship. In this phase, the moderator investigated the possible connections among participants making questions on common issues and allowing all participants to express their opinion.
- Reinforcement. During this phase, the focused topic was faced by the group through the direction and supervision of the moderator. Personal opinion emerged.
- Separation. The moderator called to mind the different topics covered during the discussion in order to induce participants to think about them objectivity.

In details, the focus group was coordinated considering specific aims:

- to investigate the reasons for muesli consumption, the daily context of intake and how it is consumed (i.e. with milk, plant beverages, yogurt);
- to consider the acceptance of pulses in a muesli promoted by the company, especially in terms of taste and reasons for an eventual purchase or disinterest for the product;
- to evaluate the appeal of the four proposed recipes (muesli with emmer, apple and crunchy soybean, muesli with whole cereal flakes, superfood muesli and muesli with dried fruits) through the panel tasting;
- to evaluate the acceptance of muesli with the company brand, especially referring to competitor muesli;
- to take into consideration participants' feedbacks on the final price of the product;
- to collect participants' opinion regarding the showed packs.

Focus groups were organized in April 2019.

Milan and Rome were selected as city of reference by the agency responsible for the focus group coordination. The focus-group coordinating agency reported that Milan and Rome are generally chosen as geographical references for marketing research on Italian population. Milan is representative for population of the North of Italy, while Rome for the population of the Centre and the South of Italy. According to the focus-group coordinating agency, Milan was selected also because of the assumption that muesli is a food product that is mainly consumed in the North of Europe, consequently, it could be interesting and useful to analyse the impact of muesli consumption in the nearest geographical area (North of Italy, in particular Milan, which is the most densely populated city in the North).

Three age groups were considered:

- 24-35 years old participants (younger group)
- 30-50 years old participants (intermediate group)

- 38-55 years old participants (older group).

By the marketing analysis, teenagers and young consumers were not involved because they do not have purchasing power [79]. Also elderly people were not included, as they are not muesli consumers generally [80].

Each group was composed by 8 participants, in accordance to the standard reference of marketing research [81] in order to allow a more fluid conversation.

Gender distribution of focus group was defined by the agency. In detail, each group was composed by 5 women and 3 men according to the marketing analysis performed by the agency considering participants who were responsible for purchases and had purchasing power at home.

Based on the evaluation of the ISTAT (Italian Institute for Statistics) (www.istat.it), the agency pointed out that the responsible for purchases in Italy are generally women. Consequently, they are the main target for market and marketing researches.

Characteristics of participants' involved in the focus group where:

- to be ≥ 18 years old,
- to be responsible for purchases at home,
- to be a regular muesli consumer,
- to eat cereals and pulses weekly.

The focus group was organized starting with 10 minutes in which participant introduced themselves and the mediator explained the topic of the discussion. The activity lasted approximately 2 hours. The time for the focus group was divided considering the time required for the discussion of the different aspects of the topic:

- Breakfast (10 minutes). In this section participants were asked to define what they expected from breakfast, the types of breakfast they image to exist and how they describe them. Participants were also asked to specify which is their favourite type of breakfast and which is the one they usually avoid.
- Muesli (20 minutes). For the muesli section a brainstorming was organized using the word "muesli" as central item. Participants were invited to describe what the word raised in their mind and to give a definition of muesli. Moreover, participants had to illustrate pros and cons of this product and the types of muesli in the market. Finally, they were asked to specify which muesli they preferentially consume and why, the frequency of consumption and the association with other foods for breakfast.

- Shelf sight (20 minutes). In this part participants were invited to stand in front of a shelf reproducing a typical market shelf of muesli. Participants had to look at the products and write down positive and negative impressions, the presence of products usually/never consumed and, in case, why they are/are not consumed with particular attention to the “Mordilanatura” Colfiorito® muesli.
- Product sight (50 minutes). In this section two mockups of the product (muesli with emmer, apple and crunchy soybean and muesli with whole cereal flakes and walnuts) were shown to the participants and they were asked to describe their opinion on them (i.e. impressions, positive and negative aspects at a first sight, opinion on pulses as an ingredient, eventual desire to buy and taste the product or feeling of apathy with respect to the product, opinion on the brand of the product, on the pack and on the other muesli competitor presented on the market, on the nutritional value). Beyond their opinion, the moderator asked participants to give suggestions to improve the recipe, the product’s packaging and name.
- Taste of the product (25 minutes). This section was dedicated to the tasting of the product. Participants had the possibility to taste the product with hot or cold milk, soy beverage or white yogurt and to add refined or brown sugar considering their preferences. Each participant was provided with a sheet to note personal impressions and evaluations. For each recipe tasted, it was required to give an opinion on the aspect, smell, texture, taste and express a mark (from 1 to 10) to obtain a ranking of preferences and to collect suggestions to improve recipes.
- Evaluation of alternative packages (5 minutes). Participants were asked to express their opinion on the two mockups shown, trying to describe if they are adequate and in line with the idea promoted by the company brand. In addition, they had to give their personal preference and suggest possible improvements of mockups.
- Conclusions (5 minutes). In the last part of the focus group, participants had to summarize the pros and cons related to the products and how to optimize the proposed recipes and the used packaging.

Results

Ingredients selection

Cereals (as flakes and/or puffed cereals), processed pulses, nuts, seeds and dried fruit were the food groups present in each formulation.

- Among cereals, it was decided to introduce rye and emmer, which are not frequently consumed in the Italian daily diet [82], to replace the use of wheat. Moreover, in 3 of the 4 mixes proposed puffed cereals such as emmer, quinoa and millet were added to promote the diffusion of minor cereals [83] and to meet the preferences of consumers, who are generally inclined to choose puffed cereals for their crispy texture [84].
- Nut and seed consumption has shown multiple benefits for the health of individuals. They were proposed in order to increase the nutritional quality of the products by adding foods rich in MUFAs, PUFAs, fibers, vitamins, minerals and bioactive compounds with demonstrated benefits on health outcomes (i.e. amelioration in the lipid profile) [85-87]. In this case nuts were considered as crumbs in order to be present homogeneously in the mix. Among seeds, sunflower and pumpkin seeds were selected as they represent a compromise between the necessity to satisfy nutritional quality parameters and to respect production costs.
- Also dried fruit consumption has been associated with cardiometabolic and other health benefits and its consumption should be promoted for public health purposes [88]. Indeed, dried fruits had a key role in influencing the taste of formulations as they allow to avoid the addition of sugar to the recipes without renouncing to get an appreciable taste. For example, cinnamon was selected for its sweet and pleasant taste but also for its nutritional properties. Indeed, thanks to the presence of active constituents, such as cinnamaldehyde, eugenol, camphor and polyphenols, cinnamon is able to display antioxidant, anti-inflammatory, antibacterial and antiproliferative effects [89-92].
- Chocolate was also taken into account as muesli ingredient. Chocolate is a highly appreciated food by consumers, especially for its taste and texture [93]. Moreover, cocoa, chocolate's main ingredient, exhibits beneficial effects on human health, for example antioxidant and anti-inflammatory effects, due to the presence of polyphenols [94, 95].

Muesli formulations

The composition of the recipes obtained in the first phase are listed in Table 4-1.

Table 4-1. Ingredients of muesli formulations, 1st phase.

Food groups	Ingredients	Quantity (% in 100g of the final product)
Muesli with emmer, apple and crunchy soybean		
<i>Cereal flakes</i>	Rye flakes	20
	Puffed emmer	10
<i>Nuts</i>	Hazelnut crumbs	20
<i>Seeds</i>	Sunflower seeds	10
<i>Dried fruits</i>	Cinnamon dried apple	5
	Dried apple	15
	Dried blueberries	10
<i>Pulses</i>	Crunchy soybeans	10
Superfood chocolate muesli		
<i>Cereal flakes</i>	Barley flakes	15
	Puffed quinoa	10
<i>Nuts</i>	Almond crumbs	20
<i>Seeds</i>	Sesame seeds	10
<i>Dried fruits</i>	Dried strawberries	15
<i>Pulses</i>	Puffed and toasted lentils	15
<i>Chocolate</i>	Dark chocolate	15
Muesli with whole cereal flakes		
<i>Cereal flakes</i>	Whole wheat flakes	10
	Whole rye flakes	10
	Whole emmer flakes	10
<i>Nuts</i>	Walnuts	20
<i>Seeds</i>	Pumpkin seeds	10
	Sunflower seeds	10
<i>Dried fruits</i>	Raisins	15
<i>Pulses</i>	Puffed and toasted lentils	15
Muesli with dried fruits		
<i>Cereal flakes</i>	Whole rye flakes	25
	Puffed millet	5
<i>Nuts</i>	Almond crumbs	20
<i>Seeds</i>	Pumpkin seeds	10
<i>Dried fruits</i>	Raisins	10
	Dried lemon	5
	Dried orange	10
	Dried strawberries	5
<i>Pulses</i>	Puffed and toasted lentils	10

Values are expressed as % in 100g of the product.

The muesli formulations obtained from the first phase (Table 4-1) evidencing a great appreciation of the recipes in terms of taste by the internal company evaluation. However, some aspects needed to be improved and an updated ingredient composition of the recipes was proposed in a second phase.

In detail, puffed quinoa taste turned out to be apparently unpleasant for the majority after the internal taste evaluation in the company. Therefore, in the second formulation it was substituted

by puffed amaranth, whose flavour was considered less vegetable and more pleasant, reminding toasted seeds flavour.

The percentage of nuts in all the formulations was reduced because nuts emerged to significantly contribute to an increase of the final product cost. Anyway, in order to maintain a similar nutritional value, the percentage of seeds was in parallel increased. Moreover, in some formulations dried fruits were modified in order to get a more pleasant taste (i.e. dried blueberries were removed from the first recipe, dried strawberries were substituted by dried blueberries in the second one and dried strawberries were removed from the fourth mix).

In addition, dark chocolate was substituted with raisins due to the fact that the combination with the other ingredients appeared to be not effective from the taste profile.

The composition of the recipes obtained after the second phase are presented in Table 4-2. Nutritional values of formulations after the second phase are presented in Table 4-3.

Table 4-2. Ingredients of muesli formulations, 2nd phase.

Food groups	Ingredients	Quantity (% in 100g of the final product)
Muesli with emmer, apple and crunchy soybean		
Cereal flakes	Rye flakes	30
	Puffed emmer	10
Nuts	Hazelnut crumbs	10
Seeds	Sunflower seeds	15
Dried fruits	Cinnamon dried apple	5
	Dried apple	20
Pulses	Crunchy soybeans	10
Superfood chocolate muesli		
Cereal flakes	Barley flakes	30
	Puffed amaranth	10
Nuts	Almond crumbs	10
Seeds	Sesame seeds	15
Dried fruits	Dried blueberries	10
Pulses	Puffed and toasted lentils	15
Chocolate	Dark chocolate	10
Muesli with whole cereal flakes		
Cereal flakes	Whole wheat flakes	15
	Whole rye flakes	15
	Whole emmer flakes	10
Nuts	Walnuts	10
Seeds	Pumpkin seeds	10
	Sunflower seeds	10
Dried fruits	Raisins	15
Pulses	Puffed and toasted lentils	15
Muesli with dried fruits		
Cereal flakes	Whole rye flakes	25
	Puffed millet	5
Nuts	Almond crumbs	10
Seeds	Pumpkin seeds	20
Dried fruits	Raisins	15
	Dried lemon	5
	Dried orange	10
Pulses	Puffed and toasted lentils	10

Values are expressed as % in 100g of the product.

Table 4-3. Nutritional values of muesli formulations, 2nd phase.

Muesli formulation	Nutritional values (in 100g of the product)								
	Energy		Fats (g)	Saturated fats (g)	Carbohydrates (g)	Sugars (g)	Fiber (g)	Proteins (g)	Salt (g)
	kJ	kcal							
Muesli with emmer, apple and crunchy soybean	1626.6	388.2	15.3	2.1	42.1	14.8	8.3	16.3	0.01
Superfood chocolate muesli	1685.5	403.0	18.0	11.1	38.7	5.2	9.8	16.7	0.01
Muesli with whole cereal flakes	1754.5	419.1	17.8	2.2	45.0	11.0	8.8	15.2	0.00
Muesli with dried fruits	1626.6	388.2	15.3	2.1	42.1	14.8	8.3	16.3	0.01

Values are expressed in 100g of the product.

After the sensory evaluation and cost analysis of muesli formulations resulted from the second phase (data can not be disclosed since they are considered confidential by the company), small changes turned out to be necessary.

The composition of the recipes obtained are presented in Table 4-4. Nutritional values of formulations after the third phase are presented in Table 4-5.

Table 4-4. Ingredients of muesli formulations, 3rd phase.

Food groups	Ingredients	Quantity (% in 100g of the final product)
Muesli with emmer, apple and crunchy soybean		
Cereal flakes	Rye flakes	30
	Oat flakes	10
	Puffed emmer	10
Nuts	Hazelnut crumbs	10
Seeds	Sunflower seeds	5
Dried fruits	Cinnamon dried apple	5
	Dried apple	20
Pulses	Crunchy soybeans	10
Superfood muesli		
Cereal flakes	Barley flakes	30
	Puffed quinoa	10
	Puffed amaranth	5
Nuts	Almond crumbs	10
Seeds	Sesame seeds	10
Dried fruits	Raisins	15
	Dried blueberries	10
Pulses	Puffed and toasted lentils	10
Muesli with whole cereal flakes		
Cereal flakes	Whole wheat flakes	15
	Whole rye flakes	15
	Whole emmer flakes	15
Nuts	Walnuts	10
Seeds	Pumpkin seeds	10
	Sunflower seeds	10
Dried fruits	Raisins	15
Pulses	Puffed and toasted lentils	15
Muesli with whole cereal flakes and strawberries		
Cereal flakes	Whole wheat flakes	20
	Whole rye flakes	15
	Whole emmer flakes	15
Nuts	Walnuts	10
Seeds	Pumpkin seeds	10
Dried fruits	Raisins	15
	Dried strawberries	5
Pulses	Puffed and toasted lentils	10
Muesli with dried fruits		
Cereal flakes	Whole rye flakes	25
	Puffed millet	15
Nuts	Almond crumbs	10
Seeds	Pumpkin seeds	10
Dried fruits	Raisins	15
	Dried apple	5
	Dried orange	10
Pulses	Puffed and toasted lentils	10

Values are expressed as % in 100g of the product

Table 4-5. Nutritional values of muesli formulations, 3rd phase.

Muesli formulation	Nutritional composition (in 100g of the product)								
	Energy		Fats (g)	Saturated fats (g)	Carbohydrates (g)	Sugars (g)	Fiber (g)	Proteins (g)	Salt (g)
	kJ	kcal							
Muesli with emmer, apple and crunchy soybean	1589.0	378.0	11.0	1.0	51.0	20.0	9.0	14.0	0.00
Superfood muesli	1624.0	387.0	11.0	1.0	54.0	18.0	10.0	12.0	0.01
Muesli with whole cereal flakes	1672.0	399.0	15.0	2.0	47.0	11.0	8.0	16.0	0.01
Muesli with whole cereal flakes and strawberries	1621.0	386.0	12.0	2.0	51.5	14.0	9.0	13.0	0.01
Muesli with dried fruits	1611.0	383.0	11.0	1.0	55.0	21.0	6.0	14.0	0.01

Values are expressed in 100g of the product.

After the third phase, the focus group was organized and small changes to the recipes were proposed to get the final formulations (Table 4-6).

After the third evaluation step, formulations were modified according to both internal and focus groups feedbacks. In particular, it was decided not to further develop the “Superfood muesli” recipe because of the feedbacks reported after the focus group. Indeed, according to the focus group participants, the taste of quinoa and amaranth was considered unpleasant. At the same time, the recipe “Muesli with dried fruits”, which was characterized by the highest amount of dried fruits, was modified to make the general taste more pleasant for consumers (adding oat flakes and pumpkin seeds and replacing dried orange with dried apricot). The muesli was renamed “Muesli with millet, oat and dried fruits”.

Nutritional values of the final muesli formulations are presented in Table 4-6. Figures of the final muesli products are reported from Figure 4-1 to Figure 4-4.

Table 4-6. Nutritional values and ingredients of muesli formulations, final recipes.

Muesli formulation	Nutritional composition (in 100g of the product)									Ingredients (% in 100g of the product)
	Energy		Fats (g)	Saturated fats (g)	Carbohydrates (g)	Sugars (g)	Fiber (g)	Proteins (g)	Salt (g)	
	kJ	kcal								
Muesli with emmer, apple and crunchy soybean	1600.0	381.0	10.9	1.1	51.9	10.6	8.9	14.2	0.01	Rye flakes (30g), oat flakes (10g), puffed emmer (10g), hazelnut crumbs (10g), sunflower seeds (5g), cinnamon dried apple (5g), dried apple (20g), crunchy soybeans (10g)
Muesli with whole cereal flakes and berries	1494.0	354.0	5.8	1.2	58.2	18.2	10.1	12.3	0.01	Whole wheat flakes (10g), whole rye flakes (30g), whole emmer flakes (15g), pumpkin seeds (10g), raisins (15g), red berries (5g), dried strawberries (5g), puffed and toasted lentils (10g)
Muesli with whole cereal flakes and walnuts	1494.0	354.0	5.8	1.2	58.2	18.2	10.1	12.3	0.01	Whole wheat flakes (15g), whole rye flakes (15g), whole emmer flakes (15g), walnuts (10g), pumpkin seeds (10g), sunflower seeds (5g), raisins (15g), puffed and toasted lentils (15g)
Muesli with millet, oat and dried fruits	1565.0	372.0	8.9	1.1	56.2	23.7	6.9	13.2	0.01	Oat flakes (15g), rye flakes (15g), puffed millet (10g), almond crumbs (5g), pumpkin seeds (10g), raisins (15g), dried apple (10g), dried apricot (10g), puffed and toasted lentils (10g)

Nutritional composition values are expressed in 100g of the product. Ingredients are expressed as % in 100g of the product.



Figure 4-1. #Mordilanatura Colfiorito® muesli with emmer, apple and crunchy soybean.



Figure 4-2. #Mordilanatura Colfiorito® muesli with whole cereal flakes and berries.



Figure 4-3. #Mordilanatura Colfiorito® muesli with whole cereal flakes and walnuts.



Figure 4-4. #Mordilatura Colfiorito[®] muesli with millet, oat and dried fruits.

Focus group results

The focus group experience revealed that for consumers the concept of a healthy diet must be considered in parallel with product taste. According to consumers, a food product must be tasty (with a good flavour) and healthy (high nutritional value, high quality raw materials, certain origin of ingredients) at the same time. Indeed, product taste is one of the key aspect to face when designing a new product as taste guide food preferences in both children and adults [96]. Moreover, food product is required to be pleasant at a first sight and in fashion. These two characteristics allow a product to be perceived good for health by the general public. In the last years there has been a growing interest in foods that are considered healthy, for example foods containing the lowest concentration of sugar as possible [97] or protein rich foods, in particular considering those rich of plant-proteins [98]. In addition, there is a great interest for superfoods, such as quinoa, red berries and seeds. It emerged that superfoods were perceived healthy, especially by participants constituting the older group in Milan (“these foods are good for health”).

Muesli appeared to be a successful product. Indeed, positive feedbacks emerged by focus group participants regarding muesli formulations: “tasty, healthy, crunchy and satisfy appetite”, “perfect for a fast and healthy breakfast without renouncing to a good taste”, “light but giving a feeling of satiety, you don’t feel guilty if you add more muesli in the yogurt as it happens for biscuits”.

An interesting aspect to take into account was that muesli was perceived as a healthy product by participants, thus to be preferred to “cappuccino and brioche” for breakfast.

Furthermore, information collected regarding the shelf sight underlined that consumers generally tend to notice those products appearing more delicious and containing particular ingredients (such as coconut, apricot, spices like cinnamon). A great attention also emerged for neat packages evoking naturalness and simplicity (“naturalness reminds to quality ingredients”) and that allow the possibility to inspect ingredients inside.

Participants’ opinions on muesli formulations:

- *Muesli with emmer, apple and crunchy soybean*. This recipe acquired encouraging results in all the 3 focus groups organized. The aspect at first sight was successful mainly due to the colour of the different ingredients. Participants particularly appreciated the crunchy texture and the taste and the smell were considered pleasant thanks to the presence of cinnamon.
- *Superfood muesli*. This variant seemed original and innovative for participants. At the same time, it resulted to divide strongly their opinion due to the presence of quinoa, which was considered to negatively influence the general taste making it be unpleasantly vegetal and toasted. Moreover, the presence of quinoa negatively influenced the texture of the product.
- *Muesli with whole cereal flakes*. This formulation was appreciated especially by those participants that usually consumed cereals in their diet. The general taste of the muesli was perceived likable although not sweet enough above all for consumers that prefer sugar taste.
- *Muesli with dried fruits*. Also in this case, the aspect at first sight appeared immediately captivating due to the colour of the different ingredients used and original in the ingredient composition. However, this recipe was the less appreciated in terms of flavour, because dried orange is not appraised unanimously by participants (“I don’t like candied fruits even in panettone”). In this case, using marketing terminology, it is possible to state that, for what concerning the taste of the product, dried orange causes the formation of “segments” in the sample population resulting in opposite opinions on the recipes appreciation.

For what concerns the brand of the company, it was evidenced that the brand was perceived as a guarantee of the quality of the product, even if product categories were different from traditional cereals and pulses sold by the company.

Moreover, participants appreciated the attention the company gave for the development of a healthy and high nutritional quality product through the engagement of nutritionist and researchers of the University of Camerino. The collaboration with an institute of research was seen as a symbol of the high quality of the product.

In parallel, it was necessary to consider the price for product proposal. Referring to participants' opinion, the price of the muesli appeared to be in line with muesli competitor in the market.

Discussion

For the development of muesli formulation a particular attention was given to the selection of the ingredients. Cereals flakes constitute a fundamental muesli ingredient. Flaking consists in the thermal treatment of cereals following by rolling to obtain flakes. It is an interesting food processing method as it induces starch gelatinization increasing amylose content [99] and, consequently, positively affecting the glycaemic index of the food [100]. Moreover, cereal flakes give a valuable contribution as a source of carbohydrates for a balanced breakfast.

In addition to cereal flakes, it was decided to add puffed cereals (emmer and millet), as they are particularly appreciated by consumers for their consistency and taste [84].

Cereal puffing implies steaming cereals at high pressure and temperature [101]. The heated vapour produced inside the grain steams the cereal and causes its controlled expansion [102]. Cereal expansion is linked to processing temperature and amylose content of the cereal [103]. Moreover, puffing does not produce remarkable changes in the nutritional composition of the product but it enhances its sensorial quality [104]. Therefore, the addition of puffed cereals appeared to be a cardinal choice not only for their nutritional quality but also in terms of taste appreciation.

The addition of pulses was a strategy to improve the nutritional quality of the product. Indeed, starting from the consideration that pulses and cereals are complementary in terms of essential amino acids [74], their combination allowed to have a balance in terms of protein quality.

Furthermore, the addition of pulses to the muesli was proposed to deconstruct the idea that pulses should be commonly consumed for lunch or dinner and to push hesitant pulses consumers to taste them in an unconventional way.

Pulses were proposed as puffed or toasted pulses. More specifically, puffed lentils and toasted soybeans were added alternatively in the four muesli formulations.

To obtain puffed pulses, pulses are firstly soaked, then they undergo a high temperature (250°C) process for 15-25 s [105]. For toasting pulses are dry heated at 150-200°C [106]. The toasting process positively influences the nutritional composition of the product: indeed, toasting induces a reduction of anti-nutritional factors [107-108].

Nuts and seeds are relevant ingredients from the nutritional perspective. Nuts contain unsaturated fatty acids [109] and fiber [110] which value the muesli mixes. Indeed, it is widely known that a

regular consumption of nuts is beneficial for health [111]. A negative aspect related to nuts use is their cost. Therefore, it was essential to find a compromise between the nutritional contribution and the cost aiming to offer to consumers a nutritionally valuable and economically affordable food product.

Dried fruits are a characteristic component of muesli formulation present in the market (i.e. raisin, berries). They may represent a source of different nutrients (i.e. dietary fiber, minerals) in the diet [112]. For their natural sweet taste, they allow to avoid the use of added sugar in recipes. Dried fruits selected for the four mixes were naturally sweet, with no sugar added.

Starting from ingredient selection, five different formulations were developed and, after internal test and focus groups, four of five were commercialized. The project carried out the formulation of four different muesli products. Four evaluation phases were necessary in order to get the final products.

Moreover, the percentage of nuts in all the formulations was reduced because, from an analysis of the costs, nuts emerged to significantly contribute to an increase of the final product cost. Anyway, in order to maintain a similar nutritional value, the percentage of seeds was in parallel increased.

In addition, in some formulations, dried fruits were substituted with each other to optimize the pleasantness of muesli taste (i.e. dried strawberries were substituted by dried blueberries in the second attempt while in the last recipe, dried lemon was substituted with dried apple as the mix of citrus fruits seemed to give a strong and unpleasant taste to the mix).

The focus group activity was essential to evaluate consumers' acceptance of muesli formulations. Indeed, through the focus group, consumers' opinion and expectations came into light.

Product taste is one of the key aspect to face when designing a new product as taste guide food preferences in both children and adults [96]. Moreover, food product is required to be pleasant at a first sight and in fashion. These two characteristics allow a product to be perceived good for health by the general public. In the last years there has been a growing interest in foods that are considered healthy, for example foods containing the lowest concentration of sugar as possible [97] or protein rich foods, in particular considering those rich of plant-proteins [98]. In addition, there is a great interest for superfoods, such as quinoa, red berries and seeds.

Regarding recipes, after the tasting, it was pointed out that ingredients appeared captivating and the taste of the four formulations was gratifying. The general feedback was that recipes proposed were more appreciated by participant consumers who looked for a healthy food product compared to the glutton ones.

In particular, the presence of pulses (puffed and toasted lentils and toasted soybeans) induced a positive impact on participants, for the growing interest in reducing the consumption of animal derived food proteins among consumers. The proposal of lentils as an ingredient was particularly appreciated, even though the crunchy taste pulses bring to muesli made it to be accepted also in the case of soybeans. The participants showing doubts on pulses flavour at the beginning changed their mind after the tasting.

According to participants, pulses added nutritional value to the product. At the same time, it emerged attention to the muesli peculiar information reported in the nutritional label, which must be consequently better highlighted and differentiated for each muesli recipe.

Studied and developed muesli are currently available in the most common supermarkets. The launch of the product registered an active feedback from consumers and a positive sale trend in the last two years, wishing to gradually increase in time (data can not be disclosed since they are considered confidential by the company).

This project was conceived to contribute to the development of food products containing pulses with optimal nutritional properties able to positively impact on the diet quality of consumers.

Pulse content seemed to raise the interest of consumers and to be appreciated in terms of taste and texture, especially by those consumers who are more sensitive to a healthy nutrition, as resulted from the focus group evaluation. Indeed, it has been shown that healthy diet has been associated to pulse consumption and to a sustainable diet [113].

In the last years, consumers are becoming more interested in plant-based diets as they are perceived healthy diets [114]. Not only, plant-based dietary regimens are environment friendly and devoted to planet health [115]. At the same time, the care for planet health has got a global urgency, as described in the Agenda 2030 [116].

In this context, finding strategies to promote plant-based products consumption has acquired a central role in the diet. Of course, it is essential to support pulse and pulse-based products intake.

In this perspective, the role of the companies selling pulses could be pivotal. The proposal of the Fertitecnica Colfiorito s.r.l. company was to promote pulse consumption going forward the trade of the most traditional pulses, trying to develop pulse-based products that could be easily used in unexpected occasions of consumption, such as breakfast or snacks. This strategy could be useful to push the most hesitant consumers to eat pulses as muesli does not need to be prepared, but just open and consumed. Indeed, according to Duarte et al. the high cooking time is one of the reasons that makes pulses to be avoided by consumers [117]. Pohjolainen et al. found that preparing

vegetarian foods represents a difficulty for consumer, consequently representing a barrier in their consumption [118]. A similar conclusion was given by Smiglak-Krajewska et al. who underlined that preparing a meal with pulses gets complicated, but the real challenge is to cook a tasty meal [119]. Therefore, it appears necessary to find new strategies to support pulse intake, starting from the consideration that pulse cooking time should be reduced and the taste should be satisfactorily hidden.

In addition, pulse-based snacks or breakfast foods could potentially represent an option to reduce the consumption of other pre-packaged products, often characterized by a high sugar content [120, 121]. In the case of #Mordilanatura Colfiorito® muesli, the presence of dried fruits allows to avoid sugar addition. Moreover, as all ingredients are of plant origin, no saturated fats are present. This aspect is fundamental considering the increasing interest of consumers for healthy products [122, 123].

Indeed, looking at the nutritional label of the muesli, it can be appreciated that the list of ingredients is short, meaning few ingredients can be found in the product: no sugars added, preservatives, additives, colorants, artificial flavourings and palm oil were used for muesli production. Moreover, thanks to the ingredient composition and combination, muesli formulations have a high nutritional quality as shown by nutritional claims reported on the food package. The reported nutritional claims are in accordance with the EU regulation no. 1924/2006 [124] and with the EU regulation no. 1047/2012 [124]. In particular, according to EU regulation no. 1924/2006 [124], food labels reported the following nutritional claims:

- source of proteins and rich in fiber (muesli with whole cereal flakes and berries),
- source of proteins and potassium (muesli with emmer, apple and crunchy soybean),
- source of proteins and fiber (muesli with whole cereal flakes and walnuts),
- source of proteins and vitamin E (muesli with millet, oat and dried fruits).

Moreover, these muesli formulations represent a healthy and adaptable food alternative for consumers independent of the age and gender. Indeed, the #Mordilanatura Colfiorito® muesli can be part of a balanced diet for children, adolescents, adults and the elderly.

Conclusions

The idea to introduce pulses in a muesli revealed to be an effective way to support eating pulses but also to improve the nutritional quality of the muesli, especially in terms of fiber and protein content.

The muesli products developed are currently available in the market and are recording a positive feedback from consumers. Indeed, after the first two years of product introduction in the market, muesli with emmer, apple and crunchy soybean and muesli with millet, oat and dried fruits showed the highest trades (27980 and 27156 pieces sold, respectively), with sales currently increasing. At present, other projects are in progress to design muesli formulations with different ingredients aiming to maintain the nutritional quality of the previous ones. New flavours will be tested in order to develop more palatable foods in line with the preferences of the pickiest consumers because following a healthy diet does not exclude enthusiasm and pleasure for foods.

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Chapter 5. Fertitecnica Colfiorito s.r.l. collaboration: secondary projects

General introduction

The collaboration with Fertitecnica Colfiorito s.r.l. led to the development and conception of three projects which were carried out during the entire duration of the PhD course.

All of them have a common objective: encourage pulse consumption through the proposal of innovative pulse-based food products, each one developed to reach different consumers' categories.

Bottega Colfiorito®: an e-commerce project to promote pulse consumption

Introduction

Today, pulse consumption is a critical aspect in the diet, despite the known health benefits derived from their intake [1]. Consumers do not choose pulses in their diet for several reasons, such as long cooking time, flatulence connected to their intake and the lack of innovative pulse-based products in the market [2-4].

In order to promote pulse consumption in the diet of consumers, it was proposed to design an e-commerce project named Bottega Colfiorito®.

The project was conceived as an on-line shop where consumers can buy customized packages of company products. The idea to allow on-line purchases was proposed to face COVID-19 pandemic, giving the consumers the possibility to choose and order products directly from the company website.

Materials and methods

For the realization of the customized packages, five different target of consumers were identified: sportsmen, families, fast cooking consumers, traditional foods consumers and organic foods consumers. The five targets were defined following structured marketing analysis.

In each pack, selected company products were proposed. For the selection of the products, the following criteria were taken into account:

- products nutritional characteristics (referring to the technical sheets of the products),
- consumer target nutritional requirements [5],
- meal occasions (breakfast, snacks, lunch and dinner),
- time for meal preparation and consumption.

Each package was also provided by general dietary suggestions to promote pulses use and to guide consumers in understanding how frequently pulses should be consumed and how to combine them to get a balanced plate, making references to the “Healthy eating plate” [6].

Hereafter some examples of dietary suggestions are reported [6, 7]:

- in order to have a healthy life style, it is essential to follow a balanced diet and to keep yourself physically active,
- consume 5 meals each day, starting from a nutritionally complete breakfast. Meals should not be skipped and snacks are fundamental to modulate satiety and energy balance during the day,
- consume balanced meals in terms of energy and nutrients, making reference to the “Healthy eating plate”,
- consume cereals and cereals derivatives on a daily basis is fundamental to have a balanced diet,
- consume pulses at least 2 times a week,
- vary food choices in the diet the most as possible,
- consume your meals in company as conviviality is essential for psychophysical wellbeing,
- try to involve children in meals preparation to encourage their interest in tasting unknown foods,
- dedicate time to meal consumption and avoid the use of technological devices during meals.

Furthermore, the project wished to support informed food choices as it is worth knowing that healthy dietary behaviors allow to contribute to a more sustainable future [8, 9].

Each package contains pulses and cereals (such as bean, peas, chickpeas, lentils, cous cous and soups) and/or innovative food formulations (such as pulse-based snacks, #Mordilatura Colfiorito® muesli, fast-cooking products). Nutritional composition of products was the starting point of products match. Products were combined according to their macronutrient composition (i.e. proteins, carbohydrates and fiber) and dedicated to the needs of target consumers. In addition, in order to cover all the daily meals, all packages contain products for specific meals (i.e. muesli for breakfast, legume-based snacks for break moments, pulses for lunch and/or dinner).

Each package contains both cereals and pulses as basic products.

Results

The goal of this project was to realize five different packages containing different company food products. All the combination developed are listed in Table 5-1.

Table 5-1. Bottega Colfiorito® packages.

Bottega Colfiorito® packages				
Sportsmen	Family	Fast cooking	Traditional foods	Organic foods
Muesli with whole cereals and red fruits	Muesli with puffed emmer, crunchy soybeans and apple	Ready to eat dish with barley, emmer, millet, Khorasan wheat	Italian lentils	Organic toasted pumpkin seeds
Snack with legume flakes, pumpkin seeds, almonds, raisin, blueberries	Snack with legume flakes, pumpkin seeds, almonds, raisin, blueberries	Ready to eat dish with barley, emmer, black beans, lentils, Khorasan wheat	Borlotti beans	Organic toasted sunflower seeds
Snack with legume flakes, pumpkin seeds, hazelnuts	Snack with legume flakes, pumpkin seeds, almonds, raisin and dry apple	Pearled emmer	Emmer soup	Organic toasted flax seeds
Cereals and legumes soup	Cannellini Beans	Cereals and legumes soup	Hulled chickpeas	Organic Italian lentils
Cous cous	Italian lentils	Vegetables soup	Hulled wild peas	Organic Italian Cannellini beans
Italian lentils	Barley soup	Snack with legume flakes, pumpkin seeds, almonds, raisin, blueberries	Cannellini beans	Organic Italian cous cous
Mixed pulses	Hulled chickpeas	Hulled yellow lentils	Mixed pulses	Organic Italian emmer soup
Hulled chickpeas	Popcorn maize	Seeds mix for salads	Hulled yellow lentils	Organic quinoa
	Cereals and legumes soup			
	Cascina soup			

“Sportsmen”

The package “sportsmen” was designed to go towards the needs of physically active consumers. In each pack some general nutritional advices were provided. In particular, pulse-based snacks and #Mordilana natura muesli can be used both for breakfast and/or for snacks. Their consumption was thought to be specifically used as post-exercise snack, in association with milk or yogurt [10], aiming to get a complete energy and nutrients recovery.

Among the company cereals available, the one chosen for this package was cous cous. It is necessary to underline that carbohydrates are essential nutrients in the diet, especially for subjects performing physical activity. Indeed, carbohydrates are needed to get energy during the performance and to

recover energy stores at the end of the performance [10]. At the same time for sportsmen, it is fundamental to modulate the general fiber intake, in particular in the meals around the exercise [10, 11]. Indeed, the proper modulation of fiber consumption is necessary before the exercise to avoid intestinal problems during the performance and after the exercise to guarantee nutrient absorption [12]. Consequently, among the company cereal available (emmer, barley, cous cous), cous cous appeared to be the optimal candidate because of the lowest fiber -content.

Considering the needs to limitate the fiber intake, the consumption of pulses should be controlled and restricted to rest days. To moderate fiber intake,-pulses with a naturally minor fiber content were selected in addition to hulled pulses, which are characterized by a general lower fiber concentration because of the elimination of the hull [13] .

The Sportsmen package, composed as described in Table 5-1, is reported in Figure 5-1.



Figure 5-1. Bottega Colfiorito® sportsmen package.

“Family”

The family package resulted by a combination of company products that can be used by consumers of different ages. #Mordilatura Colfiorito® muesli and pulse-based snacks are proposed as they can be consumed by both adults and children. The muesli and pulse-based snacks do not contain added sugars, salt and preservatives consequently they contribute to get a nutritionally balanced and healthy breakfast or snack.

Moreover, the package contains a mix of pulses and cereals and two soups to encourage all family members to consume pulses weekly, always considering the “Healthy eating plate” as reference to get a balanced plate [6]. Soups were proposed especially considering that they can be appreciated by the elderly, in terms of taste and texture.

As this package was designed for families, children were taken into account. Indeed, the package contains corn seeds useful to prepare air-popped popcorn, a cereal based healthy snack. The family package composition is reported in Table 5-1 and it is presented in Figure 5-2.



Figure 5-2. Bottega Colfiorito® family package.

“Fast cooking”

Fast cooking package was thought for consumers who do not have time for cooking. The idea was to support a healthy pulse-based diet even when subjects are in a hurry or at work. Indeed, the package contains cooked products or fast cooking product constituted by ready to eat pulses and cereals meals-

In this context, also pulse-based snacks can be useful. These snacks contain legume flakes, seeds, nuts and with dried fruits being the only ingredients to give sweetness to the product. Consequently, they are a valid snack from the nutritional perspective.

They are rapid to be consumed, for example at work, and they can be an alternative snack to prefer to processed foods available in vending machines.

In addition, this pack is characterized also by a mix of seeds for salads, that is proposed to give to fast-cooking dishes a higher nutritional value. The package can be found in Figure 5-3.



Figure 5-3. Bottega Colfiorito® fast cooking package.

“Traditional foods”

The traditional foods package was designed to respond to the needs of those consumers who prefer to choose more basic foods, consistent with Italian regional culinary tradition. Indeed, in the pack there is a mix of the most consumed pulses wrapped as single products, such as lentils, beans, peas and chickpeas. The idea was to create a pack with the most typical and basic food products of the company in order to satisfy the requests of those consumers who may be less geared in using innovative foods. The traditional food package is presented in Figure 5-4.



Figure 5-4. Bottega Colfiorito® traditional food package.

“Organic foods”

The package with organic foods was conceived for those consumers who are more sensitive to environment-related issues and to sustainability, as it has been pointed out that organic production has less environmental impact than conventional methods [14]. The pack contains cereals, pulses and seeds from organic production. Moreover, this pack is distinguished by the presence of quinoa,

a pseudocereal whose consumption has been spread in the last years for its interesting nutritional properties [15, 16]. The traditional food package is presented in Figure 5-5.



Figure 5-5. Bottega Colfiorito® organic foods package.

Discussion

The Bottega Colfiorito® project was designed aiming to increase pulse consumption and to provide to consumers opportunities to have access to pulses directly at home in the state of emergency for Covid-19 pandemic.

The project was conceived as an update of the on-line shop where consumers could buy Colfiorito products. Indeed, instead of buying single products, Bottega Colfiorito® gave the possibility to chose customized packages (characterized by several products) designed and developed considering different consumers' needs, ages and lifestyles. As previously mentioned, promoting pulse intake was the primary aim of the project, anyway nutrition education of consumers still remains one of the objectives of the company. Therefore, the Bottega Colfiorito® webpage and each package were provided of nutritional suggestions wishing to positively impact on consumers' dietary habits and lifestyle. In addition, this was proposed during COVID-19 pandemic, giving the consumers the possibility to choose and order customized packages directly from the company website.

Conclusions

Bottega Colfiorito® represents a pilot project wishing to encourage pulse consumption through the proposal of food packages directed towards specific consumers' targets. The proposal of targeted packages containing pulses underlines the possibility that pulses can be introduced in the diet of all consumers, thus fostering consumers to have healthy diet.

Piatto fatto®: Italian regional recipes for new ready to eat cereals and pulse-based products

Introduction

Today, ready to eat foods are catching out rapidly as the time dedicated to cooking is less and less due to the contemporary chaotic life-style. In this context, the risk to neglect care for healthy foods is high, possibly causing health problems in the long-term. Moreover, the consumption of pulses in particular is even less considered for long preparation and cooking time [2].

Despite the lack of time to devote to cooking, maintaining a healthy diet and life-style is fundamental and cannot be postponed. At this regard, Fertitecnica Colfiorito s.r.l. company decided to conceive and develop cereal and pulse-based ready to eat foods suitable for hurried consumers. Piatto fatto® constitutes a product line designed to have a nutritious ready to eat dish without renouncing to a good taste. These products are pulse and/or cereal-based ready to eat foods that can be consumed everywhere as they just need a warming-up.

The project was conceived to implement previous recipes with new proposals. As this project is still on going, only the general conceptualization of recipes is described.

Material and methods

Confiorito company produces a line of product called Piatto fatto®.

Actually, Piatto fatto® line includes three different recipes. The ingredients of each recipe are the following:

- buckwheat, red quinoa and chia seeds,
- red rice, barley, emmer, millet and durum wheat,
- lentils, black beans, barley, emmer and durum wheat.

The request of the company was to increase Piatto fatto® selection of products by adding new healthy recipes based on traditional dishes.

For the designing of the new Piatto Fatto® recipes, it was decided to take inspiration from the traditional Italian recipes containing pulses.

Indeed, a preliminary investigation on Italian regional recipes was performed. Recipes were selected after a marketing analysis on consumers' preferences (data can not be disclosed since they are considered confidential by the company).

To develop new Piatto fatto® products, the new recipes were based on three traditional dishes:

- Vignarola, a Roman soup,
- Onano lentils soup,

- Chickpeas soup of Marche Region.

Detailed ingredients were presented in Table 5-2.

Table 5-2. Ingredients traditionally used for the preparation of selected Italian regional recipes.

Recipes	Ingredients			
	Pulses	Vegetables	Other foods	Spices or aromatic herbs
Vignarola	Broad beans and peas	Lettuce, artichoke, onions	Cheek lard	/
Onano lentils soup	Lentils	Tomatoes, carrots, onions, celery	Sausages	Laurel oak
Chickpeas soup	Chickpeas	Tomatoes, carrots, onions, celery	Spare ribs	Rosmary

Results

After the selection of the recipes, a modification of basic ingredients was performed in accordance with the Healthy Eating Plate proposed by Harvard T.H. Chan School of Public Health [6].

With the aim of making the recipes healthier, the ingredients have been modified as shown in Table 5-3.

For the initial formulation of these recipes, both gluten-containing and gluten-free cereals were used. Pseudocereals are characterized by a high nutritional profile [17, 18], consequently their consumption should be suggested to consumers, especially as a valid substitute of wheat [19]. Starting from these considerations, pseudocereals were proposed also in the new *Piatto Fatto*[®] recipes. Indeed, recipes rimodulation included pseudocereals, like buckwheat, but also other cereals, such as spelt and barley. In addition, animal-derived products (such as cheek lard, sausages and spare ribs) were substituted with pulses (such as wild pea, red lentils and beans). The combination of cereals and pulses allowed to get a balance in terms of essential aminoacids [20] and to provide to consumers exclusively plant-based products.

Table 5-3. Chosen ingredients for each studied Italian regional recipe

Recipes	Ingredients				
	Pulses	Cereals	Vegetables	Other foods	Spices or aromatic herbs
Vignarola	Broad beans and peas	Barley	Lettuce, artichoke, onions	Wild pea	/
Onano lentils soup	Lentils	Spelt and buckwheat	Tomatoes, carrots, onions, celery	Red lentils	Laurel oak
Chickpeas soup	Chickpeas	Barley	Tomatoes, carrots, onions, celery	Beans	Rosmary

Discussion

The idea was to provide to consumers a wider selection of pulse-based products by renewing Piatto Fatto® products line, starting from the proposal of new recipes. For recipes conceptualization and elaboration, the evaluation of Italian regional recipes containing pulses and cereals were analysed and remodulated. Animal derived products were substituted by other pulse types in order to foster pulse use as the main protein source in the dish. Moreover, in all the recipes pulses were combined with cereals as suggested to get a balanced plate in terms of essential amino acids [20, 21]. Recipes rimodulation allowed to conceptualize high nutritional quality ready to eat products, with no cholesterol and a low saturated fats content and, in parallel, characterized by the exclusive presence of plant-proteins from pulses and cereals. Therefore, the substitution of animal derived products with pulses made these Italian regional recipes adequate to vegetarian and vegan consumers. Further developing steps are now ongoing in order to obtain marketable products.

Conclusions

New healthy recipes were studied in order to improve nutritional quality of existing meals but still maintain their traditional characteristics. The concept of moving toward a more plant-based diet is at the base of recipes conceptualization as the company is starting to have more interest in the development of plant-based products also suitable for vegetarian and vegan consumers.

Beginning of a new project: Fertitecnica Colfiorito s.r.l. in the weaning context

Introduction

The importance of pulse consumption is fundamental not only for adults but also for children [22-24]. Starting from the scientific evidence supporting the health benefits of pulse intake in children [23], it was conceived to encourage pulse consumption since an early age by starting a new project focusing on complementary feeding supported by the competences of a paediatrician and mine. Complementary feeding or weaning refers to the transition from a liquid-based nutrition (breast milk or infant formulas) to a mixed nutrition, including semi-solid and solid foods [25]. According to the World Health Organization (WHO) breastfeeding must be the exclusive feeding for infants for nutritional and developmental reasons [26, 27].

The European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) stated that this transition generally occurs not before the 17 week of age and not later than the 26 weeks of age, when breastfeeding starts to be flanked by the first tastes of foods [28].

At the beginning of complementary feeding breast milk is the most important source of energy and nutrients for the child and conventional foods represent just a corollary [29]. When proper conditions are established (i.e. adequate development of the gastrointestinal tract, control trunk and lean capacity, mature chewing, evident interest in tasting foods), complementary feeding becomes relevant. Consequently, adequate food choices are essential for baby nutrition. Moreover, this phase is fundamental as it constitutes part of the 1000 days of life, which are crucial ones to establish the conditions for a long-term health [30, 31].

At this regard, nutrition education of the entire family becomes fundamental to guarantee a healthy and balanced nutrition for the baby and to avoid malnutrition [32]. In this context, nutrition education directed to families and especially to mothers is of main importance [33, 34].

Complementary feeding is a delicate phase for the infant as it represents the beginning of new sensory experiences and of gradual dietary changes [35]. It affects infant growth and development and it is crucial to lay the foundations for long-term health [30].

In this context, taking into considerations infant nutritional needs becomes of primary importance. In particular, it is necessary to consider fat quality, sugar intake and above all of protein consumption and quality [30].

The intake of polyunsaturated fatty acids, especially of n-3 and n-6 fatty acids, is important for infant health, in particular for cognitive and immune system development [36, 37].

In parallel, sugar intake must be carefully moderated. Indeed, sugars provide calories without having an essential nutritional value [30]. Consequently, sugar consumption must be reduced the most as possible and sugar containing foods and beverages must be avoided as their intake has been associated with an increased obesity risk in children [38].

Numerous studies evidenced that during complementary feeding, it is pivotal to pay attention to protein consumption, indeed an association between high protein intake in this period and later obesity has emerged [30, 39-41].

As a consequence, protein intake during this phase must be carefully modulated. Moreover, it has been underlined that also the type of protein source to choose needs to be taken into account [42], actually, the choice of plant-protein sources should be preferred [43]. Pulses are consequently optimal foods to be firstly introduced in complementary feeding, therefore it is essential to divulge pulse consumption benefits to promote their use in the diet [44, 45].

The main concern related to pulses introduction regards fiber and anti-nutritional factors content. The amount of fiber intake must be modulated in complementary feeding [46] in order not to compromise nutrient absorption. At the same time, it is worth considering that fiber consumption positively impacts on obesity and diabetes development risk at adult age [47].

Beyond fiber, pulses are characterized by the presence of anti-nutritional factors, for example trypsin inhibitors, tannins, phytates, phenolic compounds and saponins [48] that can compromise nutrient absorption [49]. This condition should be prevented in order to avoid mineral imbalances during complementary feeding [50, 51].

In order to reduce the concentration of anti-nutritional compounds, it is suggested to carry out the everyday domestic processing such as soaking and cooking (i.e. boiling) [52]. Consequently, taking advantage of these techniques, pulse consumption is recommended for infants to perform an optimal weaning.

