Study acronym: CIPROMED

Spontaneous study title

CIRCULAR AND INCLUSIVE UTILISATION OF ALTERNATIVE **PRO**TEINS IN THE **MED**ITERRANEAN VALUE CHAINS **(CIPROMED**)

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1. LIST OF ABBREVIATIONS

AST	Aspartate Amino Transferase
AE	Adverse Event
ALT	Alanine Amino Transferase
ALP	Alkaline Phosphatase
AUSL	Azienda Unità Sanità Locale
BDI	Beck Depression Inventory
BMI	Body Mass Index
CAP	Controlled Attenuation Parameter
CBC	Complete Blood Count
CRP	C-Reactive Protein
CRF	Case Report Form
DIMEC	Department of Medical and Surgical Sciences
DISTAL	Department of Agricultural and Food Sciences
FLI	Fatty Liver Index
EC	European Commission
EU	European Union
GGT	Gamma-Glutamyl Transferase
HACCP	Hazard Analysis and Critical Control Points
HbA1C	Glycated Hemoglobin
HTAS	Health and Taste Attitude Scale
IgE	Immunoglobulin E
IRCCS AOUBO	Istituto di Ricovero e Cura a Carattere Scientifico Azienda Ospedaliero-Universitaria di Bologna
IPAQ	International Physical Activity Questionnaire
JATOS	Just Another Tool for Online Studies
LFP	Leeds Food Preference
LSM	Liver Stiffness Measurement (hepatic elastometry)
NDI	Nepean Dyspepsia Index
GI	Gastrointestinal
SIAN	Servizio di Igiene degli alimenti e della nutrizione
SPSS	Statistical Package for the Social Sciences
UNIBO	University of Bologna
UNIBO PSI	Department of Psychology of the University of Bologna
UNITO	University of Turin
UOC	Unità Operativa Complessa
VAS	Visual Analogue Scales

2. INTRODUCTION

2.1 Background and rationale of the study

The use of alternative proteins in food products has become increasingly relevant in recent years, in response to increased consumer awareness of environmental sustainability, animal health, and welfare [1]. These proteins offer several opportunities to meet growing demand without relying solely on animal sources such as meat, fish, and dairy products [2]. Plant-based proteins, either from legumes such as peas and beans or from grains such as wheat, rice, and oats, are among the most popular [3], with soybeans remaining one of the main sources [4;5]. Algae-and microalgae-derived proteins are emerging as a promising protein source, particularly suitable for seafood products due to their quality and nutrients such as ω-3, fiber, and vitamins [6;7]. Finally, despite some initial resistance, there is growing interest in the use of edible insects as a protein source, especially in snacks, energy bars, and protein flours [8]. Crickets, grasshoppers and larvae offer a rich source of protein, healthy fats, and other nutrients [9].

The integration of alternative proteins into bread and pasta has attracted great interest, responding to the growing consumer demand for healthier, protein-rich, and gluten-free options. Alternative protein sources, like those derived from edible insects or microalgae, are being studied for their nutritional and functional benefits [10;11]. In this context, evaluating the metabolic safety and potential health benefits of products enriched with alternative proteins is essential to fully understand their characteristics and to increase consumer trust.

The CIPROMED project is a three-year Innovation Action funded by PRIMA (Partnership on Research and Innovation in the Mediterranean Area), under Article 185 of the European Union Framework Programme, Grant Agreement 2231. It is based on the highly synergistic work of 17 partners from 10 countries. The consortium aims to innovate, validate, and demonstrate (both at pre-pilot and pilot scale) the technical, socio-economic, and environmental feasibility of a platform technology for the isolation, conversion, and valorization of value-added new proteins. These proteins will be derived from a broad palette of ingredients, including edible insects, microalgae/algae and legumes, as superior value additives for food (baked goods, meat/dairy alternatives, hybrid products, etc.) and feed products (for poultry and aquaculture), while fully complying with existing regulations for food/feed safety and security. The optimized processes, practices, and final products will be evaluated from technical, environmental, and financial perspectives, as well as from consumer acceptance points of view, thereby contributing to the creation of sustainable value chains in both conventional and emerging/alternative Agri-industrial processing sectors. The project seeks solutions towards ‘zero-waste’ operations within the concept of a Circular Economy.

The incorporation of alternative proteins into food products raises questions about their acceptability and impact on overall health. Although they have been approved for human consumption due to their microbiological and toxicological safety, there is limited and/or controversial data only on their physiological effect and potential favorable effect on metabolism and weight control. About **insect** proteins, the paper from Skotnicka et al. suggested that the addition of 30% of an insect flour to a wheat pancake considerably increased the satiating potential as compared to the control sample [12], while the systematic review from Cunha et al. [13] concluded that the consumption of insect-based products was associated with lower insulin levels. However, in this latter study, the effects on inflammatory markers and microbiota composition were deemed inconclusive and the studies did not show significant effects on appetite regulation. **Microalgae and algae** are receiving increased attention in the food sector as sustainable ingredients due to their high protein content and nutritional value. They contain up to 70% protein with the presence of all 20 essential amino acids, thus fulfilling human dietary requirement. Moreover, further health benefits of could be related to the presence of high value secondary metabolites such as pigments (e.g., phenolics, carotenoids, etc.), phytosterols and omega 3 fatty acids [14]. Another potential metabolic benefit can be derived by its iodine content, that in products for human use is around 50–60 μg/100 g wet weight, while the reference dietary allowance for adults is 150 mcg/day, and the safe upper limit is 1,100 μg /day. There is scanty data only regarding the satiety and metabolic effect of use of microalgae/algae as ingredient for human consumption.

Hemp seeds for human consumption in the European union must meet the requirements for the maximum accepted level of delta-9-tetrahydrocannabinol [15]. They are appreciated and considered a good material for fortification for several reasons. They contain between 25 and 35% lipids, of which 70–80% are polyunsaturated fatty acids. The hemp seeds contain a significant amount of essential fatty acids, respecting the ideal ratio (between 3:1 and 5:1, depending on the variety) between n-6/n-3. Proteins, rich in essential amino acids, range from 20 to 25%. Another advantage of hemp proteins is their high digestibility. The amount of total carbohydrates varies between 20 and 30%, a large part being dietary fiber, predominant being the insoluble ones. There have been documented potential benefits on improving insulin sensitivity, helping intestinal transit, reducing appetite, probiotic effect, lower total blood cholesterol, and anti-inflammatory effects. However, there is limited data on their overall physiological effects on satiety, and on their psychological effects.

On the contrary, there is solid data concerning the consumption of **legume** proteins and on their health benefits, especially on glucose metabolism and on human microbiota, and they appear to have good satiating power [16;17].

In Work package 5 of the CIPROMED project, a study structured in two distinct phases (acute and chronic) using alternative proteins is planned in healthy volunteers and overweight patients in Bologna to evaluate the acceptability and metabolic effect of the novel protein food prototypes. Also, the effects on appetite/satiety, food preference, postprandial craving, reward, prospective food intake and gut microbiota will be evaluated.

In Work package 6 of the CIPROMED project, the psychological, cognitive, emotional attitudes and responses to new foods will be investigated during the chronic phase, using psychometric assessments (including questionnaires on neophobia and mood disorders).

Healthy volunteers and overweight patients will also complete online psychological tasks assessing memory, attention, and executive functioning to explore whether diet influences cognitive performance through the microbiota-gut-brain axis, both at the beginning and at the end of the intervention.

2.2 IMPORTANCE OF THE STUDY AND ITS CLINICAL RELEVANCE

This project aims to thoroughly assess the metabolic, physiological, psychological, and behavioural responses to novel protein sources through both acute and chronic clinical evaluations involving overweight patients and healthy volunteers. Its clinical significance lies in addressing critical issues at the intersection of nutrition, sustainability, and public health by examining their physiological, health-related effects and acceptability.

The **acute phase** of the project focuses on investigating the immediate postprandial effects of bread products enriched with various protein flours (edible insects, specifically crickets (*Acheta domesticus*), edible algae, specifically spirulina (*Arthrospira platensis*), as well as hemp and legumes) compared with wheat flour. These assessments are crucial for understanding whether novel proteins affect appetite regulation or influence food choices toward less healthy options. The hypothesis is that these proteins do not negatively impact eating behaviour or promote unhealthy preferences.

The **chronic phase** complements this by studying the long-term effects of regular consumption of novel protein-based products. It evaluates changes in glucose and lipid metabolism, appetite and satiety regulation, gut microbiota composition, and psychological acceptance of novel foods. Psychometric assessments are included to explore cognitive and emotional factors that influence food choices and acceptance. The hypothesis is that these proteins exert similar metabolic, physiological and psychological effects to well established “healthy” proteins like legume- and fish-derived proteins.

By combining acute and chronic perspectives, the project provides a comprehensive and ecologically valid framework for evaluating the real-world impact of novel proteins. The isoproteic (legume- and fish-based) comparator selected for the chronic phase offers the first direct comparison in the

scientific literature between edible insect (crickets-*Acheta domesticus*) or edible algae protein alternatives and traditional "healthy" legume- and fish-based products, covering physiological, metabolic, psychological, and microbiota-related aspects.

The outcomes of this research are particularly significant within the Mediterranean context, where dietary habits are evolving, and the demand for sustainable food options is growing. The findings will contribute to the scientific evidence needed to shape public health guidelines, enhance consumer education, and inform future food innovation strategies aligned with environmental and nutritional objectives.

3. OBJECTIVES AND OUTCOMES OF THE STUDY

3.1 Primary objectives

The primary objectives of this study are designed to assess the key health and physiological effects of alternative protein-based food products in both acute and chronic phases. These objectives are as follows:

Acute phase

To assess the acute effects of various protein-based food products (bread enriched with edible algae flour, edible insect flour, hemp flour, or legume flour) on:

- Post-prandial satiety ratings and rebound hunger,
- Postprandial craving and reward,
- Food preference

The research hypothesis is that these food products do not negatively affect postprandial eating behavior or alter food preference towards less healthy choices as compared to wheat flour as control.

Chronic phase

To evaluate the long-term effects of novel protein-based foods (edible insect-protein bread or an edible seaweed burger) in a Mediterranean diet on:

- Metabolic profile (glucose, insulin, triglycerides, and cholesterol levels),
- Gastrointestinal symptoms

The research hypothesis is that edible insect (cricket-*Acheta domesticus*) or edible seaweed proteins have similar impact on the metabolic profile and on gastrointestinal symptoms when compared to control proteins (legumes and fish).

<u>Both phases of the study include overweight patients and healthy volunteers to assess differential responses across population groups.</u>

3.2 Secondary objectives

The secondary objectives of each phase of the study are outlined as follows:

Acute phase

- To estimate postprandial energy intake and energy compensation in both overweight patients and healthy volunteers with normal weight.

The research hypothesis is that alternative proteins do not increase postprandial energy intake and/or energy compensation and therefore can be recommended as part of a healthy diet, with particular reference to overweight patients.

Chronic phase

To analyze the chronic effects of long-term consumption of novel food products (bread with edible insect protein or an edible seaweed burger) on:

- Anthropometric measurements (body weight and circumferences),
- Liver function tests,
- Liver fat content,
- Renal function (creatinine, urea, microalbuminuria),
- Blood pressure,
- Serum immunoglobulin levels, including total IgE,
- Adverse events and concomitant use of medication,
- Post-prandial satiety perception,
- Changes in psychometric parameters (neophobia, mood, cognitive function),
- Changes in microbiota composition.

These secondary objectives will provide information on possible unfavorable effects which have not been specifically investigated before.

3.3 Outcomes

The study will measure specific outcomes to determine whether the objectives have been achieved. These outcomes are as follows:

Primary Outcomes:

Acute phase:

- No changes in subjective satiety scores assessed via visual analogue scales (VAS) with different protein sources,
- No changes in food preference and craving responses measured through validated psychometric tools (HTAS and LFP questionnaires and 9-point hedonic scale) with different protein sources.

Chronic phase:

- No deterioration or amelioration or maintenance within normal range of metabolic profile (glucose, insulin, triglycerides, temporal cholesterol profiles),
- Comparable incidence and severity of gastrointestinal symptoms across different protein sources, assessed using validated psychometric tool (NDI).

We expect that the novel proteins will not significantly affect any of the primary outcomes.

Secondary Outcomes:

Acute phase:

- No significant variations in postprandial energy intake and energy compensation measured via 24h dietary recalls.

Chronic phase:

- No deterioration or amelioration of liver function tests: ALT, AST, GGT and FLI,
- No deterioration or amelioration in liver fat levels and hepatic stiffness measured through FibroScan and CAP analysis,
- No deterioration or maintenance within normal range of CBC, renal function (creatinine, urea, microalbuminuria and urinary nitrogen), inflammation (C-reactive protein level), blood pressure and serum immunoglobulin levels including total IgE levels,
- No unfavorable changes in anthropometry (weight and circumferences), and possibly reduction of waist circumference in overweight patients because of a controlled diet,
- No unfavorable changes in gut microbiota composition,
- No modifications or significant changes in previous pharmacological therapy and no reports of serious adverse events,
- Similar changes across different protein sources in VAS for hunger, desire to eat, fullness, prospective food intake,
- No unfavorable changes in psychometric variables on mood (measured through BDI-II), neophobia (measured through FNS), cognitive functions (measured through Web-based cognitive and psychological tasks).

Further details are provided in the following Table.

Primary objective	**Outcome: parameter (and unit of measurement) by which the objective will be evaluated.**	**When will the outcome be measured**
Acute phase		
○ Post prandial satiety and rebound hunger	○ VAS for hunger, desire to eat, fullness, prospective food intake	○ In each of the five sensory tests
○ Postprandial craving and reward	○ Leeds Food Preference (LFP) questionnaire and 9-point hedonic scale	○ In each of the five sensory tests

o Food preference	o Health and Taste Attitude Scale (HTAS) questionnaire	o At the screening and in each of the five sensory tests
Chronic phase		
o Metabolic profile	o Glucose (mg/dl), Basal Insulin (µU/ml) for Homa Index calculation; Triglycerides (mg/dL), Total Cholesterol (mg/dL), HDL (mg/dL), LDL (mg/dL) sec. Friedewald	o Baseline, month 2 and 4 of the intervention
o G.I. symptoms	o 24-hour recall using the dyspeptic symptoms rating scale of the Nepean Dyspepsia Index (NDI)	o Month 2 and 4 of the intervention
Secondary objective	**Outcome: parameter (and unit of measurement) by which the objective will be evaluated.**	**When will the outcome be measured**
Acute phase		
o Postprandial energy intake and energy compensation	o 24h dietary recall	o 24 hours after each five sensory tests
Chronic phase		
o Anthropometric measurements	o Body weight (kg) and body circumferences (cm; waist, hip, abdominal, neck, arm)	o Baseline, month 2 and 4 of the intervention
o Liver function tests	o ALT, AST, and GGT enzymes; FLI	o Baseline, month 2 and 4 of the intervention
o Liver fat content	o CAP (dB/m) using hepatic elastometry (FibroScan®)	o Baseline, month 2 and 4 of the intervention
o Renal function	o Plasma urea (mg/dL), creatinine (mg/dL), microalbuminuria (mg/L) and urinary nitrogen (mmol/L)	o Baseline, month 2 and 4 of the intervention
o Blood pressure	o Systolic and Diastolic blood pressure (mmHg)	o Baseline, month 2 and 4 of the intervention
o Gut microbiota composition	o Genomic analysis on stool sample	o Baseline and 4 of the intervention
o Serum immunoglobulin levels	o Serum IgE levels (UI/ml)	o Baseline, month 2 and 4 of the intervention
o Adverse Events and concomitant use of medication	o Anamnestic recall of Used Medications	o Baseline, month 2 and 4 of the intervention
o Post-prandial satiety perception	o VAS for hunger, desire to eat, fullness	o Baseline, month 2 and 4 of the intervention

It is important to specify that, for healthy volunteers, all tests, instrumental assessments, laboratory analyses, and questionnaires are study-specific. For overweight patients, on the other hand, blood chemistry analyses, anthropometric measurements, pharmacological and dietary history, and instrumental assessments (e.g., hepatic elastometry) are performed as part of routine clinical practice,

while the administration of questionnaires (VAS, HTAS, LFP, 9-point hedonic scale, FNS, and BDI-II) is study-specific

4. STUDY PLAN

4.1 Study design

This is a **spontaneous, exploratory, interventional controlled open-label randomized national multicentre** study involving the use of novel food prototypes (specifically, the **acute phase** involves the **tasting** of bread enriched with alternative protein sources—such as edible insect (cricket-*Acheta domesticus)*, algae (*spirulina algae*), hemp, or legume flour—by healthy volunteers and overweight patients at the **Department of Agricultural and Food Sciences** (DISTAL); the **chronic phase** includes the at-**home consumption** of either insect-based bread (criket-*Acheta domesticus)* or an edible seaweed-based burger by healthy volunteers and overweight patients). Overweight patients will be enrolled at the UOC Endocrinologia, Prevenzione e Cura del Diabete of IRCCS AOUBO as part of routine clinical practice; the UOC will also be responsible for medical screening of all study subjects and of survelliance/reporting of adverse effects. Healthy volunteers will be enrolled at DISTAL-UNIBO and will be recruited through the posting of flyers in designated institutional spaces, both physical and online, at DISTAL and DIMEC. The posters will include a QR code and/or a link directing to an information sheet, which provides a detailed description of the study, including its objectives, procedures, the requirement for participants to be adults (≥18 years of age), and the exclusion criteria. At the end of the information sheet, an institutional email address will be provided for individuals to express their interest in participating or to request further information.

The project is supported by PRIMA (Partnership on Research and Innovation in the Mediterranean Area), under Article 185 of the European Union framework, Grant Agreement 2231. Study costs will be fully covered by PRIMA funding.

The study involves collaboration with the following entities:

- The **University of Turin**, which will perform study-specific microbiota analyses on faecal samples; costs will be covered by University of Turin and National Research Council, which are partners of the CIPROMED Consortium and which therefore will receive funding directly from PRIMA- European Commission under Grant Agreement 2231.
- The **Department of Psychology at the University of Bologna**, which will administer psychometric questionnaires (FNS, BDI-II) and online psychological tasks; their costs will be

covered by funding to UNIBO from PRIMA-European Commission under Grant Agreement 2231.