The company project was designed with the aim to valorise pulse consumption in infants' diet by introducing selected company products in stores for infants' items.

Company products were so evaluated for their suitability for weaning, considering textures and consistences.

Material and methods

At this regard, it was decided to select those company products adequate to match infants' needs. Cereals, pulses and seeds selected are described in Table 5-4, Table 5-5 and Table 5-6. A suggested timing for product administration to the infant is also provided considering infant development [53, 54].

Table 5-4. Cereals selected for the project.

Cereals	
Starting from 7th months	Starting from 10th-12th months
Cous cous	Barley
Quinoa	Pearled emmer
Hulled millet	
Hulled buckwheat	

Table 5-5. Pulses selected for the project.

Pulses	
Starting from 7th months	To introduce as complementary feeding proceeds
Yellow or red hulled lentils	Lentils
Hulled chickpeas	Green Azuki beans
Hulled peas	Peas
Pulses pureed soup (made with chickpeas, lentils or peas)	Chickpeas
	Cannelini beans
	Borlotti beans
	Broken hulled broad beans
	Broad beans

Table 5-6. Seeds selected for the project.

Seeds (sunflower seeds, pumpkin seeds, sesame seeds)	
Starting from 7th months	To introduce as complementary feeding proceeds (after the acquisition of adequate chewing competences by the infant)
It is suggested to propose seeds as a powder in the food.	It is advised to propose seeds in flat pieces according to the development of infant teething. Entire seeds must be tested only after the complete teething development.

Discussion

In complementary feeding, especially at the beginning, it is suggested to consume cereals after a prolonged cooking that allows cereals to be broken up into pieces or to be flattened to get a cream.

The company cereals that can be proposed from the 7th months are cous cous, quinoa, hulled millet and buckwheat as their consistency is ideal for the first tastes. Barley and emmer can be tested later for their fibrous texture and for the bigger shape of the grain. Indeed, it is worth underlying to present to infants only foods in a safe texture and shape.

Whole cereals can be proposed occasionally at the beginning of complementary feeding and only regularly around 2 years old because the higher presence of fiber, compared to refined cereals, may interfere with infant micronutrient absorption [53, 54].

Regarding pulses, hulled pulses should be the first pulse type to be proposed because of the lower fiber content compared to entire pulse grains. At the beginning of complementary feeding, it is suggested to consume pulses after a prolonged cooking in order to break up pulses into pieces or flatten them to get a cream. In case of entire pulse grains, it is suggested to resort to manual hulling or to the use of a vegetable mill. At this regard, pulse pureed soups could be valid alternatives to hulled pulses.

Only when complementary feeding proceeds, entire pulses can be experimented, starting with over-cooked and flattened pulses.

Cereals and pulses soups can be introduced since the beginning of weaning after a prolonged cooking and eventual flattening, to be sure that all soup ingredients are suitable for the chewing capacity of the infant.

Contrary to older nutritional studies, recent scientific evidences demonstrate that seeds can be consumed since the beginning of weaning, as they are not considered allergenic foods [55]. Moreover, seeds contribute to increase the content of healthy fats, whose intake is indispensable in babies' diet [55].

All the products selected are dried and not processed foods. Indeed, according to the nutritional guidelines, processed foods must not be consumed for infant nutrition because of the high salt or sugar content [56]. At this regard, it has been evidenced that salt and sugar intake in the weaning phase can predispose to overweight or obesity development in adult age [57]. Therefore, also salt consumption should be discouraged [58].

Additional considerations were made on other company products, for example #Mordilatura Colfiorito[®] muesli and pulse-based snacks. These products are constituted by cereal flakes, toasted pulses, nuts and dried fruits, all ingredients representing problems at the beginning of baby weaning. Firstly, these ingredients are not safe for infants for the different shapes and consistencies

they have, constituting a risk for asphyxiation. Thus, at the beginning, the mixes should be grounded prior consumption and eventually softened using hot water or yogurt.

Conclusions

Supporting the evidence that pulse intake is beneficial for consumers' health at different ages (from the young people to the adults), pulse consumption should start since weaning [23]. More in general, healthy dietary habits must be established as soon as possible in order to put the basis for the growth of healthy adults [30]. At this regard the company conceived to do the theoretical groundwork for a new project focusing on complementary feeding, which is still in a conceptual phase but aims to become a practical instrument for mothers and families by suggesting the adequate foods for weaning among the variety of company products. In this context, it may be useful to provide information and define examples of the foods to introduce in weaning.

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Chapter 6. Nutrition education to promote pulse consumption in the diet

Introduction

Promoting a healthy diet is a fundamental action to prevent chronic non communicable diseases such as diabetes, obesity, cardiovascular diseases, non-alcoholic fatty liver disease, cancer and other health problems [1, 2]. Despite it is widely known how dietary prevention is necessary, unsuitable eating habits are often common in the population [3]. Several are the factors leading to unbalanced dietary behaviours, such as the availability of processed foods, which are rich in sugar and salt [4], the reduced intake of highly quality and nutritious foods, the lack of knowledge on healthy nutrition [5], continuous snacking behaviour and the absence of physical activity [6].

Consequently, nutrition education awareness raising projects should be encouraged to promote the knowledge of people as patients and consumers. Moreover, it is worth mentioning the need to train students motivated to become nutrition experts as they will be directly in contact with patients and will be responsible for patient's dietary choices awareness [7].

Economic development and globalization led to deep changes in lifestyle, in particular in consumers' dietary choices [8]. Ready to eat meals and snacks appeared to be an easy response to the hurry and the lack of time for food care of modern lifestyle [9]. However, the great availability of these products in the market does not always correspond to high nutritional quality [10]. Consequently, it is essential to stress the importance of increasing the awareness of consumers in food choices.

In this context, university students are a category of consumers to pay attention to. In particular, the change of lifestyle routine for studying and the possibility of living independently could induce them to make unhealthy dietary choices [11]. Although a regular intake of plant-based foods is associated to positive health outcomes and to the prevention of non-communicable diseases [12], vegetables and fruits are not consumed daily among university student and their consumption does not face the recommendations of Mediterranean diet [13].

Beyond plant-based foods consumption, university students appear to commonly skip breakfast or do not pay attention to breakfast nutritional quality [14, 15]. A proper nutritional quality breakfast contributes to improve cognitive abilities and to regulate food consumption in the day [16].

Consequently, it is fundamental to dedicate nutrition education initiatives and programs to university students to support students' awareness for a healthy lifestyle, starting from the adoption of simple dietary behaviours, such as pulse weekly introduction and daily nutritional-balanced breakfast consumption. In particular, it could be of great importance to sensitize bachelor degree students in Nutrition Biology as they will be the guide for consumers' food choices.

In this perspective to support healthy dietary behaviours and to perform sustainable food choices, nutrition education becomes fundamental. Consequently, it is indispensable to educate today's consumers and, in particular, young consumers, from the adolescents to the young, who will be the future consumers.

To support young people food knowledge and dietary choices, nutrition education could be promoted through innovative digital teaching tools.

E-learning at first is considered a valuable strategy for on-line nutrition education [17, 18]. E-learning, or technology education, is based on the development of interactions among users by the employment of Information and Communication Technologies (ICTs) [19]. This methodology amply spread also for the leaning young people have for technology [19]. Technology is intrinsically part of today student life [20], indeed, it could be wisely used also in nutrition education.

Although ICTs have been used for education purposes for several years, it is undeniable that their role become even more central in the context of Covid-19 pandemic [21]. Indeed, ICTs gave the possibility to go on with didactic activities but above all they allowed students to keep in contact, demolishing real distances [22]. E-learning education has been using ICTs as a current didactic tool, indeed, they can be functional in promoting nutrition education [23].

Aim of the study

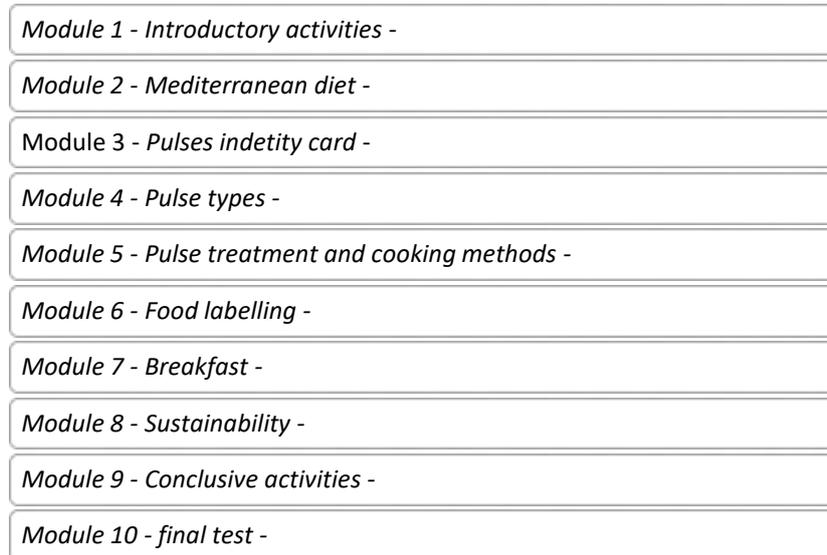
Asseveral studies and nutrition surveillance surveys pointed out that, although pulse consumption is beneficial for health, pulses are not regularly part of the diet [24], the project aimed to develop a web-based nutritional educational course to ameliorate pulse consumption by making university students conscious of pulses nutritional and health properties.

The e-learning course was firstly dedicated to bachelor degree students in Human Nutrition Biology with the aim to test the e-learning course and sensitize future nutrition trainers on this topic.

Materials and methods

The project was developed as an asynchronous on-line course to facilitate participants in accessing the platform according to their personal organization. Participants could start autonomously their distance education course with the possibility to interact with the educator through a forum but without direct relation with them [25]. The nutritional education course was tested in a group of bachelor degree student in Unicam "Human Nutrition Biology" course.

The course was developed as a 10 modules course (Figure 6-1). Each course module included a theoretical part, to provide information on the module item, and a training part, to give students the possibility to apply concretely the knowledge acquired. The training part was developed to make the user be practically involved in the course focus, wishing to ameliorate food choice and dietary behaviour.



<i>Module 1 - Introductory activities -</i>
<i>Module 2 - Mediterranean diet -</i>
<i>Module 3 - Pulses indetity card -</i>
<i>Module 4 - Pulse types -</i>
<i>Module 5 - Pulse treatment and cooking methods -</i>
<i>Module 6 - Food labelling -</i>
<i>Module 7 - Breakfast -</i>
<i>Module 8 - Sustainability -</i>
<i>Module 9 - Conclusive activities -</i>
<i>Module 10 - final test -</i>

Figure 6-1. Course modules.

Each practical activity was preceded by an explanatory session regarding the activity. The tasks proposed consisted in the development of an activity related to the module topic.

Both theoretical and practical activities were structured using the guidelines for highly readable didactic material for students with physical disabilities or learning specific disabilities provided by the UNICAM university service for the reception of students with physical disabilities or learning specific disabilities. Indeed, adequate features and contents (such as fonts, types, colour and background of the slides, text layout, tables, images, glossary) were used for the preparation of the provided materials.

The course was presented to the Nutrition Biology students in an on-line meeting held on 16th April 2021. During the meeting, the course structure, including the modules and, more specifically the theoretical and practical activities, were briefly described. Students were given two weeks to communicate their voluntary adhesion. Moreover, before adhering to the course, students were asked to confirm their participation to the study by providing a written consent.

At the end of the course (Module 10) a final set of questions to test students' acquired knowledge was also provided (Appendix II).

E-learning platform modules:

Module 1 - Introductory activities -

To have a starting snapshot of participants dietary habits and lifestyle, the compiling and the development of the food diary and the PREDIMED questionnaire [26] were proposed as introductory activities.

The food diary is a dietary report to fulfil for 7 days, based on the European Prospective Investigation of Cancer (EPIC) validated food diary [27, 28]. The student has to note the foods and beverages consumed each day, the time and the place for meal consumption and the type and duration of the eventual physical activity performed. The food diary is a useful tool to frame user's dietary behaviour.

The PREDIMED questionnaire was used to evaluate the adherence of students' diet to the guidelines of Mediterranean diet [26]. It consists of 14 questions the participant has to answer. A specific score, 1 or 0, is assigned to the question according to subject's answer according to [26]. The adherence of participants' diet to the recommendations of Mediterranean diet was defined by of the score (≤ 5 low adherence, 6-9 medium adherence, ≥ 10 good adherence).

All introductory activities proposed in Module 1 were proposed again in Module 9 in order to evaluate the efficacy of the educational intervention on participants.

Module 2 - Mediterranean diet -

The module started explaining the origin of Mediterranean diet, its nutritional characteristics and the health benefits it provides. Weekly frequency of consumption of foods were deeply described in accordance with Mediterranean diet pyramid [29].

The training part of this module consisted in the production of a student composition on the role of Mediterranean diet in the globalization context. Students were asked to express their opinion on the newness of this dietary pattern, to describe if it changed compared to the concept of Mediterranean diet by Ancel Keys and how it eventually adapted to the context of modern times (tradition, cultural exchange, migration, science and technology development, cooking leisure).

Module 3 - Pulses indetity card -

This module was dedicated to the description of pulses nutritional properties and of the positive effects of pulse consumption on health. Participants were provided with a power point presentation describing the general nutritional characteristics and health benefist of pulses. Bibliographic references were reported in each presentation.

The training part consisted making a research on pulse health benefits and in the elaboration of a presentation (using power point or other tools).

Module 4 - Pulse types -

Module 4 focused the attention on pulse types. Several pulses types, from the most common to the less known and consumed ones (chickpeas, beans, peas, lentils, soybeans, broad beans, lupin beans and wild peas) were analysed. Power point presentations describing the botanical classification, agronomic and nutritional characteristics, consumption derived health benefits, traditional cooking use and curiosities of the most common pulses, such as chickpeas, beans, peas, lentils, soybeans, broad beans, lupin beans and wild peas were provided. Bibliographic references were reported in each presentation.

The training part regarded the realization of two recipes, a traditional regional pulse-based recipe and a modern version of a traditional one. Participants were invited to prepare these recipes at home and to provide information related on recipe preparation through the development of a video, a power point presentation or a written document where all the steps were presented.

Module 5 - Pulse treatment and cooking methods -

Module 5 faced pulse pre-treatment methods, like soaking, and traditional cooking methods (boiling, pressure cooking, steam cooking and microwave cooking) considering in details the impact of each method on pulse nutritional properties. Moreover, the module presented the general characteristics of other treatment and processing methods (hulling, germination, fermentation, canning, freezing and toasting). Participants were provided with power point presentations regarding the above mentioned issues.

The training part consisted in the realization of a pulse-based ethnic dish to explore the use of pulses in other cultures and culinary traditions. Participants were invited to prepare these recipes at home and to provide information related on recipe preparation through the development of a video, a power point presentation or a written document where all the step were presented.

Module 6 - Food labelling -

Module 6 took into account food labelling making reference to EU 1169/2011 regulation [30].

This module was introduced in the course with the aim to sensitise users as consumers to help them in making conscious food choices. A power point presentation on EU 1169/2011 regulation was administered with a special attention on pulses' labels.

The training part was based on the analysis and critical comment of a pulse-based product label. In particular, participants were asked to evaluate the list of ingredients and the nutritional declaration of selected products.

Module 7 - Breakfast -

Module 7 discussed about breakfast, a fundamental nutrition item.

This module took into consideration the reasons why to have breakfast and, in particular, to consume a balanced breakfast in terms of nutrients, giving several examples. A power point presentation was provided.

The training part asked the user to propose a balanced breakfast and to give an idea of a sustainable pulse-based breakfast. Participants were invited to prepare these breakfast options at home and to provide information related on breakfast options preparation through the development of a video, a power point presentation or a written document where all the step were presented.

Breakfast consumption and breakfast nutritional quality of participants were analysed. In order to evaluate the nutrititional quality of breakfast, the indications of the Healthy eating plate were used [31].

Module 8 - Sustainability -

This module provided supplementary materials on pulse role in diet and environment sustainability. Material was administered as videos and PDF files.

Module 9 - Conclusive activities -

In Module 9 participants had to fulfil the PREDIMED questionnaire [26] and the food diary [27] again in order to evaluate changes in their dietary behaviour after the educational intervention.

Module 10 - Final test -

In module 10, participants were asked to test their acquired competences through an online test. Students were asked to answer to 30 multiple-choice questions on course topics. For each question correctly answered students got 1 point, for each uncorrect answer they got 0. The final score was obtained by the sum of all the correct answers provided. Therefore, the final mark was expressed in marks out of thirty. The final score was obtained by the sum of all the correct answers provided. Moreover, they were asked to express their opinion on the course through the completion of an on-line appreciation questionnaire.

The course included a forum section, where participants could make questions on the activities and interact one each other, and an additional material upload section, where they could upload extra

material regarding the course topic, contributing to create connections and to favour knowledge exchange.

Data analysis

Statistical analysis was performed using GraphPad Prism version 9.4.0 for Windows, GraphPad Software, San Diego, California USA. The normality of data distribution was tested through the Shapiro-Wilk test. Descriptive statistics were run and data were expressed as mean±standard deviation (SD), median and min–max range, or number and percentage (%), as appropriate. Differences between groups were estimated using the unpaired Student’s t-test and non-parametric mean comparison test, as appropriate. A p-value < 0.05 was considered statistically significant. Chi-square test or Fisher’s exact test was used to compare differences in categorical variables. A p-value < 0.05 was considered statistically significant.

PREDIMED questionnaire was scored according to Martínez-González et al. [26].

Results

Among the bachelor degree students, after the starting presentation of the course, 143 subjects declared to be interested in participating to the study. Subjects were given a formal timeline of two weeks to confirm their adhesion to the study and 126 adhesions were collected. After the adhesion, 10 subjects decided to give up for personal reasons and 116 students were officially enrolled and began the course. 38 drop out were recorded. Consequently, the course was effectively concluded by the 67.2% of the subjects.

Descriptive statistics of the sample is represented in Table 6-1. The population is mostly represented by females (66 subjects) with a median age of 22 years. Males participating to the study were 12 with a median age of 25.5 years.

Table 6-1. Sample characteristics.

	Number of subjects	Age				
		Minimum	25% percentile	Median	75% percentile	Maximum
Population	78	20.0	21.0	22.0	28.3	53.0
Females	66	20.0	21.0	22.0	27.3	53.0
Males	12	20.0	21.3	25.5	37.0	44.0

Through the evaluation of the 14 items of PREDIMED questionnaire it was possible to investigate participants dietary habits and food groups consumption highlighting the adherence of their diet to

Mediterranean diet. For each item, PREDIMED questionnaire assigns the score 1 for an optimal consumption and the score 0 for non-optimal consumption [26]. Regarding the use of extra-virgin olive oil, it emerged that all participants were found to use it every day. In addition, it was pointed out that around the 50% of participants had an optimal daily consumption of extra-virgin olive oil in terms of quantity (≥ 4 spoons/day). Concerning vegetables and fruits, opposite results were found. Indeed, the 79.5% of participants reported an optimal consumption of vegetables (≥ 2 servings/day) while a suboptimal intake of fruits was found for the 83.3% (< 3 servings/day). For red meat and meat products it was found that the 64.1% of participants had a non-optimal consumption (≥ 1 servings/day) while for butter or animal-derived seasoning fats it came into light that the 91.0% of students involved did not consume them (< 1 servings/day). In parallel, it was found that 80.8% of participants preferred white meat to red meat. Regarding drinks, it emerged that the 92.3% of participants did not consume soft drinks (< 1 servings/day) and that the 1.3% of the students drank wine weekly (≥ 7 glasses of wine/week).

For pulses and for fish, it was pointed out that their consumption was not in line with Mediterranean diet recommendations [29]. Indeed, for pulses only the 24.6% of the population reported an optimal pulse consumption (≥ 3 servings/week), while for fish only the 25.6% (≥ 3 servings/week).

The 62.8% of participants were found to consume nuts with an optimal intake (≥ 3 servings/week) and the 85.9% displayed an optimal low consumption of sweets (< 3 servings/week). Lastly, it emerged that around the half of participants consumed sautéed vegetables. Data are showed in Figure 6-2.

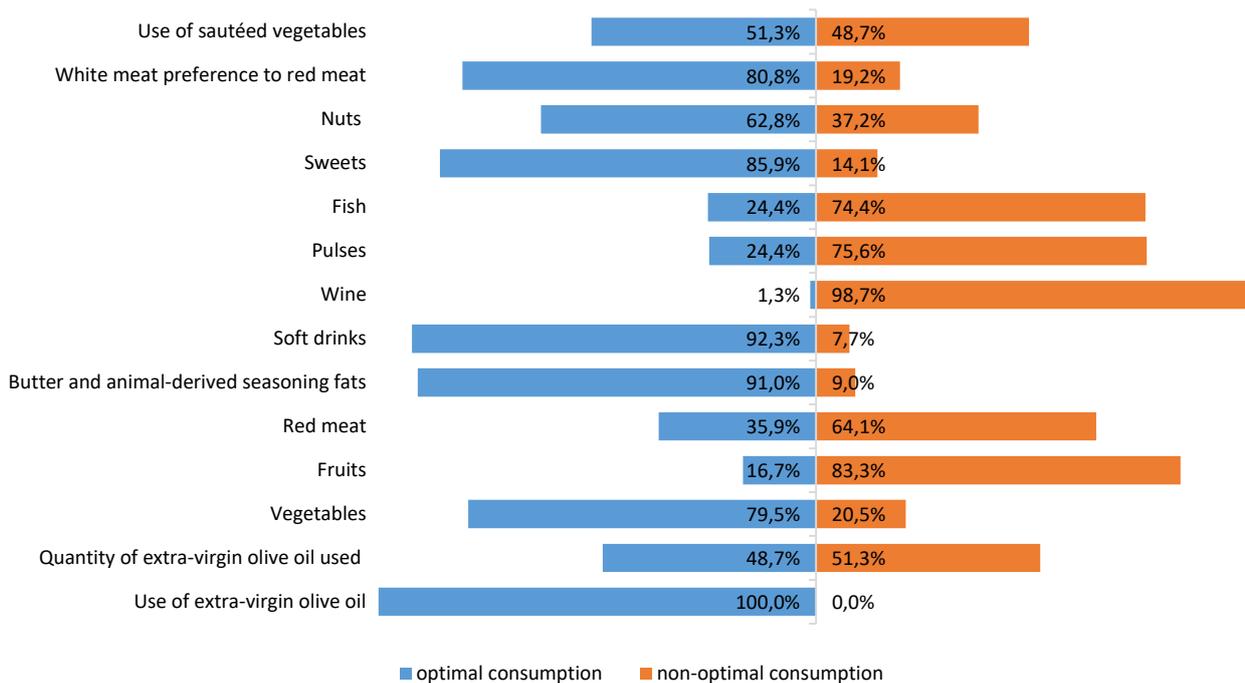


Figure 6-2. Percentages of population reporting optimal consumption (score 1) and non-optimal consumption (score 0) for food groups according to PREDIMED.

By the evaluation of PREDIMED questionnaire, it was possible to investigate the adherence to Mediterranean diet before the intervention (pre-intervention = 8.0 ± 1.8).

Interestingly, after the nutritional education intervention, the score was significantly improved (post-intervention = 8.4 ± 1.7).

However, no significant differences were found in percentage of low adherence, medium adherence and good adherence (Table 6-2).

Table 6-2. Mediterranean diet adherence from PREDIMED questionnaire before and after the intervention.

Adherence to Mediterranean diet (score)	Pre-intervention		Post-intervention	
	n° of subjects	%	n° of subjects	%
≤ 5 low adherence	4	5.1%	3	3.8%
6-9 medium adherence	59	75.6%	53	67.9%
≥ 10 good adherence	15	19.2%	22	28.2%

Data are reported as number (n) and percentage (%) of subjects obtaining low, medium and good adherence to Mediterranean diet.

The nutrition education course was thought to implement pulse consumption among students, therefore it is interesting to focus the attention on pulse consumption of participants after the

intervention. It was found that the percentage of people obtaining score 1 significantly increased post intervention (+20.5%) (Table 6-3).

Table 6-3. Pulse consumption from PREDIMED evaluation in the sample population before and after the intervention.

Subjects	Pulse consumption (PREDIMED)			
	Pre-intervention		Post-intervention	
	Score		Score	
	1	0	1	0
n	19	59	35	43
%	24.4	75.6	44.9	55.1**

score 1= pulse frequency of consumption ≥ 3 times per week, score 0= pulse frequency of consumption < 3 times per week. Chi-squared test pre vs post. ** $p < 0.01$.

To investigate more in details the types of pulses consumed by participants, food diaries were analyzed. In Table 6-4 the variations in pulse type consumption before and after the intervention are described. No statistical differences were found with the exception of soybeans that showed a decrease in percentage of consumption (Table 6-4).

Table 6-4. Types of pulses consumed before and after the intervention.

	Chickpeas	Peas	Lentils	Beans	Soybeans	Broad beans	Lupin beans
% of participant before the intervention	47.4	42.3	35.9	21.8	17.9	6.4	0.0
% of participants after the intervention	61.5	43.6	37.2	32.1	5.1*	5.1	2.6

Values are expressed as percentage (%). Chi squared test pre vs post. * $p < 0.05$.

As described in Table 6-3, PREDIMED results allowed to show variations in pulse weekly frequency of consumption before and after the intervention. It is worth mentioning that increasing pulse consumption is as important as making the right food combinations. Indeed, pulses should be consumed in association with cereals or cereal derivatives [32, 33]. Through the analysis of food diaries, it was possible to investigate both weekly pulses consumption and the proper combination of pulses with cereals. In particular, each time pulses were consumed, a check regarding the combination with cereal in that meal were performed.

To deeply analyse pulses consumption, it has been taken into account both the changes in pulses weekly consumption (Δ pulses weekly consumption = pulses weekly consumption post intervention – pulses weekly consumption pre intervention) and changes in proper combination (Δ proper

combination= pulses properly combined with cereals post intervention – pulses properly combined with cereals pre intervention). A bubble plot was constructed taken into account Δ pulses weekly consumption (y axis) and Δ proper combination (x axis), Figure 6-3.

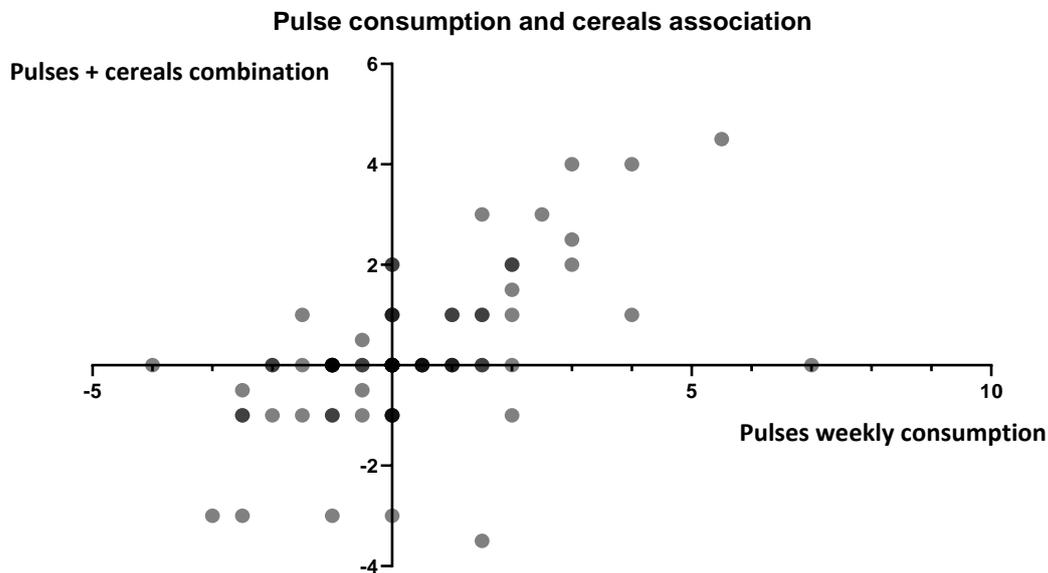


Figure 6-3. Pulse consumption and cereals association. Pulses weekly consumption and adequate weekly association (pulses + cereals) are plotted as delta values obtained before and after the intervention.

As it can be appreciated by the Figure 6-3, it is possible to observe subjects that improved both pulses consumption and proper combination, in the first quadrant. In the second quadrant those that worsening weekly pulses consumption but improved pulse association with cereals. In the third those that worsening both weekly pulses consumption and combination. In the fourth, those that improved weekly pulses consumption but worsening the combination.

In addition, through the evaluation of food diaries, it was feasible to acquire information on participants' dietary habits. The attention was focused on breakfast, both in terms of frequency of consumption and nutrient balance.

The attention given to breakfast was firstly due to the high nutritional significance it covers in the diet and secondly to the will to improve breakfast nutritional balance, for example by proposing pulse consumption. Pulses can be introduced at breakfast in the form of innovative food products (for example pulse-based or pulse containing products), overthrowing the barriers that are usually considered responsible for a reduced pulse consumption (i.e. long cooking time) [34].

Starting from these considerations, it was decided to investigate participants' habits regarding breakfast daily consumption and its nutrient balance [35]. Before the intervention breakfast was daily consumed by the 96.2% of participants while after the intervention it was consumed by the 93.6% of the subjects. Therefore, daily breakfast was consumed almost by all-participants both

Numerous recipes were pointed out, for example chickpea flour-based biscuits, “fagiotella” (a cream made of beans and chocolate) and pulse-based pancakes (Appendix III).

In module 10 participants were asked to test their acquired competences through an online test. Students were asked to answer to 30 multiple-choice questions on course topics. For each question correctly answered students got 1 point, for each uncorrect answer they got 0. The final score was obtained by the sum of all the correct answers provided.

The test was successfully passed by all students and the mean of the final mark obtained by students was 24.0 ± 2.8 .

In order to evaluate the general appreciation for the course, participants were asked to full-fill a questionnaire at the end of all the activities. The questionnaire investigated in details the degree of appreciation for the course, participants’ expectations and expectation satisfaction, usefulness and pleasantness of practical tasks and usefulness of the e-learning platform as an effective learning tool. Regarding the degree of expectation, it emerged that the most of participants (71.1%) declared to have appreciated the course (Figure 6-5).

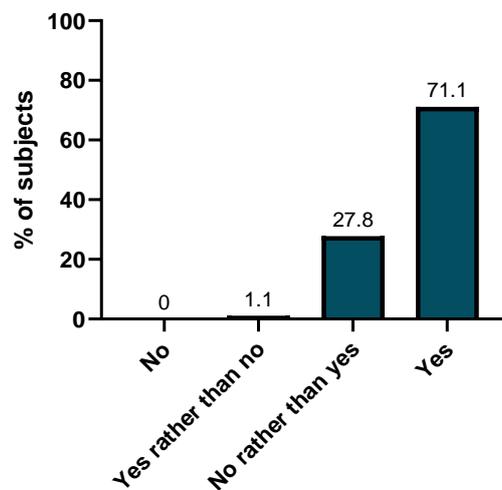


Figure 6-5. Participants' appreciation for the course.

In addition, participants were asked which were their expectations before starting the course. It was found that the 73.3% wanted to improved the knowledge on the topic and the 72.2% wished to increase the awareness of personal food choices (participants could give more than one answer at the same time. Percentages are obtained from the total answer for each option) (Figure 6-6).

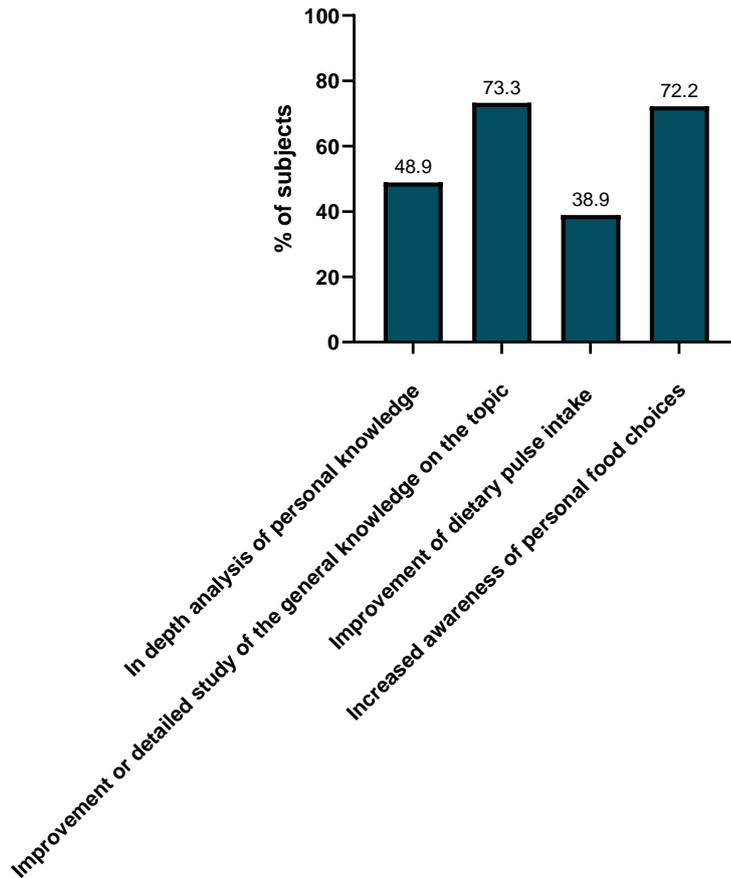


Figure 6-6. Participants' expectations before starting the course.

Concerning the satisfaction of course expectations, the 65.6% of participants stated to be satisfied. No one pointed out the course was unsatisfying (Figure 6-7).

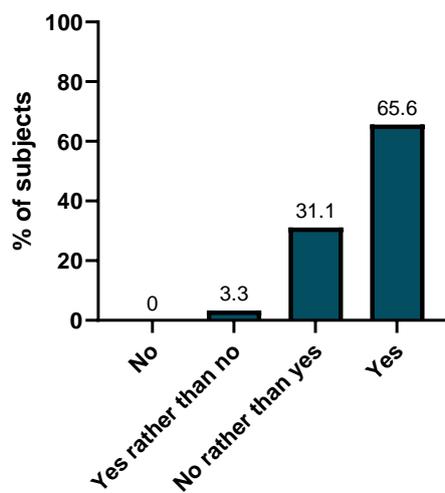


Figure 6-7. Satisfaction of participants' expectations.

Moreover, the 56.2% of participants declared practical tasks were useful and pleasant and the 74.4% said the e-learning platform was an effective tool for course administration (Figures 6-8 and Figure 6-9).

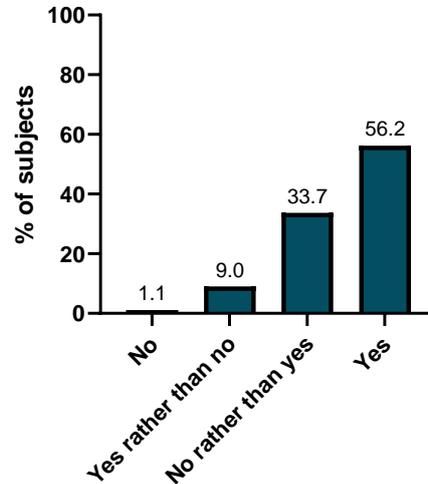


Figure 6-8. Usefulness and pleasantness of practical tasks.

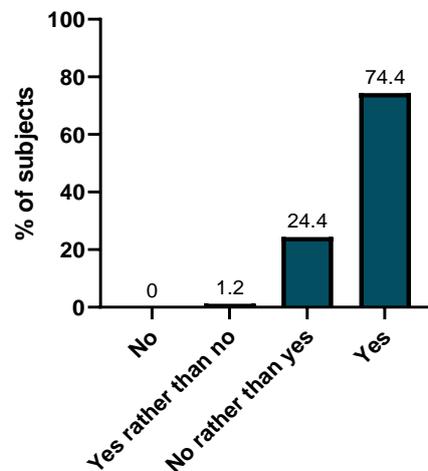


Figure 6-9. Usefulness of the e-learning tool.

Discussion

Nutrition education has been acquiring a fundamental role in supporting healthy dietary habits and lifestyle, thus also in promoting pulse consumption. In particular, pulse-based nutrition education appears to pose itself as a strategy to make participants be aware of their dietary choices starting by including pulses in the diet [36].

Several studies reported that nutrition education programs appear to positively influence children behaviours at school age [37, 38] and are confirmed to be useful also for university students, especially when considering future nutrition trainers [39]. This results of fundamental importance as Blunt et al. [39, 40] pointed out that an appropriate nutrition training is lacking at all levels of medical education. Therefore, investing resources in the education of nutrition trainers is determinant for guaranteeing a nutritional support for patients and for consumers.

In the present study, a new e-learning nutritional educational course was study, developed and tested on a group of “Human nutrition” university students. Dietary habits of university students were investigated through the analysis of PREDIMED questionnaire and food diary before and after the e-learning nutrition education intervention.

Thanks to the study of food diaries and PREDIMED questionnaire, the study firstly enable to evaluate inadequacies on specific food group consumption. In particular, it was found that the score reported by students for fruits is not in line with the recommendations of Mediterranean diet [29]. In addition, a excess consumption of red and meat products was pointed out. This was in accordance with what reported in other studies. For example, Sprake and colleagues pointed out that, in a convenience sample of British university students, the two-thirds were regular red meat eaters [41], Dinu and colleagues reported a non-optimal consumption of meat and meat products on 79% and 89% of female and male 17-19 years old students, respectively [42]. Concerning fish consumption, it emerged that around the 75% of participants did not have an optimal consumption as also reported by Thorsdottir and colleagues and Dinu and colleagues [42, 43]. Regarding soft drinks intake, it was observed that almost all of participants did not consume soft drinks. Similar results were found by Bawadi and colleagues in a study conducted on Jordanian college students [44]. In parallel, it emerged that also wine is not consumed by almost all participants. According to Mediterranean diet wine should be consumed occasionally [29], consequently PREDIMED questionnaire, which is based on Mediterranean diet guidelines, considers wine intake as a positive outcome. Anyway, it is worth mentioning that wine contains alcohol, which negatively affects health [45, 46]. Therefore, the reduced wine consumption observed in the study population is a relevant result, especially considering the high alcohol intake found in nutritional surveillance studies [47, 48].

Legumes weekly consumption was found to be optimal only in the 25% of the involved students. This percentage was slightly lower respect to the percentage reported by Dinu and colleagues (35%) regarding pulse consumption on a group of Italian students [42]. However, in both situations, a very low consumption of legumes can be underlined.

In this regard, the e-learning course focuses the attention particularly on pulses aiming to improve their consumption as pulses display an interesting nutritional composition [49, 50]. After the intervention, an improvement in the Mediterranean diet adherence score was reported and, by the analysis of PREDIMED questionnaire results, it was evidenced an increase in the number of participants consuming pulses ≥ 3 times per week. This came to light that nutrition education interventions may be useful to approach participants to improve their awareness regarding healthy food choices and pulse consumption.

Considering in general plant-proteins profile, it is worth mentioning that essential amino acids for humans are not present in the adequate amount [51]. Actually, pulses are characterized by a high content of lysine, leucine and arginine but lack of sulphur amino acids, such as methionine and cysteine, and tryptophan [52, 53]. Consequently, in order to improve the quality of pulse proteins by solving the matter of limiting amino acids for human nutrition, it is generally recommended to combine plant sources foods properly and to take into consideration the concept of amino acid complementarity [32, 33]. Therefore, during the course, participants were also informed on how to combine pulses in a meal to get the adequate nutrient balance. Indeed, the association between pulses and cereals/cereals derivatives be improved in students' diet after the course.

Starting from the importance of increasing pulse consumption and in the perspective to improve general dietary habits, further attention was focused on breakfast, which the last course module was dedicated for. Breakfast has a high nutritional significance, by contributing to energy and nutrient daily balance and consequently by exerting beneficial health effects [54, 55]. Therefore, a great attention was given to the importance of consuming a daily and a nutritionally balanced breakfast. In addition, pulses could be introduced at breakfast as whole or as ingredients in innovative food products. Pulse-based or pulse-containing food products could represent an alternative option to introduce pulses in unconventional meal consumption occasions.

As it was possible to observe from obtained data and from data obtained from national surveillance programmes [56, 57], one of the main issue to be faced in terms of breakfast is to obtain a nutritional balanced meal. Indeed, usually, breakfast appear unbalanced without proteins and health fat presence and with an excess of simple sugar and saturated fatty acids [58, 59]. From obtained data, it was possible to observe that almost all students consume breakfast daily, differently from what reported elsewhere [14, 15, 60]. However, although almost all students consume breakfast daily, it is important to study and evaluaty also breakfast quality.

Indeed, only the 12.0% of those consuming breakfast have a nutritionally balanced breakfast. The results of the present study are in accordance to the findings emerged in the study of Alexy and colleagues, conducted on German children and adolescents to investigate breakfast frequency and quality, where it was pointed out that breakfast quality was low and not in accordance with food-based guidelines [61].

After the nutrition education intervention, the percentage of subjects having a nutritionally balanced breakfast underwent an increase, even if not significant. This result underlines that it is essential to care of what constitutes breakfast, by choosing the adequate foods aiming to get necessary nutrients. In this context, also pulses may have a role by supporting the nutritional improvement of breakfast [62].

Beside legumes and breakfast consumption, the basic concepts of Mediterranean diet were analyzed. In this regard, participants were asked to express their opinion on Mediterranean diet in the globalization context in Module 2. By the word cloud obtained from the analysis of students' compositions, it emerged that Mediterranean diet can be associated not only to primary and concrete needs (such as "foods", "consumption", "products") but also to wider concepts (such as "life", "style"). Therefore, students appear to consider Mediterranean diet still current, perceiving it as lifestyle and not simply a dietary regimen, in accordance with the study of Diolintzi and colleagues [63].

Starting from the efficacy and usefulness of nutrition education interventions, it is fundamental to verify participants' feedback on the course. After completing the course, students had to fulfil a final appreciation questionnaire. It emerged that around the 70% of participants appreciated the course and no negative feedbacks were recorded. Regarding course expectations, the most of participants foreseen the course to be a way to improve or deepen personal knowledge on pulses and to increase awareness in food choices. Expectations appeared to be satisfied for the more than the half of participants while a third declared "yes rather than no", and around the the 3% assessed "no rather than yes" and no negative answer were reported.

The platform represents a pilot study whose aim was to train students motivated to become nutrition experts as they will be directly in contact with patients and will be responsible for patient's dietary choices awareness. The results of study highlighted that the course have induced an improvement in pulse consumption especially in those participants who were already sensitive to pulse intake and pulse adequate combination with cereals. Therefore, it is essential to support less attentive participants in approaching pulses world. Implementation of the course is ongoing in order

to make pulses be attracting for all participants, for example by the introduction of preliminary explanatory videos for pulse preparation and cooking to facilitate their use.

Moreover, it could be advantageous to broaden course employment to students of other university departments and to other users' categories, such as adults, families and food services managers. As healthy dietary behaviours are established during childhood in the family and social contexts, nutrition education should be promoting since an early age. Consequently, it could be possible to address the platform also to children by adapting activities to their learning abilities.

Conclusions

The e-learning course was an opportunity to deepen the knowledge of participants on pulse nutritional characteristics, preparation methods and food combination. Moreover, practical activities (i.e. pulse-based recipes preparation) were useful to approach students to pulse consumption.

In addition, this nutritional education course represents an advantageous and intriguing strategy to approach the users to pulse world for the adoption of the university e-learning platform for course administration. Indeed, today the use of digital learning tool is acquiring a growing importance in the didactic perspective and its role has been underlined even more during Covid-19 pandemic.

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Chapter 7. Effects of Mediterranean diet on body composition of healthy and physically active subjects

Introduction

Numerous studies evidenced that Mediterranean diet exerts positive effects on human health, in particular, by contributing to the prevention of chronic diseases, such as cardiovascular disease and diabetes [1-5]. In addition it has been shown that a regular consumption of vegetables, fruits/nuts, legumes, cereals and fish and less dairy and meat/poultry, induces positive outcomes for cardiovascular and cognitive health [6, 7].

The intake of foods typical of the traditional Mediterranean diet pattern is associated with a reduced risk for developing various types of cancers, including upper digestive tract, stomach, colorectal, pancreas, liver, and selected hormone-related cancers such as endometrial cancer [7, 8].

Considering the increasing global trends in overweight and obesity and the finding that most deaths attributable to overweight and obesity are due to cardiovascular disease, the adoption of a Mediterranean diet pattern can be an important cost-effective health-care measure development strategy [9].

Mediterranean diet is based on the consumption of plant-derived foods such as vegetables, fruits, cereals, nuts, seeds and pulses [10]. In parallel it is characterized by a moderate intake of fish, white meat and dairy products and by low intake of eggs, red and processed meat and sweets. Extra virgin olive oil is the main source of fat [11].

Recommended numbers of servings for these food groups are represented in the Mediterranean diet pyramid, described by the Figure 7-1 [12].



Figure 7-1. Mediterranean diet pyramid [12].

Food items that should sustain the diet and provide the highest energy intake are located at the base of the pyramid while foods to be eaten in moderate amounts, such as those of animal origin and/or rich in sugars and fats, are found on the top.

The pyramid establishes dietary daily, weekly and occasional food frequency of consumption in order to follow a healthy and balanced diet [12].

Plant-origin foods represent a cornerstone of the pyramid. They provide key nutrients, such as vitamins, minerals, antioxidant compounds and fiber that contribute to human general well-being [13-15], thus they should be consumed in high proportions and frequency.

In this context, pulses have a pivotal role. Indeed, a regular pulse consumption has been associated with an improved health status and with the prevention of non-communicable diseases [16, 17]. The benefits derived from pulse intake are related to their nutritional composition [18]. Pulses contain complex carbohydrates and fiber and they are the most important plant-based protein source in the diet [19]. According to the guidelines of Mediterranean diet pyramid, pulses should be consumed at least 2 times per week [12].

Along with recommendations regarding the proportion and frequency of food consumption, the incorporation of cultural and lifestyle elements is one of the innovations of the pyramid. Adopting a healthy lifestyle and preserving cultural elements should also be considered in order to acquire all the benefits from the diet and to preserve cultural heritage [20]. They include moderation,

socialization, culinary activities, physical activity, adequate rest, seasonality and traditional, local, eco-friendly and biodiverse products preference.

The Mediterranean diet encourages the use of a wide range of cereals, legumes, fruits and vegetables, not only from human production but also wild species. The seasonal consumption of fresh and local products, biodiversity, variety of foods (especially fruits and vegetables of different colours), traditional culinary activities, conviviality and frugality represent the cornerstones of Mediterranean diet heritage [21].

Several works have evidenced that a high adherence to Mediterranean diet has been associated to positive health benefits [1-5]. Contrarily, a lack of studies emerged regarding the impact of Mediterranean diet adherence on body composition. Given the relevance of body composition in contributing the maintenance of a healthy status [22], it results of interest to investigate the effect of the Mediterranean diet on body composition parameters.

Aim of the study

The aim of the study was to evaluate the effect of adherence to Mediterranean Diet on body composition on healthy and physically active subjects. Adherence to Mediterranean diet was also investigated.

Materials and methods

Dietary intervention

The dietary intervention proposed was a normo-caloric Mediterranean diet, nutritionally balanced and customized in line with participant's nutritional requirements. The percentages of macronutrients on the daily caloric amount were in accordance to LARN indications (45-60% of carbohydrates, 15-20% of proteins and 20-35% of fats) [23].

In order to cover nutritional requirements, the frequencies of consumption of foods categories in the dietary protocol were fixed as follows:

- fruits, 1-2 servings/day,
- vegetables, 2 servings/day,
- bread/pasta/other cereals (preferably whole grain), ≥ 2 servings/day,
- nuts and seeds, 1 serving/day,
- dairy products (preferably low fat) such as milk and yogurt 2 servings/day while fresh cheese 1 serving/week,
- legumes, 3 servings/week,

- eggs, 2 servings/week,
- fish, 5 servings/week,
- white meat, 2 servings/week,
- red meat, 1 serving/week.

Moreover, all meals were planned according to the indications of the “Healthy Eating Plate” [24] and were prepared in order to guarantee diet variety and to respect vegetables and fruit seasonality. Each dietary protocol was represented by a 7-day dietary plan to be repeated for a month.

Dietary protocols were elaborated using Winfood® software (Medimatica Srl Unipersonale, Colonnella, TE, Italia).

An example of week of dietary plan is reported in Table 7-1.

Table 7-1. Example week of dietary plan.