- The **SSD Clinical Nutrition and Metabolism** from **IRCCS AOUBO** will carry out the explanation and discussion of the patient's food diary, as well as the calculation, preparation and explanation of the nutritional schemes of different dietary interventions. These latter procedures will be study specific for healthy volunteers, while will be part of the ordinary clinical practice for the overweight patients and will be funded by IRCCS AOUBO as linked third party of UNIBO under Grant Agreement 223.
- The **Laboratorio Unico Metropolitano** (LUM) will conduct additional study-specific procedures funded by IRCCS AOUBO as linked third party of UNIBO under Grant Agreement 2231.

A study-specific insurance policy covering both healthy volunteers and patients will be activated and financed by UNIBO on PRIMA funds related to a specifically envisaged budget, due to the interventional nature of the protocol.

All acute-phase procedures (except enrolment of overweight patients which will be carried out at IRCCS AOUBO) will take place at DISTAL and be funded by UNIBO; chronic-phase procedures (except enrolment of healthy volunteers which will be carried out at DISTAL) will be conducted at IRCCS AOUBO and funded accordingly. Shipping of faecal samples to the University of Turin will be covered by UNIBO.

4.1.1 Coordination between centers

- **DIMEC** serves as the local Promotor of the EU-funded CIPROMED project and it is the Beneficiary of the financing from PRIMA-European Commission. It is also the academic afference of the responsible scientist Prof. Maria Letizia Petroni who will also ensure alignment across clinical procedures and inter-center communication.

The recruiting centers for this study are IRCCS AOUBO (UOC Endocrinologia, Prevenzione e Cura del Diabete) and DISTAL, while LUM, the UNIBO Department of Psychology, the University of Turin and SSD Clinical Nutrition and Metabolism of IRCCS AOUBO act as scientific collaborators contributing to specialized assessments.

- **IRCCS AOUBO (Coordinating Center)** – specifically the **UOC Endocrinologia, Prevenzione e Cura del Diabete** – serves as the clinical site for enrolment of overweight patients. Also, it is

responsible for carrying out the intervention procedures, both study-specific and according to routine clinical practice, during the chronic phase of the study from dedicated funding from PRIMA- European Commission. Moreover, it is responsible for conducting medical screenings in both the acute and chronic phases of the study, monitoring volunteers and patients, overseeing clinical parameters during the chronic phase, and supervising/reporting any adverse effects.

- **DISTAL (Satellite Center)** will serve as an enrolment center for healthy volunteers and is responsible for executing all acute-phase procedures, with the exception of medical screening of the volunteers which is carried out by the Coordinating Center. In addition, DISTAL is responsible for the delivery of both investigational and control food products used in the acute and chronic phases of the study, through a formal agreement with the COOP Superstore located at Meraville Shopping Park (Viale Tito Carnacini 57, 40127 Bologna, BO). DISTAL will also manage the provision of control products (which will be available for direct collection by the interested parties) for the chronic phase of the study to both volunteers and patients enrolled in the trial. Furthermore, DISTAL is responsible for the shipping of the novel food prototypes from the European partner institution to Bologna, as well as for ensuring compliance with HACCP regulations and food safety protocols throughout the study. It is funded by PRIMA- European Commission under Grant Agreement 2231.

The following institutions are involved as collaborating centers:

- **LUM** will perform study-specific laboratory analyses funded by IRCCS AOUBO, including biochemical and clinical tests relevant to the protocol.
- **UNIBO PSI** will be responsible for administering psychometric assessments (FNS, BDI-II) and for delivering online cognitive tasks to evaluate psychological and behavioural responses to the dietary intervention.
- **UNITO** conducts microbiota analyses on faecal samples collected during the study, while UNIBO covers the shipment of samples to the University of Turin.
- **SSD Clinical Nutrition and Metabolism from IRCCS AOUBO** will provide support by dieticians, who will carry out anthropometric measurements, the delivery and discussion of the patients' and volunteers' food diary, as well as the calculation, preparation and explanation of the nutritional scheme under supervision of a medical doctor of the unit. These latter procedures will be study specific for healthy volunteers, while will be part of the ordinary clinical practice for the overweight patients.

4.2 Study population

The target population for both the acute and chronic phases of the study consist of both healthy volunteers (normal weight) and patients (overweight), both men and women, aged between 18 and 65 years, with a Body Mass Index (BMI) between 18.5 and 34.9 kg/m². At least 35% of the total study participants (volunteers and patients) will be individuals with overweight (BMI > 25 kg/m²).

4.2.1 Inclusion criteria

To be eligible for participation, individuals must meet specific inclusion criteria based on their health status and weight classification.

For patients, inclusion requires:

- Providing signed informed consent,
- Being between 18 and 65 years of age,
- Having a body mass index (BMI) between 25.0 and 34.9 kg/m², inclusive, corresponding to the overweight category or, at most, class I obesity.

For healthy volunteers, inclusion requires:

- Providing signed informed consent,
- Being between 18 and 65 years of age,
- Having a body mass index (BMI) within the normal range, defined as between 18.5 and 24.9 kg/m²

These criteria ensure that the study population includes both healthy volunteers and overweight patients, allowing for a comprehensive evaluation of the effects of alternative protein-based foods in diverse metabolic conditions.

4.2.2 Exclusion criteria

The exclusion criteria, which apply to both the acute and chronic phases of the study, and to both healthy volunteers and overweight patients, include:

- Positive and documented history of allergy to legumes, insects, microalgae, shellfish, mollusks, crustaceans, snails, insect venom, house dust mites, or any component (ingredient

or additive) present in the food prototypes tested, or a confirmed or suspected diagnosis of Celiac Disease.

- History of severe allergic reactions to any type of allergen.
- Pregnancy or Breastfeeding

4.2.3 Sample Size

To assess sample size for non-inferiority, we followed the procedures illustrated by Flight and Julious [18]. Calculations have been carried out according to the following principles:

- non-inferiority trials are reduced to a simple one-sided hypothesis test
- the non-inferiority limit has been defined as the 'largest difference that is (clinically) acceptable, so that a difference bigger than this would matter in practice".

Acute phase

Based on published data for crossover studies [19], showing a standard deviation of 8.4 mm at 45 minutes following a test meal using electronic VAS, if there is truly no difference between the control and the study foods, then 38 subjects are required to be 80% sure that the lower limit of a one-sided 95% confidence interval (or equivalently a 90% two-sided confidence interval) will be above the non-inferiority limit of -6.9mm [20]. Therefore, a total of 40 subjects will be enrolled in the acute study.

Chronic phase

As a pilot exploratory study, sample sizes for continuous variables have been estimated firstly following Kieser and Wassmer's rule of thumb, which recommends a minimum of 20 subjects per treatment arm [21]. Moreover, we made a confirmatory estimate based on published data on changes in fasting glucose levels following a chronic dietary intervention [22]. For a standard deviation of the outcome equal to 9.2 mg/dl, if there is truly no difference between the control and the study meals, then 34 subjects are required to be 80% sure that the lower limit of one-sided 95% confidence interval (or equivalently a 90% two-sided confidence interval) will be above the non-inferiority limit of -8 mg/dl. The within-subjects crossover trial will therefore randomize 40 participants.

4.2.4 Randomisation mode

Random sampling and allocation will be carried out using an updated version of SPSS (Statistical Package for the Social Sciences) software.

Acute phase

Both healthy volunteers and overweight patients will repeat the tasting for five consecutive weeks, following a Latin Square cross over Design to ensure balanced exposure to all test conditions. In this design, healthy volunteers and overweight patients will taste all four breads with alternative protein sources, as well as the control wheat bread. All bread portions will be comparable in terms of energy content.

A Latin Square cross over design will be employed to assign the food sequence, with eight subjects (healthy volunteers or overweight patients) per sequence. For instance, the first eight subjects (healthy volunteers or overweight patients) will follow the sequence B1-B2-B3-B4-B5 (control), the next eight subjects (healthy volunteers or overweight patients) will follow B2-B3-B4-B5 (control)-B1, and so on. This approach will ensure that each participant (healthy volunteers or overweight patients) is exposed to every product in a systematic and unbiased manner.

The randomization will be stratified by gender and weight status, ensuring an even distribution of characteristics across all sequences, thus minimizing potential confounding variables in the study.

Chronic phase

The within-subjects crossover trial will randomize healthy volunteers and overweight patients, who may either be the same as those from the acute phase or different individuals (healthy volunteers or overweight patients), to follow one of two dietary interventions. Healthy volunteers and overweight spatients will be assigned a Mediterranean diet (Group A) with the inclusion of control products (PRODUCT A – legume bread and/or ordinary fish burger), or the same diet with the inclusion of one of the two novel food products: PRODUCT B (bread with edible insect protein *Acheta domesticus*) or PRODUCT C (edible seaweed burger). These products will be consumed five times a week for a period of two months. Products will be comparable in terms of energy content and protein content.

The Mediterranean diet may be isocaloric or moderately hypocaloric (in relation to subject’s energy expenditure), in line with normal clinical practice, for overweight patients; while it will be isocaloric for healthy volunteers.