Meals	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Breakfast	Skimmed milk or plain yogurt + Oat flakes or whole cereals + Nuts	Skimmed milk or plain yogurt + Whole bread + Nut spread	Skimmed milk or plain yogurt + Oat flakes or whole cereals + Nuts	Skimmed milk or plain yogurt + Whole bread + Jam	Skimmed milk or plain yogurt + Oat flakes or whole cereals + Nuts	Skimmed milk or plain yogurt + Whole bread + Nut spread	Skimmed milk or plain yogurt + Oat flakes or whole cereals + Nuts
Snack	Fruit + Nuts	Fruit + Plain yogurt	Fruit + Seeds and nuts	Fruit + Plain yogurt	Fruit + Nuts	Fruit + Seeds and nuts	Fruit + Plain yogurt
Lunch	Cereals/pasta/bread + White meat + Vegetables + Extra-virgin olive oil	Cereals/pasta/bread + Red meat + Vegetables + Extra-virgin olive oil	Cereals/pasta/bread + Fish + Vegetables + Extra-virgin olive oil	Cereals/pasta/bread + Legumes + Vegetables + Extra-virgin olive oil	Cereals/pasta/bread + White meat + Vegetables + Extra-virgin olive oil	Cereals/pasta/bread + Fish + Vegetables + Extra-virgin olive oil	Cereals/pasta/bread + Legumes + Vegetables + Extra-virgin olive oil
Snack	Fruit + Seeds and nuts	Fruit + Nuts	Fruit + Plain yogurt	Fruit + Nuts	Fruit + Seeds and nuts	Fruit + Plain yogurt	Fruit + Nuts
Dinner	Cereals/pasta/bread + Fish + Vegetables + Extra-virgin olive oil	Cereals/pasta/bread + Legumes + Vegetables + Extra-virgin olive oil	Cereals/pasta/bread + Eggs + Vegetables + Extra-virgin olive oil	Cereals/pasta/bread + Fish + Vegetables + Extra-virgin olive oil	Cereals/pasta/bread + Low fat fresh cheese + Vegetables + Extra-virgin olive oil	Cereals/pasta/bread + Eggs + Vegetables + Extra-virgin olive oil	Cereals/pasta/bread + Fish + Vegetables + Extra-virgin olive oil

The entire duration of the study was 4 months. Subjects were invited for a first meeting before starting the study (pre-intervention visit) during which they were asked to complete PREDIMED questionnaire [25] and body composition analysis was performed. Then, they were contacted one month later to start the dietary intervention (T0) in order to consider that months as control. During this meeting PREDIMED questionnaire [25] was fulfilled again and body composition analysis was performed.

Later on, participants were followed-up for three months (T1, T2, T3) (Figure 7-2). Each month they were recalled to repeat all the body composition measurements. Moreover, in each meeting participants were asked to fulfil the PREDIMED questionnaire [25] in order to monitor their perseverance in the adherence to the Mediterranean diet and they were asked eventual difficulties in following the dietary protocol (nutritional interview). The protocol was adjusted during follow-up visits, if necessary, according to the needs of each participant to maintain the diet isocaloric and balanced.

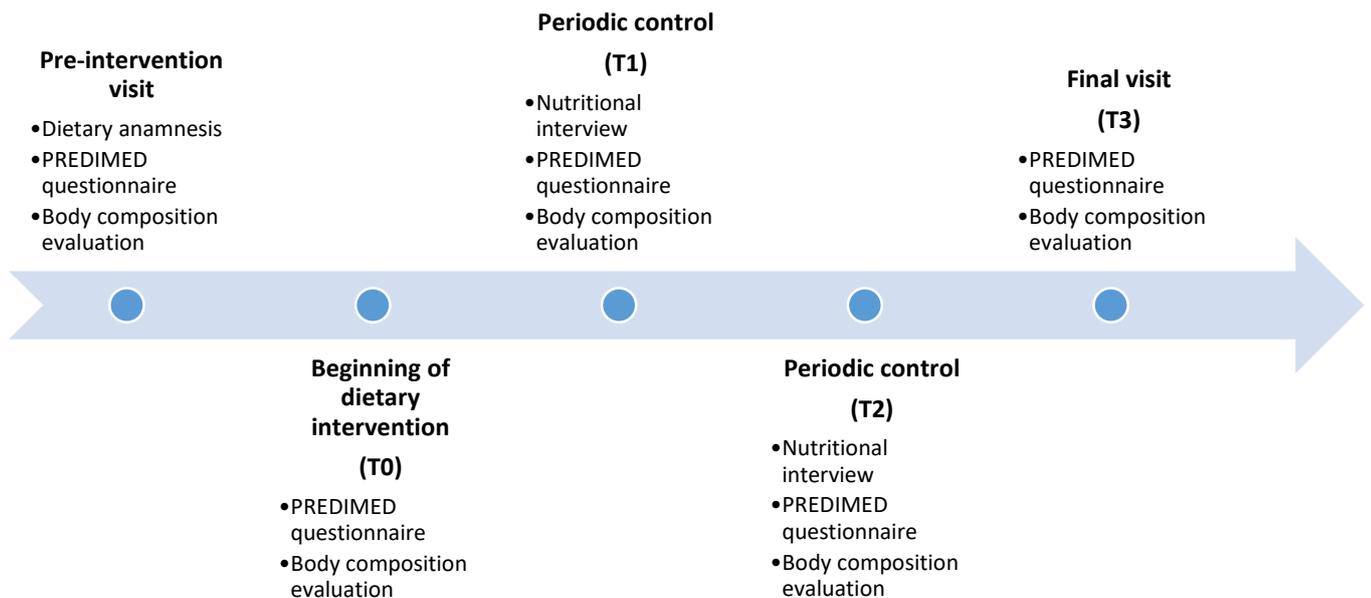


Figure 7-2. Study design.

The population sample

16 healthy adults were involved in the study aged 32.5 ± 9.56 years old. All subjects were physically active, training two times per week (cardiofitness, weight training, or both). Physical activity was settled in terms of duration and intensity before and during the intervention by a personal trainer. Subjects were selected without any gender, socio-economical and ethnical distinctions. Inclusion and exclusion criteria are illustrated hereafter.

Inclusion criteria

- signed information and consensus sheet,
- adult age,
- healthy status,
- constant levels of physical activity.

Exclusion criteria

- supplements use,

- drugs and/or antibiotics use,
- allergies and/or intolerances (lactose and/or gluten intolerance),
- diseases and injuries during the last 6 months,
- pregnancy or breastfeeding.

Each participant was requested to sign the information and consensus sheet containing all the useful details about the study: aim of the study, study's protocol and dietary intervention, length of the study, associated risks and benefits, agreement and privacy notice.

The introductory meeting consisted in the participant anamnesis. The anamnesis process represents the first step of a visit as well as the most important in order to acquire all the main information about the subject. It consists in a succession of specific questions, which are generally used for the elaboration of the personalized dietary protocols.

In addition, each participant had to fulfil a food diary for a week. A food diary is a document where each participant has to report the foods and beverages consumed daily for a week [26, 27].

The fulfilled and delivered-back food diary were then analysed with the help of the professional software WinFood[®] (Medimatica Srl Unipersonale, Colonnella, TE, Italia) in order to obtain a weekly quantitative estimation of nutrient and energy intakes.

Adherence to Mediterranean diet was evaluated by PREDIMED questionnaire (Prevención Dieta Mediterránea) [25]. It consists of 14-item questionnaire regarding the frequency of consumption of specific foodstuffs and beverages. A score (0 or 1) is assigned to each question and the sum of the different scores results in the final total score determine the adherence of participants to the Mediterranean diet. According to the final score obtained, the adherence can be classified into low (≤ 5), medium (6-9) or high (≥ 10).

PREDIMED questionnaire was proposed to participants in the pre-intervention visit, before starting the dietary intervention (T0) and at all follow-up controls (T1, T2, T3) in order to monitor their adherence to the Mediterranean diet (Figure 7-2). If any concerns related to the protocol were evidenced, customized variations were adopted, according to the needs of each participant but being certain of maintaining a good adherence to the Mediterranean diet.

Anthropometric evaluation

For body weight and height measurement, each subject was asked to be barefoot and in underwear. Body weight was measured by using the electronic Wunder[®] scale, RB cable (Trezzo sull'Adda, Milan, Italy), while the height was measured by using a portable mechanical altimeter Wunder[®], HR1 model (Trezzo sull'Adda, Milan, Italy) (Figure 7-3).

For height recording, the subject was requested to position feet at 60°, the head oriented in the horizontal Frankfurt's plane (the imaginary line that links ears with eyes), the arms laterally laid along the body, the palms toward the thighs, and the shoulder blades and buttocks in contact with the measuring bar. Height measure was read at the end of a normal expiration.



Figure 7-3. Electronic Wunder® scale (on the left) and mechanical altimeter Wunder® HR1 model.

BMI was calculated as the ratio between the body weight of the subject (kg) and the height square (m²) [28].

Waist circumferences

Waist circumferences were measured using a flexible and inelastic meter with a precision of 0.1 mm. The measure was taken with the subject in underwear clothes, at the end of a normal expiration, with the arms relaxed at the sides, at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest (hip bone) according to WHO STEPS-wise instrument guidelines [29].

Skinfold thickness

Skinfold thickness was measured using a GIMA® skinfold caliper 27320 (Gessate, Milan, Italy) with a precision of 0.2 mm (Figure 7-4).



Figure 7-4. GIMA® skinfold caliper.

Skinfold was unstaked using the thumb and forefinger, avoiding interesting the muscular tissue, and placed on the skinfold’s basis. After three seconds the measure was read from the caliper quadrant (Figure 7-5). Each skinfold thickness was measured three times and the average was considered.

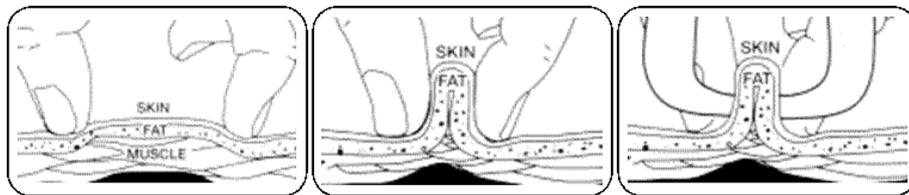


Figure 7-5. Skinfold thickness individuation with skinfold caliper.

The specific skinfolds measured are reported in the Table 7-2.

Table 7-2. Skinfold evaluated and measurement protocol.

Skinfolds	Measurement protocol
Triceps	Taken vertically on the posterior surface of the arm, on the middle point of the triceps muscle. The measurement is taken with relaxed arm.
Biceps	Taken vertically on the anterior surface of the arm, at the same level in which triceps skinfold is taken. Taken with relaxed arm.
Subscapular	Taken under the lower angle of the scapula, with a 45° inclination respect to the horizontal plane.
Supra-iliac	Taken following the skin’s cleavage along the mid-axillary line just above the iliac crest.

Measure skinfolds were then used to estimate percentage of fat mass using the specific tool on Winfood® software (Medimatica Srl Unipersonale, Colonnella, TE, Italia) by utilizing Durnin and Womersley equation to calculate body density then converted to Fat mass percentage by SIRI equation [30, 31].

Bioelectrical impedance analysis (BIA)

A single-frequency BIA (50-kHz, BIA AKERN 101®, Akern, Firenze, Italy) (Figure 7-6) with wrist-ankle tetrapolar electrode placement was used. Resistance (Rx) and reactance (Xc) were measured through tetrapolar impedance method applying a constant, low level alternating current (50 kHz) (Figure 7-7).



Figure 7-6. BIA Akern 101® for bioimpedance analysis.

The measurement was performed in standard conditions (the same moment of the day and no physical activity immediately before the analysis).

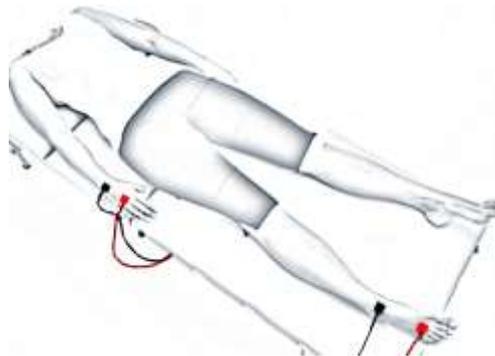


Figure 7-7. Wrist-ankle tetrapolar electrode Bioimpedance analysis.

Both Bioelectrical Impedance Analysis (BIA) and Bioelectrical Impedance Vector Analysis (BIVA) were performed. Data obtained were analyzed using BodyGram Plus® software in order to estimate body cell mass (BCM). BIVA was evaluated to confirm data estimation obtained. It resulted in the BIAVECTOR® always provided by BodyGram Plus® software. In this case, impedance was plotted as a bivariate vector from its component R_z and X_c obtained at 50 kHz and standardized by height (Figure 7-8).

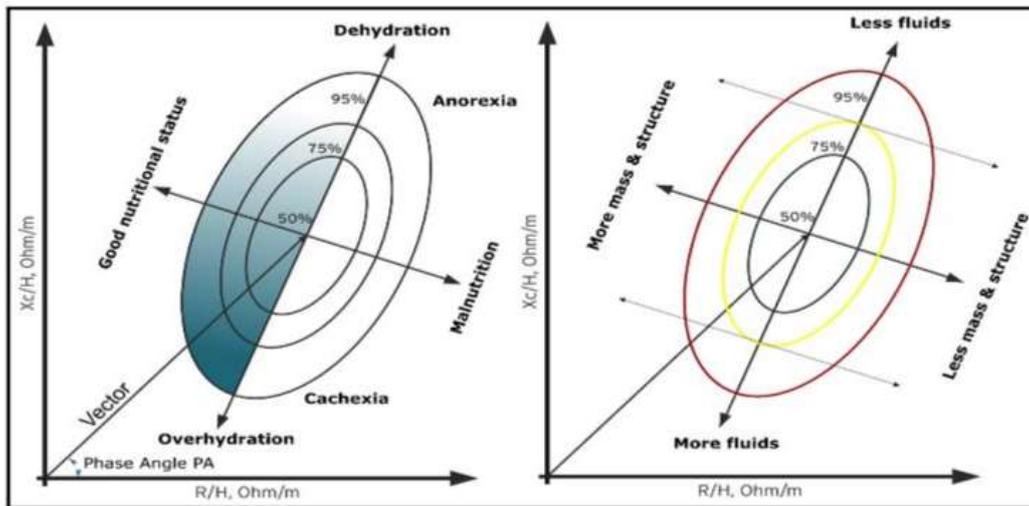


Figure 7-8. BIAVECTOR® analysis provided by BodyGram Plus® software.

Data analysis

Statistical analysis was performed using GraphPad Prism version 9.4.0 for Windows, GraphPad Software, San Diego, California USA. Data were elaborated on the total sample.

The normality of data distribution was tested through the Shapiro-Wilk test. Descriptive statistics were run and data were expressed as mean±standard deviation (SD), median and min–max range, or number and percentage (%), as appropriate. t-student test and one-way ANOVA for independent samples with multiple pairwise comparisons test were used for normal data distribution. Friedman test was used as non-parametric test for one-way repeated measures analysis of variance and Dunn's test pairwise multiple- comparison procedure when data distribution was not normal.

Results

PREDIMED score evaluation

PREDIMED score was evaluated in pre-intervention visit before starting the study (T0) and every months (T1, T2, T3). A significant increase in the score was recorder after the dietary intervention in each follow-up control (pre-intervention = 6.0 ± 1.8 , T0 = 6.1 ± 1.6 , T1 = 10.7 ± 1.9 , T2 = 11.3 ± 1.3 , T3 = 11.4 ± 1.7 , data are reported as mean ± SD. Figure 7-9).

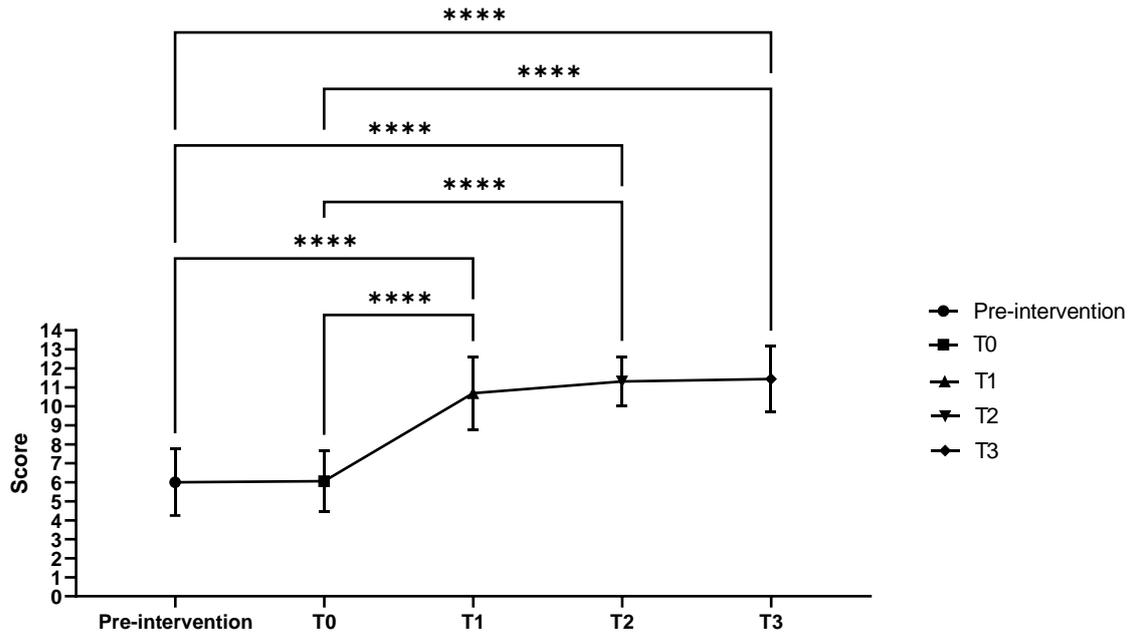


Figure 7-9. PREDIMED score during the study course. One-way ANOVA with Fisher LSD test. **** $p < 0.0001$.

Pulse weekly frequency of consumption

Weekly frequency of pulse consumption was evaluated (Figure 7-10). It significantly increased from pre-intervention to T3 (pre-intervention = 0.6 ± 1.0 , T3 = 2.4 ± 1.7) and from T0 to T3 (T0 = 0.6 ± 1.1 , T3 = 2.4 ± 1.7).

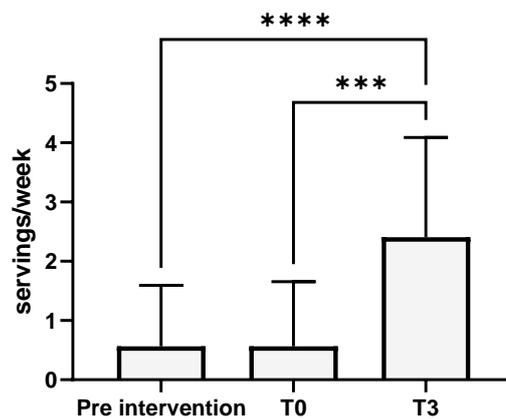


Figure 7-10. Pulses weekly intake before the intervention, at T0 and T3. One-way ANOVA with post-hoc Tukey. *** $p < 0.0001$.

Anthropometric evaluation: BMI

A significant reduction of BMI was observed after the dietary intervention in each follow-up control (T0 = 26.0 ± 3.6 kg/m², T1 = 25.4 ± 3.4 kg/m², T2 = 25.3 ± 3.4 kg/m²) (Figure 7-11).

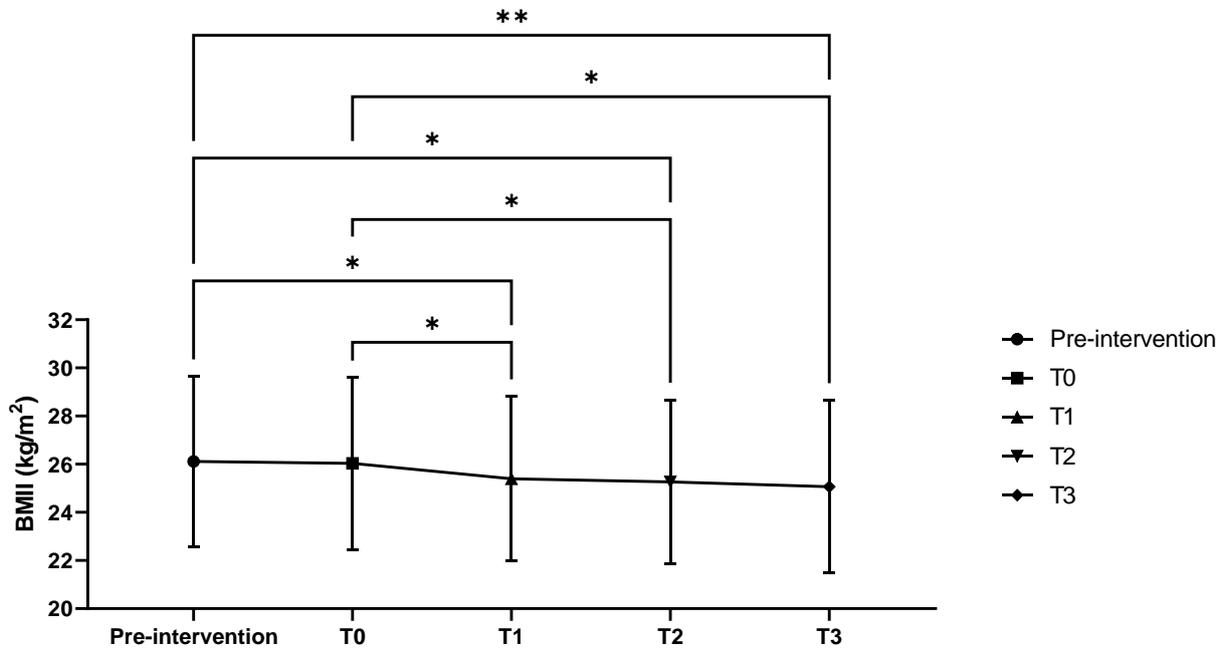


Figure 7-11. BMI variations during the study course. One-way ANOVA with Fisher LSD test. *p<0.05, **p<0.01.

Anthropometric evaluation: body circumferences and fat mass

Concerning waist circumference, a significant reduction was observed between T0 and T3 (T0 = 90.5 ± 11.7 cm, T3 = 86.7 ± 11.5 cm), with intermediate decreasing observed between T1 and T3 (T1 = 89.0 ± 11.8 cm, T3 = 86.7 ± 11.5 cm) and T2 and T3 (T2 = 88.1 ± 11.5 cm, T3 = 86.7 ± 11.5 cm), Figure 7-12.

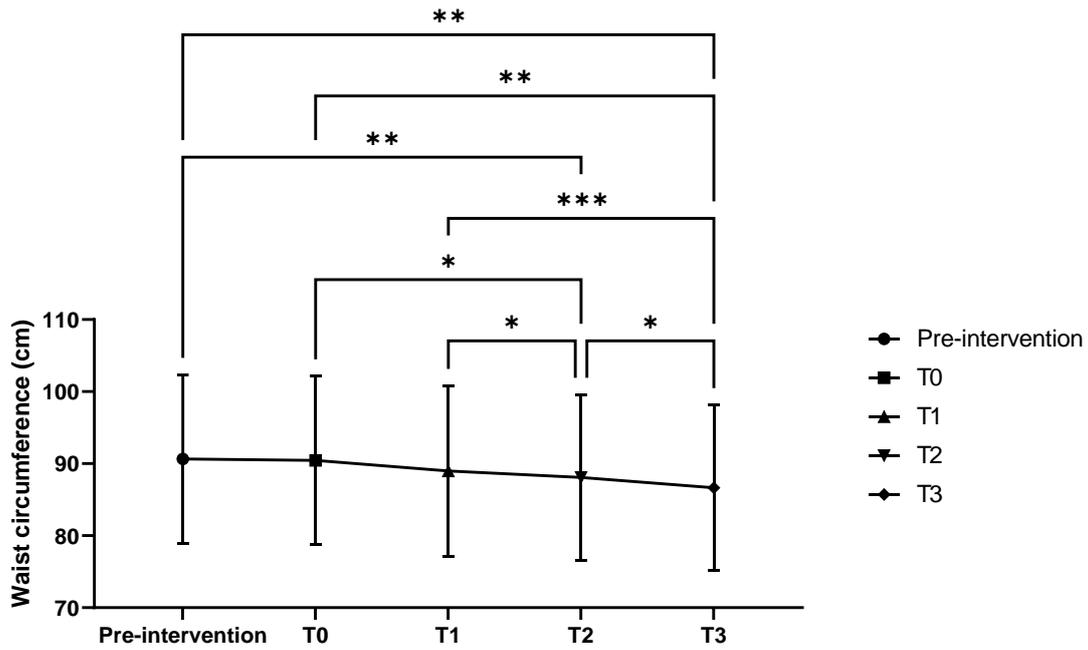


Figure 7-12. Waist circumference during the study course. One-way ANOVA with Fisher LSD test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Concerning body composition evaluation, a remarkable significant decrease of fat mass percentage (%FM) was evidenced at the end of the intervention (T3) compared to the %FM at the pre-intervention and at T0 (pre-intervention = $25.3 \pm 4.9\%$, T0 = $25.2 \pm 4.9\%$, T3 = $22.1 \pm 5.6\%$), Figure 7-13.

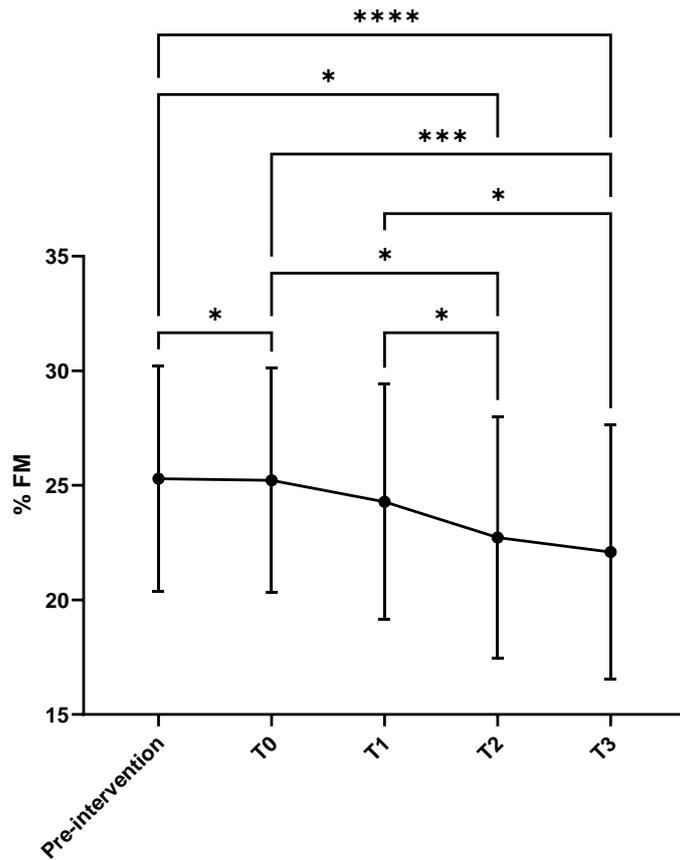


Figure 7-13. Fat mass percentage before the intervention, at T0 and T3. One-way-ANOVA with Fisher LSD test. * $p < 0.05$, *** $p < 0.001$.

BIA evaluation: BCM (body cell mass)

Body cell mass (BCM), at first, decrease from T0 to T1 and then significantly increases from T1 to T3 (Figure 7-14).

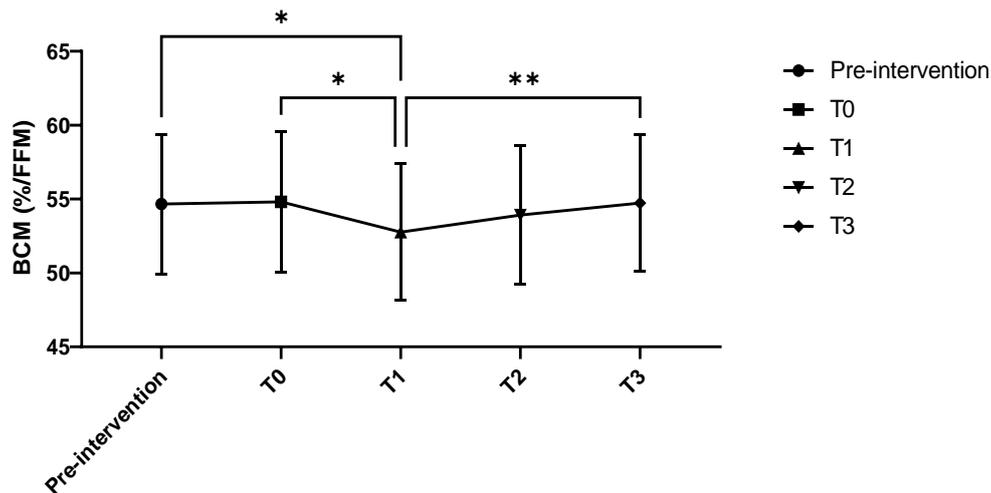


Figure 7-14. BCM during the study course. One-way- ANOVA with Fisher LSD test. * $p < 0.05$, ** $p < 0.01$.

Discussion

In this study participants were asked to follow a structured Mediterranean dietary protocol, adequately planned to cover their nutritional needs, for three months. The adherence to the diet was monitored using PREDIMED questionnaire. After the intervention (T3), PREDIMED score recorded a significant increase respect to the beginning (T0 = 6.1 ± 1.6 , T3 = 11.4 ± 1.71) with a parallel increase of pulse consumption, as suggested by Mediterranean dietary guidelines [12]. Since a regular nutritional support was offered to the subjects during the follow-up controls, it may be gathered that this action was essential to sustain their adherence to Mediterranean diet. Furthermore, follow-up controls were also useful to clarify doubts on pulse preparation and cooking, which contributed to the compliance to the dietary protocol. Indeed, it has been highlighted that nutritionist knowledge of patient dietary habits and practices is crucial in improving adherence to a diet, in different contexts and clinical conditions [32]. Moreover, it emerged that giving advices on pulse preparation and cooking methods is fundamental to support pulse consumption [33].

Regarding body composition, the nutritional intervention lead to the reduction of subjects' BMI. Several studies show that an increasing adherence to Mediterranean diet is associated to a BMI reduction and obesity risk decrease [34] but also to BMI reduction in both non-overweight and non-obese individuals [4, 34-36]. Study data show that BMI reduction is simultaneously accompanied by waist circumference and fat mass decrease [37, 38].

In this context, Mediterranean diet must be cited as it has been found to be effective in the managing of weight control [10, 39], in inducing a reduction of abdominal fat [40, 41] and consequently, in decreasing waist circumference [4, 42]. Also in the present study, a decrease of waist circumference emerged after the nutritional intervention. These results are probably due to the fact that Mediterranean diet is rich in vegetables and fruits and high in unsaturated fats, whose consumption is negatively associated to abdominal fat deposition and metabolic syndrome [43-45]. As widely demonstrated, beyond weight control and fat mass loss, Mediterranean diet is responsible for other positive health outcomes, such as non-communicable diseases prevention [46-48].

Anyway, despite these evidences, little is known on the effects of Mediterranean diet on muscle mass [49], although it is implicated in contributing and maintaining an healthy status. Indeed, data are lacking especially considering the adult population, physically active or sedentary, and contexts outside clinical settings.

Data are available for adolescents and young adults. Cobo-Cuenca and colleagues found that a adherence to Mediterranean diet in a group of Spanish university students is associated to a higher muscular fitness and cardiorespiratory fitness [50]. Moreover, Evaristo et al. pointed out that in a sample of Portuguese adolescents high physical fitness and adherence to Mediterranean diet are associated with health-related quality of life [51]. In addition, García-Hermoso et al. reported that following the recommendations of Mediterranean diet could be associated with improved physical fitness and physical activity in children and adolescents [52].

Despite a first decrease in BCM at the beginning of the intervention (T1), the results of the present study reported a subsequent increase and then maintenance of body cell mass.

It could be speculated that the initial decrease of BCM can be linked to the change of the diet and so a change in caloric intake that led also to an initial decrease on body weight (as demonstrated by the decreased BMI). However, after one months of dietary intervention BCM increased and then was maintained during the study period suggesting that an optimization of body composition was reached.

BCM is a fundamental parameter to take into consideration in the evaluation of body composition through bioimpedance analysis [53]. Indeed, BCM values are associated to a better nutritional status [22]. Consequently, adherence to Mediterranean diet and regular physical activity appear to contribute to the maintenance or improvement of BCM and of the nutritional and health status of the subjects with the concurrent reduction of fat mass. It was highlighted that the adherence to

Mediterranean diet may aid at least to maintain body cell mass in healthy and physically active adults [54].

These findings may be of interest as Mediterranean diet and physical activity together have a key role in improving health status, life quality and in preventing non-communicable diseases [55, 56].

The study has some limitations to work on. Firstly, it could be necessary to increase the number of subjects in the sample size, especially to evaluate the possible different impact of Mediterranean diet on body composition on physically active women and men, above all considering the growing relevance of gender medicine [57]. In addition, all the subjects involved were physically active, therefore further studies must be conducted on healthy and sedentary participants with a high adherence to Mediterranean diet in order to investigate the effects on body composition.

Conclusions

The impact of Mediterranean diet on body composition has been little investigated. Therefore, the results of the present study appear of a great interest, underlining that in three months Mediterranean diet positively impacts on subjects body composition. In particular, a normocaloric Mediterranean diet maintains body cell mass and determine a fat mass reduction.

Consequently, it is essential to sustain a high adherence to this dietary pattern, providing nutritional help to the population, for example by nutrition expert consultation. Moreover, it is worth mentioning that also physical activity has a pivotal role. Indeed, the synergy between a nutritionally balanced diet and physical activity contributes to the establishment of a healthy status.

In this context, awareness campaign and nutrition education interventions must be fostered to promote a healthy nutrition and an active lifestyle at all ages.

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Chapter 8. Plant-based diet: the impact of different animal/plant-proteins ratio on body composition and resting metabolic rate

Introduction

The impact of diet on the Earth system is a hot and debated topic. In this context, the Agenda 2030 for a sustainable development has a central role.

The Agenda 2030 is an action plan, approved on 25th September 2015 by 193 countries that are part of the United Nations, targeting people, the planet and prosperity and intended to promote sustainability of all dimensions of development [1, 2].

Briefly, sustainable development considers current needs avoiding to compromise those of future generations [3]. A sustainable development does not include only the environmental one. Indeed, also social and economic contexts must be taken into account and they are all connected and fundamental to guarantee a better life for everyone.

The Agenda 2030 defines 17 Sustainable Development Goals (SDGs) to be reached within 2030 that constitute a possible strategy to make the Earth more sustainable Figure 8-1 [2].

Sustainable Development Goals (SDGs)
1. No poverty
2. Zero hunger
3. Good health and well-being
4. Quality education
5. Gender equality
6. Clear water and sanitation
7. Affordable and clean energy
8. Decent work and economic growth
9. Industry innovation and infrastructure
10. Reduced inequalities
11. Sustainable cities and communities
12. Responsible consumption and production
13. Climate action
14. Life below water
15. Life on land
16. Peace, justice and strong institutions
17. Partnerships for the goals

Figure 8-1. Sustainable Development Goals (SDGs).

SDGs 2 and 3 regard physical well-being. Indeed, SDG 2 aims to end hunger, achieving food security and facing malnutrition. Adequate nutrition must be promoted to everyone by encouraging

sustainable agriculture. SDG 3 targets to ensure a healthy life at all ages guaranteeing support and therapies.

SDGs 1, 4, 5, 10 and 16 are addressed to the social sphere. SDG 1 faces poverty, with the aim to reduce the proportion of the population living below the national poverty line and to pledge access to basic services to everyone. In this perspective, SDG 4 has the goal to safeguard the completion of the education cycle and to sustain the development of personal skills, wishing everyone can find a decent job. Moreover, SDG 5 guarantees gender equality in the working context and women empowerment. SDG 10's purpose is to reduce inequalities within and among countries while SDG 16 aims to promote peaceful and inclusive societies for sustainable development and to guarantee everyone's access to justice and appeal to institutions.

SDGs 6,7, 12, 13, 14, 15 and 17 regard aspects related to environmental sustainability. SDG 6 ensures the access of safe drinking water and the management of water sanitation services, of water resources and of water-related ecosystems. In parallel, SDG 7 concerns to ensure access to all forms of energy and to promote clean energy research and renewable energy production.

In order to guarantee environmental sustainability, SDG 12 promotes a careful food consumption and the avoidance of food waste, by encouraging citizens' education policies.

SDG 13 aims to face climate change, with SDG 14 and 15 wishing to preserve the oceans, seas and marine resources and land ecosystems respectively to promote a sustainable development.

SDG 17 has the purpose to strengthen the means of implementation and revitalize the Global Partnership for Sustainable Development.

Instead, SDG 8 and 9 concern economic growth by promoting a decent work and fostering inclusive and sustainable industrialization and innovation.

SDG 11 regards the promotion of a sustainable urbanization, by favouring inclusive, safe and resilient cities and human settlements.

As it can be gathered by SDGs 2 and 3, well-being is strictly linked to an adequate nutrition, consisting in the recovery of energy and nutrient requirements for everyone. At the same time an adequate nutrition must be sustainable, meaning it derives from sustainable agriculture and productions. Indeed, agriculture and the food system are the major influential human perturbations on the environment [4].

Actually, the main issue to face for humanity is trying to contain the perturbations and consequences it caused on the Earth system. In this context, it necessary to introduce the concept of planetary boundaries. Planetary boundaries are "safe limits" that allow human societies to

continue develop in a “safe operating space” [5]. Standing within these boundaries could allow to safeguard Earth system and to guarantee system resilience. According to the analysis of Rockström et al. [6], planetary boundaries are nine, as described by Figure 8-2.

Planetary boundaries
1. Climate change
2. Ocean acidification
3. Stratospheric ozone depletion
4. Atmospheric aerosol loading
5. Biogeochemical flows (interference with P and N cycles)
6. Global freshwater use
7. Land system change
8. Biodiversity loss
9. Chemical pollution

Figure 8-2. Planetary boundaries. Adapted from [6].

Despite the alert raised by planetary boundaries inviting to take care of the Earth system, several of these limits (specifically regarding climate change, biosphere integrity, biogeochemical flows related to nitrogen and phosphorous cycles) have been crossed. One of the main responsible actor is the global food system [7]. The food system has a great environmental impact in terms of land use, biodiversity loss, depletion of freshwater resources and ecosystem pollution [8, 9]. More in detail, the problem is related to the production of the different food groups, which implicates diverse environmental impacts. Figure 8-3 by Springmann et al. [7] is a projection of the environmental pressure caused by specific food groups in 2050 compared to 2010.

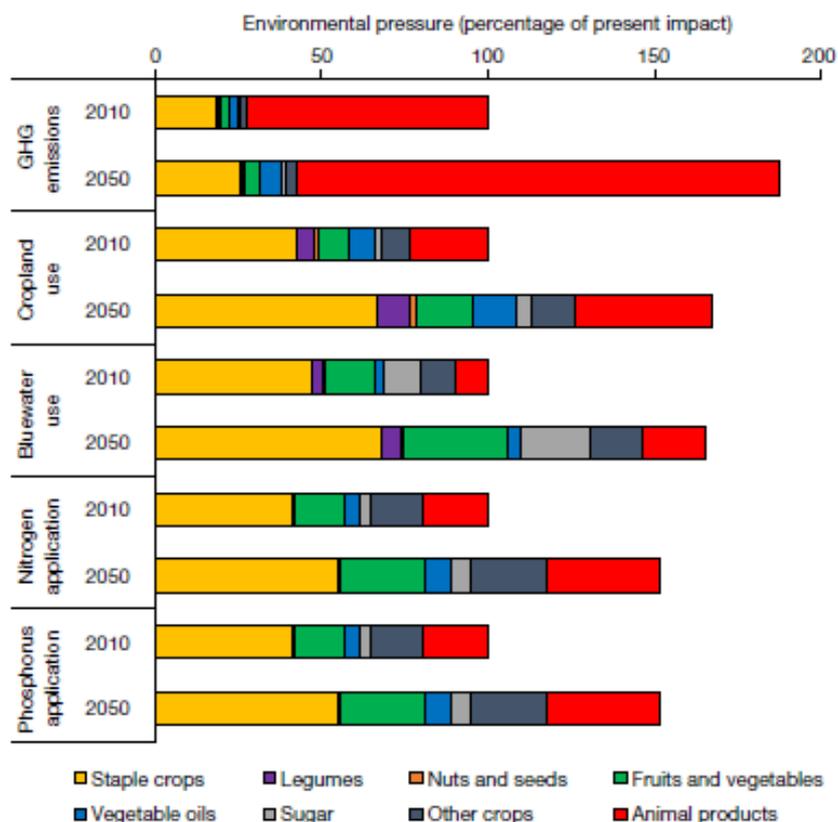


Figure 8-3. Present (2010) and projected (2050) environmental pressures on five environmental domains divided by food groups [7].

As it can be appreciated from Figure 8-3, animal products have and will be responsible for the greatest impact in terms of Green House Gas emission (GHG emission) while staple crops are and will be the most impacting in terms of land and water use.

It is interesting to note that for legumes the scenario is completely different, especially looking at the effect their production has on biogeochemical flows. Accordingly, legumes consumption should be sustained as an alternative plant-protein source and a more plant-based diet should be promoted.

Proteins are essential nutrients for human nutrition [10]. Dietary protein intake should sustain body's nitrogen balance homeostasis, whose requirement, in physiological conditions, varies according to gender, age, BMI, physical activity and to particular conditions (pregnancy and lactation) [11].

According to the LARN, the Population Reference Intake (PRI) recommended for protein for healthy adults (≥ 18 years old) is 0.9 g of protein per kg of body weight daily [12]. PRI for proteins constitutes an important nutritional reference that should be intended as the minimal intake recommendation. For the elder population, an intake of 1.1 g/kg per day is instead considered the reference value

(Suggested Dietary Target, SDT) (≥ 60 years old), recommended to prevent sarcopenia and chronic-degenerative diseases [12].

In this context, the concept of protein quantity should be considered in parallel to the one of protein quality. Protein quality refers to the capability of a given protein amount to satisfy the organism's metabolic requirements in terms of both total and essential amino acids (EAA) [11, 13]. Non-essential and essential amino acids are reported in the Figure 8-4 adapted from Lopez et al. [14].

NON-ESSENTIAL AMINO ACIDS	ESSENTIAL AMINO ACIDS
Alanine	Phenylalanine
Arginine	Isoleucine
Asparagine	Leucine
Aspartic Acid	Lysine
Cysteine	Methionine
Glycine	Threonine
Glutamic Acid	Tryptophan
Glutamine	Valine
Glycine	Histidine
Histidine	
Proline	
Serine	
Tyrosine	

Figure 8-4. Non-essential amino acids and essential amino acids for humans. Adapted from [14].

Protein quality depends on total protein content, amino acid profile and protein digestibility and absorption [15-18].

One of the major differences between food products of animal and plant origins is their protein and EAA densities. Lean protein-rich foods from animal sources provide a higher protein/energy ratio than plant sources. Indeed, it has been observed that strict plant-based diets are normally associated with lesser daily protein intake than omnivorous diets [13]. Consequently, in order to meet indispensable amino acids requirements, it may be necessary to increase the quantity of food introduced [19].

Proteins from animal sources provide all EAA and are referred to as high biological value proteins because the amino acids absorbed from these proteins meet the organism metabolic demand [11, 20]. Alternatively, animal-proteins can be also defined as complete proteins, intending a single protein contain all the nine essential amino acids [14]. In contrast, plant-proteins are low in specific

EAA, meaning they do not meet the minimal amino acidic requirements for human (Table 8-1) [11, 21]. For example, pulses have a low content of sulphur-containing amino acids (methionine and cysteine), grains lack of lysine [22, 23]. Only soy appears to have a more complete EAA profile [24]. However, combining plant foods with a complementary amino acidic profile in a plant-based diet can provide an adequate EAA supply that is equivalent to that provided by animal-proteins [22, 25]. Therefore, consuming a balanced vegan diet, based on a variety of plant-protein sources, has been found to provide a sufficient quantity of essential amino acids [25, 26].

Table 8-1. Reference amino acidic profile for Italian adult population.

Reference amino acidic profile for Italian adult population (>18 years)*								
Histidine	Isoleucine	Leucine	Lysine	Sulphur amino acids	Aromatic amino acids	Threonine	Tryptophan	Valine
15	30	59	45	22	38	23	6	39

*mg of amino acids/g of protein. Adapted from LARN [12].

Protein digestibility is the amount of food protein which is digested and absorbed by the organism. It is related to protein bioavailability, which is in turn a measure of protein quality. Protein quality is used to predict relative protein utilization [11].

Animal-proteins generally have a higher digestibility rate than proteins from plants mainly due to protein structure and anti-nutritional factors content.

Protein digestibility is also influenced by protein molecular structure, which is related to amino acid sequence [27]. In plant-proteins the main structure found is the β -sheet conformation. The high presence of β -sheets is likely related to plant-protein resistance to proteolysis in the gastrointestinal tract. Hence, the hydrophobic β -sheet structure of plant-proteins facilitates protein aggregation, resulting in a decreased digestibility [28].

Moreover, plant-based foods are characterized by the presence of anti-nutritional factors that are able to inhibit protein digestibility and absorption [29]. Indeed, plant-based protein sources contain enzyme inhibitors (i.e. trypsin inhibitor) that hinder the access of enzymes (i.e. trypsin) to proteins inducing a decrease in protein digestibility [30]. In addition, also food preparation, processing and storage may affect protein digestibility [20].

Thus, the higher protein/energy rate, the completeness in the EAA pattern and easy digestibility make animal-proteins to be high biological value proteins, suggesting their usage to support better muscle protein synthesis [31].

Anyway, considering the health and environmental benefits associated to plant-based foods production and consumption, the role of plant-based proteins should be valorised [32, 33]. In spite of that, perplexities still remain on their adequacy in supporting muscle mass maintenance. Evidence comparing the effects of different protein sources on body composition are in fact inconclusive or incomplete at today.

Beyond Mediterranean diet, other dietary regimens are based on the consumption of plant-based foods or are completely plant-based. Vegetarian diets for example include a variety of diets (i.e. lacto-vegetarian, ovo-vegetarian, lacto-ovo-vegetarian, pescetarian), all avoiding meat consumption [34]. On the contrary, vegan diets are based on the exclusion of any kind of animal product (raw and derived products) and approve exclusively the intake of plant-based foods [35]. Today vegan diets have acquired a great popularity for the increased interest of consumers for environmental issues and animal wellbeing [36].

The shift towards more plant-based diets has become necessary to respond to environmental challenges and the reduction of meat consumption and the parallel increased intake of proteins from plant sources are pivotal points [37]. Anyway, reduce or avoid meat in the diet is arduous for the most of consumers mainly because of the cultural and gastronomic importance meat has covered in the past and that it is still covering today [36]. Actually, the transition to more plant-based diets inevitably requires a deep social transition [38]. Not surprisingly, vegan diets have spread especially among the youngest, in particular thanks to the support of social media [39]. Indeed, several studies have pointed out that social networks can influence food choices and dietary behaviour [40-42].

At the same time keep informed through social networks could be insidious if the information source is not reliable and strictly evidenced-based. Therefore, it is important to avoid diets doing yourself and always refer to a nutrition expert. Indeed, vegan diets may be responsible for nutritional imbalances if they are not adequately planned [43]. Therefore, the choice to adhere to such exclusive diets requires the necessity to combine foods carefully and to consider specific supplementation use if they are followed for a longer time in order to avoid incurring nutritional deficiencies [44].

Indeed, in vegan diets the intake of specific nutrients such as vitamins B-12 and D, calcium, iodine, iron, zinc, long-chain ω 3 fatty acids and proteins must be considered cautiously [45].

It has been described that plant-protein sources are not provided by all the essential amino acids for humans [21]. As a matter of fact, it is suggested to combined plant-based foods, i.e. cereals and pulses, referring to amino acid complementarity [46, 47]. More in details, lysine is the limiting amino acid in cereal proteins while methionine and cysteine in pulse proteins, accordingly, their association allows to get a complete pattern of essential amino acids for humans [25].

It has been found that following well-balanced and planned plant-based diets exerts protective health benefits, especially in terms of body weight control and metabolic syndrome prevention [43, 48].

Instead little information is present in literature regarding the effect of plant-based diets on body composition and resting metabolic rate evaluation in physically active individuals.

Aim of the study

The study aimed to evaluate the impact of different customized dietary protocols on body composition and resting metabolic rate of physically active subjects. More specifically, the attention was focused on the protein quality of the diet evaluating different ratios between animal and plant-proteins.

Materials and methods

Dietary interventions and study design

Starting from the assumption that the distribution of protein intake in the diet is constituted by 2 parts of animal-proteins and 1 part of plant-proteins [49], dietary interventions tested were characterized by a modified animal-/plant-protein rate, always considering subject's nutritional requirements.

Each dietary protocol was defined by a specific rate between the % of animal-proteins and the % of plant-proteins on total proteins of the diet (rates are expressed as % of animal-proteins : % of plant-proteins):

- 70:30 diet, a Mediterranean-like diet in which the ~70% of total dietary proteins came from animal-based foods and the ~30% from plant-based foods,
- 30:70 diet, a diet where the ~30% of total dietary proteins came from animal-based foods and the

~70% from plant-based foods,

- vegan diet, a 100% plant-based diet. All dietary proteins came from plant-based foods.

The dietary interventions proposed were thought to be normo-caloric, nutritionally balanced and customized on participant's nutritional requirements. Moreover, energy and macronutrients intakes among the three dietary protocols were maintained quantitatively constant for each subject.

The only difference was attributed to the application of the correction factor for plant-proteins. Indeed, due to the reduced bioavailability of plant-proteins, a correction of ~20% was applied for the estimation of the overall plant-protein intake and for the elaboration of nutritionally balanced meals [50].

Each participant was subjected to three different dietary interventions, each one lasting 4 weeks. After each dietary protocol, participants were asked to follow at least a week of free diet before starting the subsequent dietary protocol.

Data from body composition (anthropometric evaluation, i.e. body weight, body mass index, body circumferences, skinfolds, and bioelectrical impedance analyses) and resting metabolic rate (RMR) were collected at the end of each dietary intervention. Body composition and resting metabolic rate evaluation methods are described in body composition evaluation and resting metabolic rate evaluation sections.

Participant's nutritional status was estimated starting from body composition evaluation, participant anamnesis and individual requirements.

Energy and macronutrients to be administered were established respecting Italian dietary Guidelines, the LARN (Livelli di Assunzione di Riferimento di Nutrienti ed energia per la popolazione italiana) [12], in order to elaborate nutritionally balanced diets. Caloric intake was distributed as follows: 45-60% of carbohydrates, 15-20% of proteins and 20-35% of fats. Regarding proteins, a particular attention was focused to the ratio between animal- and plant-proteins on total dietary proteins. Animal or plant-derived foods were differently selected in order to maintain the desired ratio (70:30, 30:70 or 100% plant-derived proteins), consequently, the daily and weekly frequency of consumption of foods was adequately managed.

The 70:30 diet followed the Mediterranean guidelines in terms of food quality and frequency of food consumption. The frequencies of consumption of foods were fixed as follows:

- fruits, 2-3 servings/day,
- vegetables, 2 servings/day,

- bread/pasta/other cereals (preferably whole grain), ≥ 2 servings/day,
- nuts and seeds, 1 serving/day,
- dairy products (preferably low fat) such as milk and yogurt 1-2 servings/day, fresh cheese 1 serving/week, aged cheese 1 serving/week,
- legumes, 3 servings/week,
- eggs, 1 serving/week,
- fish, 5 servings/week,
- white meat, 2 servings/week
- red meat, 1 serving/week,
- pizza, 1 serving/week.

The 30:70 diet was a prevalently plant-based diet. In order to reach the ratio of interest, the weekly frequency of consumption of foods rich in animal-proteins was decreased while those rich in plant-proteins was increased (i.e. legumes and derivatives, grains and derivatives) respect to the 70:30 diet.

Indeed, for the 30:70 diet, the frequencies of consumption of foods were fixed as follows:

- fruits, 2-3 servings/day,
- vegetables, 2 servings/day,
- bread/pasta/other cereals (preferably whole grain), ≥ 2 servings/day,
- nuts and seeds, 1 serving/day,
- dairy products (preferably low fat) such as milk and yogurt 1-2 servings/day. According to the needs of the subjects and in order to maintain the desired animal- and plant-proteins ratio, milk and yogurt could be substituted with soy- milk and yogurt. The introduction of fresh and aged cheese servings during the week was optional and proposed as a substitute of a serving of white meat.
- legumes, 8 servings/week,
- eggs, 1 serving/week. Egg portions could be increased to 2 to substitute one serving of white meat if desired by the subject.
- fish, 2 servings/week,
- white meat, 2 servings/week
- red meat, 0 serving/week,
- pizza, 1 serving/week.

The vegan diet was a completely plant-based diet, in which all the proteins were plant derived.

A protein supplementation was provided to fulfil the recommended intake exclusively in those cases where protein quantitative nutritional requirements were not supported entirely by foods in the diet. Ultimate Sport® isolated soya proteins (cocoa or vanilla flavoured) was selected as protein supplement.

Except for proteins supplementation, no other supplementation was provided. Indeed, as each dietary protocol was followed for a short term (4 weeks), no supplementation of vitamin B12 and other micronutrients was planned for plant-based diets [51].

For the vegan diet, the frequencies of consumption of foods were managed as follows:

- fruits, 2-3 servings/day,
- vegetables, 2 servings/day,
- bread/pasta/other cereals (preferably whole grain), ≥ 2 servings/day,
- nuts and seeds, 2 servings/day,
- soy- milk and yogurt, 1-2 servings/day,
- legumes, 10 servings/week,
- seitan or tofu, 2 servings/week,
- legume pasta 2 servings/week,
- pizza without animal derived products, 1 serving/week.

Dietary protocols were elaborated using Winfood® software (Medimatica Srl Unipersonale, Colonnella, TE, Italia).

The population sample

18 physically active subjects were enrolled for the study.

Subjects were selected without any gender, socio-economical and ethnical distinctions. Inclusion and exclusion criteria are illustrated hereafter.

Inclusion criteria

- signed information and consensus sheet,
- adult age,
- healthy status,
- constant levels of physical activity,
- $24.99 < \text{BMI} > 18.50 \text{ kg/m}^2$.

Exclusion criteria

- supplements use,
- drugs and/or antibiotics use,

- allergies and/or intolerances (lactose and/or gluten intolerance),
- diseases and injuries during the last 6 months,
- pregnancy or breastfeeding.

Each participant was requested to sign the information and consensus sheet containing all the useful details about the study: aim of the study, study's protocol and diet intervention, length of the study, associated risks and benefits, agreement and privacy notice.

The introductory meeting consisted in the participant anamnesis.

Each participant was asked to explain his/her day in terms of:

- meal organization (i.e., time and daily frequency of the different meals) and food consumption (i.e., how each meal is generally organized, which food is consumed and in which amount),
- eventual food preferences,
- specific food sensibilities,
- physical activity (i.e. type of physical activity, weekly frequency and duration of each single workout),
- daily water consumption.

Moreover, each participant had to describe:

- his/her occupation (i.e., kind of occupation, hours devoted to it in a day),
- eventual allergies and certified intolerances to specific foods or food components,
- presence of disease and/or injury during the last 6 months,
- any disease familiarity or known disease predisposition,
- drugs and/or antibiotics and supplements use.

Other questions regarded:

- bowel functionality and feces consistency,
- eventual energy drops during the day,
- degree of fatigue felt after an effort,
- resting time spent during the night,
- recent haemato-chemical analyses.

Food diary. Dietary habits of participants were evaluated by using a 7-days food diary where reporting foods and beverages consumed daily, for a week. The food diary proposed was based on the EPIC validated food frequency questionnaire [52] and each day was structured in two different sections. The first one regarded the daily food compilation. Each day was organized in 6 meals

(breakfast, morning snack, lunch, afternoon snack, dinner and evening snack) and participants were asked to indicate the qualitative and quantitative composition for each meal. In addition, each participant was request to records the information regarding food preparation. The second section was about the physical activity performed during the week. Subjects had to report the type and the duration of the activity. The fulfilled and delivered-back food diary were then analysed with the help of the professional software WinFood[®] (Medimatica Srl Unipersonale, Colonnella, TE, Italia) in order to obtain a weekly quantitative estimation of nutrients and energy intakes.

Body composition evaluation. For body weight and height measurement, each subject was asked to be barefoot and in underwear. Body weight was measured by using the electronic Wunder[®] scale, RB cable (Trezzo sull'Adda, Milan, Italy), while the height was instead measured by using a portable mechanical altimeter Wunder[®], HR1 model (Trezzo sull'Adda, Milan, Italy) (Figure 8-5). For height recording, the subject was requested to position feet at 60°, the head oriented in the horizontal Frankfurt's plane (the imaginary line that links ears with eyes), the arms laterally laid along the body, the palms toward the thighs, and the shoulder blades and buttocks in contact with the measuring bar. Height measure was read at the end of a normal expiration.



Figure 8-5. Electronic Wunder[®] scale (on the left) and mechanical altimeter Wunder[®] HR1 model.

BMI was calculated as the ratio between the body weight of the subject (kg) and the height square (m²) [53].

Waist circumference was measured using a flexible and inelastic meter with a precision of 0.1 mm. The measure was taken with the subject in underwear clothes, at the end of a normal expiration, with the arms relaxed at the sides, at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest (hip bone) according to WHO STEPS-wise instrument guidelines [54].

Skinfold thickness was measured using a GIMA® skinfold caliper 27320 (Gessate, Milan, Italy) with a precision of 0.2 mm.

Specific body sites were used for skinfold thickness individuation. The skinfold was unstacked using the thumb and forefinger, avoiding interesting the muscular tissue, and placed on the skinfold's basis. After 3 seconds the measure is read from the caliper quadrant (Figure 8-6). Each skinfold thickness was measured three times and the average was considered.

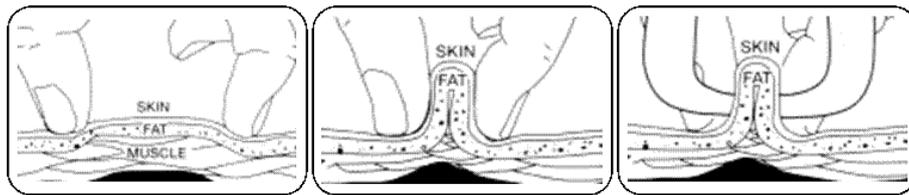


Figure 8-6. Skinfold thickness individuation with skinfold caliper.

The specific skinfolds measured are reported in Table 8-2.

Table 8-2. Skinfold evaluated and measurement protocol.

Skinfolds	Measurement protocol
Triceps	Taken vertically on the posterior surface of the arm, on the middle point of the triceps muscle. The measurement is taken with relaxed arm.
Biceps	Taken vertically on the anterior surface of the arm, at the same level in which triceps skinfold is taken. Taken with relaxed arm.
Subscapular	Taken under the lower angle of the scapula, with a 45° inclination respect to the horizontal plane.

In the study, for subcutaneous fat thickness evaluation, the sum of biceps, triceps and subscapular sites was considered. Skinfold sum was performed as described by Kavak et al. [55] and by Chatterjee et al. [56].

Bioelectrical impedance analysis (BIA) was performed using a single-frequency BIA (50-kHz, BIA AKERN 101®, Akern, Firenze, Italy) with wrist-ankle tetrapolar electrode placement measuring Resistance (Rx) and Reactance (Xc) (Figure 8-7).

The measurement were performed in standard conditions (the same moment of the day and no physical activity immediately before the analysis).

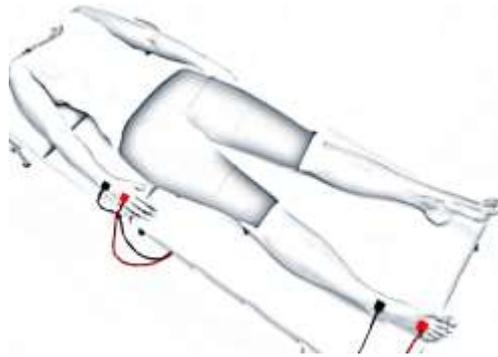


Figure 8-7. Wrist-ankle tetrapolar electrode Bioimpedence analysis.

Both Bioelectrical Impedance Analysis (BIA) and Bioelectrical Impedance Vector Analysis (BIVA) were performed. BIA was performed by evaluating the value of resistance and reactance and using BodyGram Plus® software in order to estimate total body water (TBW), intra-cellular water (ICW), extra-cellular water (ECW), body cell mass (BCM) and body cell mass index (BCMI), which expresses the BCM in relation to subject’s height.

BIVA was resulted in the BIAVECTOR® always provided by BodyGram Plus® software. In this case, impedance was plotted as a bivariate vector from its component R_z and X_c obtained at 50 kHz and standardized by height (Figure 8-8).

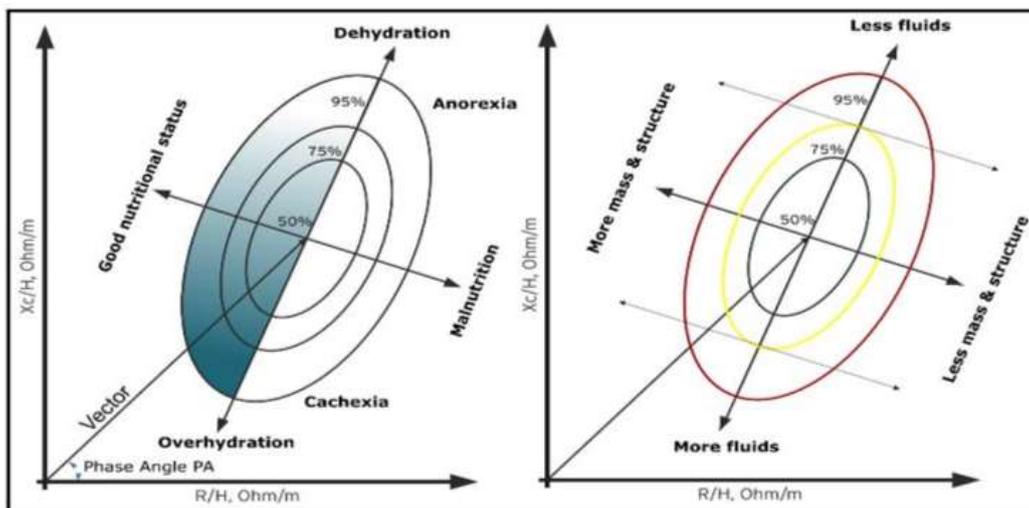


Figure 8-8. BIAVECTOR® analysis provided by BodyGram Plus® software.

Resting metabolic rate evaluation. For the analysis of the resting metabolic rate (RMR), Cortex MetaLyzer 3B® was used (Figure 8-9). A 20-minutes test was performed at the end of each dietary intervention.



Figure 8-9. Cortex MetaLyzer 3B®.

The tests were executed after each dietary intervention at the same hour of the first test and after night fasting or 6 hours of fasting.

Moreover, each participant abstained from physical activity for at least 8 hours before all measurements in accordance to Woods et al. [57].

According to the instrument protocol, each participant had to wear the POLAR® transmitter belt at the level of the thorax (Figure 8-10) and a specific mask (Figure 8-11).



Figure 8-10. Cortex MetaLyzer 3B® belt.



Figure 8-11. Cortex MetaLyzer 3B® face mask.

Later on, an adapter and a disposable turbine were assembled on the mask, then the mask and the instrument were connected through the sample line and the volume transducer (Figure 8-12 and Figure 8-13).



Figure 8-12. Cortex MetaLyzer 3B[®] mask further components (disposable turbine, adapter and sample line).



Figure 8-13. Cortex MetaLyzer 3B[®] mask assembly.

Before starting the analysis, each participant had to lie down for 5 minutes.

By breathing through the volume transducer, the instrument measure the volume of inspired O_2 and exhaled CO_2 continuously and simultaneously. These data, in addition to the heart rate, the ventilation, the room temperature and pressure were registered and processed by MetaSoft[®] software and shown in real time in the PC monitor. The software provides the estimation of the resting metabolic rate, O_2 consumption, CO_2 production and respiratory quotient (CO_2 production/ O_2 consumption).

The instrument took advantage of the method of indirect calorimetry [58, 59]. It is based on the principle that all the energy introduced in the body with food is converted to kinetics or thermal energy. At the same time, starting from a specific amount of inspired O_2 , the macronutrient oxidation produces a specific quantity of CO_2 (different among carbohydrates, lipids and proteins). Consequently, the measurement of the consumed O_2 and the produced CO_2 allows to the estimation of the heat generated during the oxidation and of the energy expenditure. Indeed, oxygen and carbon dioxide were used to calculate the respiratory quotient (CO_2/O_2) or respiratory exchange ratio (RER). Starting from this quotient, it was possible to obtain the percentage of macronutrients

used and the corresponding calories consumed per each litre of oxygen [60]. The analysis of these data resulted in the final estimation of the RMR [61].

Data analysis

Statistical analysis was performed using GraphPad Prism version 9.4.0 for Windows, GraphPad Software, San Diego, California USA. The normality of data distribution was tested through the Shapiro-Wilk test. Descriptive statistics were run and data were expressed as number and percentage (%) and as mean±standard deviation (SD), if data distribution was normal, otherwise as median and min–max range. Differences between groups were estimated using the unpaired Student’s t-test and non-parametric mean comparison test, as appropriate. A p-value < 0.05 was considered statistically significant. For the resting metabolic rate, results are expressed as percentage of oxidated substrates (carbohydrates, %CHO, or fats, %Fats) on total RMR (kcal).

Results

Participants

18 subjects adhered to the 70:30 diet (Mediterranean-like diet). The same participants were invited to test 30:70 and vegan diets. For the 30:70 dietary protocol, 5 drop out were registered during the study while for the vegan diet 4 subjects did not concluded the protocol.

Characteristics of population are reported in Table 8-3.

Table 8-3. Characteristics of the sample populations enrolled for each dietary protocol.

Dietary protocols	Number of subjects	Gender (n. of subjects)			Age (mean ± S.D.)	
		Females	Males	All	Females	Males
70:30	18	11	7	27.8 ± 4.0	27.7 ± 4.8	27.9 ± 2.4
30:70	13	7	6	28.8 ± 4.2	28.9 ± 5.5	28.8 ± 2.6
Vegan	14	8	6	28.9 ± 3.9	28.7 ± 4.9	27.9 ± 2.4

Data expressed as mean ± s.d.

Results of 70:30 dietary protocol in the total sample

In Table 8-4 body composition and resting metabolic rate parameters pre- and post- the 70:30 dietary protocol in addition to the delta values (calculated as the difference of post- and pre-values) in the total sample are presented. Data are reported as mean ± standard deviation or median, (min-max) of the total participants.

Table 8-4. Body composition and resting metabolic rate parameters before (pre) and after (post) the 70:30 diet and delta values (post-pre) in the total sample.

70:30 dietary protocol	All (n = 18)		
	PRE	POST	DELTA
BMI (kg/m ²)	23.6 ± 2.6	23.3 ± 2.6	-0.4 ± 0.4***
Waist circumference (cm)	84.3 ± 9.6	83.9 ± 9.0	-0.4 ± 2.2
Skinfold sum (mm)	42.9 ± 16.0	41.0 ± 14.3	-1.9 ± 4.9
Rz (Ω)	575.0 ± 100.0	568.0 ± 107.4	-0.7 ± 32.3
Xc (Ω)	64.9 ± 9.7	64.5 ± 11.6	0.4 ± 5.4
PhA (°)	6.4 (5.5-7.5)	6.4 (5.7-7.8)	0.0 (-0.6-0.3)
BCM (kg)	25.7 (20.9-40.2)	26.1 (21.0-39.0)	0.4 (-2.5-2.0)
BCMI (BCM kg/m ²)	9.9 ± 1.7	10.0 ± 1.6	0.1 ± 0.4
TBW (L)	34.6 (27.3-49.3)	35.4 (26.5-50.6)	0.5 (-3.9-1.6)
ECW (L)	15.8 (11.6-43.5)	16.1 (11.4-43.7)	0.2 (-1.9-1.7)
ICW (L)	19.8 ± 6.0	19.9 ± 6.1	0.2 ± 0.7
TBW (%)	53.7 (47.5-62.3)	53.7 (50.3-65.3)	1.5 (-4.4-3.8)†
ECW (%)	43.7 ± 2.2	43.6 ± 2.4	-0.1 ± 1.2
ICW (%)	56.4 ± 2.2	56.4 ± 2.4	0.0 ± 1.2
RMR (kcal/day)	1797.0 ± 552.9	1910.0 ± 526.1	112.5 ± 367.4
%CHO (%RMR)	55.5 ± 9.7	59.2 ± 9.3	3.6 ± 13.5
%FAT (%RMR)	37.1 ± 9.7	33.6 ± 9.2	-3.5 ± 13.4

Values are expressed as mean ± standard deviation or median, (min-max).

Paired t-test, *** p<0.001. Wilcoxon test, † p<0.05.

BMI, waist circumference and skinfold sum

BMI, waist circumference and skinfold sum were reported before and after the dietary intervention. After the 70:30 diet, a statistical significant decrease of BMI was recorded (Figures 8-14), mean value (-0.4 ± 0.4 kg/m²) while maintenance of waist circumference and skinfold thickness was reported (Figures 8-15 and 8-16).

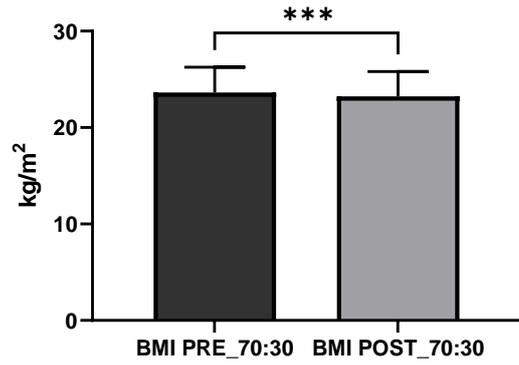


Figure 8-14. BMI before and after the 70:30 dietary protocol. Paired t-test. *** p<0.001. Data are reported as mean ± standard deviation.

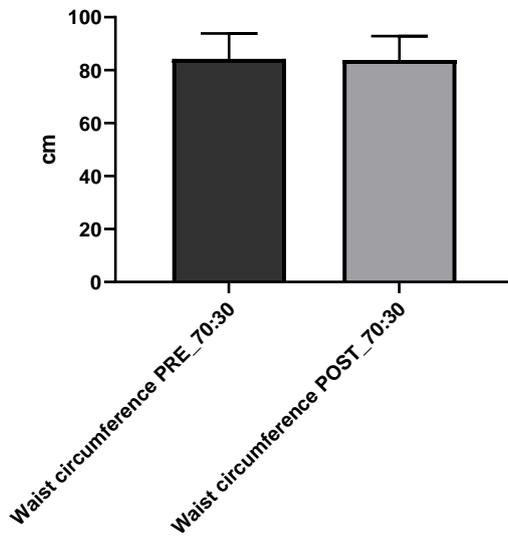


Figure 8-15. Waist circumference before and after the 70:30 dietary protocol. Data are reported as mean ± standard deviation.

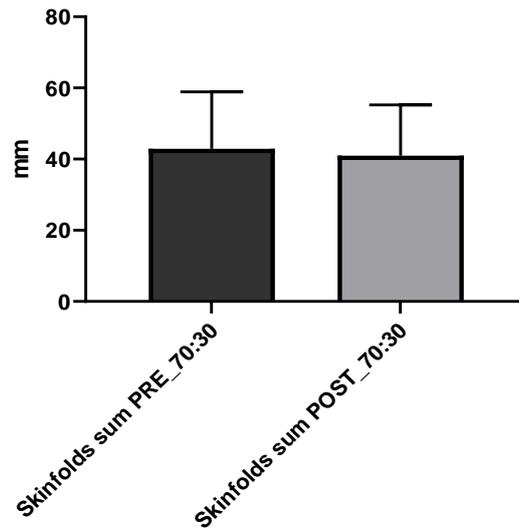


Figure 8-16. Skinfold sum before and after the 70:30 dietary protocol. Data are reported as mean \pm standard deviation.

BIA parameters

Rz, Xc, PhA, BCM and BCMI before and after the 70:30 diet were also recorded.

All parameters were maintained after the 70:30 diet (data are shown from Figure 8-17 to Figure 8-21).

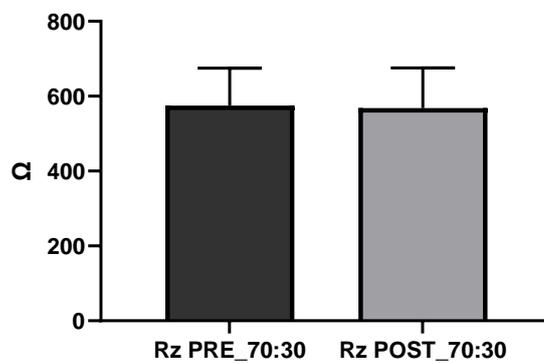


Figure 8-17. Rz before and after the 70:30 dietary protocol. Data are reported as mean \pm standard deviation.

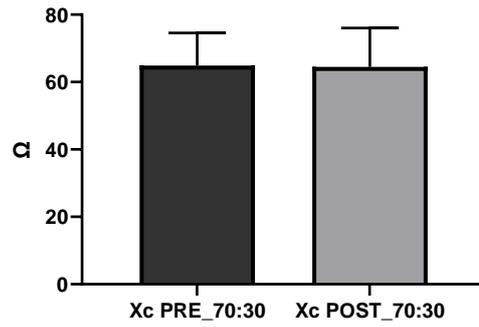


Figure 8-18. Xc before and after the 70:30 dietary protocol. Data are reported as mean \pm standard deviation.

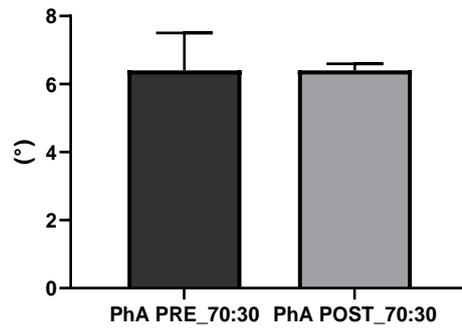


Figure 8-19. PhA before and after the 70:30 dietary protocol. Data are reported as median with min-max range.

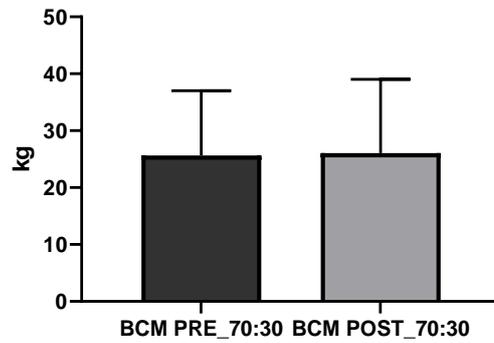


Figure 8-20. BCM before and after the 70:30 dietary protocol. Data are reported as median with min-max range.

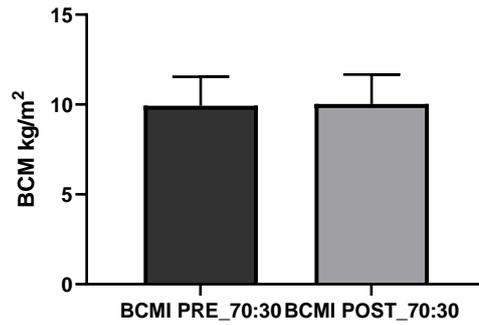


Figure 8-21. BCMI before and after the 70:30 dietary protocol. Data are reported as mean \pm standard deviation.

Results related to TBW, ECW and ICW were also reported before and after the 70:30 diet in the total sample. Data are shown as litres and as percentage. TBW is expressed as percentage of body weight while ECW and ICW as percentage of TBW. Regarding TBW (L), the median value was maintained, indeed a slight but not statistical significant difference of 0.5 (-3.9-1.6) L was found. A maintenance was reported also for ECW (L) and ICW (L). For TBW (%) a statistical significant difference of median values (1.5 (-4.4-3.8) %), was observed after the 70:30 dietary protocol, while a maintenance of mean values was found for ECW (%), and for ICW (%). Data are shown from Figure 8-22 to Figure 8-27.

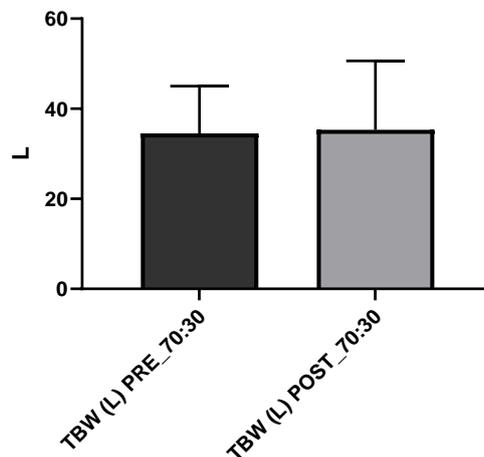


Figure 8-22. TBW (L) before and after the 70:30 dietary protocol. Data are reported as median with min-max range.

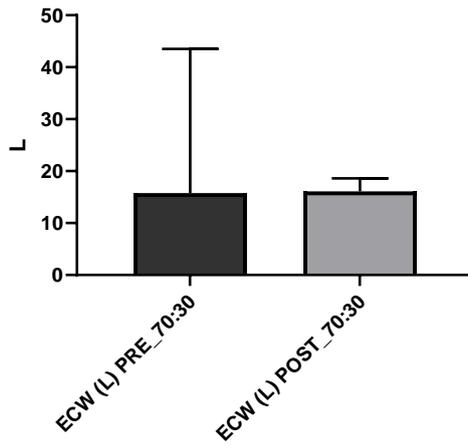


Figure 8-23. ECW (L) before and after the 70:30 dietary protocol. Data are reported as median with min–max range.

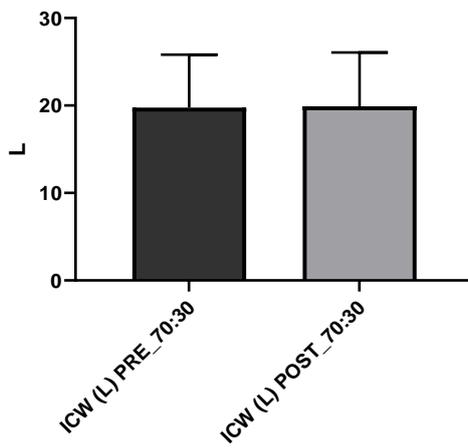


Figure 8-24. ICW (L) before and after the 70:30 dietary protocol. Data are reported as mean \pm standard deviation.

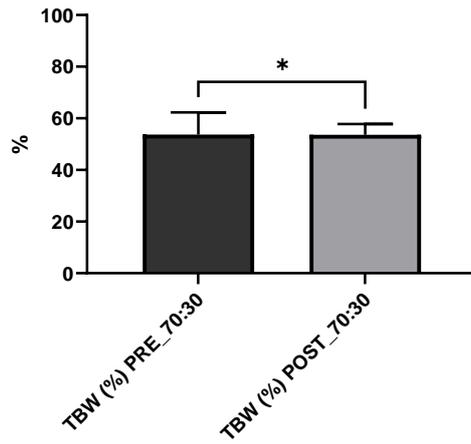


Figure 8-25. TBW (%) before and after the 70:30 dietary protocol. Wilcoxon test, * $p < 0.05$. Data are reported as median with min–max range.

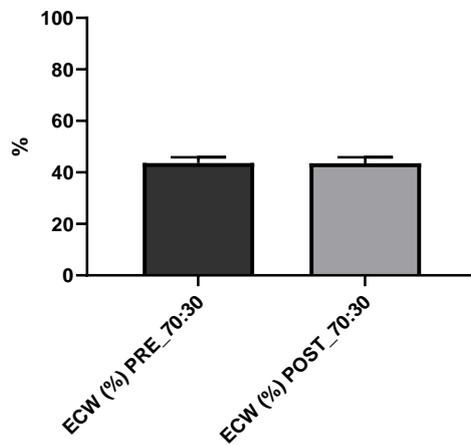


Figure 8-26. ECW (%) before and after the 70:30 dietary protocol. Data are reported as mean \pm standard deviation.

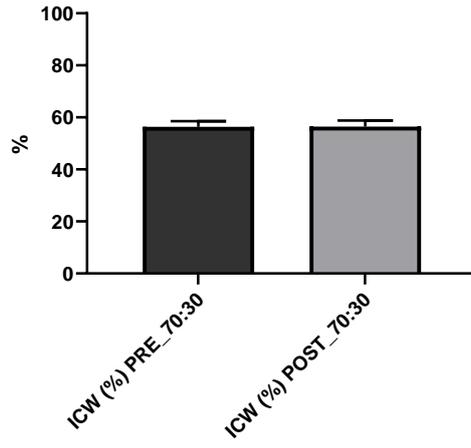


Figure 8-27. ICW (%) before and after the 70:30 dietary protocol.
Data are reported as mean \pm standard deviation.

RMR evaluation

After the 70:30 diet, RMR was maintained, indeed a slight but not statistical significant difference was reported (112.5 ± 367.4 kcal/day) (Figure 8-28). Also %CHO and %Fat were maintained after the dietary protocol (Figure 8-29).

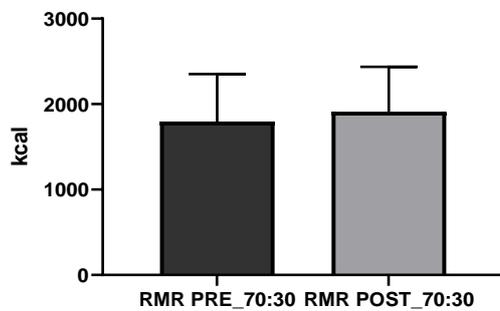


Figure 8-28. RMR before and after the 70:30 dietary protocol.
Data are reported as mean \pm standard deviation.

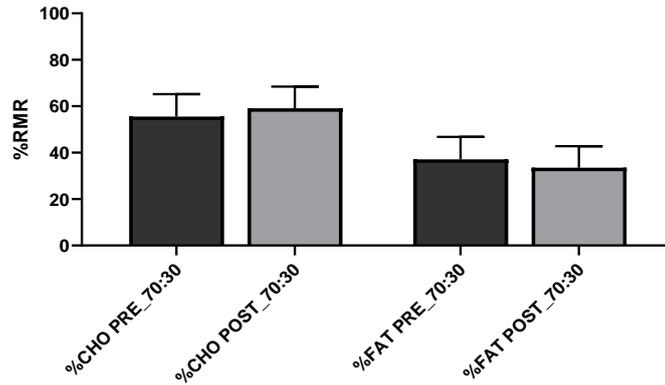


Figure 8-29. %CHO and %Fats (%RMR) before and after the 70:30 dietary protocol. Data are reported as mean \pm standard deviation.

Results of 30:70 dietary protocol in the total sample

In Table 8-5 body composition and resting metabolic rate parameters pre- and post- the 30:70 dietary protocol in addition to the delta values (calculated as the difference of post- and pre-values) in the total sample are reported.

Table 8-5. Body composition and resting metabolic rate parameters before (pre) and after (post) the 30:70 diet and delta values (post-pre) in the total sample.

30:70 dietary protocol	All (n = 13)		
	PRE	POST	DELTA
BMI (kg/m ²)	23.6 \pm 2.8	23.5 \pm 2.7	-0.1 \pm 0.4
Waist circumference (cm)	83.7 \pm 9.5	83.6 \pm 9.7	-0.1 \pm 1.8
Skinfold sum (mm)	36.2 \pm 13.7	36.1 \pm 13.0	-0.1 \pm 2.4
Rz (Ω)	540.1 \pm 98.2	534.3 \pm 92.8	-5.8 \pm 23.6
Xc (Ω)	62.0 \pm 10.8	60.1 \pm 9.3	-1.9 \pm 4.4
PhA ($^{\circ}$)	6.6 \pm 0.1	6.5 \pm 0.6	-0.1 \pm 0.3
BCM (kg)	27.1 (21.9-39.0)	26.9 (21.7-40.0)	-0.2 (-1.4-1.4)
BCMI (BCM kg/m ²)	10.4 \pm 1.7	10.3 \pm 1.8	-0.1 \pm 0.3
TBW (L)	37.1 \pm 8.3	37.3 \pm 8.5	0.2 \pm 1.4
ECW (L)	16.8 \pm 3.4	17.1 \pm 3.4	0.3 \pm 0.8
ICW (L)	20.1 (15.1-28.6)	20.0 (15.3-37.4)	0.1 (-0.8-9.1)
TBW (%)	56.0 (51.3-65.3)	56.9 (51.2-66.6)	0.9 (-1.7-1.6)
ECW (%)	43.3 \pm 2.5	43.8 \pm 2.4	0.5 \pm 1.2
ICW (%)	22.2 \pm 5.2	22.9 \pm 6.7	0.7 \pm 2.6

RMR (kcal/day)	2162 ± 440.8	2022 ± 338.2	-139.5 ± 234.9
%CHO (%RMR)	62.8 ± 7.9	55.4 ± 6.2	-7.4 ± 12.0
%FAT (%RMR)	30.0 ± 7.8	37.3 ± 6.1	7.3 ± 11.9

Values are expressed as mean ± standard deviation or median, (min-max).

BMI, waist circumference and skinfold sum

BMI, waist circumference and skinfold sum were reported before and after the dietary intervention. After the 30:70 diet, a maintenance of BMI was reported as for waist circumference and skinfolds thickness sum (Figures 8-30, 8-31, 8-32).

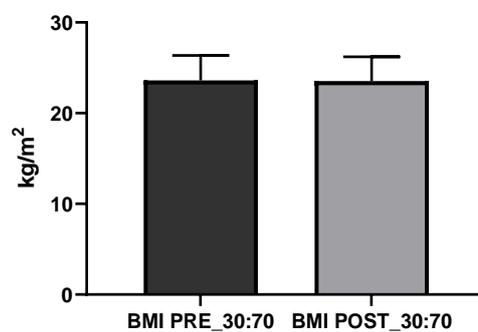


Figure 8-30. BMI before and after the 30:70 dietary protocol. Data are reported as mean ± standard deviation.

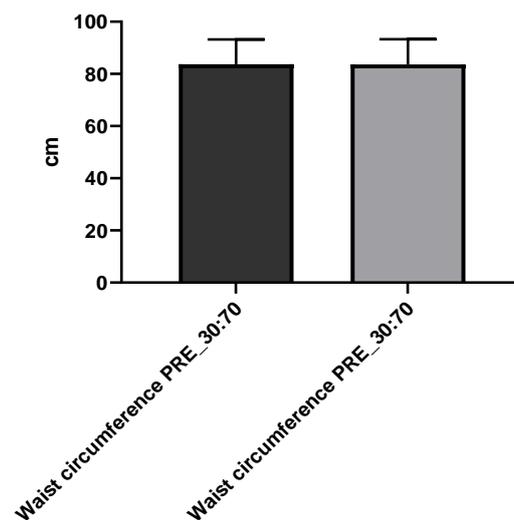


Figure 8-31. Waist circumference before and after the 30:70 dietary protocol. Data are reported as mean ± standard deviation.

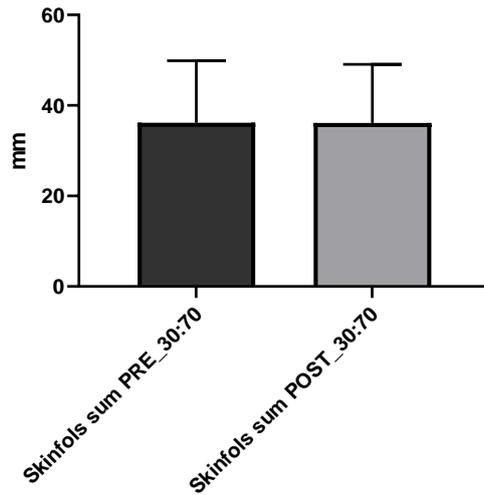


Figure 8-32. Skinfolds sum before and after the 30:70 dietary protocol. Data are reported as mean \pm standard deviation.

BIA parameters

The maintenance of Rz, Xc, PhA, BCM and BCMI before and after the 30:70 diet was observed. (data are shown from Figure 8-33 to Figure 8-37).

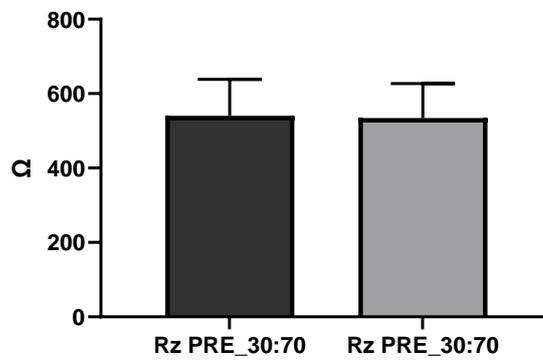


Figure 8-33. Rz before and after the 30:70 dietary protocol. Data are reported as mean \pm standard deviation.

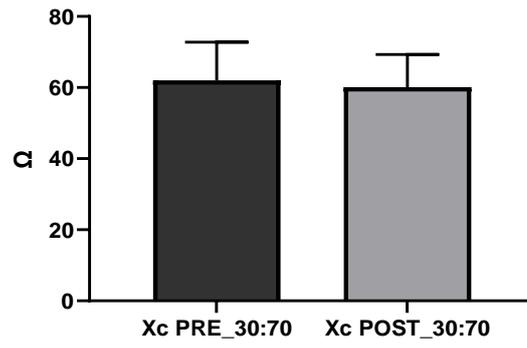


Figure 8-34. Xc before and after the 30:70 dietary protocol. Data are reported as mean \pm standard deviation.

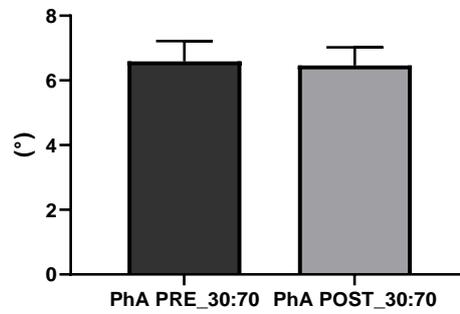


Figure 8-35. PhA before and after the 30:70 dietary protocol. Data are reported as mean \pm standard deviation.

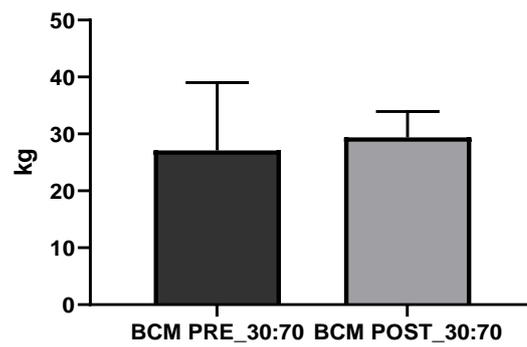


Figure 8-36. BCM before and after the 30:70 dietary protocol. Data are reported as median with min-max range.

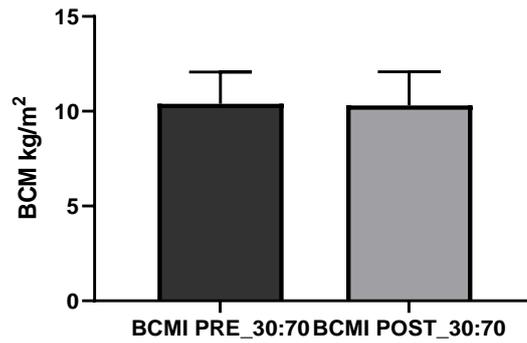


Figure 8-37. BCMI before and after the 30:70 dietary protocol.
Data are reported as mean \pm standard deviation.

Results related to TBW, ECW and ICW were reported. Data are shown as litres and as percentage. TBW is expressed as percentage of body weight while ECW and ICW as percentage of TBW. TBW, ECW and ICW were maintained after 30:70 diet. Data are shown from Figure 8-38 to Figure 8-43.

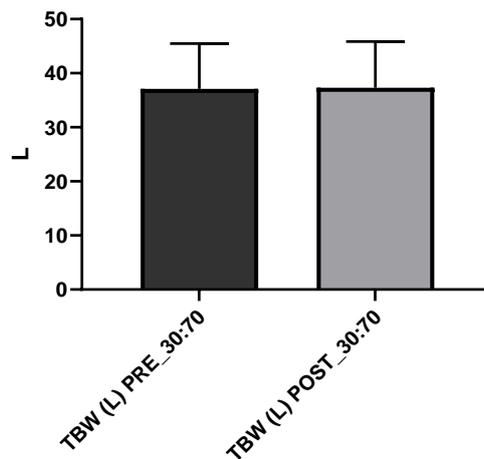


Figure 8-38. TBW (L) before and after the 30:70 dietary protocol.
Data are reported as mean \pm standard deviation.

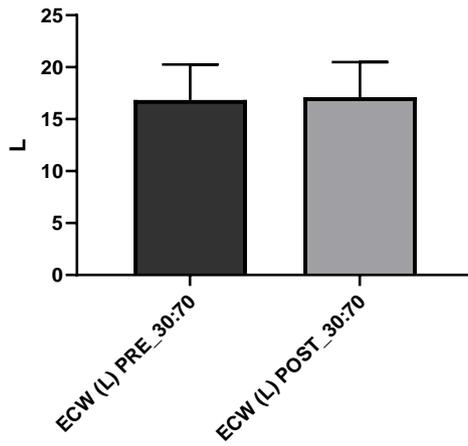


Figure 8-39. ECW (L) before and after the 30:70 dietary protocol. Data are reported as mean \pm standard deviation.

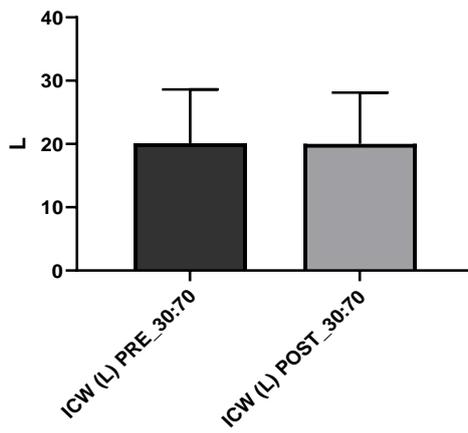


Figure 8-40. ICW (L) before and after the 30:70 dietary protocol. Data are reported as median with min-max range.

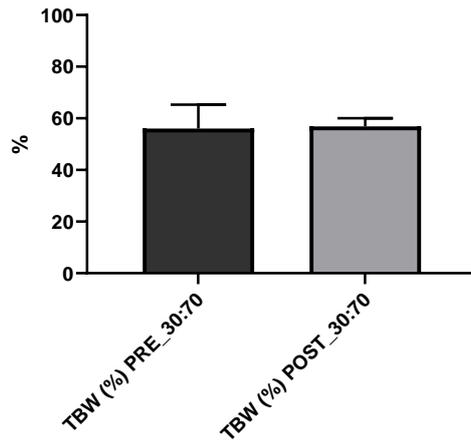


Figure 8-41. TBW (%) before and after the 30:70 dietary protocol. Data are reported as median with min–max range.

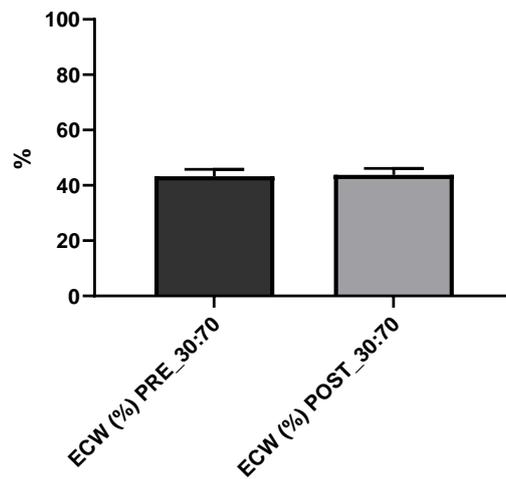


Figure 8-42. ECW (%) before and after the 30:70 dietary protocol. Data are reported as mean \pm standard deviation.

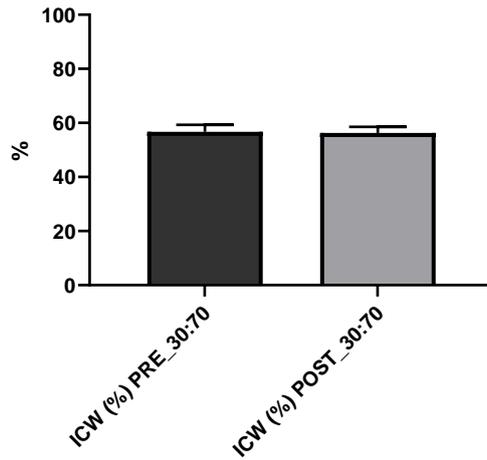


Figure 8-43. ICW (%) before and after the 30:70 dietary protocol.
Data are reported as mean \pm standard deviation.

RMR evaluation

RMR evaluation was carried out (Figure 8-44). Although no statistically different, a decrease of RMR (-139.5 ± 234.9 kcal/day) was reported. In addition, differences on %CHO and %Fat utilization were observed resulting in -7.4 ± 12.0 %RMR and 7.3 ± 11.9 %RMR, respectively (Figure 8-45).