A Latin Square cross over design will be used to assign the food sequence, with 10 subjects (healthy volunteers or overweight patients) per sequence. For example, the first 10 subjects (healthy volunteers or overweight patients) will follow the sequence A-B, the next 10 will follow the

sequence B-A, the third 10 will follow the sequence A-C, and the last 10 will follow the sequence C-A. This design will ensure a balanced comparison of the products across healthy volunteers and overweight patients.

Each sequence will be stratified by gender and weight status (normal weight/overweight), enabling an effective analysis of potential group differences. The study will compare health and dietary parameters at three key time points: baseline, month 2, and month 4 of the intervention.

4.3 Study intervention

4.3.1 Novel Food Definition and Regulations

Novel foods are defined by Regulation (EU) 2015/2283 [23] and listed in Regulation (EU) 2017/2470 [24]. According to these regulations, a 'novel food' is defined as food that was not consumed to a significant degree by humans in the EU before 15 May 1997, the date when the first Regulation on novel food came into force. Novel foods can include newly developed and innovative foods, foods produced using new technologies and production processes, and foods traditionally eaten outside of the European Union (EU). These foods must be safe for consumers, properly labeled, and not nutritionally disadvantageous if intended to replace another food. The European Food Safety Authority (EFSA) has assessed the microbiological and toxicological safety of specific farmed insects. EFSA has concluded that a type of house cricket (Acheta domesticus) is safe for human consumption and has approved its use in food in frozen, dried, and powder forms for the general population, in accordance with Regulation (EU) 2015/2283 [25]. However, EFSA has also noted the potential for allergic reactions in individuals with allergies to crustaceans, mites, and molluscs (cross-reactivity), and the possible presence of gluten allergens from insect feed in the final food product. Regarding microalgae, six microalgae species were already consumed as food in the EU before May 1997 and are therefore not considered novel foods. Nevertheless, their potential as a low-cost, low-environmental impact protein source is attracting increasing attention from consumers and the food industry [26].

The specific novel foods – approved by EFSA - to be evaluated within the scope of this research protocol are described in detail below. Additionally, the technical data sheets for each of the aforementioned novel foods are provided as annexes to the present document.

Acute phase

Participants will consume different food prototypes, including **bread with the inclusion of edible algae flour (*spirulina algae*), insect flour (cricket-*Acheta domesticus*), hemp flour, or legume**

flour. Each participant will repeat the tasting for five consecutive weeks, following a Latin Square Design. This ensures that each participant will taste all four breads with alternative protein sources (edible algae, edible insect, and hemp) or legume flour and the control bread made of ordinary wheat flour.

Chronic phase

Participants will follow a within-subjects crossover trial, randomized into sequences. They will consume a Mediterranean diet, either with control products (PRODUCT A) or including one of the two novel food prototypes: PRODUCT B (**bread with edible insect protein *Acheta Domesticus***) or PRODUCT C (**edible seaweed burger**). These meals will be consumed five times a week for 2 months.

Chronic study meals:

- 50g legume bread + ordinary fish burger (both PRODUCT A or control products),
- 50g bread with edible insect protein (cricket-*Acheta domesticus*) (PRODUCT B) + ordinary fish burger (PRODUCT A or control product),
- 50g legume bread (PRODUCT A or control product) + edible seaweed burger (PRODUCT C).

To minimize any risk to volunteers or patients related to the potential allergenicity of alternative proteins (such as those derived from edible insects, edible algae, or hemp) included in the novel foods, this protocol will only involve proteins that have undergone thermal processing, aimed at reducing protein cross-allergenicity. Furthermore, individuals with a documented history of allergy to any of the study food components, known cross-allergens, or any form of severe allergy will be excluded from participation. A medical doctor equipped with an anaphylaxis emergency kit will be present during the initial administration of each food prototype. The novel food prototypes will be supplied by the European partner institution within the PRIMA consortium.

4.3.2 Control intervention/treatment

Acute phase

Control intervention will be represented by ordinary wheat bread.

Chronic phase

Control intervention will be represented by 50g legume bread + ordinary fish burger (“PRODUCT A”).

Legume bread (which has a well-established safety profile) has been chosen as control to allow a control with similar energy and protein content which would not be possible using ordinary wheat bread.

4.3.3 In vitro studies on human tissues

Procedures for Fecal Sample Collection, Processing and Microbiome Analysis

Fecal samples will be self-collected from all participants, including both volunteers and patients, at 2 time points for microbiome analyses. Samples will be shipped on dry ice to UNITO, where they will be labeled with a unique identification code to ensure pseudonymization. Data collected during the experimentation phase may be used for this research and potential future studies to enhance knowledge. Data will only be shared with prior authorization, in either aggregated (multiple subjects) or individual form, maintaining pseudonymization, and used exclusively for scientific purposes. DNA extraction from fecal samples will be performed following the International Human Microbiome Consortium's Standard Operating Procedures (SOPs), followed by meta-genomics analysis. Read quality filtering, taxonomical and functional annotation will be conducted using a standardized in-house procedure. This analysis will determine variations in microbiome structure and function based on dietary intervention and time of exposure. Statistical analyses will be used to define taxonomic differences in the microbiome affected by dietary intervention. Expected results include the assessment of the differential distribution of microbial genes and functions from meta-genomics to link them to specific ingredients.

4.3.4 Questionnaires and scales

All scales and questionnaires are administered in Italian, using an appropriate font and size for optimal readability. They either have a validated Italian version or have been carefully translated from English as faithfully as possible. The questionnaires will be completed either in paper format or through the UNIBO REDCap platform.

For healthy volunteers, all administered scales and questionnaires are study-specific. In the case of overweight patients, the completion of the IPAQ questionnaire is part of routine clinical practice, while all other administered instruments are study-specific.

- **Level of physical activity**: To determine the level of physical activity, the IPAQ questionnaire is used [27]. The IPAQ questionnaire is available in a translated and validated Italian version [28].
- **Risk factors for metabolic diseases**: The FINDRiskis a simple, fast, inexpensive, noninvasive, and reliable tool to identify individuals at high risk for type 2 diabetes [29]. The use of the FINDRisk questionnaire to assess the risk of type 2 diabetes in primary prevention settings has been validated within the framework of the PRE.DI.CO project (Prevention of Diabetes in the Val

di Cornia area) [30]. In the CIPROMED project, the Italian translation of the questionnaire, provided by the Deutsche Diabetes-Stiftung, will be administered.

The MASLD Risk Assessment Questionnaire is a non–laboratory-based self-assessment score that may be useful for identifying individuals at high-risk of Metabolic Associated Steatotic Liver Disease (MASLD) [31]. Since no validated Italian translation of the MASLD Risk Assessment questionnaire is currently available, a study-specific version will be administered, translated as closely as possible to the original text.

- **Risk factors for dietary habits**: The PREDIMED questionnaire is a tool developed within the PREDIMED clinical study (PREvención con DIeta MEDiterránea) to assess adherence to the Mediterranean diet. It is used in clinical studies and nutritional practices to evaluate the risk of cardiovascular diseases and other diet-related conditions [32]. The Italian version of the PREDIMED questionnaire, as used in a previous study [33], will be administered in this project.
- **Food preference**: HTAS is used in consumer behavior research, nutrition studies, and food marketing to understand how people balance health concerns and taste preferences when making food choices. HTAS consists of six subscales, grouped into two main dimensions: Health-related attitudes (general health interest, light product interest, natural product interest) and Taste-related attitudes (craving for sweet foods, pleasure orientation, using food as a reward) [34]. A validated Italian version of the HTAS questionnaire will be administered [35].
- **Postprandial craving and reward:** For this complex variable, we will use two tools: the 9-point hedonic scale and the Leeds Food Preference Questionnaire.

  The 9-Point Hedonic Scale is a standardized tool used to measure the sensory liking of food, beverages, and other products. It is based on a scale from 1 to 9, where:

  1 = Extremely unpleasant

  5 = Neither pleasant nor unpleasant

  9 = Extremely pleasant [36].

  Within the CIPROMED study, a study-specific version of the 9-point hedonic scale will be administered to evaluate the test foods during the acute phase (novel foods vs control product).

  The Leeds Food Preference Questionnaire is a psychometric tool developed to objectively measure food preferences and motivation towards food. The LFP questionnaire uses food images to assess two key components of eating behavior:

  "Liking" (how pleasant a food is perceived to be) and "Wanting" (the motivation to consume food at a given moment).

  Widely used in nutritional research, eating behavior psychology, and obesity studies, the LFP questionaire helps to understand food preferences and their impact on appetite regulation and

dietary choices [37]. The Italian version of the questionnaire was provided directly by Prof. Graham Finlayson from Leeds University following cultural adaptation to Italian dietary habits by Prof. Paola Vitaglione from the University of Naples 'Federico II'.