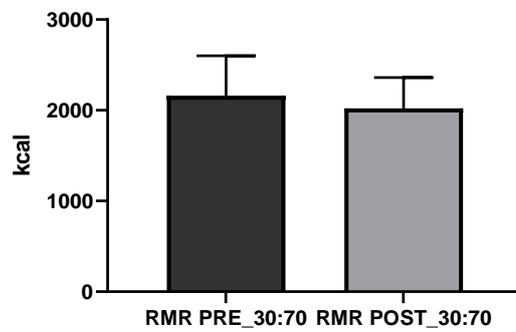


Figure 8-44. RMR before and after the 30:70 dietary protocol.
Data are reported as mean \pm standard deviation.

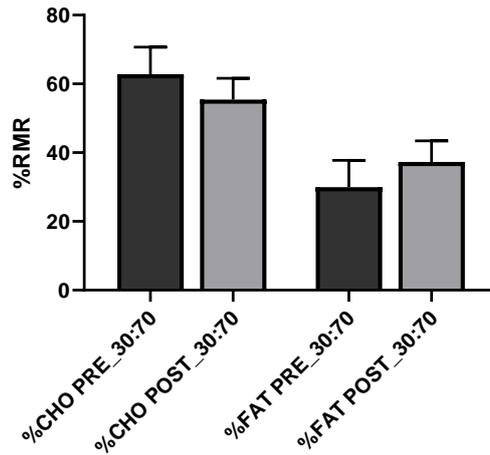


Figure 8-45. %CHO and %Fats (%RMR) before and after the 30:70 dietary protocol. Data are reported as mean \pm standard deviation.

Results of vegan dietary protocol in the total sample

In Table 8-6 body composition and resting metabolic rate parameters pre- and post- the vegan dietary protocol in addition to the delta values (calculated as the difference of post- and pre-values) in the total sample are presented.

Table 8-6. Body composition and resting metabolic rate parameters before (pre) and after (post) the vegan diet and delta values (post-pre) in the total sample.

Vegan dietary protocol	All (n = 14)		
	PRE	POST	DELTA
BMI (kg/m ²)	23.9 \pm 2.4	23.7 \pm 2.5	-0.3 \pm 0.4
Waist circumference (cm)	84.5 \pm 9.5	84.3 \pm 9.6	-0.2 \pm 2.2
Skinfold sum (mm)	38.8 \pm 15.2	37.2 \pm 14.1	-1.6 \pm 3.4
Rz (Ω)	548.4 \pm 88.9	539.7 \pm 86.1	-8.7 \pm 40.0
Xc (Ω)	62.6 \pm 7.6	61.7 \pm 6.9	-0.9 \pm 5.2
PhA ($^{\circ}$)	6.6 \pm 0.7	6.6 \pm 0.6	-0.0 \pm 0.4
BCM (kg/m ²)	26.6 (21.0-39.9)	27.0 (20.9-42.2)	-0.3 (-2.6-5.3)
BCMI (BCM kg/m ²)	10.3 \pm 1.8	10.4 \pm 1.7	0.0 \pm 0.6
TBW (L)	36.3 (27.1-49.5)	35.5 (28.5-50.6)	-0.1 (-1.7-4.7)
ECW (L)	16.3 \pm 3.1	16.5 \pm 3.4	0.2 \pm 1.0
ICW (L)	19.6 (15.3-29.0)	19.9 (15.3-30.8)	-0.2 (1.6-3.8)
TBW (%)	55.4 \pm 4.7	56.5 \pm 5.1	1.1 \pm 2.3

ECW (%)	43.3 ± 2.8	43.3 ± 2.4	-0.0 ± 1.8
ICW (%)	56.7 ± 2.8	56.1 ± 3.5	-0.6 ± 3.0
RMR (kcal/day)	1925 ± 446.0	1944 ± 295.0	18.7 ± 207.7
%CHO (%RMR)	58.2 ± 5.7	62.8 ± 7.2	4.6 ± 6.1
%FAT (%RMR)	34.5 ± 5.7	29.7 ± 6.8	-4.8 ± 5.9

Values are expressed as mean ± standard deviation or median, (min-max).

BMI, waist circumference and skinfold sum

BMI, waist circumference and skinfold sum were reported before and after the dietary intervention. After the vegan diet, BMI evaluated as mean values was preserved, indeed a slight but not statistical significant difference was recorded ($-0.3 \pm 0.4 \text{ kg/m}^2$) (Figure 8-46). Waist circumference and skinfold sum were preserved (Figure 8-47 and Figure 8-48).

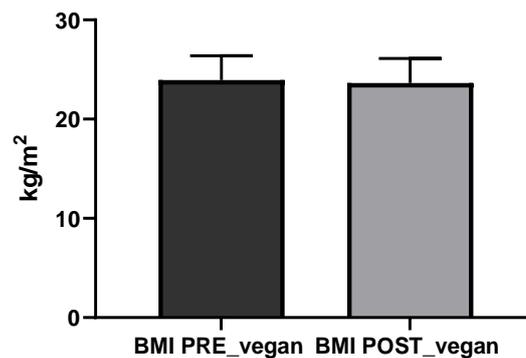


Figure 8-46. BMI before and after the vegan dietary protocol.
Data are reported as mean ± standard deviation.

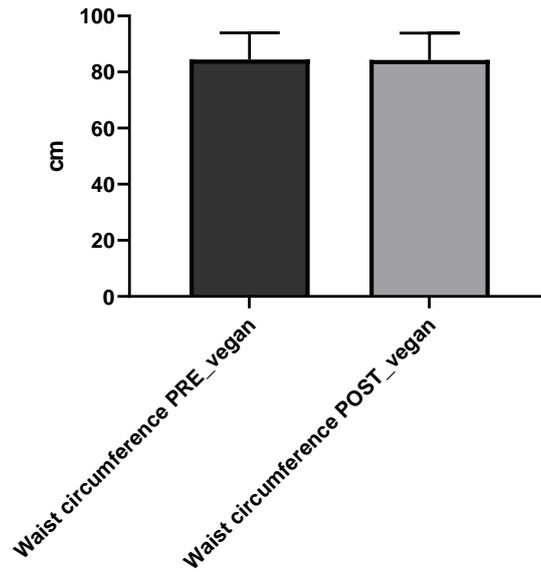


Figure 8-47. Waist circumference before and after the vegan dietary protocol. Data are reported as mean \pm standard deviation.

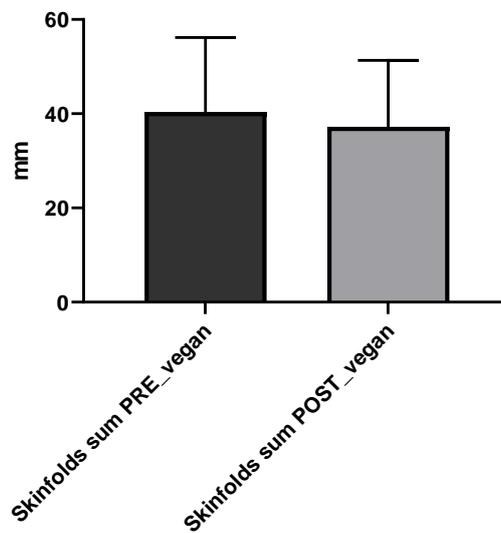


Figure 8-48. Skinfolds sum before and after the vegan dietary protocol. Data are reported as mean \pm standard deviation.

BIA parameters

Rz, Xc, PhA, BCM and BCMI before and after the vegan diet were also reported. A maintenance of mean values for Rz and Xc was observed and median value of PhA of 6.6° was preserved. BCM and BCMI were also maintained. Data are shown from Figure 8-49 to Figure 8-53, respectively.

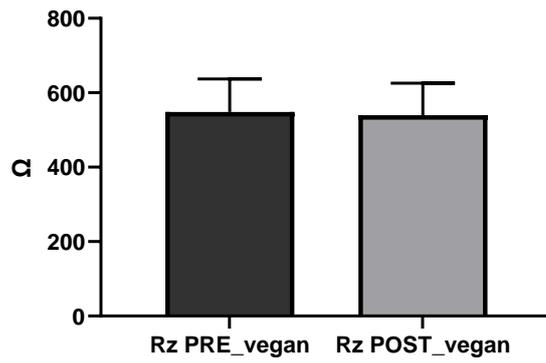


Figure 8-49. Rz before and after the vegan dietary protocol.
Data are reported as mean \pm standard deviation.

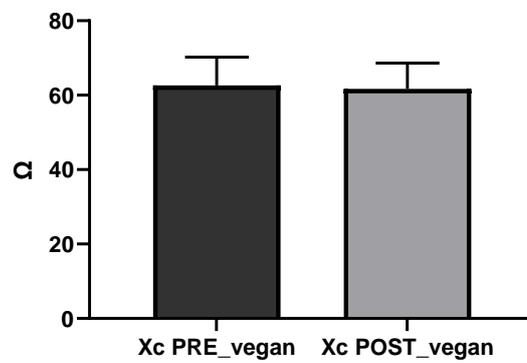


Figure 8-50. Xc before and after the vegan dietary protocol.
Data are reported as mean \pm standard deviation.

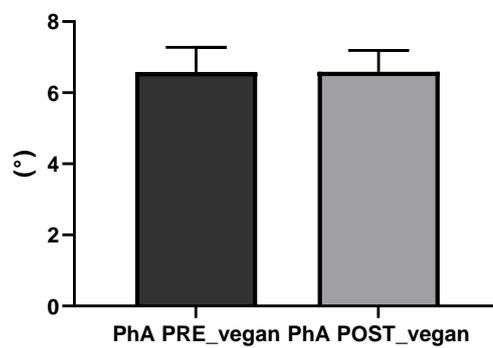


Figure 8-51. PhA before and after the vegan dietary protocol.
Data are reported as mean \pm standard deviation.

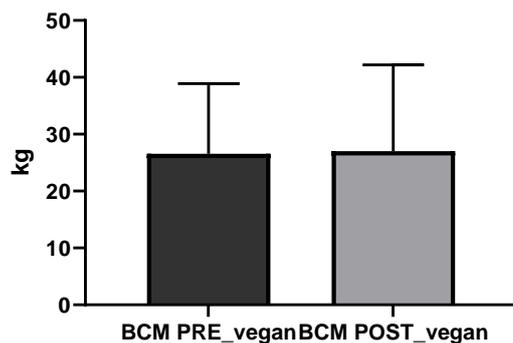


Figure 8-52. BCM before and after the vegan dietary protocol. Data are reported as median with min–max range.

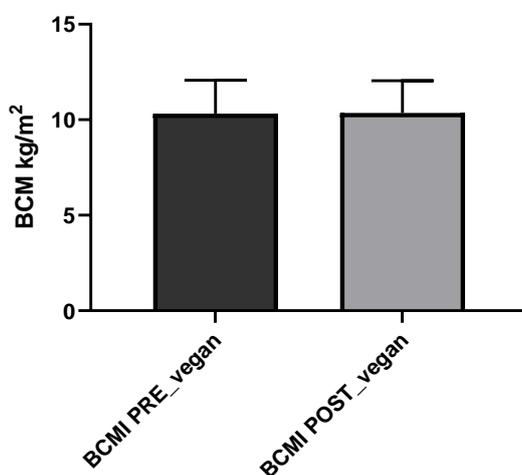


Figure 8-53. BCMI before and after the vegan dietary protocol. Data are reported as mean \pm standard deviation.

Results related to TBW, ECW and ICW were also reported. Data are shown as litres and as percentage. TBW is expressed as percentage of body weight while ECW and ICW as percentage of TBW. Regarding TBW (L), the median value was preserved. A maintenance of ECW (L) and ICW (L) was also reported. A slight difference of mean values of TBW (%) (1.1 ± 2.3 %) was observed after the vegan dietary protocol although no statistical significant. Also ECW (%) and ICW (%) were maintained. Data are shown from Figure 8-54 to Figure 8-59.

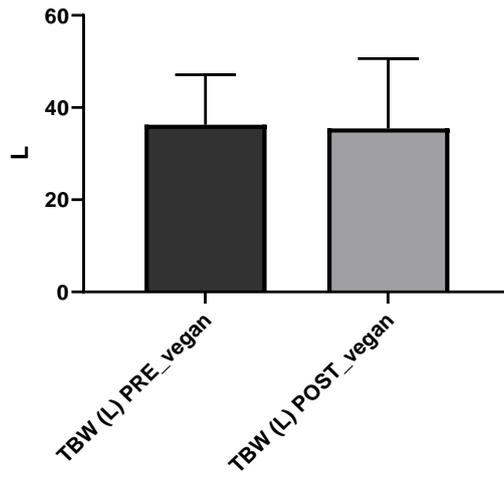


Figure 8-54. TBW (L) before and after the vegan dietary protocol. Data are reported as median with min–max range.

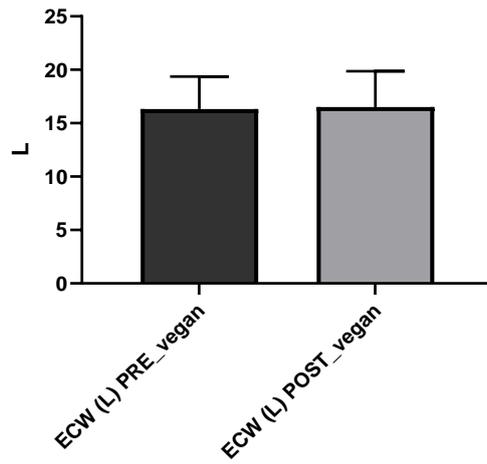


Figure 8-55. ECW (L) before and after the vegan dietary protocol. Data are reported as mean \pm standard deviation.

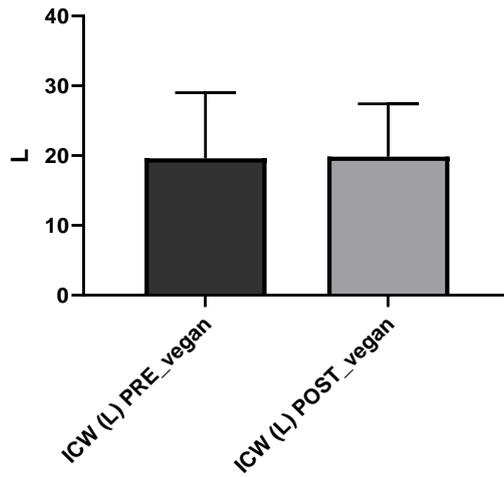


Figure 8-56. ICW (L) before and after the vegan dietary protocol. Data are reported as median with min–max range.

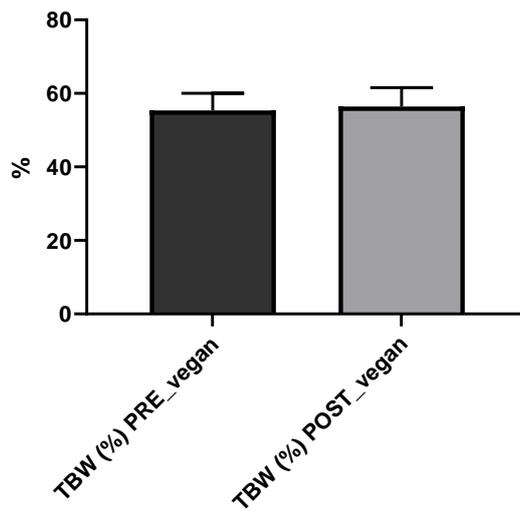


Figure 8-57. TBW (%) before and after the vegan dietary protocol. Data are reported as mean \pm standard deviation.

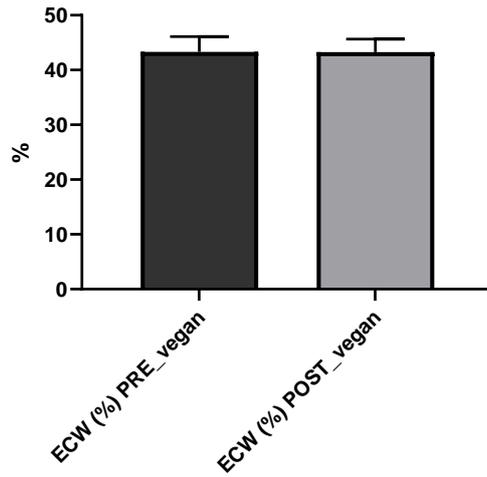


Figure 8-58. ECW (%) before and after the vegan dietary protocol. Data are reported as mean \pm standard deviation.

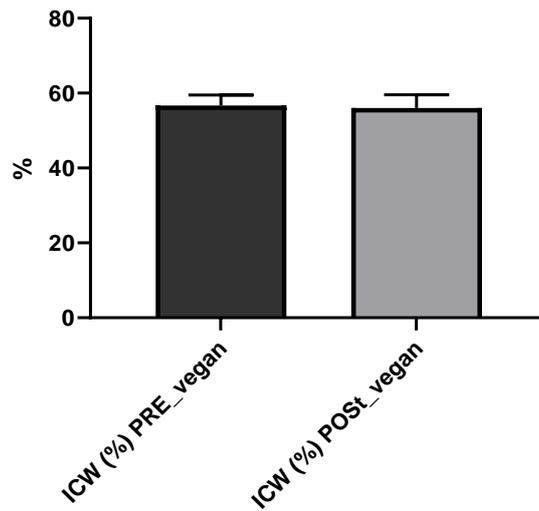


Figure 8-59. ICW (%) before and after the vegan dietary protocol. Data are reported as mean \pm standard deviation.

RMR evaluation

RMR evaluation was carried out. RMR remains constant (Figure 8-60) as well as no variation in terms of used substrate was observed (Figure 8-61).

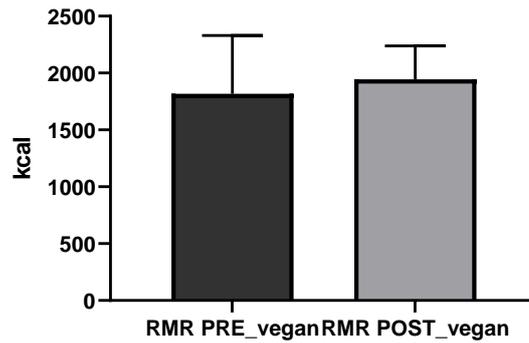


Figure 8-60. RMR before and after the vegan dietary protocol. Data are reported as mean \pm standard deviation.

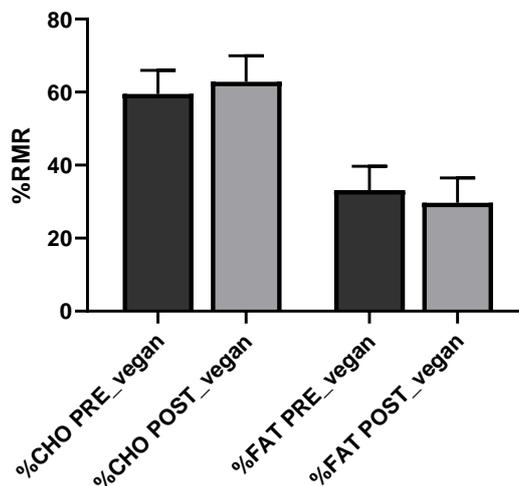


Figure 8-61. %CHO and %Fats (%RMR) before and after the vegan dietary protocol. Data are reported as mean \pm standard deviation.

Results of 70:30 dietary protocol in females

In Table 8-7 body composition and resting metabolic rate parameters pre- and post- the 70:30 dietary protocol in addition to the delta values (calculated as the difference of post- and pre-values) in females are presented. Data are reported as mean \pm standard deviation or median, (min-max) of the females participants.

Data related to TBW, ECW and ICW are shown as litres and as percentage. TBW is expressed as percentage of body weight while ECW and ICW as percentage of TBW.

Table 8-7. Body composition and resting metabolic rate parameters before (pre) and after (post) the 70:30 diet and delta values (post-pre) in females.

70:30 dietary protocol	Females (n = 11)		
	PRE	POST	DELTA
BMI (kg/m ²)	22.5 ± 2.5	22.1 ± 2.3	-0.4 ± 0.4**
Waist circumference (cm)	80.7 ± 8.9	80.1 ± 8.6	-0.5 ± 2.1
Skinfold sum (mm)	47.5 ± 12.8	46.8 ± 11.9	-0.7 ± 3.9
Rz (Ω)	640.8 ± 64.2	634.5 ± 79.4	-6.3 ± 33.4
Xc (Ω)	70.3 ± 8.3	69.9 ± 10.7	0.4 ± 5.8
PhA (°)	6.3 ± 0.4	6.3 ± 0.3	-0.0 ± 0.3
BCM (kg)	23.6 ± 2.3	23.8 ± 2.1	0.2 ± 0.8
BCMI (BCM kg/m ²)	8.8 ± 0.7	8.9 ± 0.6	0.1 ± 0.3
TBW (L)	31.1 ± 3.2	31.2 ± 3.8	0.1 ± 0.9
ECW (L)	13.9 ± 1.7	13.9 ± 2.1	0.1 ± 0.8
ICW (L)	17.2 ± 1.8	17.3 ± 1.8	0.1 ± 0.4
TBW (%)	51.3 (47.5-55.9)	52.9 (50.3-57.8)	1.3 (-2.4-3.8)
ECW (%)	44.6 ± 1.8	44.5 ± 1.5	-0.1 ± 1.3
ICW (%)	55.4 ± 1.8	55.5 ± 1.5	0.1 ± 1.3
RMR (kcal/day)	1529 ± 508.5	1640 ± 363.9	111.3 ± 368.0
%CHO (%RMR)	57.4 ± 11.3	57.4 ± 11.1	0.0 ± 15.2
%FAT (%RMR)	35.2 ± 11.3	35.3 ± 11.0	0.1 ± 15.0

Values are expressed as mean ± standard deviation or median, (min-max).
Paired t-test, ** p<0.01.

BMI, waist circumference and skinfold sum were registered before and after the dietary intervention (Table 8-7). After the 70:30 diet in females, a statistical significant decrease of BMI was recorded, mean value (-0.4 ± 0.4 kg/m²). Regarding waist circumference mean values were preserved after the protocol, indeed a slight but not statistical significant difference was observed, (-0.5 ± 2.1 cm). Skinfolds data are presented as the sum (mm) of triceps, biceps and subscapular skinfolds and a slight decrease in the total mean was reported, but not significant (-0.7 ± 3.9 mm). Rz, Xc, PhA, BCM and BCMI before and after the 70:30 diet were also reported (Table 8-8). A maintenance of mean values for Rz and Xc was observed ((-6.3 ± 33.4 Ω) and (0.4 ± 5.8 Ω), respectively). In addition, mean value of PhA of 6.3° was preserved. BCM and BCMI values were maintained after 70:30 diet.

Results related to TBW, ECW and ICW were also reported in females (Table 8-7). A maintenance of TBW, ECW and ICW was observed.

Although not statistically significant in RMR, a difference of mean values (111.3 ± 368.0 kcal/day) was reported after the 70:30 diet, while for %CHO and %Fat mean values were maintained.

Results of 70:30 dietary protocol in males

In Table 8-8, body composition and resting metabolic rate parameters pre- and post- the 70:30 dietary protocol in addition to the delta values (calculated as the difference of post- and pre-values) in males are presented. Data related to TBW, ECW and ICW are shown as litres and as percentage. TBW is expressed as percentage of body weight while ECW and ICW as percentage of TBW.

Table 8-8. Body composition and resting metabolic rate parameters before (pre) and after (post) the 70:30 diet and delta values (post-pre) in males.

70:30 dietary protocol	Males (n = 7)		
	PRE	POST	DELTA
BMI (kg/m ²)	25.5 ± 1.7	25.1 ± 1.7	-0.3 ± 0.3*
Waist circumference (cm)	90.1 ± 8.0	89.7 ± 6.5	-0.3 ± 2.5
Skinfold sum (mm)	35.7 ± 18.8	31.9 ± 13.5	-3.7 ± 6.1
Rz (Ω)	471.6 ± 32.3	463.4 ± 36.2	-8.1 ± 33.0
Xc (Ω)	56.4 ± 3.8	56.0 ± 7.0	-0.4 ± 5.2
PhA (°)	6.8 ± 0.5	6.9 ± 0.7	0.1 ± 0.2
BCM (kg)	36.7 ± 2.3	37.0 ± 2.2	0.3 ± 1.5
BCMI (BCM kg/m ²)	11.7 ± 0.9	11.8 ± 1.0	0.1 ± 0.5
TBW (L)	46.7 ± 2.5	47.0 ± 2.2	0.3 ± 1.9
ECW (L)	19.7 (17.6-43.5)	19.9 (17.8-43.7)	0.2 (-1.9-1.5)
ICW (L)	26.9 (5.6-29.4)	27.4 (5.5-28.5)	0.6 (-2.0-1.4)
TBW (%)	58.4 ± 3.9	59.5 ± 4.3	1.2 ± 2.6
ECW (%)	42.5 (39.6-44.9)	43.7 (38.5-44.6)	-0.3 (-1.2 ± 1.9)
ICW (%)	57.9 ± 1.9	57.9 ± 2.8	-0.0 ± 1.1
RMR (kcal/day)	2268 ± 196.1	2383 ± 435.7	114.8 ± 423.1
%CHO (%RMR)	52.2 ± 6.0	62.2 ± 4.9	10.0 ± 8.1
%FAT (%RMR)	40.4 ± 5.9	30.6 ± 4.8	-9.8 ± 7.9

Values are expressed as mean ± standard deviation or median, (min-max).

Paired t-test, *p<0.05.

BMI, waist circumference and skinfold sum were registered before and after the 70:30 dietary intervention (Table 8-8). A statistical significant decrease of BMI was recorded, mean value ($-0.3 \pm 0.3 \text{ kg/m}^2$). Regarding waist circumference, mean values were maintained, indeed a slight no statistical significant difference was pointed out ($-0.3 \pm 2.5 \text{ cm}$). Skinfolds data, presented as the sum (mm) of triceps, biceps and subscapular skinfolds, were maintained.

Rz, Xc, PhA, BCM and BCMI before and after the 70:30 diet were also reported (Table 8-8). After the 70:30 diet, a maintenance of mean values for Rz and Xc was observed as for PhA.

For BCM and for BCMI mean values were preserved after the protocol, indeed a slight but not statistical significant difference was found for BCM ($0.3 \pm 1.5 \text{ kg}$), and for BCMI ($0.1 \pm 0.5 \text{ kg/m}^2$), after the 70:30 diet.

No effects on body water (TBW, ICW and ECW) were observed after dietary intervention.

Regarding RMR evaluation in males, a difference of mean values ($114.8 \pm 423.1 \text{ kcal/day}$) was reported, although not statistical significant, while %CHO and %Fat were maintained.

Results of 30:70 dietary protocol in females

In Table 8-9 body composition and resting metabolic rate parameters pre- and post- the 30:70 dietary protocol in addition to the delta values (calculated as the difference of post- and pre-values) in females are presented. Data related to TBW, ECW and ICW are shown as litres and as percentage. TBW is expressed as percentage of body weight while ECW and ICW as percentage of TBW.

BMI, waist circumference and skinfold sum were reported before and after the dietary intervention (Table 8-9). After the 30:70 diet in females, for BMI the mean value was maintained as for waist circumference and skinfolds thickness sum.

Rz, Xc, PhA, BCM and BCMI before and after the 30:70 diet were also reported (Table 8-9): a maintenance of mean values for Rz, Xc and PhA was observed.

For BCM and for BCMI, the mean values were maintained, indeed a slight but not statistical significant difference of ($-0.4 \pm 0.5 \text{ kg}$) was observed for BCM and of ($-0.2 \pm 0.2 \text{ kg/m}^2$) for BCMI.

Results related to TBW, ECW and ICW were also reported (Table 8-9). Mean value of TBW (L) of 32.2 L was preserved. Also ECW (L) and ICW (L) were maintained. TBW (%), ECW (%) and ICW (%) were also preserved after dietary intervention.

Table 8-9. Body composition and resting metabolic rate parameters before (pre) and after (post) the 30:70 diet and delta values (post-pre) in females.

30:70 dietary protocol	Females (n = 7)		
	PRE	POST	DELTA
BMI (kg/m ²)	22.7 ± 2.7	22.0 ± 2.4	-0.1 ± 0.4
Waist circumference (cm)	78.8 ± 9.3	78.1 ± 9.0	-0.6 ± 1.7
Skinfold sum (mm)	43.1 ± 13.7	42.8 ± 12.9	-0.2 ± 2.7
Rz (Ω)	606.7 ± 80.0	601.7 ± 61.8	-5.0 ± 31.0
Xc (Ω)	66.9 ± 11.1	64.9 ± 9.1	-2.0 ± 5.6
PhA (°)	6.3 ± 0.4	6.1 ± 0.4	-0.1 ± 0.2
BCM (kg)	24.5 ± 2.3	24.1 ± 2.0	-0.4 ± 0.5
BCMI (BCM kg/m ²)	9.1 ± 0.7	8.9 ± 0.7	-0.2 ± 0.2
TBW (L)	32.2 ± 4.3	32.2 ± 3.6	0.0 ± 1.0
ECW (L)	14.4 ± 2.4	14.6 ± 2.0	0.2 ± 0.8
ICW (L)	17.8 ± 2.0	17.6 ± 1.7	-0.2 ± 0.4
TBW (%)	54.1 ± 2.4	54.5 ± 3.0	0.4 ± 1.1
ECW (%)	43.3 ± 2.5	43.8 ± 2.4	0.5 ± 1.2
ICW (%)	55.5 ± 1.6	55.1 ± 1.7	-0.5 ± 1.2
RMR (kcal/day)	1760 (1689-2297)	1679 (1566-2067)	-181.0 (-230.0-69.0)
%CHO (%RMR)	61.9 ± 5.4	58.0 ± 3.8	-3.8 ± 12.4
%FAT (%RMR)	30.9 ± 10.8	34.7 ± 3.7	3.8 ± 12.2

Values are expressed as mean ± standard deviation or median, (min-max).

Although not statistical significant, a difference of RMR median values of -181.0 (-230.0-69.0) kcal/day was reported while for %CHO and %Fat the mean values were maintained after the 30:70 diet.

Results of 30:70 dietary protocol in males

In Table 8-10 body composition and resting metabolic rate parameters pre- and post- the 30:70 dietary protocol in addition to the delta values (calculated as the difference of post- and pre-values) in males are presented. Data are reported as mean ± standard deviation or median, (min-max) of the males participants. Data related to TBW, ECW and ICW are shown as litres and as percentage. TBW is expressed as percentage of body weight while ECW and ICW as percentage of TBW.

Table 8-10. Body composition and resting metabolic rate parameters before (pre) and after (post) the 30:70 diet and delta values (post-pre) in males.

30:70 dietary protocol	Males (n = 6)		
	PRE	POST	DELTA
BMI (kg/m ²)	25.3 ± 1.6	25.3 ± 1.7	-0.0 ± 0.5
Waist circumference (cm)	89.4 ± 6.3	90.0 ± 6.0	0.6 ± 1.9
Skinfold sum (mm)	28.2 ± 9.1	28.3 ± 8.3	0.1 ± 2.3
Rz (Ω)	462.3 ± 44.9	455.7 ± 48.2	-6.7 ± 13.6
Xc (Ω)	56.3 ± 7.7	54.5 ± 6.0	-1.8 ± 3.1
PhA (°)	7.0 ± 0.7	6.8 ± 0.5	-0.1 ± 0.3
BCM (kg)	37.3 ± 2.0	37.3 ± 1.7	0.0 ± 1.2
BCMI (BCM kg/m ²)	11.9 ± 1.0	11.9 ± 1.2	3.0 ± 0.4
TBW (L)	47.1 ± 2.0	47.5 ± 2.4	0.4 ± 1.1
ECW (L)	19.7 ± 1.8	20.1 ± 1.7	0.4 ± 0.8
ICW (L)	27.6 (24.9-28.6)	27.6 (25.8-37.4)	0.8 (-0.8-9.1)
TBW (%)	59.5 ± 5.2	60.1 ± 5.1	0.6 ± 0.8
ECW (%)	41.8 ± 2.7	42.2 ± 2.0	0.4 ± 1.3
ICW (%)	58.2 ± 2.7	57.8 ± 2.0	-0.4 ± 1.3
RMR (kcal/day)	2445 ± 401.2	2297.0 ± 113.3	-148.3 ± 331.6
%CHO (%RMR)	63.8 ± 5.0	52.8 ± 7.6	-11.0 ± 12.3
%FAT (%RMR)	29.0 ± 4.9	39.9 ± 7.5	10.8 ± 12.1

Values are expressed as mean ± standard deviation or median, (min-max).

BMI, waist circumference and skinfold sum were reported before and after the dietary intervention (Table 8-10). Mean value of BMI of 25.3 kg/m² was preserved. Regarding waist circumference, a difference of mean values (0.6 ± 1.9 cm) after the protocol was observed, although not statistically significant. Skinfolds data were maintained before and after the dietary intervention.

Bioimpedance analysis values Rz, Xc, PhA, BCM and BCMI were also preserved.

Regarding TBW (L), a maintenance of around 47 liters was observed. Similar mean values were reported for ECW (L) and for ICW (L). For TBW (%) mean values were maintained as for ECW (%) and ICW (%).

Although not statistically significant, a decrease in RMR was reported and fat substrate utilization results to be enhanced.

Results of vegan dietary protocol in females

In Table 8-11 body composition and resting metabolic rate parameters pre- and post- the vegan dietary protocol in addition to the delta values (calculated as the difference of post- and pre-values) in females are presented. Data are reported as mean \pm standard deviation or median, (min-max) of the females participants. Data related to TBW, ECW and ICW are shown as litres and as percentage. TBW is expressed as percentage of body weight while ECW and ICW as percentage of TBW.

Table 8-11. Body composition and resting metabolic rate parameters before (pre) and after (post) the vegan diet and and delta values (post-pre) in females.

Vegan dietary protocol	Females (n = 8)		
	PRE	POST	DELTA
BMI (kg/m ²)	22.8 \pm 2.4	22.6 \pm 2.4	-0.3 \pm 0.4
Waist circumference (cm)	80.3 \pm 9.5	80.1 \pm 9.5	-0.2 \pm 1.9
Skinfold sum (mm)	47.6 \pm 13.9	44.1 \pm 13.8	-3.5 \pm 3.2
Rz (Ω)	608.8 \pm 62.7	601.9 \pm 50.0	-6.9 \pm 46.2
Xc (Ω)	65.6 \pm 8.4	66.5 \pm 4.2	0.9 \pm 5.0
PhA ($^{\circ}$)	6.2 \pm 0.4	6.3 \pm 0.4	0.2 \pm 0.2
BCM (kg)	23.7 \pm 2.1	24.1 \pm 2.2	0.4 \pm 0.9
BCMI (BCM kg/m ²)	9.2 \pm 0.7	0.2 \pm 0.4	-9.0 \pm 0.7
TBW (L)	31.7 \pm 3.5	31.8 \pm 2.8	0.0 \pm 1.2
ECW (L)	14.2 \pm 2.1	14.0 \pm 1.4	-0.2 \pm 0.8
ICW (L)	17.5 \pm 1.7	17.7 \pm 1.6	0.2 \pm 0.9
TBW (%)	52.8 \pm 3.1	53.7 \pm 4.0	0.9 \pm 2.4
ECW (%)	45.1 \pm 1.9	44.3 \pm 1.7	-0.8 \pm 1.2
ICW (%)	54.9 \pm 1.9	54.5 \pm 3.4	-0.4 \pm 3.7
RMR (kcal/day)	1572 \pm 46.6	1733 \pm 159.9	161.0 \pm 130.2
%CHO (%RMR)	56.4 \pm 1.8	64.7 \pm 4.9	8.3 \pm 3.3
%FAT (%RMR)	36.3 \pm 1.9	28.0 \pm 4.9	-8.4 \pm 3.2

Values are expressed as mean \pm standard deviation or median, (min-max).

BMI, waist circumference and skinfold sum were reported before and after the dietary intervention in females (Table 8-11). After the vegan diet, for BMI the mean values were maintained. Regarding waist circumference a maintenance of mean values was observed. Skinfolds data are presented as

the sum (mm) of triceps, biceps and subscapular skinfolds thickness and a not statistical significant change was reported.

Rz, Xc, PhA, BCM and BCMI before and after the vegan diet were also recorded (Table 8-11). A maintainance of mean values for Rz and Xc was observed) as for PhA, BCM and BCMI.

Results related to TBW, ECW and ICW were also reported (Table 8-11). TBW (L) was maintained. For ECW (L) and for ICW (L), mean values were preserved. Also for TBW, ECW and ICW (%), mean values were maintained.

After the vegan dietary protocol, it was found that RMR, %CHO and %Fat were maintained.

Results of vegan dietary protocol in males

In Table 8-12 body composition and resting metabolic rate parameters pre- and post- the vegan dietary protocol in addition to the delta values (calculated as the difference of post- and pre-values) in males are presented. Data are reported as mean ± standard deviation or median, (min-max) of the males participants. Data related to TBW, ECW and ICW are shown as litres and as percentage. TBW is expressed as percentage of body weight while ECW and ICW as percentage of TBW.

Table 8-12. Body composition and resting metabolic rate parameters before (pre) and after (post) the vegan diet and delta values (post-pre) in males.

Vegan dietary protocol	Males (n = 6)		
	PRE	POST	DELTA
BMI (kg/m ²)	25.4 ± 1.7	25.1 ± 1.7	-0.3 ± 0.5
Waist circumference (cm)	90.1 ± 6.6	89.8 ± 6.9	-0.3 ± 2.8
Skinfold sum (mm)	28.4 ± 9.4	29.1 ± 10.2	0.7 ± 2.0
Rz (Ω)	468.0 ± 38.2	456.8 ± 37.2	-11.2 ± 34.2
Xc (Ω)	58.7 ± 4.2	55.3 ± 3.7	-3.3 ± 4.8
PhA (°)	7.1 ± 0.5	7.0 ± 0.7	-0.2 ± 0.6
BCM (kg)	37.8 ± 2.5	37.4 ± 3.1	-0.3 ± 2.9
BCMI (BCM kg/m ²)	12.1 ± 1.0	12.0 ± 1.1	-0.1 ± 0.9
TBW (L)	46.8 ± 2.3	47.3 ± 3.3	0.5 ± 2.3
ECW (L)	19.1 ± 1.2	19.8 ± 2.0	0.7 ± 1.1
ICW (L)	27.6 ± 1.7	27.5 ± 2.2	-0.1 ± 2.0
TBW (%)	58.8 ± 4.3	60.1 ± 4.1	1.3 ± 2.4
ECW (%)	40.9 ± 1.9	41.8 ± 2.6	0.9 ± 2.2
ICW (%)	59.1 ± 1.9	58.1 ± 2.6	-0.9 ± 2.2

RMR (kcal/day)	2396.0 ± 116.0	2225.0 ± 122.6	-171.0 ± 97.9
%CHO (%RMR)	60.6 ± 8.8	60.3 ± 10.1	-0.3 ± 5.8
%FAT (%RMR)	32.1 ± 8.8	32.0 ± 9.4	-0.1 ± 5.6

Values are expressed as mean ± standard deviation or median, (min-max).

BMI, waist circumference and skinfold sum were reported before and after the dietary intervention in males (Table 8-12). For BMI mean values were maintained. Regarding waist circumference, the maintenance of mean values was observed. Skinfolds data are presented as the sum (mm) of triceps, biceps and subscapular skinfolds thickness and a not statistical significant change was reported after the vegan diet.

Bioimpedance analysis values, Rz, Xc, Pha, BCM and BCMI, were maintained after the vegan diet. TBW, ECW and ICW were also preserved.

Also RMR evaluation was carried out (Table 8-12). A difference of mean values was found (-171.0 ± 97.9 kcal/day) for RMR, even if not statistical significant. No effects on the utilized substrates were reported.

Body composition parameters changes among the three dietary protocols in the total sample

In order to evaluate differences on the impact of the three dietary protocols on body composition and resting metabolic rate parameters, delta values (calculated as the difference of post- and pre-mean values) for the total sample are reported. Data are reported as mean ± standard deviation or median, (min-max) of the total participants. Data related to TBW, ECW and ICW are shown as litres and as percentage. TBW is expressed as percentage of body weight while ECW and ICW as percentage of TBW.

In Table 8-13 delta values of 70:30, 30:70 and vegan dietary protocols in the total sample are reported.

Table 8-13. Delta values (post-pre) of 70:30, 30:70 and vegan dietary protocols in the total sample.

All	70:30 dietary protocol (n = 18)	30:70 dietary protocol (n = 13)	Vegan dietary protocol (n = 14)
ΔBMI (kg/m ²)	-0.4 ± 0.4	-0.1 ± 0.4	-0.3 ± 0.4
ΔWaist circumference (cm)	-0.4 ± 2.2	-0.6 ± 1.7	-0.2 ± 2.2
ΔSkinfold sum (mm)	-1.9 ± 4.9	-0.2 ± 2.7	-1.6 ± 3.4
ΔRz (Ω)	-0.7 ± 32.3	-5.0 ± 31.0	-8.7 ± 40.0
ΔXc (Ω)	0.4 ± 5.4	-2.0 ± 5.6	-0.9 ± 5.2
ΔPhA (°)	0.0 (-0.6-0.3)	-0.1 ± 0.2	-0.0 ± 0.4

Δ BCM (kg)	0.4 (-2.5-2.0)	-0.4 \pm 0.5	-0.3 (-2.6-5.3)
Δ BCMI (BCM kg/m ²)	0.1 \pm 0.4	-0.2 \pm 0.2	0.0 \pm 0.6
Δ TBW (L)	0.5 (-3.9-1.6)	0.0 \pm 1.0	-0.1 (-1.7-4.7)
Δ ECW (L)	0.2 (-1.9-1.7)	0.2 \pm 0.8	0.2 \pm 1.0
Δ ICW (L)	0.2 \pm 0.7	-0.2 \pm 0.4	-0.2 (1.6-3.8)
Δ TBW (%)	1.5 (-4.4-3.8)*	0.4 \pm 1.1	1.1 \pm 2.3
Δ ECW (%)	-0.1 \pm 1.2	0.5 \pm 1.2	-0.0 \pm 1.8
Δ ICW (%)	0.0 \pm 1.2	-0.5 \pm 1.2	-0.6 \pm 3.0
Δ RMR (kcal/day)	112.5 \pm 367.4	-181.0 (-230.0-69.0)	18.7 \pm 207.7
Δ %CHO (%RMR)	3.6 \pm 13.5	-3.8 \pm 12.4	4.6 \pm 6.1
Δ %FAT (%RMR)	-3.5 \pm 13.4	3.8 \pm 12.2	-4.8 \pm 5.9

Values are expressed as mean \pm standard deviation or median, (min-max).

As it is possible to observe from Table 8-13, no statistical significant differences were reported among delta parameters for the three dietary protocols. From Figure 8-62 to Figure 8-77 the comparison among the obtained results from the three dietary interventions is reported.

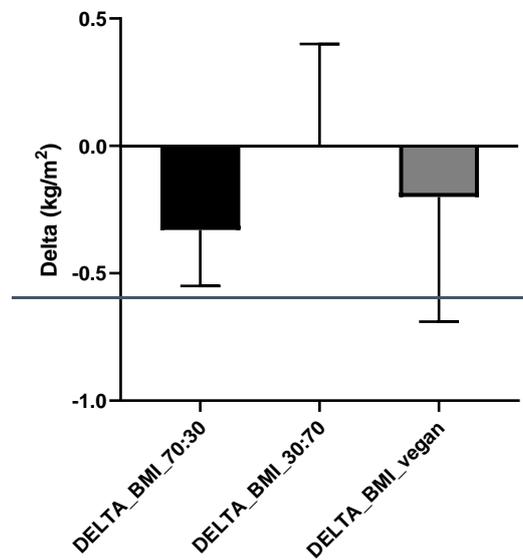


Figure 8-62. Comparison between the differences of BMI observed post- and pre- intervention with the 3 dietary protocols in the total sample.

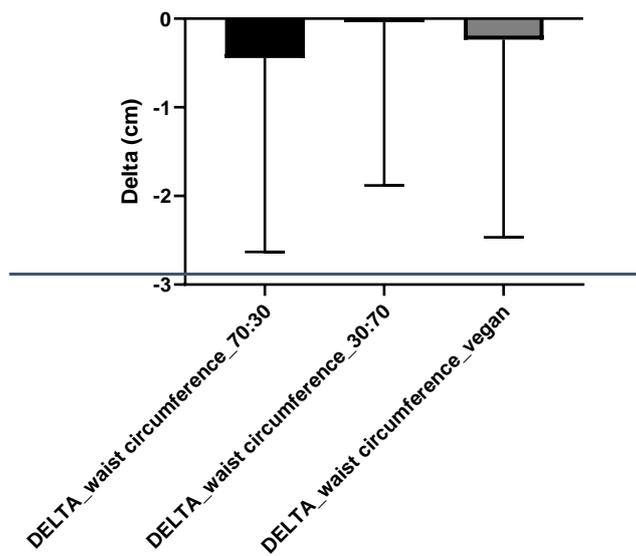


Figure 8-63. Comparison between the differences of waist circumferences observed post- and pre- intervention with the 3 dietary protocols in the total sample.

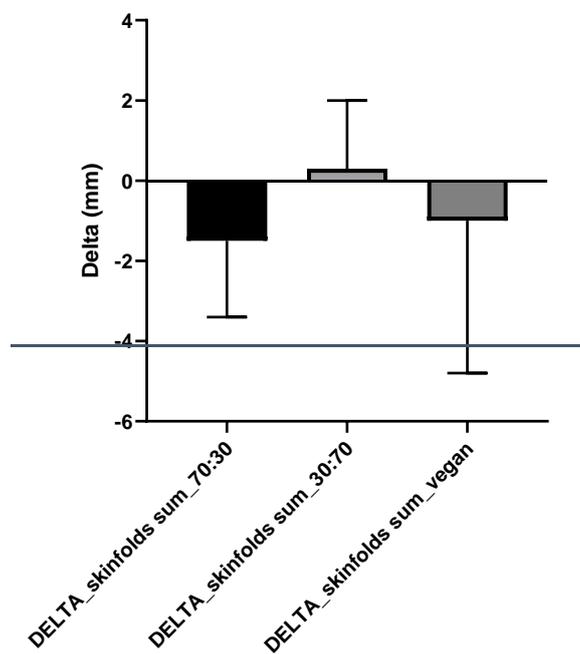


Figure 8-64. Comparison between the differences of skinfold sum observed post- and pre- intervention with the 3 dietary protocols in the total sample.

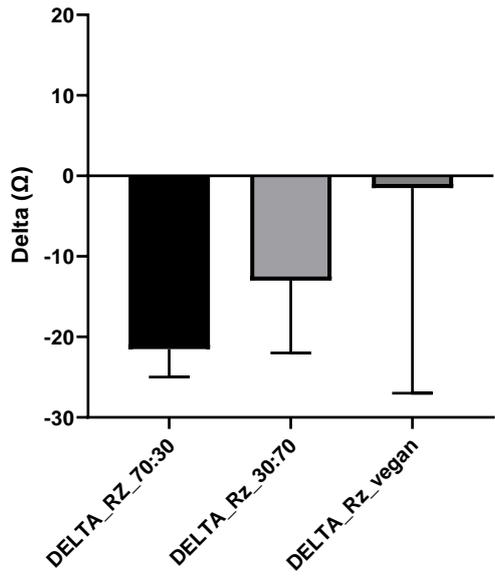


Figure 8-65. Comparison between the differences of Rz observed post- and pre- intervention with the 3 dietary protocols in the total sample.

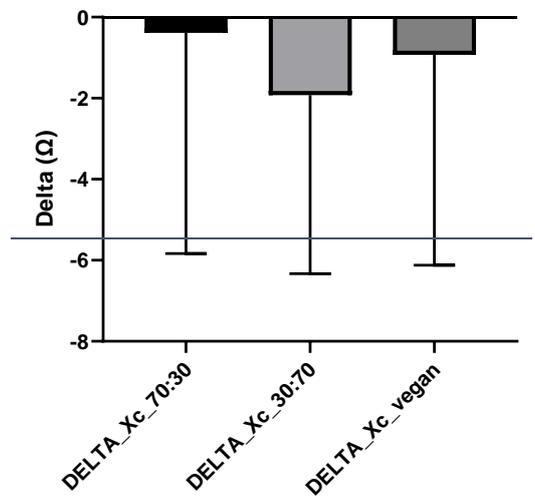


Figure 8-66. Comparison between the differences of Xc observed post- and pre- intervention with the 3 dietary protocols in the total sample.