- **Post-prandial satiety ratings, including rebound hunger**: The Visual Analogue Scale is a psychometric tool used to measure the subjective perception of a specific sensation or state, such as hunger, desire to eat, satiety, and prospective food intake. To assess this variable, a customized study specific 100 mm VAS scale is used [38].
- **Gastrointestinal Symptoms**: The Nepean Dyspepsia Index is a validated questionnaire designed to assess symptoms, quality of life, and the impact of functional dyspepsia on daily living [39]. The questionnaire has undergone validated translation from Australian English to Italian [40].
- **Food neophobia**: Food Neophobia Scale, to measure neophobia, or the reluctance to taste new foods. The questionnaire consists of 10 items (e.g., "I constantly taste new and different foods" or "If I don't know what a food is, I won't try it") with responses on a 7-point Likert scale: from "strongly disagree" to "strongly agree", for an individual total neophobia score ranging from 8 to 40, with increasing neophobic attitude [41]. The Food Neophobia Scale has been translated into Italian and psychometrically validated [42], including its revised form [43].
- **Mood disorders:** The Beck Depression Inventory-Second Edition is a widely used psychological assessment tool designed to measure the severity of depressive symptoms in adults and adolescents. It consists of 21 multiple-choice items [44]. The BDI-II has been translated into Italian and its psychometric properties have been validated [45].

The estimated time required to complete all questionnaires administered during the acute phase of the study is approximately 45 minutes. The estimated time required to complete all questionnaires administered during the chronic phase of the study is approximately 50 minutes.

4.3.5 Web-based cognitive and psychological tasks

The study subjects will perform an online pictorial dot-probe task to examine food-related attentional bias as a predictor of dieting success.

The dot-probe task is a cognitive psychology paradigm used to assess selective attention, particularly attentional biases. It measures how quickly someone responds to a visual stimulus (the "probe") that appears in a location previously occupied by another stimulus (in our case pictures of healthy and unhealthy food). With this task, it is possible to investigate whether people have an attentional bias towards one of the two types of stimuli. Faster reaction times to probes appearing in the location of the emotionally salient food stimulus suggest that the participant's attention is drawn to that stimulus. Previous research has demonstrated that dwell time and reaction time indices derived from the dot-probe task provide reliable and valid measures of food-related attentional bias [46].

The tasks will be administered using the JATOS (Just Another Tool for Online Studies) platform. JATOS is a free and open-source web-based platform designed to help researchers create, manage, and run psychological experiments and surveys online and is interfaced with the Qualtrics platform of UNIBO.

Each subject will access the platform using a pseudo-anonymization code.

The estimated time required to complete the online tasks is approximately 10 minutes.

4.3.6 Transient Elastography (FibroScan®) and CAP™

Following a fasting period of at least 6 hours, liver stiffness and hepatic fat quantification using the CAP™ method will be performed with a FibroScan 630 Expert ® (Echosens, Paris, France) in both healthy volunteers and overweight patients.

The physical principle, known as "Young's Modulus," on which these indirect techniques for quantifying fibrosis and hepatic steatosis are based, is the ability of a mechanical wave to propagate through a medium at different speeds, depending on the elasticity of the medium.

The probe of the ultrasound device used for elastography is placed on the skin over the anatomical location of the liver. It consists of a small transducer that produces a mechanical wave with constant frequency, amplitude, and shape, emitting a minimal amount of energy. The speed of wave propagation in the medium, in this case, the hepatic parenchyma, is studied by emitting ultrasound from the probe itself. The acoustic energy needed to track the propagation of the mechanical wave is also very low and non-harmful. The data regarding the wave's propagation in the hepatic parenchyma are analyzed by the software included in the device through sophisticated algorithms, which yield the liver elasticity value and quantifies liver fat. The procedures for performing elastometric techniques via FibroScan® will be in accordance with the manufacturer's instructions and EFSUMB European guidelines [47-48].

Hepatic steatosis using CAP ™ will be quantified in DB/m at baseline and at each visit.

Hepatic stiffness will be measured at baseline to rule out significant hepatis fibrosis (liver stifness measurement ≤ 7kPa); the presence of fibrosis found by chance does not represent a contraindication to the protocol, and in this unlikely case, the subject will be referred by medical staff for appropriate evaluation.

4.3.7 Adverse effects

An adverse event (AE) is defined as any adverse medical event which occurs to the subjects who are participating in a clinical study including the use of a product, not necessarily with a cause-effect relationship with the product. Therefore, an AE may be any sign (i.e. an anomaly in laboratory values), symptom or infirmity which is non-intentional and which is occurring in

the time period when a product is taken, even if a cause-effect with the product assumption is not thought to exist.

The subjects who will experience AEs which will determine interruption or suspension of the treatment with the study products, or subjects who will experience AEs which will persist until the end of treatment will have to follow a procedure. In particular, any AEs which will lead to permanent suspension of the treatment or which will persist until the end of treatment will be communicated as soon as reasonably possible to the SIAN (Servizio di Igiene degli alimenti e della nutrizione – Food hygiene Service) of the AUSL (Azienda Unità Sanità Locale) of Bologna, and following their assessment, to the Italian Ministry of Health (through the dedicated e-mail address alimenti@salute.it), particularly if the clinical investigator will consider it related or probably or even possibly related to the ongoing study treatments.

Adverse Reaction (AR)

An adverse reaction (AR) is an adverse event (any untoward medical occurrence in a human using the Product) that with a reasonable possibility may have been caused by the novel food.

Serious Adverse Event (SAE)

A serious AE is defined as any untoward medical event which:

- led to a death,
- is life-threatening (an event in which the subject was at risk of death at the time of the event itself (i.e. it does not refer to an event which hypothetically might have caused death if it were more severe), or
- resulted in a persistent or significant disability/incapacity, or
- required in-patient hospitalization or prolongation of existing hospitalization, or
- is an important medical event, that may not be immediately life-threatening or results in death or hospitalization but may jeopardize the subject or may require intervention to prevent one of the outcomes listed above, or
- led to fetal distress, fetal death or a congenital abnormality or birth defect.

Serious Adverse Reaction (SAR)

Based upon EFSA documents [22-25] the following AR are considered as listed:

- Allergic reactions

Relationship of AEs to the study product

The relationship of each AE to the study products is to be determined by the investigator based on the following definitions:

- Certain: when a certain cause-effect relationship exists between the AE and the study product. For example, when the medical event improves at treatment suspension and reappears if treatment is restarted (in case this is clinically possible)
- Probable: when a reasonable cause-effect relationship exists between the AE and the study product. The medical event improves at treatment suspension.
  Possible: when a reasonable cause-effect relationship exists between the AE and the study product, but there is no information or non-clear information about the improvement of the medical condition at treatment suspension.
  Improbable: when a temporal relationship exists between the occurrence of the AE and administration of the study product, but without a reasonable cause-effect relationship with the administration of the product.
- Non-related: when there is neither a temporal relationship nor reasonable cause-effect relationship between the AEs and administration of the study product.

The categories “certain, probable, possible” will have to be recorded as “related” to the study product; the categories “improbable, non-related” will have to be recorded as “not related” to the study product.

Duration of Reporting of Adverse events and Serious adverse events

The AEs and SAEs will be reported from initiation of study until the last visit of the study.

Documentation of safety assessments

The recording of AE will have to start immediately after the signature of consent form. The AE may be spontaneously reported by the patient anytime or during the scheduled visits and phone call (when the Investigators will specifically enquire about this topic). All the AE will have to be fully recorded in the source document and transcribed in the proper section (Serious AE or non-serious AE) of the CRF.

Any AEs which is rated as non-serious will be recorded in the section “non-serious AEs” of the CRF. These AEs will undergo follow-up until their resolution; in case they will become serious AEs, they will then be reported in the section of SAEs.

If there is a worsening of a medical condition that was present before starting the study, this should be considered as a new AE and a complete evaluation should be recorded.
The medical investigator from UOC Endocrinologia IRCCS AOUBO is responsible for ensuring the follow-up of any patient who experiences an SAE during the study. The medical investigator must re-examine the patient at regular intervals until the symptoms have completely disappeared or stabilized.
Certain, probable, and possible SAEs will be communicated as soon as reasonably possible to the SIAN (Servizio di Igiene degli alimenti e della nutrizione – Food hygiene Service) of the AUSL (Azienda Unità Sanità Locale) of Bologna, and following their final assessment, to the Italian Ministry of Health (through the dedicated e-mail address alimenti@salute.it)
It is the responsibility of the investigator in complying to these regulations.

4.4 Duration of the study

Study phases	Time (weeks/months/years)
Patients' and volunteers' enrolment/selection	Acute phase: July 2025 - January 2026 Chronic phase: July-November 2025
Treatment phase	Acute phase: 5 weeks Chronic phase: 8 + 8 weeks
Follow-up for treatment outcome evaluations	Acute phase: 24 and 72 hours Chronic phase: at the end of each treatment phase
Data analysis	April 2026
Total study duration	36 months

4.5 Treatments, visits and assessments

Flow chart acute phase

Procedure	**Screening**	**Novel food challenge**					**24 h post-ingestion**	**72 h post-ingestion**
Timing of test (weeks)	**0**	**1**	**2**	**3**	**4**	**5**	**From week 1 to week 5**	**From week 1 to week 5**
Anthropometry[a]	**x**							
Physical Activity[b]	**x**							
Risk factors for metabolic diseases and dietary habits[c]	**x**							
Randomization		**x**	**x**	**x**	**x**	**x**		
Questionnaire about food preference [d]	**x**	**x**	**x**	**x**	**x**	**x**		
Questionnaires about craving and reward [e] and satiety rating [f]		**x**	**x**	**x**	**x**	**x**		

Post prandial Food Intake[g]							**x**	
G.I. side effects (questionnaire)[h]							**x**	
Allergic/Hypersensitivity reactions[i]								**x**

[a] Weight, height, abdominal circumference
[b] International Physical Activity Questionnaire (IPAQ)
[c] FINDRisk (type 2 diabetes), MASLD Risk Assessment Questionnaire and PREDIMED
[d] HTAS questionnaire
[e] 9-point hedonic scale and LFP questionaire
[f] VAS for hunger, desire to eat, fullness, prospective food intake
[g] 24h dietary recall
[h] NDI
[i] 72h recall

Flow chart chronic phase

Procedure	Baseline visit	Month 2 of the intervention	Month 4 of the intervention
Timing of test (weeks)	**0**	**8-9**	**17-18**
Timing of test (days)	**0**	**56-63**	**119-126**
Anthropometry[a]	**x**	**x**	**x**
Anamnestic recall of Used Medications	**x**		**x**
Physical Activity[b]	**x**		
Risk factors for metabolic diseases and dietary habits[c]	**x**		
Randomization	**x**		
Blood pressure measurement	**x**	**x**	**x**
Stool collection for microbiota analysis	**x**		**x**
Hepatic Elastometry (FibroScan®)	**x**	**x**	**x**
Blood Sampling [d]	**x**	**x**	**x**
24-HR Urine collection [e]	**x**	**x**	**x**
Questionnaires [f]	**x**	**x**	**x**
Provision of a nutrition plan [g]	**x**	**x**	
Food Intake (diary)		**x**	**x**
G.I. side effects (questionnaire) [h]		**x**	**x**
Web-based cognitive and psychological tasks assessing memory, attention, and executive functioning [i]	**x**		**x**

[a] Weight, height; abdominal, waist, hip, arm and neck circumferences.
[b] International Physical Activity Questionnaire (IPAQ)
[c] FINDRisk (type 2 diabetes), MASLD Risk Assessment Questionnaire and PREDIMED
[d] Complete Blood Count, Glucose, insulin (for HOMA index calculation),HbA1c, Lipid profile (Total cholesterol, HDL, LDL, triglycerides), Serum uric acid, plasma urea, creatinine, Liver function tests (AST, ALT, GGT, ALP, total protein, albumin, total and direct/indirect bilirubin, FLI), Microalbuminuria, high-sensitivity CRP and serum IgE levels
[e]Urinary nitrogen (24-hour sample),
[f]VAS for hunger, desire to eat, fullness; Food Neophobia Scale (FNS) and Beck Depression Inventory (BDI)-II;
[g] Dietary plans, personalized according to the nutritional needs of each volunteer and patient, based on the Mediterranean diet model. These plans include the consumption of edible insect-based bread or edible seaweed burgers and/or legume-based bread and/or fish-based burgers five times per week, to be followed for a duration of two months.
[h]NDI;

[i]The tasks will be administered using the JATOS platform.

The study will involve both healthy volunteers (normal weight) and patients (overweight), with all procedures conducted after obtaining informed consent. The assessments will be divided into two phases: acute and chronic.

Acute phase

The acute study will begin with a screening phase, during which volunteers and patients will undergo the following procedures:

- **First of all, a medical interview will be conducted during which the participant will provide detailed information regarding their medical history. In addition, specific inquiries about any prior allergic reactions—including mild reactions and those unrelated to food—will be addressed. After confirming that no exclusion criteria apply, the participant will provide written informed consent under medical supervision**.
- Anthropometric measurements: weight, height, and waist circumference.
- Administration of questionnaires: IPAQ, FINDRisk, MASLD, PREDIMED, and HTAS.

Subsequently, volunteers and overweight patients will participate in one sensory evaluation session per week, for a total duration of 5 weeks, with each session scheduled after an overnight fasting period. In each session, healthy volunteers and patients will be presented with a different product (bread enriched with edible insect, edible algae, hemp, or legume proteins, or a control bread made with wheat flour). After consumption, volunteers and patients will provide feedback on the following:

- Postprandial food preference (HTAS)
- Craving and reward (9-point hedonic scale and LFP questionnaire)
- Postprandial satiety (VAS scale for hunger, desire to eat, fullness, and prospective food intake)
- Postprandial food intake (24-hour dietary recall)

To ensure volunteers and patients safety:

- A medical screening will occur at both Coordinating Center (UOC Endocrinologia IRCCS AOUBO) and Satellite Center (DISTAL) before the start of the acute phase of the study to ensure proper assessment of exclusion criteria, in terms of history of allergies
- A 24–72 hour follow-up will be conducted to monitor for potential gastrointestinal symptoms (NDI) or allergic reactions. A physician will be present during each test session to oversee the safety and well-being of both volunteers and patients. Additionally, a medical doctor will be available 24/7 by phone to provide immediate assistance in the event of gastrointestinal

complaints or, although unlikely given that only non-allergic individuals will be enrolled, any allergic reactions following food ingestion.

For volunteers, all procedures, including anthropometric measurements, completion of scales and questionnaires, 24-hour dietary recall, and 72-hour allergy follow-up, are study-specific procedures. For overweight patients, the completion of the IPAQ questionnaire, anthropometric measurements, and 24-hour dietary recall are standard clinical practices, while the remaining evaluations are study-specific.

Chronic phase

The chronic study will involve both volunteers and patients following a Mediterranean diet plan, which will be isocaloric or moderately hypocaloric (in relation to subject's energy expenditure) for overweight patients or isocaloric for healthy volunteers, with either control products or one of the two novel food prototypes. These will be consumed five times per week over a period of 4 months. For those who did not participate in the acute phase, product tasting will occur first to assess tolerance and acceptability. Assessments will take place at three key time points: baseline, mid-study (after 2 months), and the final visit (after 4 months).

- **First of all, a medical interview will be conducted during which the participant will provide detailed information regarding their medical history. In addition, specific inquiries about any prior allergic reactions—including mild reactions and those unrelated to food—will be addressed. After confirming that no exclusion criteria apply, the participant will provide written informed consent under medical supervision**.
- **Anthropometric measurements** (baseline, mid-study, final): weight, height, and body circumferences (abdominal, waist, hip, arm, and neck).
- **Medication history** (baseline and final).
- **Questionnaires and scales**: IPAQ, FINDRisk, MASLD, and PREDIMED (baseline); VAS scale (baseline, mid-study, final); NDI questionnaire for gastrointestinal symptoms (mid-study and final); BDI-II and Food Neophobia Scale to assess mood and food-related fears (mid-study and final).
- **Blood pressure measurement** (baseline, mid-study, final).
- **Fecal sample collection** for microbiota analysis, to be sent to UNITO (baseline and final).
- **Liver elastography** using FibroScan® (baseline, mid-study, final).
- **Biochemical tests** (baseline, mid-study, final): complete blood count, glucose, insulin, HbA1c, lipid profile, uric acid, urea, creatinine, liver profile, microalbuminuria, CRP, and serum IgE.

- **24-hour urine collection** (baseline, mid-study, final) for the assessment of urinary nitrogen excretion.
- **Dietary plan** (baseline and final): both volunteers and patients will receive a personalized, Mediterranean-based dietary plan at both the baseline and mid-study visits, tailored to their caloric needs. The plan, which may include innovative or standard food products, will be randomized and followed for two months, until the next follow-up visit.
- **Food diary** (baseline and final): Completed by volunteers and patients and discussed with a research dietitian at mid-study and final visits.
- **Web-based cognitive and psychological tasks** (baseline and final): Cognitive function assessment.

To ensure volunteers and patients safety:

- A medical screening will occur at the Coordinating Center (UOC Endocrinologia IRCCS AOUBO) before the start of the chronic phase of the study to ensure proper assessment of exclusion criteria, in terms of history of allergies
- A doctor will be available 24/7 by phone to provide immediate assistance in case of gastrointestinal complaints or, although unlikely, any allergic reactions following food ingestion (given that only non-allergic individuals will be enrolled).

For healthy volunteers, all the above-mentioned procedures are study-specific. However, for overweight patients, anthropometric measurements, medication history, completion of the IPAQ questionnaire, blood pressure measurement, liver elastography, biochemical testing (blood sampling), and completion of the food diary are part of routine clinical practice. All remaining procedures are considered study-specific for overweight patients.

4.5.1 Assessments at the First Visit

Acute phase – Screening Assessments:

- General Assessment: Medical interview to collect history and allergies, informed consent under supervision, followed by anthropometric measurements.
- Outpatient Visits: Detailed allergy history to exclude contraindications and ensure no allergic reactions to the test products.
- Administration of Questionnaires: IPAQ, FINDRisk, MASLD Risk Assessment, PREDIMED and HTAS.