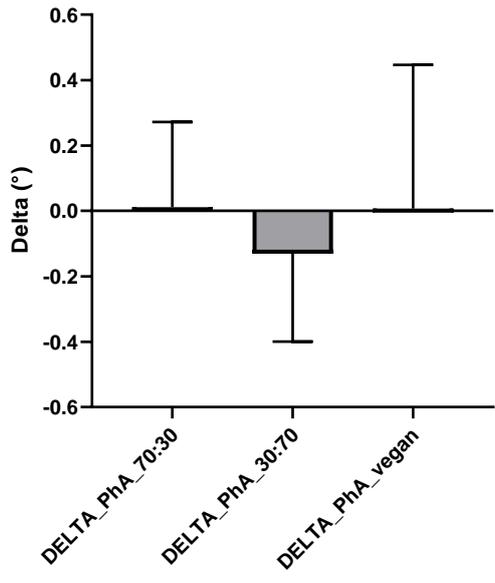


Figure 8-67. Comparison between the differences of PhA observed post- and pre- intervention with the 3 dietary protocols in the total sample.

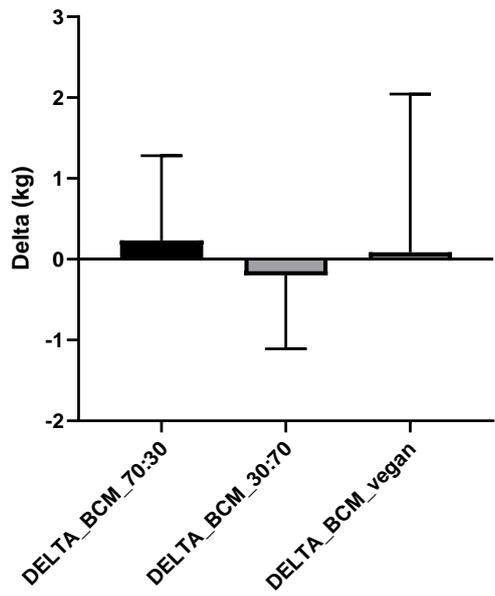


Figure 8-68. Comparison between the differences of BCM observed post- and pre- intervention with the 3 dietary protocols in the total sample.

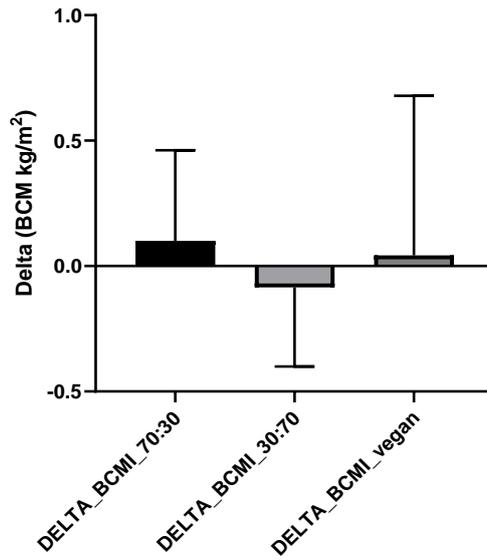


Figure 8-69. Comparison between the differences of BCMI observed post- and pre- intervention with the 3 dietary protocols in the total sample.

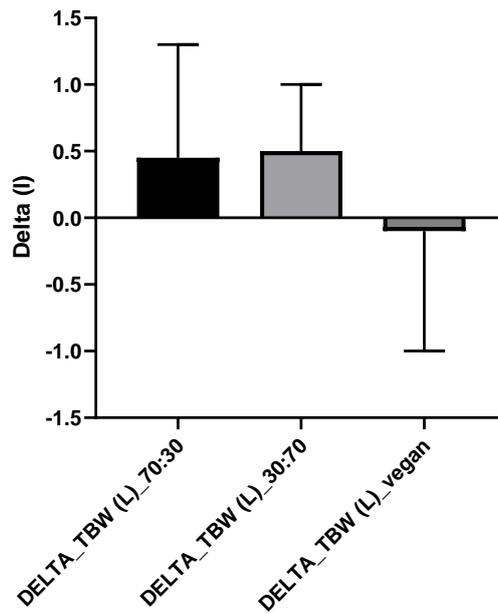


Figure 8-70. Comparison between the differences of TBW (L) observed post- and pre- intervention with the 3 dietary protocols in the total sample.

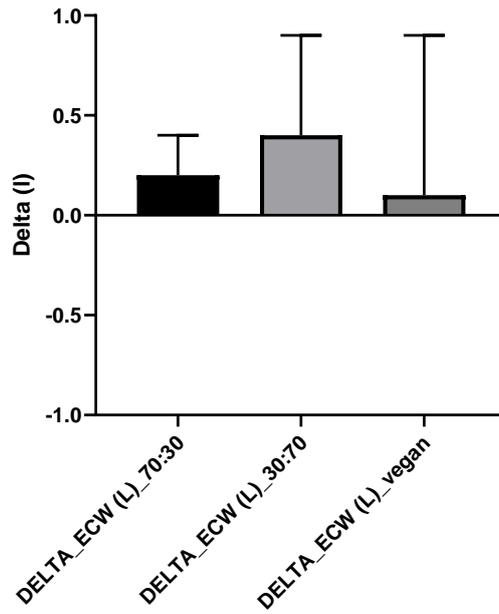


Figure 8-71. Comparison between the differences of ECW (L) observed post- and pre- intervention with the 3 dietary protocols in the total sample.

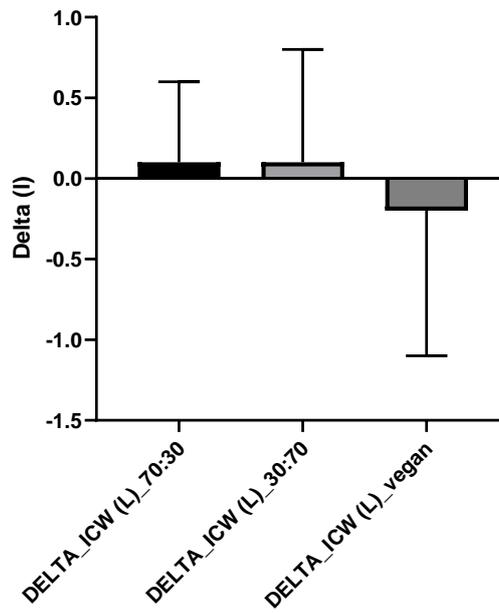


Figure 8-72. Comparison between the differences of ICW (L) observed post- and pre- intervention with the 3 dietary protocols in the total sample.

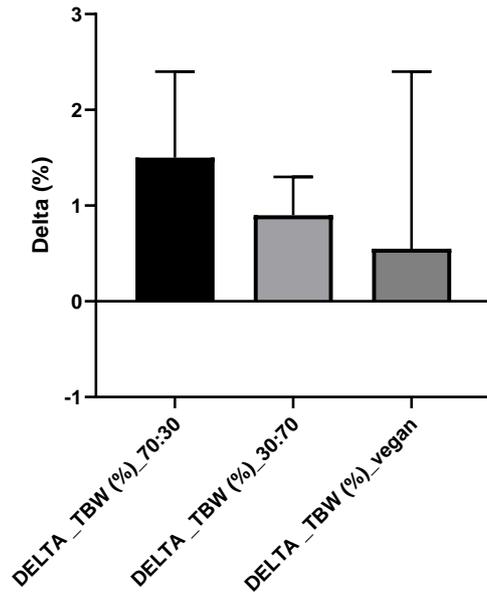


Figure 8-73. Comparison between the differences of TBW (%) observed post- and pre- intervention with the 3 dietary protocols in the total sample.

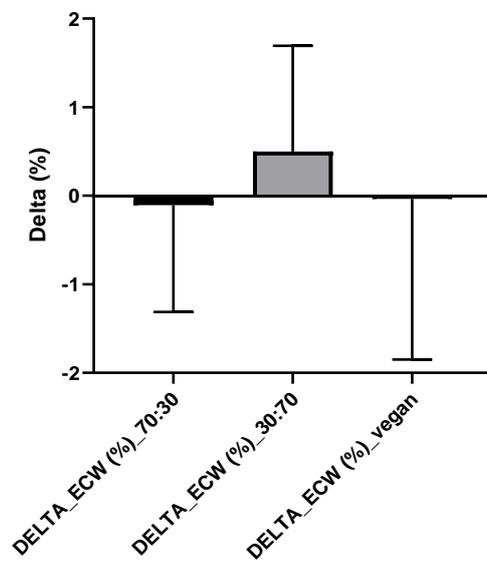


Figure 8-74. Comparison between the differences of ECW (%) observed post- and pre- intervention with the 3 dietary protocols in the total sample.

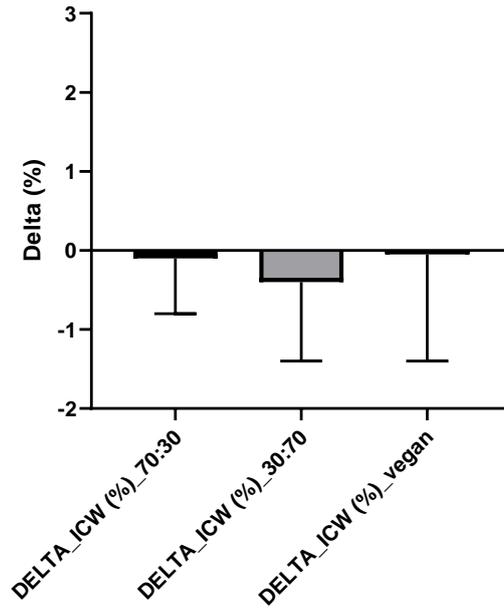


Figure 8-75. Comparison between the differences of ICW (%) observed post- and pre- intervention with the 3 dietary protocols in the total sample.

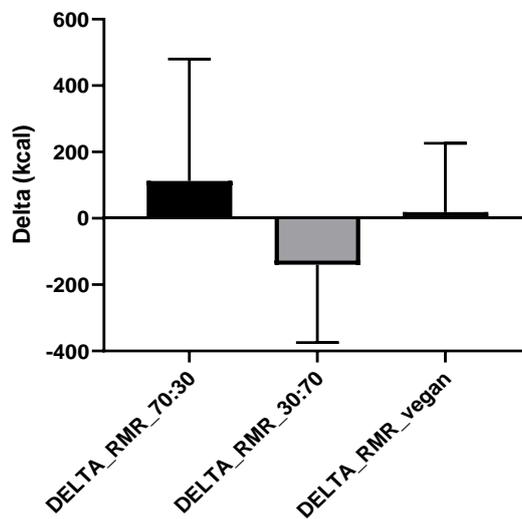


Figure 8-76. Comparison between the differences of RMR observed post- and pre- intervention with the 3 dietary protocols in the total sample.

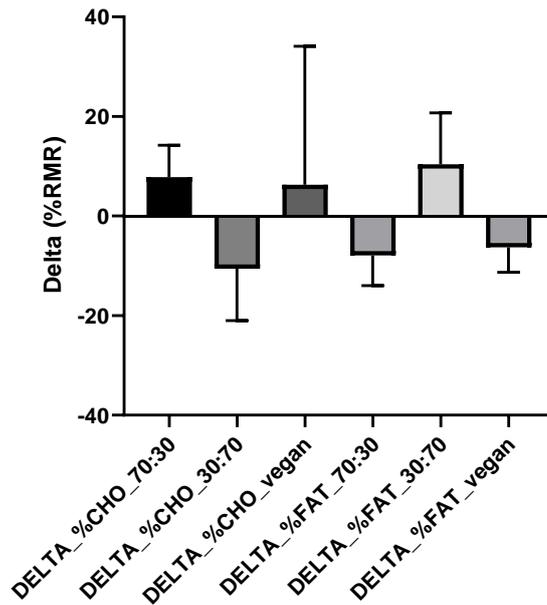


Figure 8-77. Comparison between the differences of %CHO (%RMR) and %Fats (%RMR) observed post- and pre-intervention with the 3 dietary protocols in the total sample.

Table 8-14 and Table 8-15 reported differences on the impact of the three dietary protocols on body composition and resting metabolic rate parameters, delta values (calculated as the difference of post- and pre- mean values) for females and males.

Table 8-14. Delta values (post-pre) of 70:30, 30:70 and vegan dietary protocols in females.

Females	70:30 dietary protocol (n = 11)	30:70 dietary protocol (n = 7)	Vegan dietary protocol (n = 8)
Δ BMI (kg/m ²)	-0.4 ± 0.4	-0.1 ± 0.4	-0.3 ± 0.4
Δ Waist circumference (cm)	-0.5 ± 2.1	-0.6 ± 1.7	-0.2 ± 1.9
Δ Skinfold sum (mm)	-0.7 ± 3.9	0.5 ± 2.0	-3.5 ± 3.2
Δ Rz (Ω)	-6.3 ± 33.4	-5.0 ± 31.0	-6.9 ± 46.2
Δ Xc (Ω)	-0.4 ± 5.8	-2.0 ± 5.6	0.9 ± 5.0
Δ PhA (°)	-0.0 ± 0.3	-0.1 ± 0.2	0.2 ± 0.2
Δ BCM (kg)	0.2 ± 0.8	-0.4 ± 0.5	0.4 ± 0.9
Δ BCMI (BCM kg/m ²)	0.1 ± 0.3	-0.2 ± 0.2	0.2 ± 0.4
Δ TBW (L)	0.1 ± 0.9	0.0 ± 1.0	0.0 ± 1.2
Δ ECW (L)	0.1 ± 0.8	0.2 ± 0.8	-0.2 ± 0.8
Δ ICW (L)	0.1 ± 0.4	-0.2 ± 0.4	0.2 ± 0.9
Δ TBW (%)	1.1 ± 1.7	-0.5 ± 1.2	0.9 ± 2.4
Δ ECW (%)	-0.1 ± 1.3	0.6 ± 1.2	-0.8 ± 1.2
Δ ICW (%)	-0.1 (-2.8-1.7)	-0.4 (-2.4-0.8)	0.4 ± 3.7
Δ RMR (kcal/day)	111.3 ± 368.0	-130.8 ± 136.2	161.0 ± 130.2
Δ %CHO (%RMR)	0.0 ± 15.2	-3.9 ± 12.4	8.3 ± 3.3
Δ %FAT (%RMR)	0.1 ± 15.0	3.8 ± 12.2	-8.4 ± 3.2

Values are expressed as mean ± standard deviation or median, (min-max).

Table 8-15. Delta values (post-pre) of 70:30, 30:70 and vegan dietary protocols in males.

Males	70:30 dietary protocol (n = 7)	30:70 dietary protocol (n = 6)	Vegan dietary protocol (n = 6)
ΔBMI (kg/m ²)	-0.3 ± 0.3	-0.0 ± 0.5	-0.3 ± 0.5
ΔWaist circumference (cm)	-0.3 ± 2.5	0.6 ± 1.9	-0.3 ± 2.8
ΔSkinfold sum (mm)	-3.7 ± 6.1	0.1 ± 2.3	0.7 ± 2.0
ΔRz (Ω)	-8.1 ± 33.0	-6.7 ± 13.6	-11.2 ± 34.2
ΔXc (Ω)	-0.4 ± 5.2	-1.8 ± 3.1	-3.3 ± 4.8
ΔPhA (°)	0.1 ± 0.2	-0.1 ± 0.3	-0.2 ± 0.6
ΔBCM (kg)	0.3 ± 1.5	0.0 ± 1.2	-0.3 ± 2.9
ΔBCMI (BCM kg/m ²)	0.1 ± 0.5	3.0 ± 0.4	-0.1 ± 0.9
ΔTBW (L)	0.3 ± 1.9	0.4 ± 1.1	0.5 ± 2.3
ΔECW (L)	0.2 (-1.9-1.5)	0.4 ± 0.8	0.7 ± 1.1
ΔICW (L)	0.6 (-2.0-1.4)	0.8 (-0.8-9.1)	-0.1 ± 2.0
ΔTBW (%)	1.2 ± 2.6	0.6 ± 0.8	1.3 ± 2.4
ΔECW (%)	-0.3 (-1.2 ± 1.9)	0.4 ± 1.3	0.9 ± 2.2
ΔICW (%)	-0.0 ± 1.1	-0.4 ± 1.3	-0.9 ± 2.2
ΔRMR (kcal/day)	114.8 ± 423.1	-148.3 ± 331.6	-171.0 ± 97.9
Δ%CHO (%RMR)	10.0 ± 8.1	-11.0 ± 12.3	-0.3 ± 5.8
Δ%FAT (%RMR)	-9.8 ± 7.9	10.8 ± 12.1	-0.1 ± 5.6

Values are expressed as mean ± standard deviation or median, (min-max).

Discussion

In recent year, interest and awareness on plant-based diet has increased due to both growing research into the health effects and its environmental sustainability [62, 63].

Plant-based diets have been studied for their effects on several conditions (i.e. type II diabetes and cardiovascular disease) and have been linked to several beneficial nutritional, metabolic and health outcomes [64].

In this context, the study of the impact of these diets on body composition has become increasingly interesting above all in terms of body weight management and obesity fighting [64].

However, plant-based diet could be lacking in certain macro- and micronutrients if not adequately planned. Thus, careful attention should be paid in diet planning and in particular to proteins' quantity and quality [65-68].

Due to the central role of proteins' quality, this study aimed to investigate short-term effects of three normocaloric dietary protocols with same protein quantity but different ratio of animal- and plant-proteins on body composition and resting metabolic rate of physically active and normal weight adults. In details, the three tested dietary approaches were characterized by 70% of animal-proteins and 30% of plant-proteins (70:30, Mediterranean-like diet), 30% of animal-proteins and 70% of plant-proteins (30:70) and 100% of plant-proteins (vegan diet).

From the present study, both the 30:70 and the vegan diet short-term dietary approaches were able to maintain body composition and hydration status. Thus, it is possible to speculate that in the short term, a more plant-based diet, carefully planned, can be followed without affecting body composition. Similar results were reported in a study conducted by Losasso and colleagues in which no significant differences were found among omnivorous, vegetarians and vegans individuals in terms of BMI, body lean mass and body fat mass [69]. Though, several studies reported an impact of plant-based diet on body composition, in particular a reduction of the BMI, waist circumference and body fat mass over time [70, 71]. In particular, the systematic review of Tran et al. reported that plant-based diet can improve weight status in some patients group. However, this impact was always linked to fat intake restriction and, as a consequence, of decreased energy intake applied in many analysed studies [70]. In the present study, no fat reduction nor energy restriction were applied. The aim was to observe the impact of a normocaloric plant-based diet on body composition and resting metabolic rate in order to study their applicability and sustainability.

In the present study, gender stratification was performed aiming to deeply investigate the impact of the three dietary protocols on females and males due to the recent relevance of gender medicine [72-74].

Obtained results suggest that those diets can be considered dietary strategies suitable to be followed in the short term without compromising body composition both in male and in female. This could be useful in order to make diet more varied and to increase consumption of plant-based proteins while decreasing animal ones with positive effect on both health status and environment. Interesting applications of these short-term dietary approaches can be evaluated.

An example could be the application on athletes' diet. Indeed, during the season of competitions, athletes follow a low-fiber and high quality proteins diet in order to optimize body composition and performance and avoid intestinal discomfort [75, 76]. Consequently, such a diet is often limited in daily food choices. Therefore, in off-season periods diets like the 30:70 or vegan are of great interest

as they allow athletes to vary food choices, shifting towards a more plant-based diet, and, in parallel, to maintain body composition in the short term.

Also concerning body fluids, TBW, ECW and ICW were preserved. In a study of Dawczynsk et al., it was pointed out that vegan diet induces a higher reduction of body water (l) compared to an omnivorous diet in all participants enrolled [77]. However, in short term approach, it seems that those strategies can be considered suitable to be followed without compromising body composition and hydration status.

It could be interesting to observe that a slight decrease of ECW and increase of ICW was reported in female but an opposite situation was found in males.

It may be hypothesized that female sex hormones may have a role in this different dietary feedback on fluid balance as they influence the equilibrium between intra- and extra-cellular fluids [78, 79]. However, there is a lack of data concerning the impact of vegan diet on body fluids according to gender, therefore, further studies are needed to deepen this aspect due to the importance of body fluid balance in the establishment of body hydration [80].

Also in terms of resting metabolic rate, no significant differences were found among the three dietary approaches. These results were in accordance with data reported by Ashworth and colleagues in their study in which similar RMR was found between a group of vegetarians and the one of omnivores [81].

Also Bissoli and colleague found no significant differences in RMR comparing RMR and the thermogenic effect of food in a group of vegetarians and in a group of subjects following a Mediterranean diet [82]. On the other hand, in the study of Nadimi et al. the association of vegan diet and body composition and resting metabolic rate was investigated in 20 vegetarians and 20 non-vegetarians founding that non-vegetarians displayed a higher absolute resting metabolic rate than vegetarians while no differences were pointed out in term of body composition [83].

From the present study, RMR was preserved with short-term vegan dietary approach. However, more studies are needed to evaluate in deep this aspect.

In addition to body composition and RMR, it could be useful to report feedbacks of subjects following the three different dietary approaches. The 70:30 diet did not registered complaints by participants, indeed, all of them expressed a general positive feeling and no fatigue was perceived, despite participants have an active lifestyle. A high grade of acceptance emerged for 30:70 diet. Indeed, at the control visit almost all of participants expressed the willing to continue with this dietary protocol in their daily life. However, several participants reported negative feedbacks

referring to vegan diet. Indeed, almost half of the participants stated to be relieved at the control visit for finishing the protocol, as the diet appeared to be monotonous to them. No complaints for bloating sensations after meals were described while general fatigue was reported by some of the participants, although no adverse effects emerged from body composition evaluation.

Adequately planned short-term plant-based diets were found to be able to maintain body composition in physically active subjects.

So, these results demonstrated that a shift on a more plant-based diet could be favourable and feasible. In addition, the 30:70 approach underlined that a good strategy could be the one related to the presence of both animal and plant-based proteins shifted toward more plant-based proteins and less animal-proteins. This shift is of fundamental importance considering the importance of reducing animal-proteins consumption and making the diet more sustainable also for the environment [30, 32].

Therefore, adequately structured plant-based diets could support and maintain an adequate body composition and healthy status in physically active subjects and managing the ratio animal-proteins/plant-proteins results a key strategy.

The importance of properly manage nutrients intake was also confirmed by previous studies conducted in our laboratory to investigate vegan's diet quality in which it was pointed out that vegan diets are generally characterized by inadequate protein intakes and by at least one daily meal contained only cereal based foods (data not shown). Thus, these results highlighted the necessity to avoid improvising vegan diets, in order not to incur into nutritional deficiencies and to compromise body composition and healthy status. This underlines also the importance of properly managing daily protein intake also by considering and by applying the correction factor for plant-proteins to guarantee an adequate total daily protein intake.

In the present study, plant-based dietary protocols were not provided for supplementations except for proteins supplementation. It is fundamental to note that, as the protocol was followed in the short term (4 weeks), no supplementation of vitamin B12 and other micronutrients was planned for plant-based diets. Indeed, also Waldmann et al. confirmed that the duration of the adherence to a vegan diet may be the discriminating factor in suggesting an eventual specific supplementation. Therefore, if the duration of vegan diet adherence is prolonged, specific supplementation (i.e. vitamin B12) may be required to prevent nutritional imbalances [51].

Some limitation of the study are present. The study was a pilot and the number of subjects was limited, based on other studies conducted to evaluate the effect of vegetarian diet on body

composition [82-84]. Therefore, it could be interesting to evaluate the impact of such dietary protocols on a larger sample and deeply investigate their impact on females and males.

Moreover, it may be important to investigate the effects of plant-based dietary protocols, like the 30:70 diet, on body composition and resting metabolic rate in the long-term.

Conclusions

Adequately planned short-term plant-based diets appears to be able to maintain body composition in physically active subjects demonstrating that a shift on a more plant-based diet could be favourable and feasible.

This shift represents a key factor considering the importance of reducing animal-proteins consumption and making the diet more sustainable also for the environment.

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Chapter 9. Nutritional deficiencies and management of celiac disease

Preface

Celiac disease has become a world-wide health problem [1], affecting around 1% of the general population [1, 2].

It is a chronic immune mediated enteropathy of the small intestine that is triggered by dietary gluten proteins exposure in individuals who are genetically predisposed [1, 2].

The only effective resolution therapy is the avoidance of dietary gluten [3] and consequently the exclusion of gluten-containing foods from the diet, causing the apparent reduction of diet variety.

For this reason, it is necessary to give more and more value to the concerns related to gluten-free diet, wishing to guarantee a high quality of life to celiac patients.

Celiac disease patients show an altered intestinal mucosa, due to the disassembly of enterocyte tight junctions (which, in physiological conditions, contributes to making the intestinal mucosa a barrier against external agents) [4], causing an impaired nutrient absorption.

The complete histological recovery of the intestine surface is of course essential to ensure nutrient absorption, but this re-establishment requests a long time, in particular in adult patients, even with a strict gluten-free diet [5]. Inevitably, celiac patients can be subjected to nutrient deficiencies, especially at the beginning of their adherence to a gluten-free diet [6].

In this context, nutrient deficiencies require a special consideration [7]. Deficiencies of iron, folate and calcium are the most common [8] but also the incidence of vitamin B12 deficiency has been reported [9, 10]. Calcium, phosphorous and vitamin D deficiencies can turn up because of the eventual lactose intolerance which is concurrent to celiac disease [11].

Moreover, imbalances could emerge because of the difficulty in strictly adhering to a gluten-free diet. Indeed, it is appraised that the adherence to the gluten-free diet is around 45-80% [6, 12].

Another aspect responsible for deficiencies is that the careless choice of gluten-free foods could entail a decrease of the diet quality and that dissatisfaction for gluten-free foods contributes to compromise nutrient balance of gluten-free diet and life quality [13].

Thus, attention must be devoted in the selection of both processed and naturally gluten-free foods. As it is widely known, processed gluten-free foods are characterized by a higher content of sugars, saturated fats and salt and a lower content of proteins and fiber compared to the corresponding gluten-containing foods [14, 15].

Often, the lower quality of the nutritional composition of gluten-free foods is due to issues related to food production. In spite of that, thanks to the development of food technologies and food

processing, today several highly nutritious options are available in the market [16, 17]. Anyway, celiac consumers should spend time in the carefully reading of food labels.

In parallel, processed gluten-free foods are not the only alternatives to be considered in a gluten-free diet. Naturally gluten-free cereals such as rice, maize, quinoa, amaranth, buckwheat, millet and sorghum are valid wheat substitutes, especially for their nutritional composition [8]. Indeed, these minor cereals contain essential micronutrients for the diet [18, 19]. Unfortunately, their use is not common among celiac consumers because of lack of knowledge on their nutritional value and especially for the flavour, which is not generally appreciated [20].

Moreover, it must be considered that there is a general trend among all consumers to avoid using cereal grains and to prefer grain derived or refined foods [21, 22].

Beyond cereal grains, pulses can be enumerated as naturally gluten-free foods [23].

Pulses have an interesting nutritional profile that goes forward the absence of gluten. Indeed, pulses contain complex carbohydrates and fiber, which are essential nutrients for the organism [24, 25]. Indeed, fiber consumption contributes to the reduction of the incidence of type 2 diabetes and of obesity-related disorders [26, 27]. Moreover, they are a plant-based source of proteins [28, 29]: if properly combined with cereals, a pulse-based dish can be a valid substitute of animal-protein sources. As it has been widely described in literature, pulses and cereals are complementary foods in terms of proteins composition [30]. More in details, pulse and cereal proteins have a complementary amino acidic pattern, consequently their combination allows for the optimization of protein quality of the single foodstuffs [31].

In addition, pulses are an environment friendly food product [32-34]. Consequently, their intake should be promoted not only among celiac consumers but among the general public of consumers. Furthermore, pulse use has come into light in the field of gluten-free foods production, in particular for their rheological properties [35, 36]. Indeed, pulses flours are currently utilized to improve the flavour and the texture of several gluten-free products [37]. At the same time pulses nutritional characteristics are important to optimize their nutritional profile. For example, pulse flour can be used for the biofortification of breads and other bakery products [38].

In addition, pulse flour can be employed in the preparation of snacks and pasta, that can be used as an alternative to the gluten-containing one [39, 40]. Therefore, pulses represent a functional ingredient in the gluten-free diet as they improve its nutritional quality and increase its variety.

Despite the nutritional properties of pulses and their numerous application in food technology, pulse consumption is not regular among both celiac and standard consumers [41].

Several are the reasons for a reduced pulse intake, such as disregard for pulses taste, lack of knowledge on pulse cooking and conviction that pulses are foods for vegetarians [42, 43].

These motivations, considered in the current context, direct consumers to the choice of fast-cooking foods and of refined foods [44]. The frequent use of refined foods and cereals, which have a lower nutritional value than the whole equivalents [45], may be responsible for the lower nutritional adequacy of the diet and of nutritional deficiencies.

In addition, the nutrient composition of a food can be definitely affected by the cooking method used [46-48]. Indeed, cooking methods have a different impact on nutrient content depending on the matrix considered. Therefore, it becomes essential to deepen the knowledge on natural gluten-free cereals preparation to promote their use and to sensitize consumers in picking whole cereals when possible because of their notable nutritional value.

Contemporarily, it is fundamental to encourage celiac patients to cautiously select foods and foods preparation methods in order to guarantee a balanced diet.

Nutrient imbalances have been shown to be secondary consequences of celiac disease [5].

Despite it is known that several are the triggers for celiac disease [49], it may be hypothesize that specific nutrient deficiencies may have a role in celiac disease aetiology. Indeed, nutrients are involved in different mechanism, at both a cellular and tissue level, for example in the modulation of inflammation and immune mechanisms [50]. Consequently, the lack of these molecules can be the concurrent cause of pathologic outcomes, as in the specific of celiac disease.

The following paragraphs discuss and deepen the issues previously described by analysing the possible role of a specific micronutrient, vitamin D, in celiac disease onset. At the end the development of a nutrition education course for the management of gluten-free diet is described.

Possible role of vitamin D in celiac disease onset [51]

Abstract

Beside skeletal system maintenance and protection, possible extra-calcium roles of vitamin D have been recently described. In particular, studies have investigated possible roles of vitamin D as key modulator of inflammation and immune mechanisms and of the intestinal mucosa barrier.

In this regard, vitamin D has been considered as a factor that affects different conditions as in immune-mediated diseases. The new emerging role of vitamin D and its involvement in immune modulation has led to consider it as a possible key factor involved in celiac disease (CD) onset.

CD is a chronic immune mediated enteropathy of the small intestine that is triggered by dietary gluten protein exposure in individuals who are genetically predisposed. However, along with gluten, other environmental factors are involved in CD onset. The renewed interest in a molecule that offers great possibilities for new roles has led to an increase in studies, although there remains a lack of studies aimed at contextualizing the role of vitamin D on CD.

The possible role of vitamin D in CD onset as it is presently understood, taking into account the potential links among vitamin D, immune system and CD will be discuss.

Introduction

Vitamin D belongs to the steroid hormone family. It has two major forms, vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol), both of which can be found in foods or supplements, although only vitamin D3 is synthesized in skin [52]. Indeed, vitamin D3 is mainly produced endogenously in the skin by converting 7-dehydrocholesterol through the action of ultraviolet light B (UVB) of the sun or other UVB sources [53, 54]. Oral intake of vitamin D is generally limited (oily fish and eggs, vitamin D fortified food), while the majority of it is derived from UVB light action. This links its content not only to dietary intake, but above all to seasonal changes, sun exposure, geographic locations and skin pigmentation [55, 56]. The steroid hormone precursor vitamin D3 is synthesized within skin and is biologically inactive. Two hydroxylation reactions are required to activate it. The first one forms the 25-hydroxyvitamin D3 [25(OH)D3 or calcidiol] in the liver, then 25(OH)D3 is converted to 1,25-dihydroxyvitamin D3 [1,25(OH)2D3 or calcitriol] by the 1-hydroxylase CYP27B1 in the kidney. CYP27B1 enzyme is stimulated by parathyroid hormone (PTH) [55, 56]. Vitamin D has been known for its role in the maintenance and safeguarding of skeletal system integrity. Indeed, biologically active vitamin D enhances calcium intestinal absorption by

regulating calcium transport proteins in the small intestine, stimulating osteoclastic maturation and helping bone growth, which in turn supports collagen matrix mineralization [52, 54, 57, 58].

The vitamin D receptor (VDR), a nuclear receptor hormone, mediates the biological activity of 1,25(OH)₂D₃ [55, 56]. The highly polymorphic VDR belongs to the steroid receptor super family and enables vitamin D to exert its genomic actions [52, 58, 59].

Recently, vitamin D has received increased attention, as it was discovered that tissues and cells not involved in mineral and bone metabolism present VDR and vitamin D-activating enzymes.

VDR has been reported to be expressed on cell types as antigen-presenting cells and lymphocytes, underlining a possible role of vitamin D as a key modulator of immune and inflammation mechanisms, and suggesting VDR gene polymorphisms to be markers of protection from or susceptibility to autoimmune diseases [54, 58, 59]. Pleiotropic actions on chronic diseases such as cardiovascular disease, diabetes, neurological disease and autoimmune disease are now under study. Over 900 genes have been reported as regulated by vitamin D [52, 54]. Although its effect on the immune system and infection is an emerging topic, there has been an increased understanding related to vitamin D as a regulator and signalling component on the immune system [57, 60, 61].

Celiac disease (CD) is a chronic small intestinal immune-mediated enteropathy triggered by exposure to dietary gluten proteins among individuals genetically predisposed, which affects around 1% of the general population [1, 2, 7, 62]. CD is a unique autoimmune disease for which gluten has been identified as the environmental trigger. For the disease to occur, the key genetic elements human leukocyte antigen HLA-DQ2 and HLA-DQ8, and the involvement of autoantigen (tissue transglutaminase (tTG)) are required [63, 64].

The inducers of the disease are gluten and the alcohol-soluble gliadins it comprises, which are mainly present in specific cereals such as wheat, barley and rye [65].

In CD patients, the gluten contained in food products is broken down by specific gastrointestinal enzymes into peptides, which pass through the intestinal epithelial barrier to reach the mucosal lamina propria. The enzyme designated to convert gluten peptides is transglutaminase 2, which recognizes glutamine residues. Glutamine residues are converted into glutamic acid, leading the production of deamidated gluten peptides, which are now able to strongly bind to HLA-DQ2/-DQ8 molecules (the surface restriction elements for gluten-specific CD4⁺ T lymphocytes). Therefore, gluten peptides behave like antigens inducing an amplified immune response [66].

The cited HLA-DQ2/DQ8 molecules are the products of an expression of class II genes by the main histocompatibility complex (HLA system) [67].

Beyond genetically predisposed HLA-DQ2/DQ8 haplotypes, another characteristic in CD patients is the autoantibody response expressed against transglutaminase 2. The role of antibodies for transglutaminase 2 in the pathophysiology of CD is not clear, but their serum concentration in CD patients is increased. Autoantibodies play a role in the diagnosis of CD and in monitoring the progression of the disease after beginning a gluten-free diet, which generally reduces serum autoantibodies after a period of months [68].

Moreover, celiac disease patients show an altered intestinal mucosa, due to the disassembly of enterocyte tight junctions (which, in physiological conditions, contributes to making the intestinal mucosa a barrier against external agents) [4].

Tight junctions are localized in the apical part of enterocytes and are responsible for their connection. Tight junctions are made up of integral membrane proteins such as occluding, claudins and scaffolding proteins (e.g., zonulin) [69]. Thanks to their capacity to connect enterocytes, tight junctions form a barrier to regulate the transfer of molecules through the intestine. Consequently, a variation in tight junction structure can lead to increased intestinal permeability [4].

The characteristic damage that occurs during CD due to gluten exposure develops gradually [70]. The appearance of lesions characterized by intensified increased intraepithelial lymphocytes, crypt hypertrophy and progressive villous atrophy are the first typical histologic sign of CD. At a macroscopic scale, celiac disease results in malabsorption, diarrhea, bloating and (to variable degrees) undernutrition [71]. According to Livshits et al., the most widespread intestinal clinical traits are malnutrition, chronic diarrhea and nutritional deficiencies. Today, these traits are disappearing, making way for extra-intestinal presentations such as skin, endocrine, skeletal, hepatic, hematological, thrombophylic, gynecological, fertility, dental, cutaneous, neurological and behavioral abnormalities [72]. Other extra-intestinal manifestations are osteopenia, osteoporosis, fractures, arthritis and arthralgia [73]. Currently, gluten-driven symptoms and injuries are occurring at more advanced ages, and latent, hypo-symptomatic or asymptomatic manifestations are becoming amply present [74].

Diagnosing CD requires several factors: the presence of clinical signs and symptoms, seropositivity for endomysial or tissue transglutaminase autoantibodies (tTGA), the existence of HLA-DQ2/DQ8 haplotypes and, last but not least, the appearance of histologic lesions characterized by increased intraepithelial lymphocytes, crypt hypertrophy and progressive villous atrophy. However, none of these four criteria are individually sufficient to confirm CD diagnosis [75].

While gluten exposure and the presence of specific HLA antigen genotypes are necessary elements for the occurrence of CD, the disease risk is also strictly related to the timing or quantities of gluten consumed, and to the role of other potential pathophysiological factors. Therefore, the genetic predisposition is not enough on its own to be considered the triggering factor of the disease [76].

Aside from genetic predisposition, there are additional factors that play important roles in CD. Indeed, genetic background is a mandatory determinant of CD development, but environmental factors (e.g., viral infections) also contribute, such as loss of intestinal barrier function, inappropriate adaptive immune response and an imbalance in gut microbiome [64]. The role of environmental factors in relation to celiac disease onset is of great interest. In this regard, Ludvigsson and Green have introduced the concept of a “missing environmental factor” [77].

Other than gluten, which is the most important recognized environmental trigger factor, emerging data underline the possible roles of microbiota, time of gluten introduction to children, delivery method, history of breastfeeding, acute viral gastrointestinal infections and micronutrient deficiency in the development of CD [62, 78, 79]. The emerging extra-calcium role of vitamin D and the increasing interest in its involvement in immune modulation led Tanpowpong and Camargo to postulate that, in genetically predisposed individuals, vitamin D deficiency can play an important role in CD onset in children. In particular, this deficiency can result in a dysregulated immune response that can contribute to an abnormal intestinal mucosa, increasing susceptibility to acute gastrointestinal infection [62]. Even if the role of vitamin D in celiac disease pathogenesis is not completely known, its potential role in immune regulation could link vitamin D deficiency to this condition, considering that vitamin and mineral deficiencies have been noted both in newly diagnosed CD patients and in CD patients with a gluten free diet (GFD) [7, 62, 80].

The role of vitamin D in celiac disease onset, considering key findings from literature regarding vitamin D effects on the immune system, 25(OH)D plasma levels, vitamin D supplementation both in pregnancy and in infancy and CD development will be discuss.

Methods

Searches at MEDLINE/PubMed were performed in February 2020 using a combination of keywords addressing “celiac disease”, “vitamin D”, “25-hydroxyvitamin D”, “cholecalciferol”, “vitamin supplement”, “vitamin supplementation”, “pregnancy” and “children”, looking for articles published between 1 January 2010 and 7 February 2020. The following search algorithms were used:

((vitamin D) OR (vit. D) OR (25(OH)D) OR (25-hydroxyvitamin D) OR (cholecalciferol)) AND (celiac disease), which retrieved 428 papers, reduced to 213 with the “last 10 years” filter.

((vitamin D) OR (vit. D) OR (25(OH)D) OR (25-hydroxyvitamin D) OR (cholecalciferol)) AND (celiac disease) AND ((supplementation) OR (supplement)), which retrieved 80 papers, reduced to 57 with the “last 10 years” filter.

((vitamin D) OR (vit. D) OR (25(OH)D) OR (25-hydroxyvitamin D) OR (cholecalciferol)) AND (celiac disease) AND ((pregnancy) OR (pregnant)), which retrieved 21 papers, reduced to nine with the “last 10 years” filter.

((vitamin D) OR (vit. D) OR (25(OH)D) OR (25-hydroxyvitamin D) OR (cholecalciferol)) AND (celiac disease) AND ((infancy) OR (pediatric)), which retrieved 84 papers, reduced to 68 with the “last 10 years” filter.

((vitamin D) OR (vit. D) OR (25(OH)D) OR (25-hydroxyvitamin D) OR (cholecalciferol)) AND (celiac disease) AND ((molecular) OR (in vivo) OR (in vitro)), which retrieved 13 papers, reduced to six with the “last 10 years” filter.

After compiling all of the papers obtained via the research algorithms and eliminating duplicates, the full text of the remaining 44 articles were extensively reviewed on the basis of originality and relevance of each paper. Thirty-five papers were selected; moreover, 25 papers were added by searching the reference lists of the 35 papers reviewed. A total of 60 articles were considered.

Discussion

Vitamin D and Immune System

The role of vitamin D on immune function has been the subject of increasing interest, and studies have reported effects on both innate and adaptive immune responses. Vitamin D exerts effects on different aspects of immune functions, and it helps in promoting innate immunity by maintaining and improving defences against infection whilst, in parallel, regulating T-cells and dendritic cells (DCs) via a possible role in the mitigation of T-cell-mediated autoimmune disease [57-60]. The enzyme to convert 25-hydroxyvitamin D to its active form is expressed in macrophages and monocytes. It has been reported that vitamin D can participate in infection control thanks to its effect on macrophages, which enhance the antimicrobial effect. Indeed, the activation of Toll-like receptors regulates VDR expression, as bacteria lead Toll-like receptors to activate VDR expression and 25-hydroxyvitamin D-1-hydroxylase activity to be increased, with the latter stimulating cathelicidins [57, 81]. Moreover, vitamin D has shown an effect on dendritic cell activity by inhibiting

monocyte differentiation into dendritic cells and decreasing IL-12 production. Effects on both B and T lymphocytes have also been demonstrated. Influence on B-cell function has been reported as a consequence of differentiation and proliferation inhibition, apoptosis promotion and decreases in immunoglobulin production (autoantibodies included). It also reduces T-cell function by both decreasing T helper cell proliferation and differentiation, promoting a shift from a pro-inflammatory to a more tolerogenic immune status [57, 81]. These new findings on the role of vitamin D have been underlined by studies reporting the increased risk of respiratory infection and of autoimmune disease in cases of low levels of vitamin D [60, 82]. VDR is also expressed in the intestine, with a crucial role in cell proliferation, differentiation and apoptosis-induction regulation [83]. A literature revision by Masri underlined an association between vitamin D deficiency and incidences and activity of inflammatory bowel disease (IBD), with a focus on a possible role of vitamin D replacement therapy in IBD clinical practice, even if large intervention trials are needed [83]. Recently, the role of vitamin D in intestines has been described by Malaguarnera, who reported the role of vitamin D in the maintenance of gut homeostasis through a local synthesis of $1\alpha,25(\text{OH})_2\text{D}_3$ and VDR expression, emphasizing that an optimal $1\alpha,25(\text{OH})_2\text{D}_3$ status is fundamental, as it participates in several regulatory activities regarding not only calcium absorption but also infection protection, epithelial barrier function preservation and gut microbiota modulation. This role has received increased attention, as an unbalanced microbiota could be linked to several negative health disorders such as inflammation, allergic reactions, autoimmune diseases, heart diseases, obesity and metabolic syndrome [84].

Celiac disease is characterized by an immune response to undigested gliadin peptide fragments (e.g., pepsin–trypsin-resistant gliadin) in the small intestine. In susceptible individuals, the food-derived antigens of these fragments result in activation of the immune system and CD onset. In CD, ingested gluten leads to an immune response characterized by an interplay between innate and adaptive response. Gluten peptides are transferred to mucosal lamina propria and deamidated by tissue transglutaminase. The transfer from epithelial barrier to mucosal lamina propria could occur via either epithelial transcytosis or increased permeability of tight junctions. After the deamidation, gluten peptides bind to human leucocyte antigens (HLA-DQ2 and HLA-DQ8) on antigen-presenting cells. This step induces a response by activating CD4+ T-cells. An inflammatory response occurs, as CD4+ cells mainly secrete Th1 cytokines (as INF-gamma) that result in small bowel enteropathy, which is characterized by mucosal remodeling, villus atrophy and increasing cytotoxicity of intraepithelial lymphocytes or natural killer (NK) T-cells. Increases of intraepithelial lymphocytes and

epithelial cell proliferations with crypt hyperplasia, as well as reduced enterocyte differentiation, characterize pathological lesions. In addition, CD4⁺ cells participate to the activation of B cells throughout Th2, resulting in the production of antibodies to gluten and transglutaminase [66, 85-88].

Although the main external trigger is gluten, which is able to activate both innate and adaptive immune systems, the intestinal barrier plays a key role. Indeed, in susceptible individuals, an early disruption of the gut barrier will result in an increased permeability that could precede the onset of immune events induced by gluten [66, 85].

The intestinal barrier is a complex structure that aims to prevent harmful material from passing through intestinal mucosa that could reach the lamina propria. Its dynamic interaction with environmental factors such as pollutants, microorganisms and other materials are at the base of its crucial role. Its functionality depends on several factors, including epithelial layer integrity, gut-associated lymphoid tissue and intestinal microbiota homeostasis [66, 85, 89].

Tight junctions (TJs) are the major junctions responsible for intestinal mucosa barrier regulation. Indeed, under physiological conditions, they limit the passage of macromolecules (including gliadin peptides) across the intestinal barrier. TJs are multiprotein junctional complexes that regulate intestinal permeability. Injuries of TJs and consequent junction disassembly are considered initial processes that lead to the entry of gliadin peptide fragments and subsequent immune response activation. In this regard, increased intestinal permeability appears to be an early biologic change that precedes the onset of autoimmune diseases, including CD [85, 90, 91].

Gliadin has been reported as causing an increase in the permeability of the intercellular tight junctions of intestinal epithelial cells. This has been linked to an enhancement in zonulin release. Zonulin is a family of molecules that is responsible for the structural disassembly of tight junctions and consequently enhancement of intestinal permeability [64].

Lammers et al. have reported that the disassembly of gliadin-induced tight junctions is caused by the binding of gliadin to chemokine receptor CXCR3 and the subsequent MyD88-dependent release of zonulin. They also underlined that CXCR3 is expressed more abundantly at the intestinal level (i.e., epithelium and lamina propria) in patients with CD than in non-CD individuals [90, 91].

Fasano reported the upregulation of zonulin induced early in the disease by exposure to the disease's antigenic trigger gliadin impacts on TJs, causing an increased opening resulting in the passage of antigens (including gliadin) [92].

Thomas et al. proposed a model in which the increased intestinal permeability caused by gluten exposure and the release of zonulin gain access to submucosa through gliadin. Gliadin stimulates macrophages in a MyD88-dependent way to promote Th1 cytokine pooling (i.e., IL-12 p70, IL-15) through up-regulation of proinflammatory gene expression and cytokine secretion [93]. The role of vitamin D is of great interest in this context, as gut epithelial cells highly express vitamin D receptor (VDR), which mediates 1,25(OH)₂D₃ biological activity, and immunomodulatory properties have been reported [94, 95].

The expression and kinetics of vitamin D-related genes in human-activated T lymphocytes has been investigated by Baeke et al. They showed that 1,25(OH)₂D₃ effectively triggered VDR signalling, but only when introduced to T lymphocytes expressing high levels of VDR. These findings show that an enhanced degree of VDR signalling correlates with a stronger inhibition of cytokines, describing human T lymphocytes as direct targets of 1,25-dihydroxyvitamin D₃ in the immune system [95].

In addition, the role of dendritic cells (DCs) in the interface between innate and adaptive immunity is of great interest in CD. Phenotype and function of DCs can be conditioned by several factors, one of which is represented by 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). The potentially positive role of vitamin D on DCs has recently been underlined, demonstrating a strong relation between suboptimal vitamin D level and the occurrence and progression of many autoimmune diseases [96]. Ferreira et al. have demonstrated that *in vitro* treatment with 1,25-dihydroxyvitamin D₃ alters the phenotype and behavior of murine bone marrow-derived dendritic cells, converting their immunogenicity into a tolerogenic profile for a reduction of T-cell responsiveness and an increased production of regulatory cytokines [97].

Another key aspect of the role of vitamin D in CD is its effect on TJ injuries.

A protective effect has been reported by several studies in different intestinal TJ injuries caused by dextran sulfate sodium and alcohol [98, 99]. In the CD context, an interesting impact on TNF- α has been reported by Chen et al. They investigated the effect of 1,25(OH)₂D₃ on TNF- α induced barrier dysfunction in Caco-2 cell monolayers. Their results describe a protective effect of vitamin D, which suppresses NF- κ B p65 by mediating the activation of the myosin light-chain kinase (MLCK)-P-MLC signalling pathway [100]. In particular, MLCK phosphorylates MLC, which is a mediator of actin dynamics, thus leading to a contraction of the actomyosin cytoskeleton and disruption of TJs in the intestine [101].