Chronic phase – Baseline Assessments:

- General Assessment: Anthropometric and body composition measurements and blood pressure measurement are conducted. For those who didn't participate in the acute study, a detailed allergy history will be collected to exclude contraindications.
- Previous Therapies: Review of ongoing treatments or pharmacotherapy, past medical conditions, and any history of metabolic or gastrointestinal disorders.
- Laboratory Tests: Blood sampling.
- Outpatient Visits and/or Specialized Tests: Liver fat and fibrosis measurement (FibroScan® with CAP™ technology) and fecal microbiota sample.
- Administration of Questionnaires: IPAQ, FINDRisk, MASLD Risk Assessment, VAS for hunger, desire to eat, fullness, Food Neophobia Scale and BDI-II.
- Administration of Scales: Psychometric and cognitive function assessments (Web-based cognitive and psychological tasks).
- Other: Randomization and provision of a food diary and a dietary plan to be followed during the first two months

4.5.2 Assessments at follow-up visits and timelines

Acute phase– Follow-up Assessments

- No blood or laboratory tests are scheduled,
- **Data collection tools:** Validated questionnaires (HTAS, 9-point Hedonic Scale, LFP Questionnaire) and scales (VAS for Hunger, Desire to eat, Fullness and Prospective food intake),
- **Dietary intake assessment:**
    - 24-hour dietary recall (to assess food intake post-ingestion)
    - 72-hour recall (to monitor delayed allergic or hypersensitivity reactions)
- **Gastrointestinal symptom assessment:** Nepean Dyspepsia Index (NDI), completed within 24 hours post-ingestion

Chronic phase – Follow-up Assessments

During the chronic study, clinical and instrumental evaluations will be conducted at mid-study (2 months) and at the final visit (4 months):

- **Laboratory tests:** Blood samples and 24-hour urine collection will be collected.
- **Fecal microbiota:** At the final visit, a fecal sample will be collected and sent to UNITO for analysis.
- **Liver fat content:** Assessed using hepatic elastography (FibroScan® with CAP technology).
- **Outpatient visits and physical measurements:** Anthropometric assessments (weight, height, body circumferences) and blood pressure measurements.

- **Provision of a food diary and a dietary plan to be followed during the next two month** at mid-study visit.
- **Medication use:** Medication history will be collected at the final visit.
- **Cognitive functions:** Assessed through online tests at the end of the study.
- **Questionnaires and scales:** Nepean Dyspepsia Index (NDI), Food Neophobia Scale (FNS), Beck Depression Inventory (BDI-II), and Visual Analogue Scales (VAS) for post-prandial satiety, hunger, and desire to eat.

The following tables, divided into acute and chronic study phases, summarize all procedures included in the protocol, indicating whether each is study-specific or part of standard clinical practice, and distinguishing between healthy volunteers and overweight patients.

Acute phase

Type of assessment	**Description**	**Assessment time (days/months/years)**	**Is it clinical practice or study-specific?**	**If already in clinical practice, is the timing different?**
Anthropometric Measurements	Weight, height, and abdominal circumference will be measured to assess body composition.	Assessments are conducted at Week 0	Clinical practice for overweight patients; Study-specific for healthy volunteers	No
Physical Activity Evaluation	The IPAQ Questionnaire will be administered to assess participants' habitual movement and exercise patterns.	Assessments are conducted at Week 0	Clinical practice for overweight patients; Study-specific for healthy volunteers	No
Metabolic Risk Assessment	The FINDRisk and MASLD Risk Assessment Questionnaires will be used to evaluate the likelihood of developing metabolic disorders, including type 2 diabetes and metabolic-associated fatty liver disease (MASLD).	Assessments are conducted at Week 0	Study-specific for both healthy volunteers and overweight patients	
Dietary Habits Analysis	The PREDIMED Questionnaire will be administered to assess adherence to the Mediterranean diet and provide insights into overall dietary quality.	Assessments are conducted at Week 0	Study-specific for both healthy volunteers and overweight patients	

Food Preferences and Eating Attitudes	The HTAS questionnaire will be used to evaluate participants' initial perceptions of food based on health-related and sensory attributes, establishing a baseline for comparison with post-consumption responses.	Assessments are conducted at Weeks 0, 1, 2, 3, 4, and 5	Study-specific for both healthy volunteers and overweight patients	
Postprandial Cravings and Rewards	Participants will report their postprandial cravings and rewards using a 9-point hedonic scale and the Leeds Food Preference Questionnaire.	Assessments are conducted at Weeks 0, 1, 2, 3, 4, and 5	Study-specific for both healthy volunteers and overweight patients	
Post-prandial satiety ratings, including rebound hunger	Hunger, desire to eat, fullness, and prospective food intake will be assessed using Visual Analogue Scales (VAS).	Assessments are conducted at Weeks 0, 1, 2, 3, 4, and 5	Study-specific for both healthy volunteers and overweight patients	
Postprandial Food Intake	A 24-hour dietary recall will be conducted to assess participants' food intake following the consumption of the test product, allowing researchers to track both the types and quantities of food consumed.	Assessments are conducted at 24 hours into weeks 1, 2, 3, 4, and 5	Clinical practice for overweight patients; Study-specific for healthy volunteers	
Gastrointestinal Symptoms	Participants will report any symptoms of gastrointestinal discomfort, bloating, or dyspepsia occurring within the first 24 hours after ingestion using the Nepean Dyspepsia Index, a standardized tool for assessing digestive issues.	Assessments are conducted at 24 hours into Weeks 1, 2, 3, 4, and 5	Study-specific for both healthy volunteers and overweight patients	
Allergic or Hypersensitivity Reactions	Participants will complete a 72-hour recall questionnaire to document any adverse reactions they may have experienced following product consumption.	Assessments are conducted at 72 hours into Weeks 1, 2, 3, 4, and 5	Study-specific for both healthy volunteers and overweight patients	

Chronic phase

Type of assessment	Description	Assessment time (days/months/years)	Is it clinical practice or study-specific?	If already in clinical practice, is the timing different?
Anthropometric Measurements	Weight, height, and circumferences (abdominal, waist, hip, arm, and neck) will be recorded to assess body composition.	Assessments are conducted at Week 0 (Day 0), Weeks 8–9 (Days 56–63), and Weeks 17–18 (Days 119–126).	Clinical practice for overweight patients; Study-specific for healthy volunteers	No
Anamnestic Recall of Used Medications	Participants will be asked to provide a detailed account of any medications they are currently taking.	Assessments are conducted at Week 0 (Day 0) and Weeks 17–18 (Days 119–126).	Clinical practice for overweight patients; Study-specific for healthy volunteers	No
Physical Activity Evaluation	The IPA Questionnaire will be administered to assess participants' habitual movement and exercise patterns.	Assessments are conducted at Week 0	Clinical practice for overweight patients; Study-specific for healthy volunteers	No
Metabolic Risk Assessment	The FINDRisk and MASLD Risk Assessment Questionnaires will be used to evaluate the likelihood of developing metabolic disorders, including type 2 diabetes and metabolic-associated fatty liver disease (MASLD).	Assessments are conducted at Week 0	Study-specific for both healthy volunteers and overweight patients	
Dietary Habits Analysis	The PREDIMED Questionnaire will be administered to assess adherence to the Mediterranean diet and provide insights into overall dietary quality.	Assessments are conducted at Week 0	Study-specific for both healthy volunteers and overweight patients	
Blood Pressure Measurements	Both systolic and diastolic blood pressure will be measured to monitor cardiovascular health and track any potential changes that may occur during the intervention,	Assessments are conducted at Week 0 (Day 0), Weeks 8–9 (Days 56–63), and Weeks 17–18 (Days 119–126)	Clinical practice for overweight patients; Study-specific for healthy volunteers	No
Fecal Microbiota Sample	A fecal sample will be collected to examine any changes in microbiota composition, providing valuable insights into how dietary intervention may influence gut health. The sample will be sent to UNITO for analysis using DNA-based whole-genome shotgun sequencing.	Assessments are conducted at Week 0 (Day 0) and Weeks 17–18 (Days 119–126)	Study-specific for both healthy volunteers and overweight patients	
Liver Fat Content	Liver fat will be assessed using CAP technology, which employs hepatic elastometry	Assessments are conducted at Week 0 (Day 0), Weeks 8–9	Clinical practice for overweight patients; Study-	Yes: in clinical practice this non-invasive assessment

	(FibroScan®). This will allow for the tracking of any changes in liver fat, a key indicator of metabolic health.	(Days 56–63), and Weeks 17–18 (Days 119–126)	specific for healthy volunteers	is carried out every 1-2 years
Biochemistry Tests	A blood sample will be collected to measure a variety of biomarkers, including CBC; Glucose, insulin and HbA1c levels; Lipid profile; Serum uric acid, plasma urea, and creatinine; Liver function tests; Microalbuminuria; CRP and serum IgE levels.	Assessments are conducted at Week 0 (Day 0), Weeks 8–9 (Days 56–63), and Weeks 17–18 (Days 119–126)	Clinical practice for overweight patients; Study-specific for healthy volunteers	In clinical practice they are repeated at time intervals ranging between 4 to 12 months
24-hour urine collection	Urinary nitrogen	Assessments are conducted at Week 0 (Day 0), Weeks 8–9 (Days 56–63), and Weeks 17–18 (Days 119–126)	Study-specific for both healthy volunteers and overweight patients	
Post-prandial Satiety Ratings, including rebound hunger	Hunger, desire to eat, fullness, and prospective food intake will be assessed using Visual Analogue Scales (VAS).	Assessments are conducted at Week 0 (Day 0), Weeks 8–9 (Days 56–63), and Weeks 17–18 (Days 119–126)	Study-specific for both healthy volunteers and overweight patients	
Dietary plan	Dietary plan, personalized according to the nutritional needs of each volunteer and patient, based on the Mediterranean diet model. These plans include the consumption of edible insect-based bread or edible seaweed burgers and/or legume-based bread and/or fish-based burgers five times per week, to be followed for a duration of two months.	Assessments are conducted at Week 0 (Day 0) and Weeks 8–9 (Days 56–63)	Study-specific for both healthy volunteers and overweight patients	
Food Diary	Participants will maintain a food diary throughout the study, which will be reviewed by a researcher dietitian to ensure adherence to the prescribed dietary intervention. This will help researchers monitor compliance and evaluate the impact of the diet on health outcomes.	Assessments are conducted at Weeks 8–9 (Days 56–63), and Weeks 17–18 (Days 119–126)	Clinical practice for overweight patients; Study-specific for healthy volunteers	No
Gastrointestinal Side Effects	Participants will report any gastrointestinal discomfort, bloating, or dyspepsia using the Nepean Dyspepsia Index. This standardized tool will help track any gastrointestinal symptoms associated with the food prototypes consumed during the study.	Assessments are conducted at Weeks 8–9 (Days 56–63), and Weeks 17–18 (Days 119–126)	Study-specific for both healthy volunteers and overweight patients	