Dong et al. demonstrated that vitamin D 1,25 had a beneficial effect on TJ injuries induced by pepsin-trypsin-resistant gliadin (PT-G) both in a Caco-2 monolayer model and in a gluten-sensitized

mouse model. They reported that in all their results, VD3 was able to suppress zonulin release signalling pathway activity, upregulate TJ protein expression and attenuate increases in Caco-2 monolayer permeability. Among three different studied concentrations (10^{-7} , 10^{-8} and 10^{-9} M), 10^{-8} M showed a protective effect on PT–G-induced TJ injury, underlining that it may be dose dependent. At this concentration, vitamin D was linked to an upregulation of TJ protein expression and to zonulin release suppression. Moreover, this concentration inhibited the MyD88 expression involved in zonulin release. They confirmed these findings by performing an in vivo study using gluten-sensitized mice. The mice were sensitized with PT–G for 30 consecutive days, which resulted in significant increases in small intestinal permeability, significant decreases in TJ protein expression and MyD88-dependent zonulin release signalling pathway activation. A vitamin D3 oral treatment for 7 days attenuated these changes, confirming in vivo the protective role of vitamin D against PT–G-induced intestinal mucosal barrier injuries. The authors of this study did not explore the mechanism by which vitamin D blocks the zonulin release signalling pathway, but they did hypothesize that vitamin D3 may inhibit that pathway by binding to its ligand [85].

These findings support the hypothesis that vitamin D may have a key role in CD onset by being involved both in immune response regulation (through action on dendritic cells and T-cells, above all) and on intestinal permeability by regulating inflammatory cytokines and zonulin release pathway-two key factors related to CD.

Celiac Disease and Environmental Factors

The importance of defining environmental factors that are able to influence CD onset relies on the possibility of improving and studying new strategies in terms of primary prevention. In this regard, factors that are able to influence CD onset have been the subject of several studies. In particular, season of birth has become a new field of study, as a seasonally dependent fluctuation related to CD onset has been suggested (Table 9-1) [102-106]. A study conducted in Sweden showed an increased CD risk in children born during the summer compared with those born in winter. In particular, authors reported that in study group of 2151 children with verified CD, risk significantly increased in children (<2 years old) born during summer, with this seasonal pattern maintained during 10-year epidemics of CD. Moreover, it was underlined that this seasonally dependent risk decreased with age, as if this exposure only exhibited an effect in the very first year of life. Study findings suggest a possible environmental exposure characterized by seasonal patterns, and authors has hypothesized a role of viral infection or time of gluten intake [102]. In addition, Lewy et al.

examined the medical records of 431 children with CD (239 girls and 192 boys), and reported a seasonal pattern in CD that was different from the general population, with peaks in September. Moreover, different seasonality was found depending on age (< or > 24 months), sex and family history of CD. The author linked study findings with perinatal virus infection as the trigger factor [103]. More recently, a case-control study conducted in Sweden using biopsy reports from 28 Swedish pathology departments with 144,522 controls matched for gender, age, calendar year and country reported an association between summer birth and increased risk of CD, but the excess risk was small and largely limited to children diagnosed before age 2. The authors of that study focused their attention on the need for further studies to test the possible mechanisms related to seasonality. In this context, they described not only the possible role infection could play in potentially increasing the risk of CD (e.g., by influencing microbiota and compromising mucosal barrier function), but also the role of low 25(OH)D levels. In particular, they considered both low 25(OH)D levels in mothers due to lack of UV light exposure during pregnancy, and in children at the time of gluten introduction and viral infection [104]. A population-based study conducted in the US analyzed CD prevalence in people living in northern latitudes compared with people living in southern latitudes. Data on gluten-related conditions were analyzed from the US National Health and Nutrition Examination Survey, from 2009 through 2014, using 22,277 participants that were 6 years and older. Authors reported a North-South gradient in CD with higher proportion on people living at latitudes of 35° North or greater independently from race or ethnicity, socioeconomic status, or body mass index. This pattern resembles the north-south gradient in the disease occurrence of autoimmune diseases, including inflammatory bowel disease, multiple sclerosis and rheumatoid arthritis [105]. In a survey conducted at two Italian centers for CD in Rome and Bari using the data of CD patients born between 2003 and 2010 were retrospectively examined to investigate whether the season of birth could be associated with CD onset in Italy. In this study, 596 children were compared, with a reference group of 439,990 controls, and data showed that children born in the summer in Italy were at higher risk of developing CD. From these findings, the authors hypothesized that there could be some factors related to a pattern of seasonality. In particular, they underlined that children born in summer are introduced to gluten during the winter, with a concomitant increased probability of rotavirus infection. Moreover, it was highlighted that 25(OH)D levels can vary during the year due to different UV exposures [106].

Table 9-1. Seasonally dependent fluctuation related to celiac disease (CD) onset.

	Country and Year	Design	Study Population	Method	Outcomes
Ivarsson et al. [102]	Sweden, 2003	Retrospective and prospective study	2151 CD children below 15 years of age.	CD incidence rates were calculated by month of birth, stratified for age at diagnosis.	The risk for celiac disease was significantly higher if born during summer compared to winter in children below 2 years of age at diagnosis. This relative seasonal risk pattern prevailed during a 10-year epidemic of celiac disease, although incidence rates varied threefold. The incidence was constantly higher among girls, but boys showed a more pronounced seasonal variation in risk.
Lewy et al. [103]	Israel, 2009	Retrospective study	431 CD children (239 girls, 192 boys). 138 girls and 81 boys were under the age of 24 months.	Medical records were analyzed to obtain both statistical significance and parameters of rhythms.	Boys and girls with CD were found to have different seasonality of month of birth. Girls diagnosed before age 24 months (peak July–August) had a different seasonality birth from those diagnosed after age 24 months (no rhythm) and showed a different seasonality from boys with diagnosis above 24 months (peak July). Different seasonality was found in children with a family history of CD.
Lebwohl et al. [104]	USA, 2013	Case-control study	351,403 biopsy reports, of which 29,096 from patients with CD. Up to 5 controls for each CD individual were identified by Statistics Sweden (total: 144,522).	The association between summer birth (March–August) and later CD diagnosis was examined through conditional logistic regression.	54.10% of individuals with CD vs 52.75% of controls were born in the summer months. Summer birth associated with a small increased risk of later CD. Stratifying CD patients according to age at diagnosis, the highest OR was found in those diagnosed before age 2 years, while summer birth was not associated with a CD diagnosis in later childhood (age 2–18 years), but had a marginal effect on the risk of CD in adulthood.
Unalp-Arida et al. [105]	USA, 2017	Population-based study	22,277 participants 6 years and older.	Analyzed data on gluten-related conditions from the US National Health and Nutrition Examination Survey, from 2009 through 2014 identifying persons with CD.	0.7% of participants were found to have CD. Celiac disease was more common among individuals who lived at latitudes of 35–39° North or at latitudes of 40° North or more than individuals who lived at latitudes below 35° North, independent of race or ethnicity, socioeconomic status or body mass index.
Capriati et al. [106]	Italy, 2015	Retrospective study	596 CD patients children (age range 3.5) compared with 439,990 controls	Survey conducted at two Italian referral centers for CD in Rome and Bari. The CD database was created to enable retrospective examination of the data of all the consecutive patients born between 2003 and 2010 who had received a diagnosis of CD.	A summer birth preponderance was observed in CD patients compared to controls. Stratifying the case by gender, the summer birth preponderance was maintained for females.

CD = celiac disease, OR = odds ratio.

Vitamin D Status in Celiac Disease

In order to evaluate the involvement of vitamin D in CD onset, attention was focused on vitamin D status not only after CD diagnosis, but also before, especially during pregnancy and first years of life (Table 9-2).

In 2011, O'Malley reviewed the vitamin D statuses and supplementations in a pediatric population with gastrointestinal diseases, reporting that children with gastrointestinal disease were more likely to have a vitamin D deficiency, possibly due to malabsorption. The review also highlighted the importance of vitamin D status monitoring of children, and the need for possible supplementation in some instances [107].

The importance of introducing routine testing to evaluate vitamin deficiencies led Imam et al. to conduct a retrospective medical record review of CD patient, measuring fat-soluble vitamin levels at the time of diagnosis in order to identify the frequency of deficiencies. However, data obtained showed that fat-soluble vitamin D deficiencies were uncommon in pediatric CD, even if the study had some limitations (such as the small sample size (83 patients) and data coming from a single tertiary center) [108]. Similar results were obtained by Villanueva et al. that found no differences in vitamin D status between CD children and no-CD children in a retrospective study of 74 prepubertal children (age 3–12 years old) divided into 24 CD and 50 no-CD [109]. Further, Lerner et al. compared the vitamin D status of CD children (Israeli CD children and Spanish CD children, group 1 and group 5, respectively) to children with no-specific abdominal pain (group 2), their parents (group 3) and Spanish adult CD patients (group 4). No vitamin D deficiency was found in the children. Conversely, in the adult CD population, a significant vitamin D deficiency was reported. This was explained by the authors as being related to the fact that children with CD had an increased intake in terms of vitamin D, due to the routine supplementation during the first year of their lives, as well as increased sun exposure and GFD compliance. Interestingly, they observed that vitamin D deficiency was age dependent, but this seems not to be related to degree of small bowel injury in CD. From these findings they concluded that a routine check of vitamin D status in CD patients is of fundamental importance, especially in adults [110]. A study was conducted to investigate if the prevalence of autoimmune diseases was higher in patients with CD and low vitamin D than in patients with CD but normal vitamin D. Both groups had a similar prevalence, however psoriasis risk was higher in the low vitamin D group. Although this study reported that low vitamin D was not predictive of autoimmune disease among CD individuals, it was underlined that vitamin D deficiency is common in CD, and that assessment of its values should be taken into account in clinical practice [111]. The importance of nutrient assessment in CD was also underlined by Caruso in a review that aimed to evaluate the impact of CD on iron, folate, vitamin B12, vitamin D and calcium [2]. Micronutrients are commonly below optimal levels in treated CD, and this deficiency could be related to extra-intestinal symptoms or signs [2, 7].

In order to study a possible correlation among micronutrient deficiency in CD, serum tissue transglutaminase (tTG) immunoglobulin A (IgA) antibody titers and degree of mucosal damage at diagnosis, Deora et al. evaluated the prevalence of micronutrient deficiencies in children both at diagnosis and at 6 and 18 months after the start of a GFD. Examining the medical records of 140 children with CD, a 70% deficit in vitamin D was found. In addition to this, the data showed no correlation between degree of villous atrophy or serum titers of anti-tTG IgA molecules and micronutrient deficiency. Moreover, almost all micronutrient deficiencies found at the diagnosis were reported to have normalized after 6 and 10 months of a GFD. Only vitamin D and ferritin still remain under optimal levels after 18 months, highlighting the importance of their long-term assessments. In this regard, the authors of the study reported that vitamin D status evaluation should be performed annually in children [112].

As nutritional deficiencies are commonly found in gastrointestinal disease, Ahlavat et al. reviewed the prevalence of vitamin D deficiency in pediatric gastrointestinal disease. They reported that even if the exact role of vitamin D is not fully understood, the actual knowledge in bone health and the role in immune regulation mean that determining vitamin D status is very important to screening for diseases such as IBD and celiac disease. From a practical point of view, they screened for low vitamin D status at disease onset and, if needed, treated for any level greater than 30 ng/mL but less than 100 ng/mL. However, large randomized controlled trials are needed in order to further investigate the role of vitamin D in disease remission and the maintenance of optimal 25(OH)D levels [80]. Recently, a cross-sectional study conducted with the aim of screening for vitamin D status reported no significant difference between newly diagnosed CD patients and no-CD controls in regards to vitamin D status. However, inadequate 25(OH)D levels were found in both groups. Moreover, vitamin D levels were highly associated with estimated vitamin D intake in both the CD and control groups. From these findings, the authors recommended a vitamin D status screening in children at diagnosis time [113].

The impact of vitamin D status on CD onset has also been investigated in pregnant women, as low concentrations of it have been associated with offspring autoimmune disease. In particular, Marild et al. tested whether low maternal and neonatal 25(OH)D levels could be a predictor of increased CD risk in a cohort of pregnant norwegian women. In particular, mid-pregnancy and post-partum blood analysis and cord plasma were performed, comparing data of 416 children who developed CD and 570 children without CD. In addition to this, mothers and children were genotyped for established celiac disease and vitamin D metabolism variants. Obtained results showed no

significant difference of 25(OH)D levels between the two group, and genetic variants for deficiency did not associate with CD. The authors concluded that even if they could not exclude the presence of unmeasured confounders, level of 25(OH)D seems not to be associated with pediatric CD onset. However, randomized controlled trials will be needed to further investigate this topic [60]. In 2019, a cross-sectional study was conducted to evaluate whether severe vitamin D deficiency is associated with CD by evaluating 200 Saudi adolescent girls. Data obtained showed CD as a potential a risk for severe vitamin D deficiency and, above all, that low levels of vitamin D (<12.5 nmol/L), in absence of obvious causes, could be a sign of the need for CD screening [114].

In terms of supplementation, Bittker reported that oral vitamin D consumption among children could be a significant risk factor in inducing CD. Bittker related this to the fact that vitamin D tends to elevate the activity of Th2 cytokine, which upregulates immune reactions to external stimuli, reporting that vitamin D has been described as a risk factor for allergic diseases including asthma and atopic dermatitis, with high comorbidity with CD [115]. Recently, the same author reported that the “missing environmental factor” could be significant oral vitamin D exposure with its consequent increase in plasma. Oral vitamin D in large doses upregulates cytokines, chemokines and Toll-like receptors that are also upregulated in CD. Moreover, epidemiologically higher CD prevalence has been reported in countries with aggressive supplementation policies [116]. As vitamin D could be considered an immunomodulator, and its role in CD has been received increased importance, Bittker conducted a case-control epidemiological survey among parents living in the US with at least one biological child between 3 and 12 years old to determine if nine variables could be associated with CD in children. Among these, vitamin D drop exposure in infancy and vitamin supplement exposure between 2–3 years were evaluated. Data obtained from 332 children diagnosed with CD (cases) and 241 without CD (controls) suggest that infants who received oral vitamin D drops for longer than 3 months were at increased risk of subsequently developing CD [117]. On the contrary, a longitudinal prospective observational study on 6627 children reported that maternal use of dietary supplements during pregnancy is not associated with CD. In particular, maternal use of vitamin D, n-3 fatty acids and iron were not associated with CD risk, concluding that dietary supplementation during pregnancy could be a tool to improve nutrient intake [118].

Table 9-2. Vitamin D status in celiac disease.

Country and Year	Design	Study Population	Methods	Vit. D Supplement	Outcomes
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Imam et al. [108]	USA, 2014	Retrospective study	83 CD patients: 51 girls and 32 boys (average age at diagnosis, 12.8 and 13.0, respectively).	Medical record review of CD patients and fat-soluble vitamin levels measured at diagnosis between 1995 and 2012 at Mayo Clinic.	None receiving vitamin supplements at the time of diagnosis.	Average 25(OH)D levels 32.8 ng/mL; 9 patients had mild-to-moderate vitamin D deficiency, 31 patients showed insufficiency of 25(OH)D.
Villanueva et al. [109]	USA, 2012	Retrospective study	24 prepubertal CD children and 50 no-CD (age, 3–12 years).	25(OH)D level measured by chemiluminescence immunoassay. Height and body weight measured to calculate BMI.	No supplementation.	No difference in 25(OH)D level between CD and no-CD. Non-obese CD had a significantly higher 25(OH)D level than the obese no-CD. No difference in 25(OH)D level in non-obese.
Lerner et al. [110]	Israel and Spain, 2012	Case-control study	272 individuals in five groups. Group 1: 51 Israeli children with CD (age, 6 ± 4 years); Group 5: 59 Spanish children with CD (age, 4 ± 4 years); Group 2: 56 Israeli children with nonspecific abdominal pain (age, 8 ± 5 years); Group 3: 84 adults, parents of group 2 (age, 39 ± 8 years); Group 4: 22 Spanish adults with CD (age, 44 ± 13 years).	Vitamin D serum levels investigated in CD populations compared to children with nonspecific abdominal pain, their parents and Spanish adult CD patients. 25(OH)D checked by chemiluminescent immunoassays.	No supplementation.	Groups 5 and 1 had the highest levels compared to groups 4 and 3. Levels in groups 1 and 2 were comparable. Concerning 25(OH)D sera levels, only the difference between groups 5 and 4 was statistically significant. Vitamin D sera levels negatively correlated with age.
Tavakkoli et al. [111]	USA, 2013	Retrospective cross-sectional study	530 CD adult patients	Compared patients with normal vitamin D level (≥30 mg/dL) against those with vitamin D insufficiency (20–29 mg/dL) and vitamin D deficiency (<20 mg/dL) with regards to prevalence of autoimmune disorders.	Patients were not excluded from the study if they were taking vitamin D supplements, but there was no knowledge of supplementation.	25% showed vitamin D deficiency. Similar prevalence of AD among those with normal vitamin D (11%), insufficiency (9%) and deficiency (12%). Vitamin D deficiency was not associated with AD. Risk of psoriasis was higher in patients with vitamin D deficiency.

Deora et al. [112]	Canada, 2017	Single-center cohort study	Medical records of 140 CD children (mean age at diagnosis 7.8 ± 4.01 years).	Analysis of the medical records of all children with CD. Routine celiac blood tests carried out at diagnosis 6 months after starting GFD, then on an annual basis. Histopathological changes of duodenal biopsies at diagnosis documented using modified MARS classification. Diet assessment with proper teaching of GFD at diagnosis, 6 months and 18 months after diagnosis.	During each clinic visit, an experienced dietitian evaluated the nutritional status and need for micronutrient supplementations.	70% of subjects with serum vitamin D deficiency. No correlation between micronutrient deficiencies at diagnosis and serum TtG IgA antibody titers or the degree of villous atrophy. The majority of serum levels of micronutrients normalized after 6 months after beginning GFD, except for vitamin D, which improved but remained subnormal.
Ahlawat et al. [113]	USA, 2019	Cross-sectional study	38 newly diagnosed CD patients (10.4 ± 3.0 years old) and 82 controls (11.2 ± 4.2 years old).	25(OH)D levels drawn in children with newly diagnosed CD compared with pediatric outpatients with functional abdominal complaints. Anthropometric data and vitamin D intake based on milk and multivitamin ingestion were recorded.	Patients were excluded if they used single-preparation vitamin D supplements within 3 months of study enrollment. Multivitamin use was allowed.	Both groups were similar except for average daily D intake and BMI. No statistical difference in mean 25(OH)D levels between CD and controls. Both groups had high percentages of suboptimal D status. 25(OH)D levels significantly correlated with age ($r = -0.262$) and estimated vitamin D intake ($r = 0.361$).
Mårild et al. [60]	Norway, 2017	Case-control study	416 children who developed celiac disease, 570 randomly selected controls and their mothers.	25(OH)D examined in maternal blood from mid-pregnancy, post-partum and cord plasma. Mothers and children genotyped for established celiac disease and vitamin D metabolism variants.	Participants completed a food frequency questionnaire covering the period from start of pregnancy until completion around week 22, to evaluate their vitamin D intake while also considering supplements.	No significant difference in average 25(OH)D between cases and controls and no significant linear trend. Genetic variants for vitamin D deficiency were not associated with CD.

Sulimani [114]	Saudi Arabia, 2019	Cross-sectional study	200 adolescent females aged 13–19 years old with vitamin D deficiency	Female adolescent screened for IgA tTG (anti-tissue transglutaminase antibodies).	No vitamin D supplement.		Of the 200 girls, 9 (4.5%) were positive for IgA tTG antibodies; all of whom had serum 25(OH)D < 12.5 nmol/L. A strong significant inverse association was found between tTG antibody levels and serum 25(OH)D ($R = -0.53$) among antibody negative participants. Positive IgA tTG antibodies were 37.2 times higher among participants with 25(OH)D < 12.5 nmol/L than those whose vitamin D status was higher.
Bittker [117]	USA, 2019	Case-control study	332 parents with CD children + 241 parents with no-CD children (controls)	An Internet-based survey was conducted among parents living in the US with at least one biological child between 3 and 12 years old in order to determine if 9 variables are associated with CD, among these: vitamin D drop exposure in infancy and vitamin D supplement exposure between 2–3 years old.	Two questions examined supplemental vitamin D exposure. One focused on the duration of exposure to vitamin D drops in infancy. The other on vitamin D supplementation between 2–3 years of age.		In this dataset, only vitamin D drops administered for more than 3 months was associated with CD children.
Yang et al. [118]	USA, Finland, German, Sweden, 2017	Longitudinal prospective observational study	6627 children (years range 0.9–10.0)	Examined the association between maternal use of vitamin D, <i>n</i> -3 fatty acids (FA) and Fe supplements during pregnancy and risk for CD autoimmunity (CDA) and CD in the offspring.	Use of supplements containing vitamin D, <i>n</i> -3 FA and Fe was recalled by 66%, 17% and 94% of mothers, respectively, at 3–4 months post-partum. The mean cumulative intake over the entire		Of 6627 enrolled children, 1136 developed CDA at a median 3.1 years of age (range 0.9–10) and 409 developed CD at a median 3.9 years of age (range 1.2–11). No statistically significant association between the intake of

<p>pregnancy was 2014 µg vitamin D (SD 2045 µg), 111 g n-3 FA (SD 303 g) and 8806 mg Fe (SD 7017 mg).</p>	<p>vitamin D, n-3 FA or Fe, and risk for CDA or CD. Dietary supplementation during pregnancy did not seem to modify the risk for the disease in the offspring.</p>
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AD = autoimmune disorder, BMI = body mass index, CD = celiac disease, CDA = celiac disease autoimmunity, FA = fatty acids, GFD = gluten free diet, tTG = anti-tissue transglutaminase, 25(OH)D = 25-hydroxyvitamin D.

Vitamin D and International Guidelines

Even if the interest in the role of vitamin D in CD is increasing, previously reported data have been controversial, and specific studies and indications regarding optimal serum levels and supplementation strategies are still lacking. Controversy remains regarding the optimal level of serum vitamin D, with governmental agencies reporting sufficiency at levels of 25(OH)D >20 ng/mL (> 50 nmol/L). However, evidence related to both skeletal and non-skeletal outcomes has defined the vitamin D deficiency threshold as 30 nmol/L [113, 119-121].

The American College of gastroenterology recommended the assessment of micronutrient deficiencies for, but not limited to, iron, folic acid, vitamin D and Vitamin B12 in newly diagnosed CD [122]. The British Society of Gastroenterology (BSG) CD guidelines suggest measuring calcium, alkaline phosphatase, vitamin D levels and parathyroid hormone both at diagnosis and when necessary [123]. Specifically, for pediatric patients, the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) recommends vitamin D assessment at diagnosis and annually [124]. An Italian consensus suggests serum vitamin D levels be evaluated in CD patients at diagnosis and after 6–12 months of GFD if deficiency has been found [125]. A panel of experts published recommendations for managing CD in children, and suggested screening vitamin D at diagnosis; however, the quality of evidence was considered low and the strength weak. They suggested considering age-appropriate counselling regarding calcium and vitamin D supplementation at diagnosis and during follow up (evidence grade high, statement strength strong) [126]. The Endocrine Society Clinical Practice Guideline advises evaluating vitamin D status in patients at risk of deficiency, suggesting supplementation either with vitamin D2 or D3 for deficient patients [119].

Recommendations from the National Institute for Health and Care Excellence (NICE) on the recognition, assessment and management of celiac disease based on systematic reviews of available evidence suggests the possible needs of vitamin D supplementation in cases of insufficient dietary

intake. However, they reported very low-quality randomized controlled trials the basis of this recommendation [127].

Governmental agencies, including the Institute of Medicine (IOM) in North America and the European Food Safety Authority (EFSA) in the EU, have set the estimated average daily intake of vitamin D for healthy adults (with minimal or no sunlight exposure) at 400 IU per day [128, 129]. In particular, IOM stated that for vitamin D (assuming minimal sun exposure), the estimated average requirement (EAR) is 400 IU/day for ages older than 1 year, and the recommended dietary allowance (RDA) is 600 IU/day for ages 1 to 70 years and 800 IU/day for 71 years and older. The tolerable upper intake level (UL) ranges from 1000 to 4000 IU daily for vitamin D: 1000 IU for infants 0–6 months, 1500 IU infants 6–12 months, 2500 IU for children 1–3 years, 3000 IU for children 4–8 years and 4000 IU for children 9 years and older [128]. An EFSA panel set the adequate intake (AI) for adults at 15 µg/day, for children aged 1–17 years at 15 µg/day and for infants aged 7–11 months at 10 µg/day. For pregnant and lactating women, the panel set the same AI as for non-pregnant non-lactating women, (i.e., 15 µg/day). The UL for adults (including pregnant and lactating women) was set at 100 µg/day. In children and adolescents aged 11–17 years, the UL was adapted to 100 µg/day. For children aged 1–10 years a UL of 50 µg/day was proposed, and for infants it was set at 25 µg/day. A subsequent update set the upper limit at 25 µg/day for infants aged up to 6 months, but a UL of 35 µg/day for infants 6–12 months [129-131].

However, specific guidelines and indications relating to both optimal vitamin D status and supplementation specifically related to CD in terms of primary and secondary prevention are still lacking. More studies, and randomized controlled trials in particular, are needed to further and better evaluate the role of vitamin D in CD onset and during the follow up.

Conclusions

Considering the importance of environmental factors in CD pathophysiology, several aspects are currently under study in order to find a possible association with CD onset. Presently, one important focus of attention relies on birth seasonality, as CD risk seems to increase in infants born during the summer. This aspect could be related to other factors, such as time of gluten ingestion, virus infections, different UV exposures or mother's vitamin D status during pregnancy, as well as a concomitant presence of these factors. In particular, the emerging extra-calcium role of vitamin D and the increasing interest on its impact on the immune system and intestinal barrier permeability leads to its consideration as an important factor possibly involved in CD onset.

However, there have only been a few fragmentary studies, with different focuses and studied populations. Moreover, the role of supplementation is still uncertain. It is common to routinely consider vitamin D supplementation; however, this has sometimes been considered ineffective. In light of this, clarifying the role of vitamin D is of great importance. There is a need for larger studies that would take into account variables that can have a role in CD onset (i.e., vitamin D levels of pregnant women, vitamin D supplementation and/or UV exposure), as well as involving different countries. This would be of fundamental importance in order to better explore possible associations and, in particular, to study and evaluate the possible role of vitamin D that could therefore play a key role in terms of prevention and be part of possible new CD prevention strategies.

Development of a nutritional education course for the management of gluten-free diets

Considering that celiac disease has become a world-wide health problem [1] and the only effective resolution therapy is the strict adherence to a gluten-free diet [3] which is not easy to follow and that patients may encounter some difficulties in following the right nutritional choices and macro- and micronutrients imbalances can emerge [6, 7, 12]. Possible nutrient deficiencies (such as fiber and specific micronutrients) or nutrient excess (i.e., saturated fats and simple sugars) can be also related to the lower nutritional quality of commercially available gluten-free products compared to the gluten-containing counterparts [7].

In addition, the lack of nutritional knowledge on the gluten-containing foods and careless dietary choices could entail a decrease of diet quality [13].

Thus, attention must be devoted in nutrition education of celiac disease patients and to those caring for their diet in order to guarantee nutrient balance and avoid deficiencies.

Nutrition education is necessary to aid all consumers in making healthy dietary choices [132]. Indeed, nutrition education allows to create a background of knowledge and information on nutrients and foods which let the consumer to adequately plan daily meals and to consciously read food labels before buying a food product [133]. Given the role of nutrition education for consumers, it becomes even more important to underline its relevance for celiac consumers.

Celiac disease patients and their caregivers need to be guided in the choice of gluten-free foods and to be supported in gluten-free meals preparation. Numerous processed and ultra-processed gluten-free foods are available in the market, anyway their nutritional profile is not often adequate,

especially for the high content of sugars, salt and fats and low content of proteins [134]. Therefore, in order to make adequate food choices, celiac consumers must be informed on unprocessed naturally gluten-free food and on their preparation. Indeed, Sangwan et al. pointed out that training celiac disease patients on the preparation of gluten-free meals through lectures cum demonstrations and leaflets improves the knowledge of celiac participants [135]. Nutrition education should represent a strategy to voluntarily adhere to healthy dietary behaviours, empowering consumers and making them choose consciously and independently [132].

In this perspective, a nutrition education course was developed. It was specifically addressed to celiac patients and their parents, in addition to the restaurant owners and chefs with the aim to sensitize all figures involved in the preparation of gluten-free meals.

This nutrition education path was thought and developed on the basis of the results obtained from the nutrition education course described in chapter 6, after having verified the feasibility of the platform as a food educational tool.

The new nutritional educational path developed on digital platform is available. It will be used in collaboration with the “Marche section” of Italian Association for Celiac Disease (AIC) starting from September 2022.

Course structure

The course opens with a series of welcome activities and celiac patients are invited to participate in order to collect information concerning their food choices.

Specifically, celiac disease subjects are asked to compile a weekly food diary. The food diary is a 7-days food record where reporting foods and beverages consumed daily for a week. The food diary proposed was based on the EPIC validated food frequency questionnaire [136] and each day was structured in two different sections. The first one regarded the daily food compilation. Each day was organized in 6 meals (breakfast, morning snack, lunch, afternoon snack, dinner and evening snack) and for each meal participants were asked to indicate the qualitative and quantitative composition of the meal. Each participant was asked to report details on food preparation and to compile an online questionnaire (Google forms®) on their eating habits and lifestyle (Appendix IV).

The course is divided into modules including both theoretical and practical activities.

The theoretical part of each module consists in a series of interacting videos concerning: the principles of a healthy diet and how to structure it (module 1), how to choose gluten-free products by starting from the evaluation of nutritional labels (module 2), how to effectively manage gluten-free diet (module 3).

Course modules

Module 1: "Feed ourselves...with healthy!"

In this module, videos focus the attention on the general principles of the Mediterranean diet [130]. The goal is to guide food choices to achieve and maintain a good health status. For the practical activity, subjects are invited to propose a nutritionally balanced meal (breakfast, snacks, lunch or dinner), starting from the analysis of the "Healthy Eating Plate" [137].

Module 2: "At the discovery of the gluten-free"

In this module, the videos underline the importance of diet quality beyond gluten removal in celiac disease treatment. At this regard, the crucial role of "Associazione Italiana Celiachia (AIC)" played in the celiac disease patients' daily life is shown. Moreover, the videos provide indications on how to make a correct and careful reading of the food labelling aiming to make healthy and aware food choices in everyday life. The practical activity consists in comparing the food label of three gluten-free products indicating which of them is better from a nutritional point of view.

Module 3: "Celiac disease: practical guide...in-out of home"

In this module, the videos provide some practical and useful general information to facilitate the management of the gluten-free diet. Indeed, general advices, ranging from how to properly go food shopping to meals preparation and gluten-free diet management outside the home, are supplied. The practical activity consists in proposing an example of meal that celiac consumers would like to find in a restaurant's menu.

At the end of the course, additional materials for users are also provided:

- 4 weekly menu proposals, one for each season of the year, offering some examples of gluten-free and nutritionally balanced meals (an example of a gluten-free menu is reported in Appendix V),
- a collection of recipes, explained step by step, for preparing gluten-free healthy and tasty foods (an example of a gluten-free recipe is reported in Appendix VI).

The development of nutrition education activities is fundamental to increase nutrition awareness in the general population and even more in celiac consumers. Indeed, conscious and smart dietary choices are necessary to avoid nutrient deficiencies and to guarantee a healthy status.

Moreover, these activities can be useful to improve the nutritional quality of gluten-free products available in the market or to develop gluten-free products with high nutritional quality.

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Chapter 10. Nutritional Properties of Rice Varieties Commonly Consumed in Italy and Applicability in Gluten Free Diet [1]

Abstract

Gluten-free diets are often characterized by an inadequate intake of nutrients and are generally monotonous for the limited number of products celiac patients can use. As rice is the most used cereal by celiac consumers, studying rice varieties nutritional characteristics is of interest to manage diet quality and variety.

Proteins, total carbohydrates and amylose content of six rice varieties (Ribe, Vialone Nano, Carnaroli, Arborio, Basmati, and Fragrance) were analyzed. Analyses were performed in raw products and after boiling, stewing, and microwaving.

A decrease of proteins and total carbohydrates amount was observed in cooked rice. The same was reported for amylose content with boiling showing the highest loss (average retained amylose 53%). Considering amylose percentage with respect to total carbohydrates, each variety showed either an increase or a decrease depending on cooking method. The highest values were obtained with stewing above all for Basmati rice and Arborio rice. However, exceptions can be underlined as Carnaroli rice, showing the highest percentage when boiled.

In this context, nutritional characteristics of cooked rice varieties appear to be of great importance to increase specific nutritional knowledge to better manage gluten-free diets.

Introduction

Celiac disease is a T cell mediated intestinal disorder, genetically inherited, which affects the small intestine by damaging intestinal villi and consequently compromising nutrient absorption [2]. The trigger responsible for an abnormal immune response in genetically susceptible individuals is wheat protein gluten [3]. The only effective treatment for celiac disease patients is to follow a life-long gluten-free diet [4]. A gluten free diet is necessary for celiac patients, but such a specific diet could be complicated by the effort involved in selecting high quality gluten-free foods and in maintaining a balanced and varied dietary regimen [5]. The use of commercial gluten-free foods, often rich in fats and hypercaloric, can contribute to the risk of overweight and obesity [6]. Moreover, according to Agarwal et al., the risk of developing metabolic syndrome and fatty liver disease in celiac patients following a gluten-free diet is high, especially with the consumption of commercially available gluten-free products [7]. As a gluten-free diet is followed by celiac patients for their entire life, it is essential to guarantee nutrient balance and to avoid deficiencies in order to maintain a healthy status [8, 9].

Indeed, gluten-free diets are often characterized by an inadequate intake of nutrients, such as excess fats and sugar, and a lack of vitamins (i.e., vitamin B12, folate, vitamin C) and minerals (i.e., calcium, selenium, zinc and magnesium) [10].

Therefore, to avoid nutrient deficiency, it is fundamental to follow a healthy balanced diet [11]. Mediterranean diet (MD) has been widely described to positively impact health status [12]. According to the MD dietary guidelines, it is necessary to consume different food categories every day to guarantee nutrient balance in the diet [13, 14]. Cereals are one of the pillars of the Mediterranean diet and they should be part of every daily meal [15, 16]. In this perspective, it is fundamental to vary among different types of cereals available and for each type, changing current varieties. This aspect is fundamental to follow a balanced diet, especially for celiac consumers who have a limited number of products to select. Among cereals, rice is the mostly used by celiac consumers [17, 18]. Consequently, it can be useful to better exploit nutritional characteristics of rice varieties. In general, cereals contain carbohydrates, proteins, vitamins, minerals, antioxidants and phytochemicals [19, 20]. However, attention should be paid to carbohydrate contents of different cereals and varieties and, in particular, to amylose, one of the components of starch. Amylose has a slightly branched structure [21] while amylopectin, the other starch component, has a highly branched structure [22]. Amylose is less available for degradation by digestive enzymes because of the reduced surface area per molecule than amylopectin, thus inducing a lower impact on glycaemic response [23-25]. At the same time, it is evident that high amylose starches are less available for digestion than low amylose starches [23]. Moreover, amylose is converted into resistant starch, which is involved in improving the parameters of glucose and insulin homeostasis and has an hypocholesterolaemic effect [26, 27].

Beyond amylose content, amylopectin content plays a role in starch digestion rate. For its highly branched structure, amylopectin is more available for degradation than amylose [23-25]. Anyway, the degree of branching and the length of chains of amylopectin molecule must be considered as an important parameter in starch digestion [28, 29].

Although a high amylose content ameliorates the nutritional characteristics of rice, it is essential to consider that it influences rice taste and consequently consumer acceptance [30]. Indeed, different studies highlight that a low amylose concentration improves the texture and the eating quality of rice [31-33].

It can be gathered that a high amylose concentration negatively impacts on rice taste but in parallel exerts nutritional benefits. Accordingly, selecting a high amylose rice results the preferable choice

for health [34]. Moreover, it must be considered that cereals, including rice, constitute a part of a balanced plate in which other foods, such as fruits and vegetables, plant-based fats, healthy proteins, and nuts, contribute to improve the quality of the meal [35].

Furthermore, it is widely known that the quality of the diet and nutrient balance depend also on the cooking methods used. Indeed, cooking methods differently affect the nutritional profile of foods [36-39]. The knowledge of cooking methods impact on nutritional quality of cereals could guide nutritionist or expert in nutrition in the choice of the appropriate cooking method to better manage glucose response, thus improving the general quality of the diet [40].

Consequently, the study of the nutritional characteristics of cereals could be of great interest for selecting them according to specific and personalized nutritional needs. In the case of celiac patients, this knowledge can contribute to better manage the quality of the diet, to make it more varied and balanced [41].

This study aims to analyse protein and carbohydrates content, in particular amylose content, of different varieties of rice commonly consumed in Italy in order to ameliorate the quality of gluten-free diets, to promote healthy dietary habits, but also to guide food choices for specific nutritional requirements (i.e., daily diet, physical exercise) in celiac patients.

Materials and Methods

Materials

Six varieties of rice were purchased from an Italian supermarket: Ribe (Contiriso Riseria Conti Francesco S.R.L), Vialone Nano (Selex S.p.a.), Carnaroli (Selex S.p.a.); Arborio (Selex S.p.a.); Basmati (Selex S.p.a.), Fragrance (Selex S.p.a.) (Table 10-1). Sulphuric acid (analytical grade), D (+) –glucose anhydrous, iodine and potassium iodide were purchased from J.T.Baker (Holland). Anthrone analytical standard (purity > 97%), amylose (from potato) and Bovine Serum Albumin (BSA) were purchased from Sigma-Aldrich (USA). Bradford reagent was from Bio-Rad Laboratories, Inc., Italy.

Table 10-1. Nutritional facts of the six selected raw rice varieties from nutritional labels.

Nutrition Facts (for 100 g of Raw Rice)					
Rice Varieties	Energy (kcal)	Total Fat (g)	Total Carbohydrates (g)	Dietary Fiber (g)	Total Protein (g)
Ribe	350	0.6	76.9	0.9	7.4
Basmati	359	1.3	77.3	1.3	8.8
Carnaroli	348	1.6	75.9	0.7	7.2

Vialone Nano	346	1.1	77.2	1.3	6.2
Fragrance	350	1.2	76.5	1.2	7.8
Arborio	351	1.3	77.0	1.9	6.9

Sample Preparation

For raw rice samples (uncooked), rice grains were ground into a fine flour using a mechanical grinder (Robert Bosch Engineering Company, Germany) before analysis.

Rice was also processed using three cooking methods: boiling, stewing and microwaving.

For boiling: 50 g of rice were immersed in 500 mL of water preheated at 100 °C and boiled for the time indicated on product's label (Table 10-2). Then, the cooking water was removed, and cooked rice was collected in a filter paper.

For stewing: 50 g of rice was placed in a pot and toasted for 30 s. Then, aliquots of water were added every 3 min for the cooking time indicated on product's label (Table 10-2). The water utilized for this cooking method depends on different ability to absorb water.

For microwaving: 50 g of rice were placed in a suitable container and cooked in a microwave oven at a power of 750 W using 166 mL of water for the time indicated in Table 10-2. The cooked materials from the three methods were dried in an oven at 37 °C until complete drying and then ground into a fine flour using a mechanical grinder (Robert Bosch Engineering Company, Germany) before analysis.

Table 10-2. Cooking time (min) and water (mL) used for each rice variety for the three cooking methods (boiling, stewing and microwaving).

Rice varieties	Boiling		Stewing		Microwaving	
	Cooking Time (min)	Water (mL)	Cooking Time (min)	Water (mL)	Cooking Time (min)	Water (mL)
Ribe	18	500	18	250	8	166
Basmati	15	500	15	150	8	166
Carnaroli	15	500	15	150	7	166
Vialone Nano	14	500	14	150	7	166
Fragrance	14	500	14	175	8	166
Arborio	14	500	14	175	9	166

Protein Content Determination (Bradford Assay)

Total protein content in rice samples was determined by the Bradford assay. For the analysis, 0.010 g of the samples were weighted out and 10 mL of NaOH 0.1 M was added and heated at 50 °C for 20 min. The analysis was performed using 60 µL of the sample, Coomassie Blue as reagent and BSA for the calibration curve ($y = 0.0060x + 0.0169$; $R^2 = 0.993$). The analysis was carried out according to the reported procedure by Bradford [42] and protein content was quantified spectrophotometrically at 595 nm. Analyses were performed in triplicates.

Total Carbohydrate Determination (Anthrone Assay)

Anthrone assay is a spectrophotometrical method for the determination of the total carbohydrates content. For the test, 0.010 g of the sample were dissolved in NaOH 0.1 M at 50 °C for 20 min and diluted up to 100 mL with ultrapure water. Then, 2 mL of the anthrone reagent solution (0.040 g of anthrone in 20 mL of concentrated sulfuric acid) were added to 1 mL of sample diluted solution and boiled for 10 min in a water bath. After cooling down at room temperature, the absorbance of solution was measured spectrophotometrically at 630 nm. The total carbohydrate content of the samples was determined by building up a calibration curve using pure glucose standard solutions ($y = 0.0140 + 0.0267x$; $R^2 = 0.990$). Analyses were performed in triplicates.

Amylose Content Determination (Iodine Assay)

Iodine colorimetric method was used to determine the amylose content. For the test, 0.010 g of the sample were dissolved in NaOH 0.1 M at 50 °C then neutralized with HCl 4 M and dilute up to 50 mL with ultrapure water. The iodine reagent solution was diluted in ultrapure water of a stock solution containing 7% w/w iodine and 5% w/w potassium iodide in a hydroalcoholic mixture (90% v/v of ethanol). Then, 0.5 mL of the iodine reagent solution (0.2% w/w of iodine in water) were added to 10 mL of sample diluted solution. After mixing, the absorbance of solution was measured spectrophotometrically at 625 nm. The amylose content of the samples was determined by building up a calibration curve using pure amylose standard solutions ($y = 0.1828 + 0.1320x$; $R^2 = 0.994$). Analyses were performed in triplicates.

Data Analysis

Data obtained were recorded in GraphPad Prism version 9.1.0 for Windows, (GraphPad Software, San Diego, CA, USA) and Two-way ANOVA followed by Tukey's multiple comparisons test was performed taking into account rice varieties and cooking methods. Each p value was adjusted to

account for multiple comparison with family-wise alpha threshold and confidence levels set at 0.05 (95% confidence interval).

Results

Protein Content Determination

Protein amounts detected in raw rice were comparable with ones reported on the nutritional labels (Table 10-1) and range from 5.89 to 8.71 g per 100 g of product among the different varieties (Table 10-3). Protein contents of analyzed raw rice were statistically significantly different each other. All selected cooking methods reduced detectable proteins amount in all rice in comparison with the corresponding raw rice (Table 10-3).

In general, boiling causes the highest loss of detectable protein content with an average amount of retained proteins of 79%, with Basmati rice showing the lowest percentage ($63.67 \pm 0.40\%$) and Arborio rice showing the highest ($87.70 \pm 0.38\%$).

An average of 88% of retained proteins was observed after stewing. Also in this case, Basmati rice showed the lowest percentage ($79.01 \pm 0.45\%$) but Vialone Nano rice showed the highest ($96.10 \pm 0.30\%$). After the microwaving cooking process, the average of detectable proteins (around 89%) was similar to the one detectable after stewing process.

In this case, the highest value was associated to Ribe rice ($97.89 \pm 0.21\%$) while the lowest to Fragrance rice ($81.40 \pm 0.41\%$).

Comparing different cooking methods process impact on detectable proteins amount, differences were observed among all the analyzed rice underlining the influence of both cooking methods and rice varieties on proteins amounts.

Table 10-3. Detectable protein content (g/100 g of rice) of both raw and cooked rice and percentage of retained proteins after the three cooking methods processes.

Rice Varieties		Raw	Boiling	Stewing	Microwaving
Ribe	g/100g	7.36 ± 0.01	$5.73 \pm 0.01^*$	$6.59 \pm 0.04^*$	$7.20 \pm 0.01^*$
	% ac		77.90 ± 0.26	89.60 ± 0.64	97.89 ± 0.21
Basmati	g/100g	8.71 ± 0.03	$5.55 \pm 0.02^*$	$6.88 \pm 0.02^*$	$7.18 \pm 0.01^*$
	% ac		63.67 ± 0.40	79.01 ± 0.45	82.45 ± 0.42
Carnaroli	g/100g	7.14 ± 0.03	$5.83 \pm 0.01^*$	$6.41 \pm 0.01^*$	$6.23 \pm 0.01^*$
	% ac		81.66 ± 0.50	89.69 ± 0.43	87.26 ± 0.39
Vialone Nano	g/100g	5.89 ± 0.01	$5.03 \pm 0.01^*$	$5.66 \pm 0.01^*$	$5.65 \pm 0.01^*$
	% ac		85.30 ± 0.27	96.10 ± 0.30	95.93 ± 0.30

Fragrance	g/100g	7.70 ± 0.03	6.06 ± 0.01*	6.43 ± 0.01*	6.26 ± 0.01*
	% ac		78.80 ± 0.46	83.52 ± 0.41	81.40 ± 0.41
Arborio	g/100g	6.71 ± 0.02	5.88 ± 0.01*	6.12 ± 0.01*	6.07 ± 0.01*
	% ac		87.70 ± 0.38	91.23 ± 0.45	90.48 ± 0.33

Value reported as mean ± standard deviation, % ac = percentage of remained protein content after cooking treatment, * statistically different from raw ($p < 0.05$).

Total Carbohydrates

Carbohydrates amounts in raw products were comparable to those reported on product's labels for all analyzed rice (Table 10-1) ranging from 75.50 to 77.06 g/100 g of rice (Table 10-4).

Table 10-4. Total carbohydrates content (g/100 g) of both raw and cooked rice and percentage of retained total carbohydrates after the three cooking methods processes.

Rice Varieties		Raw	Boiling	Stewing	Microwaving
Ribe	g/100g	76.23 ± 0.03	40.42 ± 2.99*	50.03 ± 0.11*	68.80 ± 0.06*
	% ac		53.02 ± 3.94	65.63 ± 0.11	90.26 ± 0.05
Basmati	g/100g	77.01 ± 0.01	49.32 ± 0.58*	51.62 ± 0.34*	70.33 ± 0.23*
	% ac		64.05 ± 0.75	67.04 ± 0.44	91.32 ± 0.28
Carnaroli	g/100g	75.51 ± 0.01	38.65 ± 0.58*	67.54 ± 0.24*	70.61 ± 0.28*
	% ac		51.18 ± 0.76	89.45 ± 0.30	93.51 ± 0.38
Vialone Nano	g/100g	77.06 ± 0.01	36.72 ± 0.23*	43.04 ± 0.43*	64.52 ± 0.37*
	% ac		47.64 ± 0.30	55.85 ± 0.56	83.72 ± 0.48
Fragrance	g/100g	76.01 ± 0.01	37.26 ± 0.85*	56.48 ± 0.42*	68.65 ± 0.07*
	% ac		49.03 ± 1.11	74.31 ± 0.56	90.31 ± 0.11
Arborio	g/100g	76.86 ± 0.04	41.95 ± 0.80*	52.70 ± 0.26*	70.21 ± 0.23*
	% ac		54.58 ± 1.01	68.56 ± 0.37	91.35 ± 0.27

Value reported as mean ± standard deviation, % ac = percentage of remained total carbohydrates content after cooking treatment, * statistically different from raw ($p < 0.05$).

No differences were observed comparing raw rice varieties. However, a decrease in carbohydrates content occurred after each cooking process.

Boiling process caused the highest decrease in total carbohydrates with an observed average of remained carbohydrates of 53%. In this case, Vialone Nano rice showed the lowest percentage ($47.64 \pm 0.30\%$) and Basmati rice showed the highest ($64.05 \pm 0.75\%$).

Stewing led to an average of 70% of retained carbohydrates after treatment. Also in this case, Vialone Nano rice showed the lowest percentage ($55.85 \pm 0.56\%$) while Carnaroli rice showed the highest ($89.45 \pm 0.30\%$). Microwaving was the cooking methods that preserved the highest

percentage of carbohydrates, average of 90%. Vialone Nano rice showed the lowest percentage ($83.72 \pm 0.48\%$) while Carnaroli rice the highest ($93.51 \pm 0.38\%$) (Table 10-4).

Comparing the impact of the different cooking methods on detectable total carbohydrates amount, differences were observed among all the analyzed rice with only few exceptions regarding stewed Ribe rice compared with boiled Basmati rice, stewed Ribe rice compared to stewed Carnaroli rice, stewed Carnaroli rice compared with microwaved Fragrance rice and stewed Vialone Nano rice compared with boiled Arborio rice.