Questionnaires on Neophobia and Mood Disorders	Participants will complete the Food Neophobia Scale to assess food-related fears and the Beck Depression Inventory (BDI-II) to evaluate mood disorders throughout the study. These questionnaires will help assess how the intervention may impact on participants' psychological well-being and food-related behaviors.	Assessments are conducted at Week 0 (Day 0), Weeks 8–9 (Days 56–63), and Weeks 17–18 (Days 119–126)	Study-specific for both healthy volunteers and overweight patients	
Cognitive Function Assessments	Participants will complete Web-based cognitive and psychological tasks designed to assess memory, attention, and executive functioning. These tasks will help determine whether the dietary intervention influences cognitive performance through the microbiota-gut-brain axis, providing valuable insights into the potential cognitive benefits of the diet.	Assessments are conducted at Week 0 (Day 0) and Weeks 17–18 (Days 119–126).	Study-specific for both healthy volunteers and overweight patients	

4.6 Funding

Is the study funded?

☐ No

☐ Yes, with internal funds

☑ Yes, by institutional third parties

☐ Yes, by private third parties

The project is funded by PRIMA (Partnership on Research and Innovation in the Mediterranean Area), under Article 185 of the European Union framework. Study costs will be fully covered by dedicated funding.

4.7 Data management

The clinical data required by the protocol will be collected in pseudonymised form by the staff designated by the Principal Investigator in an electronic Data Collection Form (CRF) and will be managed through the UNIBO REDCap platform.

4.8 Statistical Analysis Plan (SAP)

4.8.1 Analysis methodology.

A full descriptive analysis of the sampled data will be carried out. Categorical data will be reported as absolute number and percentages. The normality distribution of the continuous variables will be tested by Shapiro-Wilk test. The normally distributed variable will be described as mean+/-standard deviation, while not normally distributed ones by median (95% Confidence Intervals). All the analyses will be repeated by randomization group, gender and weight status.

Acute phase.

Primary outcomes: analyses will include repeated measures ANOVA or mixed-effects regression models adjusting for covariates, to compare novel protein food product conditions vs. ordinary food product control in the within subject design. A non-inferiority testing approach will be used in the analysis, with a 95% confidence one-side interval to reject the null hypothesis, e.g. the novel food product produces a significantly lower subjective satiety score than the control food product.

Secondary outcomes: analyses will be as for the primary end-points and/or descriptive statistics.

Chronic phase.

Primary outcomes: analysis of temporal changes in metabolic profile (glucose, insulin, triglycerides, cholesterol) will be carried out by a repeated measures ANOVA to assess differences between experimental and control diet. Comparative incidence and severity of gastrointestinal symptoms will be assessed by means of repeated measures ANOVA for each of the two subscales (symptoms and quality of life). A 95% confidence two-sides interval will be considered to reject the null hypothesis.

Secondary outcomes: analyses will include descriptive statistics; repeated measures ANOVA or multiple regression models, to compare non-categorical variables; Mc Nemar and Mantel-Haenszel Chi-square tests, and logistic regression to compare categorical variables.

Fecal microbiota analysis (Turin): Correlation analyses, sample clustering and statistical analyses will be carried out in R environment. Kruskal–Wallis and Mann–Whitney tests will be used to find significant differences in microbial taxa abundance, and alpha diversity according to the different types of diets.

4.8.2 Risk factors, confounders and effect modifiers.

Acute phase. All bread portions will be comparable in terms of energy content in order to minimize effect modifiers related to energy density.

Chronic phase. All study products will have similar energy as well as protein content, to minimize effect modifiers in body composition. Also, the Mediterranean diet plans will have similar bromatological (macronutrient) composition in order to reduce - to a reasonable extent - variability in metabolic parameters.

4.8.3 Criteria for Selection and Matching of Controls

Acute phase

Each subject will be her/his own control, since a Latin Square cross over design will be employed to assign the food sequence, with eight subjects (healthy volunteers or overweight patients) per sequence. For instance, the first eight subjects (healthy volunteers or overweight patients) will follow the sequence B1-B2-B3-B4-B5 (control), the next eight subjects (healthy volunteers or overweight patients) will follow B2-B3-B4-B5 (control)-B1, and so on. This approach will ensure that each participant (healthy volunteers or overweight patients) is exposed to every product in a systematic and unbiased manner. The randomization will be stratified by gender and weight status, ensuring an even distribution of characteristics across all sequences, thus minimizing potential confounding variables in the study.

Chronic phase

Each subject will be her/his own control, since this is a within-subjects crossover trial. Healthy volunteers and overweight patients will be randomized, in order to be assigned to a Mediterranean diet (Group A) with the inclusion of control products (PRODUCT A – legume bread and/or ordinary fish burger), or the same diet with the inclusion of one of the two novel food products: PRODUCT B (bread with edible insect protein, Acheta domesticus) or PRODUCT C (edible seaweed burger).

A Latin Square cross over design will be used to assign the food sequence, with 10 subjects (healthy volunteers or overweight patients) per sequence. For example, the first 10 subjects (healthy volunteers or overweight patients) will follow the sequence A-B, the next 10 will follow the sequence B-A, the third 10 will follow the sequence A-C, and the last 10 will follow the sequence C-A. This design will ensure a balanced comparison of the products across healthy volunteers and overweight patients.

Each sequence will be stratified by gender and weight status (normal weight/overweight), enabling an effective analysis of potential group differences. The study will compare health and dietary parameters at three key time points: baseline, month 2, and month 4 of the intervention.

4.8.4 Data sharing

The anonymized data will be uploaded to the Zenodo repository [https://zenodo.org/], which is the Open repository for EU-funded research outputs from Horizon projects, according to the specific request of the PRIMA-European Commission.

The data may be made available to other researchers in the form of aggregated and/or anonymized data.

5. ADMINISTRATIVE PROCEDURES AND DECLARATIONS

5.1 Informed consent and consent to personal data processing

The study protocol, any amendments to the protocol, informed consent, consent to the processing of personal data and any other patient information must be approved by the Ethics Committee.

To participate in the study, each patient must provide written informed consent as well as consent to the processing of their personal data.

5.1.1 Procedure for obtaining informed consent and consent to process personal data

For enrolled patients, Informed Consent and study participation will be obtained during a visit scheduled as part of the normal care pathway at the Endocrinology Unit.

For healthy volunteers, Informed Consent and study participation will be obtained at DISTAL, and the signing of the informed consent will take place in the presence of a medical doctor.

5.1.2 Phase of the study during which consents will be sought

☑ Screening

☐ Baseline

☐ First follow-up visit.....

5.2 Study-specific insurance

A study-specific insurance policy will be activated due to the interventional nature of the study and the use of specific food products.

5.3 Amendments to the protocol and changes to the conduct of the study

Any changes to the protocol will be made in the form of an amendment, to be submitted to the Ethics Committee. No other changes to the protocol are permitted during the study period. Any unforeseen changes in the conduct of the study will be recorded in the Clinical Study Report.

5.4 Study pubblication

The investigator undertakes to:

- notify the conclusion of the study
- submit the study for publication to a peer-reviewed journal within 12 months of study completion and regardless of the nature of its results

To this purpose, the study will be registered on an Open Science platform (i.e clinicaltrials.gov). Any formal submission or publication of data derived from this study must be understood as a publication by the Investigator.

5.4.1 Strategies for disseminating study results

The results of the clinical study will be presented at national and international conferences in the form of aggregated and/or anonymized data. Additionally, the findings will be published in peer-reviewed, indexed journals, with the data always reported in an aggregated and/or anonymized format.

5.5 Documentation archive

The investigator is responsible for the archiving and preservation of the essential documents of the study, before, during and after the conduct and completion or termination of the study, in accordance with the provisions of the current regulations and GCP and their timelines.

The data collected in the CRF will be in strictly pseudonymous form and the subject will only be identified by a number/code.

The Investigator should keep the original patient data and the signed written informed consent in a safe place to ensure confidentiality and privacy is maintained. In particular, the original signed consent will be stored in the Investigator Site File (ISF).

5.6 Inspections/audits

If a Regulatory Authority requests an inspection, the investigator must immediately inform the Ethics Committee.

5.7 Reference persons

The phone numbers and emails of the contact people for conducting the study can be found in the Investigator Folder at the centers.

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