Amylose Content

Raw amylose content ranges between 24.36 and 32.64 g/100 g. Among rice varieties, amylose content of raw products was statistically different with the exception of Fragrance rice compared to Arborio rice (adjusted p value = 0.9993) and Carnaroli rice compared to Vialone Nano rice (adjusted p value > 0.9999).

The loss of amylose after cooking followed a similar trend as observed for total carbohydrate with boiling process showing the highest loss followed by stewing and then microwaving (Table 10-5).

The average percentage of remained amylose after boiling resulted around 53% with the highest value obtained in case of Carnaroli rice ($63.07 \pm 2.17\%$) while the lowest in case of Arborio rice ($42.01 \pm 0.56\%$). Stewing caused an average amylose remaining of 76% with Ribe rice showing the lowest value (68.76 ± 0.69) and Fragrance rice the highest ($85.25 \pm 1.28\%$). Microwaving led to the highest average amylose remaining (average of 88%) with the lowest value obtaining for Basmati rice ($79.23 \pm 0.08\%$) and the highest with Fragrance rice (92.97 ± 0.92).

Moreover, in the case of amylose content, significant differences were found comparing different rice and different cooking methods with the exception of microwaved Ribe rice compared to stewed Basmati.

Table 10-5. Amylose content (g/100 g) of both raw and cooked rice and percentage of retained amylose after the three cooking methods processes.

Rice Varieties		Raw	Boiling	Stewing	Microwaving
Ribe	g/100g	26.66 ± 0.01	$13.83 \pm 0.14^*$	$18.33 \pm 0.19^*$	$22.66 \pm 0.15^*$
	% ac		51.87 ± 0.53	68.76 ± 0.69	85.00 ± 0.53
Basmati	g/100g	32.64 ± 0.02	$19.34 \pm 0.29^*$	$23.26 \pm 0.42^*$	$25.86 \pm 0.04^*$
	% ac		59.27 ± 0.86	71.27 ± 1.25	79.23 ± 0.08
Carnaroli	g/100g	24.41 ± 0.02	$15.40 \pm 0.53^*$	$17.39 \pm 0.26^*$	$21.75 \pm 0.15^*$

	% ac		63.07 ± 2.17	71.26 ± 1.02	89.09 ± 0.65
Vialone Nano	g/100g	24.36 ± 0.01	13.22 ± 0.09*	17.64 ± 0.35*	21.82 ± 0.13*
	% ac		54.28 ± 0.36	72.42 ± 1.45	89.58 ± 0.50
Fragrance	g/100g	27.51 ± 0.02	12.36 ± 0.01*	23.46 ± 0.37*	25.58 ± 0.27*
	% ac		44.93 ± 0.06	85.25 ± 1.28	92.97 ± 0.92
Arborio	g/100g	27.76 ± 0.02	11.66 ± 0.16*	23.37 ± 0.27*	25.18 ± 0.15*
	% ac		42.01 ± 0.56	84.20 ± 0.94	90.71 ± 0.45

Value reported as mean ± standard deviation, % ac = percentage of remained amylose after cooking treatment, * statistically different from raw ($p < 0.05$).

Amylose Percentage with Respect to Total Carbohydrates

Amylose percentage with respect to total carbohydrates was calculated both in raw and in cooked rice (Table 10-6).

Table 10-6. Amylose percentage respect to total carbohydrates in raw rice and after the three cooking methods application.

Rice Varieties	Raw	Boiling	Stewing	Microwaving
Ribe	34.97 ± 0.01	34.32 ± 2.11	36.64 ± 0.32	32.93 ± 0.19*
Basmati	42.38 ± 0.02	39.22 ± 0.41*	45.06 ± 0.96*	36.77 ± 0.13*
Carnaroli	32.32 ± 0.02	39.83 ± 0.86*	25.75 ± 0.44*	30.80 ± 0.26
Vialone Nano	31.61 ± 0.01	36.01 ± 0.37*	40.99 ± 1.15*	33.82 ± 0.15*
Fragrance	36.20 ± 0.04	33.19 ± 0.74*	41.53 ± 0.55*	37.26 ± 0.40
Arborio	36.12 ± 0.01	27.80 ± 0.55*	44.36 ± 0.39*	35.87 ± 0.16

Value reported as mean ± standard deviation, * statistically different from raw ($p < 0.05$).

Percentage of amylose with respect to total carbohydrates ranges from 31.61% to 42.38% in raw products. Statistically significant differences were found in raw products depending on rice varieties with few exceptions regarding Ribe rice compared with Fragrance rice and Arborio rice, Carnaroli rice compared with Vialone Nano rice, and Fragrance rice compared with Arborio rice.

After cooking process application, differences were found in terms of percentage of amylose with respect to total carbohydrates depending on both rice variety and cooking methods.

In particular, a significant decrease in the percentage of amylose with respect to total carbohydrates was observed after boiling in case of Basmati rice, Fragrance rice and Arborio rice while no statistical

changes were observed in case of Ribe rice. On the other hand, a statistically significant increase was found considering Carnaroli rice and Vialone Nano rice.

However, comparing boiled rice varieties, Basmati rice and Carnaroli rice showed the highest percentage of amylose with respect to total carbohydrates (39.22 ± 0.41 ; 39.83 ± 0.86 ; respectively), both statistically different from the other rice varieties. On the contrary, Arborio rice showed the lowest percentage of amylose (27.80 ± 0.55) with respect to total carbohydrates, statistically significant different from all the others.

In case of stewing, it was possible to observe a general increase in terms of percentage of amylose with respect to total carbohydrates with the exception of Ribe rice in which the percentage is maintained and Carnaroli rice in which it significantly decreased. Basmati rice resulted again to have the highest percentage of amylose with respect to total carbohydrates (45.06 ± 0.96) significantly different from all the other rice varieties except for Arborio rice that, in this case, showed one on the highest percentage of amylose respect to total carbohydrates (44.36 ± 0.39). The lowest value was shown by stewed Carnaroli rice (25.75 ± 0.44).

Microwaving cooking process did not alter percentage of amylose with respect to total carbohydrates compared to raw products in Carnaroli, Fragrance and Arborio rice. The only rice variety which showed a significant increase in the percentage of amylose with respect to total carbohydrates was Vialone Nano while Ribe and Basmati rice underwent a decrease.

Comparing the percentage of amylose with respect to total carbohydrates among microwaved rice varieties, Basmati rice again showed one of the highest values with Fragrance rice and Arborio rice (36.77 ± 0.13 ; 37.26 ± 0.40 ; 35.87 ± 0.16 ; respectively). As in stewing, Carnaroli rice showed the lowest percentage of amylose with respect to total carbohydrates (30.80 ± 0.26).

Therefore, the percentage of amylose with respect to total carbohydrates was associated to both rice variety and cooking method. Indeed, from the obtained results, it was possible to observe that each rice variety can show either an increase or a decrease in percentage of amylose with respect to total carbohydrates depending on cooking method.

Discussion

This study underlined the differences in nutritional characteristics of rice varieties and, particularly, the effects of cooking methods on their nutritional quality. Obtained data prove to be of remarkable interest considering the importance of varying foods for health and the frequent use of rice in gluten free diets [37, 43, 44].

Differences have emerged in terms of protein, total carbohydrates and amylose content among the six analyzed rice varieties (Ribe rice, Basmati rice, Carnaroli rice, Vialone Nano rice, Fragrance rice and Arborio rice) both as raw product and after cooking (boiling, stewing and microwaving).

Protein content of analysed raw rice (ranged from 5.89 to 8.71 g/100 g) was in line with ranges reported by other studies 6–12 g/100 g [43, 45] or 7–9 g/100 g [46]. Differences in terms of protein content have emerged considering either rice variety or cooking method in accordance with data reported in literature [43]. In particular, according to Khatoon et al., the protein content of different rice varieties changes depending on the type of rice considered and the cooking method applied [43].

By the analysis of obtained data from the present study, all cooking methods in general induced a decrease in protein content with boiling showing the highest effect, followed by stewing and microwaving.

Moreover, carbohydrate content is modified after cooking [37, 47]. In particular, in the study of Adi et al., total carbohydrates show a reduction after different cooking methods application (conventional cooking, rice cooker and steaming) in all the analysed rice varieties (Black rice, Brown rice, semi-organic white rice and organic white rice) [37]. The carbohydrate content of the six evaluated rice varieties was around 76% with no significant differences among raw rice varieties and close to percentages reported in literature (about 80%) [48]. However, remarkable differences were found among rice considering both the variety of rice and the cooking method used. In particular, boiling caused the highest loss in terms of total carbohydrates, compared to the other cooking methods considered, followed by stewing. On the contrary, microwaving preserves the highest content of total carbohydrates.

Among carbohydrates, it is interesting to focus the attention on amylose content.

Different rice varieties contain different amounts of amylose, and this content results in diverse nutritional characteristics, health impacts, cooking properties, and organoleptic features of rice [49]. Amylose content influences water absorption, volume expansion and stickiness of rice grains influencing rice sensory properties. Indeed, the higher the amylose content, the lower the stickiness of rice grains [49].

Furthermore, high amylose starch is resistant to digestion and acts as dietary fiber [23, 27, 50-52]. Dietary fiber is associated with many health benefits as lower glycaemic index, promoting satiety and acting as prebiotic [27, 53]. Thus, amylose content contributes to manage the glycaemic index of the food [54] and postprandial glycaemic response [51]. Glycaemic index is used to measure blood

glucose response after the ingestion of carbohydrate-containing foods [55] aiding to evaluate the quality of dietary carbohydrates [56]. This knowledge is particularly interesting from a nutritional point of view because carbohydrate composition can guide product selection in case of specific nutritional needs. In diabetes and metabolic disorders, for example, medium and low glycaemic index foods must be preferred than high glycaemic ones [57]. For this reason, selecting a rice variety with a high amylose content could be a strategy to better manage the glycaemic response after a meal [40].

From the present study, amylose content of the six raw analysed rice varieties ranges between 24.36 and 32.64 g/100 g. According to Acquistucci et al. amylose content varies from 15.5 to 25.2 g/100 g of dry weight [58]. Anyway, it is necessary to consider that amylose content changes depending also on the ambient temperatures during the growth of cereal grains. Indeed, lower ambient temperature during rice development induces an increase in amylose content [59, 60].

Considering amylose content, rice varieties can be classified depending on amylose amount in 100 g of product as waxy (0–5%), very low (5–12%), low (12–20%), intermediate (20–25%), and high (25–33%) [61]. Moreover, Suwannaporn et al. found that rice varieties available in the market can be classified according to amylose content in 100 g of products as low (less than 20% amylose), medium (21–25%) or high (26–33%) [62]. According to this, Table 10-7 shows the classification of the six evaluated rice varieties depending on their amylose content. Since the impact of cooking methods on nutritional quality of cereals has been reported in literature [63, 64], it remains of great interest to further exploit this aspect to better study cereals nutritional quality.

In this perspective, Table 10-7 shows a classification based on amylose content both for raw rice and after the application of the three studied cooking methods.

Table 10-7. Rice classification depending on amylose content of the six analyzed rice varieties as raw and after the application of the three considered cooking methods (according to Suwannaporn et al. classification for raw rice).

Rice Type	Raw	Boiling	Stewing	Microwaving
Ribe	High	Low	Low	Medium
Basmati	High	Low	Medium	High
Carnaroli	Medium	Low	Low	Medium
Vialone Nano	Medium	Low	Low	Medium
Fragrance	High	Low	Medium	High
Arborio	High	Low	Medium	Medium

The six raw rice varieties studied can be classified as medium (Carnaroli rice and Vialone Nano rice) and high (Ribe rice, Basmati rice, Fragrance rice and Arborio rice). However, after cooking processes differences can be found.

The marked loss of amylose due to boiling classified all rice varieties as low. The same table underlines that stewing has a negative impact on amylose content.

Microwaving resulted the cooking method with the less impact in terms of amylose content.

Among rice varieties considered, Basmati rice and Fragrance rice are of interest as they maintain a medium-high classification when stewed or microwaved. Similar results were pointed out by Rahim et al., suggesting a preference for Basmati and brown rice, especially for diabetic patients [65], and by Simonelli et al. regarding Fragrance rice [49].

From obtained data, differences were found in terms of total carbohydrates depending on cooking method. For this reason, amylose content was also evaluated referring to total carbohydrates in order to observe possible changes of percentage of amylose content with respect to total carbohydrates.

In raw products amylose percentage with respect to total carbohydrates varies according to the variety of rice considered. In particular, Basmati rice is the one with the highest percentage. Beyond rice variety, also in this case, it is necessary to consider the specific cooking method applied.

From the obtained results, it may be possible to point out the importance of considering cooking methods impact on nutritional quality of product taking into account not only amylose content but also percentage of amylose with respect to total carbohydrates content.

Due to the importance of the percentage of amylose with respect to total carbohydrates from a practical point of view, in Figure 10-1, a graphical representation of obtained results is proposed to be used as a tool in nutritionist practice.

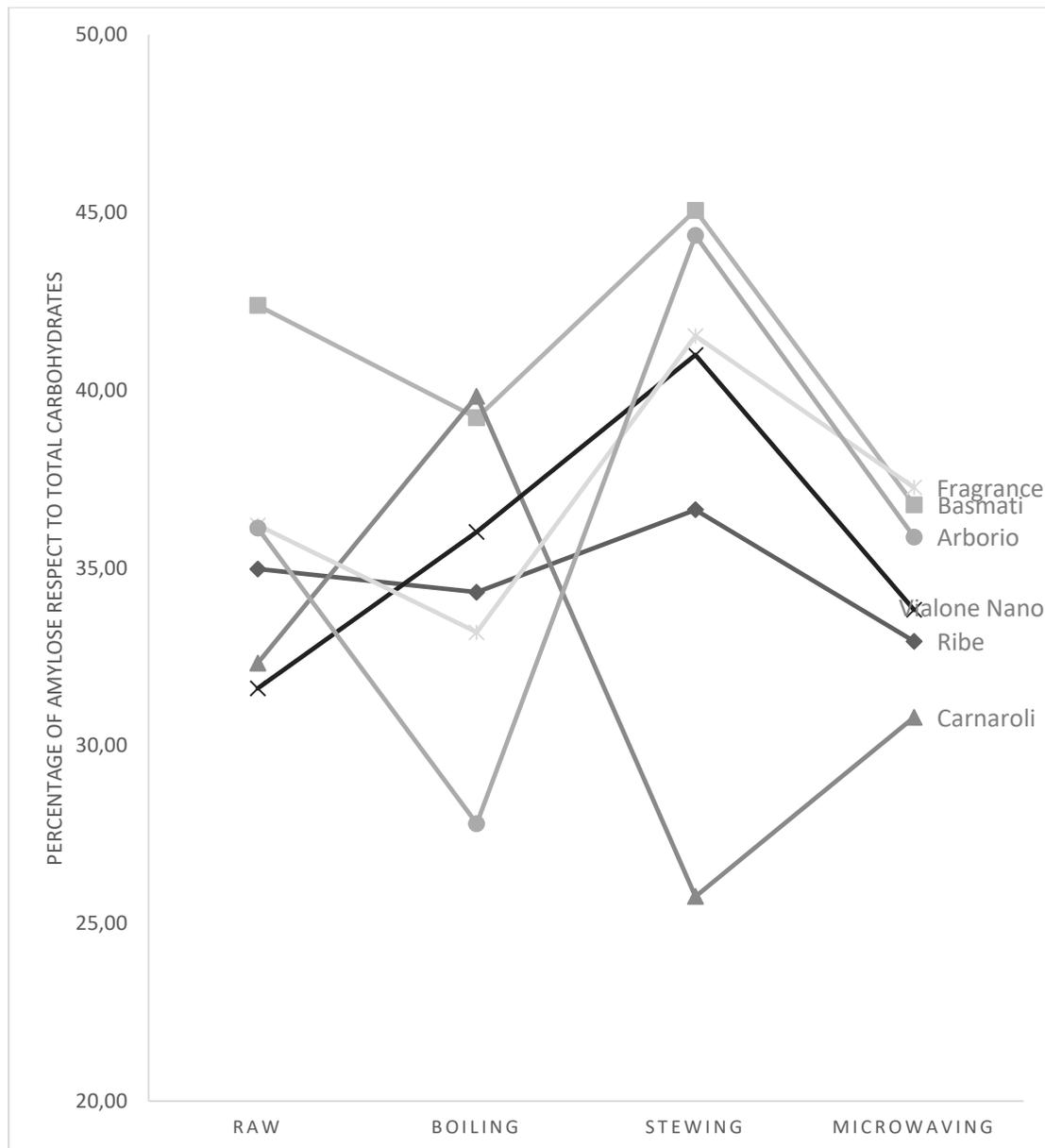


Figure 10-1. Graphical representation of amylose percentage with respect to total carbohydrate changes depending on cooking methods and rice varieties.

In particular, considering percentages, generally the highest values were obtained with stewing (above all for Basmati rice and Arborio rice), however exceptions can be underlined as in case of Carnaroli rice in which the highest percentage was associated with boiling.

In this context, the role of amylose content and amylose percentage with respect to total carbohydrates appears to be of great importance to better manage nutritional quality of diet and to focus the attention on glycaemic index of products and, consequently, glycaemic response. For example, in the study, it is shown in that for Basmati rice and Arborio rice, it could be suggested to use stewing in order to improve the percentage of amylose with respect to total carbohydrates. On the contrary, in other contexts, it could be useful to favor cooking methods that induce a reduction

of the percentage of amylose with respect to total carbohydrates, in order to increase carbohydrates availability and to contribute to recovery after physical exercise [66, 67].

Beside proteins and carbohydrates content of rice, fiber, minerals as manganese, selenium, iron and phosphorus and vitamins as folic acid, thiamine and niacin [68, 69] should also be mentioned. However, contribution in nutrient intakes is strictly related to proportion of germ, bran and endosperm [69]. For example, although energy and macronutrients contents are similar between white and brown rice, brown rice contains considerably more fiber and is higher in some minerals, particularly magnesium [69].

As reported in Table 1, the fiber content of the six analyzed rice ranged from 0.7 to 1.9 g of 100 g of raw product, with Carnaroli rice showing the lowest value, while Arborio rice the highest.

Nutrients, and in particular mineral content, depend not only on rice processing (i.e., polishing, milling, and parboiling), but are also influenced by rice variety (i.e., differences can be found in terms of potassium, phosphorus and magnesium among long grain rice, brown rice, white basmati, and wholegrain basmati rice) and cooking methods [69-72]. For example, a study by Mwale and colleagues, reported a significant loss of minerals when rice was cooked using a rice-to-water ratio of 1:6 above all of potassium [72].

Thus, encouraging the use of naturally gluten-free products and varying food choices are fundamental aspects related to the improvement of a gluten-free diet quality.

Rice is an important staple food for more than half of the world's population [73], representing one of the main sources of carbohydrate with an important role in a healthy diet [69]. However, it should be considered as part of a balanced and varied diet [13, 14]. Indeed, cereals, including rice, should be present at each meal together with healthy proteins, plant-based fat, vegetables, and fruits [35].

Conclusions

This study, where the most used rice varieties in Italy have been analysed, represents an important starting point to guide adequate food and cooking method choices in order to better manage the diet and improve its nutritional quality, especially in specific conditions, like celiac disease. In literature, a lack of informations on how to choose adequate food and cooking methods in the case of celiac patients emerged.

These results can be useful in the practice of nutritionists or experts in nutrition to counsel patients who require specific recommendations.

Moreover, this knowledge can aid consumers, in particular celiac patients, to make healthy and responsible dietary selections to establish a balanced diet.

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Chapter 11. General discussion

New insights toward more sustainable dietary patterns

A balanced diet is fundamental for maintaining a healthy status [1]. At present a healthy diet must be also a sustainable diet [2]. In the context of the current global socio-demographic changes, finding an alternative protein source to that of animal-based foods becomes more and more urgent [3]. Reducing meat consumption appears a strategy to hinder climate change and to preserve biodiversity [4]. The shift towards a more plant-based diet, even with the partial substitution of animal-proteins sources with plant-based ones, contributes to sustain environment and human health [5].

Among protein plant foods, pulses cover an essential role as they contribute to both human and planet health [6]. Indeed, pulses exert a key aspect in the prevention of non-communicable diseases [7, 8]. Moreover, from the environmental side, pulses are involved in the achievement of the 17 Sustainable Development Goals (SDGs) described in the Agenda 2030, for example helping facing hunger and malnutrition and supporting sustainable agriculture productions [9].

In chapter 2 the health benefits and nutritional characteristics of these seeds are reported.

Although the motivations for pulse consumption are numerous, consumers do not have the propensity in choosing pulses in their weekly diet [10, 11].

The long time required for pulse preparation (due to soaking and cooking) is generally advanced as an apparent justified excuse to avoid pulses, especially in the modern schedule of daily activities [10]. Another barrier for pulse intake is the lack of information about the health benefits derived from their consumption among consumers [12]. In chapter 3, it was possible to analyse pulse consumption in the population and the barriers accountable for the reduced intake evidenced.

Given the importance to promote more plant-based diets and pulse intake, the development of plant-based foods results of fundamental importance. Pulse-based and pulse-containing foods have spread increasingly in the market and have acquired a great popularity [13]. Therefore, it could be useful to design usable and sustainable pulse-based products with a valuable nutritional profile able to satisfy consumers' requests.

In accordance with this, in chapter 4 the design of a pulse-containing muesli was presented. In chapter 5 it was described the development of three projects: Bottega Colfiorito[®], Piatto fatto[®] and Fertitecnica Colfiorito s.r.l. in the weaning contexts. Bottega Colfiorito[®] consisted of an e-commerce project based on the development of 5 targeted consumers' packages containing company products. It was conceived to promote pulse consumption in the diet of consumers during COVID-

19 emergency. Piatto Fatto® project represented the initial conceptualization for the renewal of Piatto fatto®, a company ready-to-eat pulse-based product.

The last project described is dedicated to the analysis of company products adequate for infant weaning and it stated as the first company step in the promotion of pulses in the weaning context. Promoting a healthy diet is a fundamental action to prevent chronic non communicable diseases such as diabetes, obesity, cardiovascular diseases and other health problems [14, 15]. Despite it is widely known how dietary prevention is necessary, unsuitable eating habits are often common in the population [16]. Therefore, nutrition education awareness raising projects should be encouraged to promote the knowledge of people as patients and consumers.

In chapter 6 the development of a nutrition education platform and its application through a pilot nutrition education project designed to ameliorate pulse consumption was firstly tested in university students as they will become nutrition experts and will be responsible for patient's dietary choices awareness.

In the context of promoting a healthy diet and lifestyle, Mediterranean diet must be cited.

Indeed, Mediterranean diet exerts positive effects on human health, in particular, by contributing to the prevention of chronic diseases, such as cardiovascular disease and diabetes [17-21]. Given the relevance of body composition in contributing the maintenance of a healthy status [22], it is interesting to evaluate the effect of Mediterranean diet on body composition. Chapter 7 was conceived to face this aspect. The study aimed to evaluate the effects of adherence to Mediterranean diet, followed for three months, on body composition in 16 healthy and physically active subjects. Results pointed out that BMI and waist circumference were significantly decreased at the end of the study. In addition, fat mass (%FM) was significantly reduced and BCM (%FFM) was maintained.

In chapter 8, the effect of three different short-term normocaloric dietary protocols on body composition were evaluated. More specifically, maintaining constant the protein quantity across the dietary protocols, the attention was focused on the protein quality of diets, starting from the evaluation of different ratios between animal- and plant-proteins, in the perspective to support more environment-sustainable diets and in parallel to maintain body composition. The study was conducted on 18 healthy and physically active subjects to test the impact of a Mediterranean-like (70:30 diet), 30:70 diet and vegan diet, on body composition and resting metabolic rate. Results highlighted that body composition and resting metabolic rate were preserved independently from the three tested animal- and vegetal-protein ratio.

As pulses can be enumerated as naturally gluten-free foods [23], they can be employed in the diet of celiac consumers. It has been reported that strictly adhering to a gluten-free diet is complex for celiac patients, therefore nutritional imbalances could occur [24]. At this regard, chapter 9 investigates the possible role of a specific micronutrient, vitamin D, in celiac disease onset. Moreover, the development of an on-line nutritional education course for the management of gluten-free diets is described. The course is directed towards celiac patients and their parents but also to restaurant owners and chefs to sensitize all figures involved in the preparation of gluten-free meals.

In chapter 10 the attention was focused on the nutritional properties of rice, one of the most commonly consumed cereal among celiac patients, and its applicability in gluten-free diet.

In chapters presented the role of plant-based diets for human and planet health was described and, in this context, the importance of pulses was underlined through the design of innovative usable pulse-based food products and nutritional education strategies to promote their consumption. Further strategies should be implemented in order to sustain pulse intake and to sensitize consumers towards the positive benefits of plant-based diets.

Practical implications and future perspectives

- Plant-based diets exert a positive effect on human health and on the environment. Therefore, promoting the consumption of plant foods is of fundamental importance.
- Concerning plant foods, pulses emerge as an important protein source and as a nutritionally valuable and sustainable alternative to animal derived foods.
- Fostering pulse consumption is a key aspect in supporting the shift towards more plant-based diets. In this perspective, the evaluation of consumers' pulse intake habits represents a starting point to deal with. In particular, facing the barriers accountable for a reduced pulse intake needed to be taken into account. On-line questionnaires constitute a useful tool for this aim.
- Given the importance to promote more plant-based diets and pulse intake, the development of usable plant-based foods results of fundamental importance.

- Nutrition education can be used as a strategy to approach consumers to pulse world by making them become aware of pulse nutritional characteristics and of the health benefits derived from pulse consumption.
- Mediterranean diet is based on the consumption of plant foods, therefore posing itself as healthy and environment-friendly dietary pattern. The evaluation of the impact of Mediterranean diet on body composition results fundamental.
- Plant-based diet impact on body composition is essential to sustain a healthy status. Supporting plant-based diets for health results necessary in order to responde to environment sustainability. Study results demonstrate that adequately planned 30:70 diet (a diet with the 30% of animal derived proteins and the 70% of plant-proteins on total proteins) and vegan diet (100% plant-proteins) allow to maintain body composition and resting metabolic rate in a group of physically active subjects in the short term. Further studies will be needed to specifically evaluate the effects of more plant-based diets on body compositions parameters in athletes and in specific disciplines.
- Pulse are a gluten-free food, therefore constituting a valuable food choice for celiac consumers. Gluten-free diets are perceived as complicated to be followed, thus increasing the risk to develop nutritional deficiencies in consumers. Gluten-free diet sustainability results essential to avoid nutritional inadequacies that may be implicated in celiac disease onset.
- As adhering to gluten-free diets is often difficult for celiac patients, nutrition education activities are necessary to guide them and all figures involved in the preparation of gluten-free meals (such as parents, restaurant owners and chefs) in making adequate dietary choices to avoid nutritional deficiencies.
- The knowledge of gluten-free foods is fundamental for the preparation of healthy and nutritionally balanced gluten-free meals and gluten-free ready to eat products, for example starting from the combination of gluten-free cereals and pulses. Thus, the characterization of rice, one of the most used gluten-free cereal, and its applicability in gluten-free diet result fundamental.

Conclusions

Plant-based diets have an unquestionable role in the assessment of human health [25]. At the same time, they are protagonist in contributing to face environmental challenges [26]. Pulses represent one of the crucial element of plant-based diets emerging as an important protein source and as a nutritionally valuable and sustainable alternative to animal derived foods [9].

At the same time, it is worth mentioning that plant-proteins are one of the possible options to meat. Indeed, also insects can be considered as a valuable and less conventional meat substitute which is acquiring more and more relevance [27].

From the nutritional perspective, insects' proteins have a balanced aminoacidic composition, making them be an interesting protein source to cover nutritional requirements for humans [27]. Moreover, insects' proteins digestibility appears to be promising, moving from 60% to 95%, depending on the species and the order of the insect considered [28].

Furthermore, a study of Hermans et al. [29] highlighted the post-prandial metabolic responses and anabolic properties of insects' proteins compared to milk proteins, underlying their rapid digestion. Starting from these preliminary considerations, insects' proteins appears a strategy to respond to world protein demand and to face environmental issues related to protein production. Anyway, further studies are needed to deepen these aspects.

Beyond proteins, insects display an interesting nutritional profile, also in terms of fats and micronutrients [30, 31]. Regarding fats, it was pointed out that insects generally have more unsaturated fats than saturated ones [32], despite it is necessary to consider that fats composition varies according to insect species and diet [33].

Micronutrient content is still underexplored. Usable data show insects contain group B vitamins (B1, B2, B6), vitamin C, D and E [34]. Edible insect are also sources of minerals, such as magnesium, copper, iron and zinc [35]. However, other studies are necessary to investigate the presence of anti-nutritional compounds that can compromise protein and mineral bioavailability and absorption.

It is anyway necessary to mention that insects contribute to dietary protein sources diversification as they are economically affordable and environmental sustainable. Indeed, insects appear have a lower environmental impact than beef, pork and chicken [36], requiring less space, feed and water for their production [37]. Despite these advantages, research studies are needed to deepen their role in the diet [38].

In order to encourage entomophagy, an adequate insects processing appears necessary [39]. Burger and insects-based snacks represent possible proposals for insects' consumption and seem to be

more appreciated than products with original ingredients mixed together [40, 41]. A study conducted by Van Thielen et al. [42] used the survey methodology to ask to a representative sample of Belgian population their opinion about insects-based products. In the survey it emerged that potential insects' consumers would prefer products with an invisible inclusion of insects such as energy shakes and bars, burgers, soups, spreads and snacks.

Similarly, De Oliveira et al. [43] found that adding cinereous cockroaches' flours in bread production improves its nutritional quality and consumers' acceptance. Thus, further research is required to study and develop insects-based foods and to work on consumer acceptance.

Indeed, it is necessary to underline that insects acceptance by consumers is a critical aspect as insect consumption is a cultural dietary habit [44]. Therefore, work must be done to increase insect consumer's acceptance, especially in Western countries, and to develop communication strategies to inform consumers on insects' nutritional properties and on environmental impact of insects production.

Beyond insects, studies are focusing their attention on other alternative and sustainable protein sources like microbial proteins (or single-cell proteins), including microalgae, fungi and bacteria [45]. Microorganism production needs a reduced amount of lands compared to plantations and livestock [46]. Moreover, their production can be combined with waste treatment as it may be used as a substrate for microorganism growth, fostering a circularity approach [45]. It has been found that microbial proteins can be used as foods or as ingredients to add to food preparations to improve the functional properties of the final product [47]. Although these captivating aspects, it is necessary to consider the food safety hazards associated to these products, in particular referring to the presence of toxins or heavy metals (microalgae) or to their behaviour as allergens (mycoproteins) [48].

Lastly, it is interesting to note that also cultured meat appears to be a frontier in the research for alternative protein sources. Cultured meat is produced from animal stem cells cultivated in laboratories [48]. However, research is needed to study the technological, environmental, safety and regulatory issues linked to these products.

However, at the moment, plant-proteins constitute a viable and easy strategy to predominantly apply at least in the short term.

Therefore, starting from these considerations it is fundamental to foster pulse consumption among consumers. In this perspective, the development of usable plant-based foods could involve consumers in introducing pulses in their diet. In parallel, nutritional education interventions can be

a useful strategy to make consumers approach to pulses, for exemple by providing information on pulse nutritional composition and health benefits.

In parallel, deepening the knowledge on the effect of plant-based diets on body composition is important as it contributes to establishing a healthy status [22]. This work represents a starting point that allowed to deepen pulse consumption habits and knowledge among consumers to study strategies to promote pulse intake and to design new projects in order to develop pulse-based nutritionally valuable products able to satisfy consumers' requests and to sensitize consumers on pulse intake through nutrition education projects. At the same time this works underlined the importance of studying the effects of Mediterranean diet and the impact of more plant-based dietary patterns on body composition to support the diffusion of more environment sustainable diets. Further studies are needed to investigate the effects of more plant-based diets in healthy subjects, in different physiological conditions and in athletes, according to the specific discipline, aiming to support the adherence to more plant-based and sustainable diets.

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Appendix I: Pulses, yes or not? That is the problem

FIRST SECTION

Age

Gender

- F
- M
- X

SECOND SECTION

How many times per week do you consume pulses?

- 0
- 1
- 2
- 3
- ≥ 3

Which pulses do you prefer? (provide your grade of appreciation by giving a score from 0 to 5 for the following pulses. If you have never tasted a kind of specific pulse, you can check the answer "Never tasted").

Type of pulse	Grade of appreciation						Never tasted
	0	1	2	3	4	5	
Lentils							
Chickpeas							
Peas							
Beans							
Broad beans							
Wild peas							
Soybeans							
Lupin beans							

Which pulse form do you consume?

Pulse form	Always	Usually	Sometimes	Rarely	Never
Fresh legumes					
Pulses					
Canned legumes					
Frozen legumes					
Ready-to-eat pulse-based frozen dish					
Ready-to-eat pulse-based dish coming from refrigerator sector					

Do you use pulse flours or pulse flour-based products?

- Never
- Rarely
- Sometimes
- Often
- Always

In your opinion, which is the recommended weekly frequency of pulse intake?

- 1
- 2
- Almost 2
- 3
- >3
- I do not know

Which of the following nutrients are contained in pulses? (you can give more than one answer)

- Carbohydrates
- Fiber
- Proteins
- Fats
- Vitamins
- Minerals

Are you available to increase pulse consumption in your diet?

- Yes, if pulses are beneficial for health

- Yes, if I can eat pulses as I prefer
- Yes, but only if I am obligated
- No, I am not

In your opinion, which are the reasons for the reduced pulses intake in the population?

- Pulses dislike
- To forget to buy or to prepare pulses
- Lack of pulse consumption habit
- Lack of family appreciation for pulses
- Others

Which are the foods you consume pulse with? (you can give more than one answer)

- Pasta, cereals or bread
- Meat
- Fish
- Cheese
- Eggs
- Vegetables
- None. I consume pulses alone

Appendix II: Nutrition education course – Final test

1. Mediterranean diet pyramid suggests to consume pulses:
 - 2 times per week
 - ≥ 2 times per week
 - 3 times per week
 - at least 3 times per week

2. In the context of Mediterranean diet pyramid, wine:
 - is excluded from meals
 - can be consumed weekly according to social habits
 - can be consumed moderately and according to social habits
 - can be consumed daily

3. At the basis of Mediterranean diet pyramid which concepts and/or behaviours are defined?
 - regular physical activity and consumption of exotic foods
 - conviviality and traditional and local products
 - conviviality, biodiversity, non-traditional and local products
 - physical activity but especially prolonged rest

4. In how many parts is the “Healthy Eating Plate” divided?
 - 3 (whole cereals, healthy proteins, fats, fruits and vegetables)
 - 4 (whole cereals, healthy proteins, fruits and vegetables)
 - 4 (whole cereals, healthy proteins, fats and vegetables)
 - 3 (refined cereals, healthy proteins and fruits and vegetables)

5. Do pulses contain fiber?
 - yes, they do
 - no, they do not

6. To complete the pattern of essential amino acids of pulse proteins, it is necessary to combine pulses with:
 - meat
 - fish
 - cereals and derivatives
 - vegetables

7. Do pulses contain minerals?
 - yes, they do

no, they do not

8. Regarding anti-nutritional factors in pulses:

- their concentration is reduced thanks to soaking and thermal treatments
- they inhibit the activity of numerous human digestive enzymes
- all the previous answers are correct
- none of the previous answers is correct

9. The toxic substance found in lupin beans is called:

- lupidine
- lupinine
- luparine
- lupinidine

10. Is the consumption of wild peas toxic?

- yes, it is
- yes, but only if wild peas are consumed in a high and regular amount during time
- no
- no, but only after a prolonged soaking and a double cooking

11. The anti-nutritional factors present in pulses:

- can exert a positive role for human health
- only have a negative effect on human health, therefore it is necessary to eliminate them through soaking and cooking
- are not eliminated with soaking and cooking
- can be consumed without problems as they are not absorbed at the intestinal level

12. Soaking:

- has a different duration according to the type of pulse considered
- if it is prolonged, it is responsible for a higher loss of minerals
- it is not a necessary step for consuming all pulse types
- all the previous answers are correct

13. Hulling:

- modifies the nutritional quality of pulses
- reduces the fiber content of pulses
- ameliorates pulses digestibility
- all the previous answers are correct

14. Germination:

- improves the nutritional quality of pulses
- can be conducted in light or dark
- is an easy, usable and low cost process
- all the previous answers are correct

15. Canned legumes:

- must be washed adequately before consumption because they can contain salt
- must not be consumed, it is better to prefer dry seeds
- can substitute pulses or fresh legumes
- always contain additives and preservatives, therefore it is necessary to avoid their consumption

16. The allergens to highlight in the nutrition label are:

- 11
- 12
- 13
- 14

17. In the nutrition declaration, for 100g of product, it is mandatory to indicate:

- energy content, unsaturated fats, carbohydrates (simple sugars), proteins and salt
- energy content, saturated fats, carbohydrates (simple sugars), proteins and sodium
- energy content, saturated fats, carbohydrates (simple sugars), proteins and salt
- energy content, saturated fats, carbohydrates (complex carbohydrates), proteins and salt

18. In the nutrition declaration ingredients should be reported:

- in a growing order of weight
- in a decreasing order of weight
- in a scattered order
- in alphabetic order

19. The nutrition label:

- should not mislead consumers
- does not have to attribute healing properties to the products
- should be understandable
- all the previous answers are correct

20. An adequate breakfast:

- allows to better regulate satiety
- entails an increase of body weight
- provides energy for the entire day
- causes a fullness and heavy sensation during the morning

21. Breakfast should provide:

- little energy, carbohydrates, proteins and fats
- little energy, prevalently carbohydrates, proteins and fats
- energy, carbohydrates, proteins and fats
- little energy, carbohydrates, prevalently proteins and fats

22. A salty breakfast:

- is better than the sweet one as it contains fewer simple sugars
- is a valuable alternative to sweet breakfast
- should be avoided because it is rich in salt
- is not healthy

23. Breakfast should provide:

- the 30% of daily energy requirement
- from the 25% to the 30% of daily energy requirement
- only from the 15% to the 25% of daily energy requirement
- the 10% of daily energy requirement

24. The primary ingredients of the original muesli recipe are:

- oat flakes, lemon juice, apple and walnuts
- rye flakes, dried blueberries, hazelnuts and honey
- rye flakes, dried papaya, hazelnuts and honey
- oat flakes, raisins and chocolate

25. The Food and Agriculture Organization (FAO) declared that the International Year of Pulses was:

- 2015
- 2016
- 2017
- 2018

26. Pulses are important because:

- they contribute to face malnutrition ("Zero hunger" SDG)
- they contribute to food security
- they contribute to make agriculture production more sustainable
- all previous answers are correct

27. Pulses:

- are a source of plant-proteins
- do not contain cholesterol
- have a low glycaemic index

- all the previous answers are correct

28. Pulse consumption:

- causes bloating, therefore it should be avoided
- is suggested only to those who follow a plant-based diet
- aids to prevent diabetes and cardio-vascular diseases
- can be substituted by the consumption of other foods (i.e. cereals)

29. In the “Healthy Eating Plate” pulses represent:

- a source of healthy proteins
- vegetables
- a source of carbohydrates
- they are not necessary

30. Do pulses contain gluten?

- yes, they do
- no, they do not

Appendix III: pulse-based recipes for breakfast

Chickpeas pancakes

Ingredients

For 10 pancakes

- 90 g of chickpea flour
- 140 ml of water
- A pinch of salt
- Extra-virgin olive oil as required
- A teaspoon of baking powder
- A pinch of baking soda.

Procedure

Take a bowl and pour chickpea flour, add water and mix the two ingredients. Add a pinch of salt and one of baking soda and continue to mix. In the meantime, warm up a pan and nebulize the pan bottom with extra-virgin olive oil. Take a spoon of the mixture, pour it on the pan and cook the pancake on one side. After few minutes, turn the pancake on the other side. Once cooked, you can serve the pancakes with fruits, nuts, nut spread or jam (Figure 1).



Figure 1. Chickpeas pancakes.

Fagiotella

Ingredients

- 240 g of canned beans
- 60 g of brown sugar
- 30 g hazelnuts flour
- 30 g of cocoa powder
- A teaspoon of vanilla extract
- Plant-based soy beverage as required.

Procedure

Strain beans to get a purée. Pour the purée in a bowl and add sugar, hazelnut flour, cocoa powder and vanilla extract and mix these ingredients together. Lastly, add the plant-based soy beverage as required until making the mixture be creamy. Fagiotella can be put into hermetically closed cans and preserved in the fridge for 4 or 5 days (Figure 2).



Figure 2. Fagiotella.

Chickpea biscuits

Ingredients

- 210 g of chickpea flour
- 30 g of oat or rice flour
- 30 g of almonds or hazelnuts
- 75 g of brown sugar
- 2 tablespoons of cinnamon
- 100 ml of water
- 90 ml of sunflower oil.

Procedure

Mix sugar, almonds/hazelnuts and cinnamon together. Pour the mixture in a bowl and add chickpea flour, oat/rice flour, water and sunflower oil and mix these ingredients until getting a homogeneous mixture. In the meantime, heat up the oven at 180°. Roll out the dough (around 5 mm) on a support and use oat flour if needed to avoid the dough sticks. Use the moulds you prefer to get the biscuits and arrange them on a baking tray covered by parchment paper. Cook the biscuits in the oven for 10 minutes (Figure 3).



Figure 3. Chickpeas biscuits.

Appendix IV: survey on food habits and lifestyle

1. Email

2. Date of birth

3. Gender

- F
- M
- X

4. In your opinion, your weekly diet is:

- monotonous
- varied
- other

5. Which meal, among the following, causes you the most problems in varying food choices?

- breakfast
- mid-morning snack
- lunch
- mid-afternoon snack
- dinner

6. Do you think your diet is healthy and nutritionally balanced?

- yes, it is
- no, it is not
- I do not know

7. If you answered "no, it is not" to question 6, why do you think your diet is not healthy and balanced? (you can give more than one option)

- I think it is difficult to eliminate gluten from the diet and in parallel to follow a healthy diet
- I do not vary the types of foods to choose
- I often have a little time for meal preparation and consumption
- It may occur I skip meals
- I often eat ready to eat meals
- I do not eat vegetable and fruits daily
- I do not choose seasonal vegetables and fruits
- I do not eat pulses
- I do not consume milk and dairy products
- I do not consume fish
- I have an excessive intake of sweets, confectioned snacks and soft drinks
- others

8. Do you have breakfast in the morning?

- every day
- 2-3 times per week

- rarely
- never

9. If you do not have breakfast every day, explain why:

- I am not hungry
- I have no time to eat
- other reasons _____

10. Indicate which are the foods you eat for breakfast:

11. Do you have a snack in the morning?

- yes, I have
- no, I do not
- sometimes

12. If you do not have a snack in the morning, explain why:

- I am not hungry
- I have no time to eat
- other reasons _____

13. Indicate which are the foods you eat for a snack:

14. Do you have lunch?

- yes, I have
- sometimes
- never

15. If you never have lunch, explain why:

- I am not hungry
- I have no time to eat
- other reasons _____

16. Indicate which are the foods you eat for lunch:

17. Do you have a snack in the afternoon?

- yes, I have
- no, I do not
- sometimes

18. If you do not have a snack in the afternoon, explain why:

- I am not hungry
- I have no time to eat

other reasons _____

19. Indicate which are the foods you eat for a snack:

20. Do you have dinner?

- yes, I have
- sometimes
- never

21. If you do not have dinner, explain why:

- I am not hungry
- I have no time to eat
- other reasons _____

22. Indicate which are the foods you eat for dinner:

23. How much water do you drink daily?

- <500 ml
- >1 l and <1.5 l
- 1 l
- 2 l
- >2 l

24. Which beverage do you drink during meals?

- water
- wine
- soft drinks
- others

25. Do you perform physical activity during the week?

- yes, I do
- no, I do not

26. Which type of physical activity do you perform?

27. How many times per week do you perform physical activity?

- every day
- 4-5 times per week
- 2-3 times per week
- once a week
- others

28. Do you consume meals out of home (i.e. at the restaurant)?

- yes, I do
- no, I do not
- it depends

29. When you are out of home, do you manage your gluten-free diet easily?

- yes, I do
- no, I do not
- it depends

30. When you are out of home, do you feel to have limited food choices?

- yes, I do
- no, I do not
- it depends

31. When you go out for lunch/dinner, do you:

- always choose the same restaurants
- change the type of restaurant
- others

32. Which reasons guide you to choose a specific restaurant?

- confidence
- restaurant adherence to AIC (Italian Association for celiac disease)
- others

33. What do you eat when you go out for lunch/dinner?

- a starter
- a first course
- a second course
- vegetables
- pizza
- others

34. Do you think your gluten-free diet is an obstacle for your social life?

- yes, I do
- no, I do not

35. The gluten-free foods you consume more in your gluten-free diet are:

- naturally gluten-free
- confectioned gluten-free products
- both

36. Once you got the celiac disease diagnosis, did you receive indications to:

- eliminate gluten from the diet
- follow a healthy and nutrient balanced gluten-free diet
- how to choose gluten-free foods by adequately reading food labels

37. Do you usually consult the food manual of AIC?
- yes, I do
 - no, I do not
 - I do not know about this manual
38. Do you buy confectioned gluten-free products?
- yes, I do
 - no, I do not
 - sometimes
39. If you consume confectioned gluten-free foods, where do you usually buy them?
- at the pharmacy
 - at over the counter pharmacy
 - at gluten-free foods shops
 - at the supermarket
 - others
40. Do you use the vouchers for gluten-free products purchase supplied by the Italian national healthcare system?
- yes, I do
 - no, I do not
41. Do you think the vouchers are adequate for your needs?
- yes, I do
 - no, I do not
42. Which gluten-free products do you usually purchase?
- biscuits
 - muesli and breakfast cereals
 - bread
 - pizza
 - salty snacks
 - sweets
 - others
43. Do you usually read the food label of gluten-free products before purchasing?
- yes, I do
 - no, I do not
44. Which is the information you look at? (you can give more than one option)
- gluten-free brand
 - eventual presence of gluten among the ingredients
 - kcal content
 - nutrient content
 - salt content
 - list of ingredients

- origin
- expiry date
- preservation modality
- preparation modality
- others

45. Do you think it could be useful to increase the availability of gluten-free products in the market?

- yes, I do
- no, I do not

46. Which gluten-free product would you like to find in the market?

47. Do you think it could be useful to have a nutrition expert to point at for doubts regarding the gluten-free diet?

- yes, I do
- no, I do not

Appendix V: autumn gluten-free weekly menu

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Breakfast	Yogurt, chia seeds and banana, coffee	Milk, puffed quinoa and dark chocolate	Milk, puffed rice, pears and almonds	Yogurt, pear and dark chocolate	Rice cakes, ricotta cheese, pears and dark chocolate	Pancakes made with rice or pulse flours	Milk, corn flakes and nuts
Snack	Rice cakes with peanut butter	Mixed berries and nuts	Yogurt and seeds	Rice cakes with peanut butter	Yogurt and seeds	Mixed berries and nuts	Kiwi and dark chocolate
Lunch	Gluten-free pasta with ricotta cheese and dried tomatoes, salad	Basmati rice with salmon and radicchio	Pulse-based pasta with pesto, salad	Risotto with pumpkin and mushrooms, salad and seeds	Millet with broccoli and anchovy	Rice with marinated tofu, dried tomatoes and spinach	Gnocchi (made with rice flour) with tomatoe sauce and mozzarella cheese, salad
Snack	Nuts and dark chocolate	Creamy tofu with cocoa powder and honey	Banana with cinnamon and almonds	Yogurt and seeds	Crunchy chickpeas with cinnamon and dark chocolate	Yogurt and seeds	Pop corn
Dinner	Pureed soup with pumpkin, potatoes and mushrooms	Lentils burger with cauliflower and chestnuts	Baked codfish with potatoes and broccoli	Buckwheat bread with peas cream, salad and seeds	Eggs, baked pumpkin, salad and rice cakes	Gluten-free pizza	Chicken breast with radicchio, oranges, olives and buckwheat bread

Appendix VI: quinoa and eggs salad

Ingredients

- quinoa
- boiled eggs
- carrots
- cucumbers
- tomatoes
- zucchini
- peppers
- capers
- extra-virgin olive oil
- spices (i.e. black pepper or chili pepper)
- a pinch of salt, if desired.

Procedure

Boil quinoa and eggs in two separated pots. Once cooked, rinse quinoa with fresh water. In the meantime, shell eggs and cut them. Cut all vegetables (carrots, cucumbers, tomatoes, zucchini and pepper. If you prefer, you can choose other types of vegetables, according to seasonality). Finally, add capers, extra-virgin olive oil and spices. Mix the ingredients in a bowl and serve (Figure 1).



Figure 1. Quinoa and eggs salad.