UNIVERSITY OF THE
AEGEAN
DEPARTMENT OF FOOD SCIENCE AND NUTRITION

Nutritional Intervention-Clinical Trial Protocol

Title: Postprandial Inflammatory and Metabolic Responses Induced by the Authentic Ladotyri Mytilinis PDO Cheese and Italian Parmesan PDO Cheese

NCT IDENTIFIER: NCT05788887

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1. Protocol Summary

This is the protocol of a randomized, blinded and controlled nutritional intervention – clinical study to investigate the postprandial effect of consuming Authentic Ladotyri Mytilinis, PDO cheese on the lipid, glycemic profile and the oxidative status of the blood of healthy volunteers.

The acute nutritional intervention – clinical study is a cross over design (acute study), with two axes. The purpose of this study was the investigation of the postprandial metabolic responses, after the intake of a traditional, Ladotyri Mytilinis cheese, compared to corresponding responses after an Italian Parmesan cheese consumption, in healthy participants.

The study volunteers will be healthy, aged 18-65 years, residing during the intervention in Lemnos. The study will take place in the specially designed space of the Human Nutrition Unit, University of the Aegean and in voluntary collaboration with the General Hospital of Limnos. The duration of the study will be 7 days.

Participants will be randomly divided into two groups. During the first day, the first group will consume Italian Parmesan, PDO cheese (control group) and the second group will consume Authentic Ladotyri Mytilinis, PDO cheese (intervention group), together with two slices of bread, while 1 glass of water will be available for each volunteer [1st test period]. After a 7-day washout period, the same volunteers will come to the specially designed area and the process will be repeated, with the difference that the control group of the 1st day will belong to the intervention group, and the intervention group will belong to the control [2nd test period].

In each test period, blood samples will be taken from all consecutive volunteers in a fasted state, as well as 30 minutes, 1.5 and 3 hours after consuming meals. This will be followed by analysis of the samples and statistical processing of the results of the analyzes to present data regarding the acute effect of meal consumption on the postprandial biomarkers of lipemia, glycemia and oxidative stress in healthy volunteers.

2. Theoretical background and study objectives

2.1. Argumentation, Research evidence, Rigor of study

The existing scientific evidence indicates a correlation of the levels of postprandial lipid, glycemic biomarkers and oxidative stress markers, with chronic diseases, such as cardiovascular diseases, diabetes, etc. (Koutelidakis and Dimou, 2016). Recent nutritional interventions by the team in healthy volunteers, but also participants at high metabolic risk, have shown a beneficial effect of consuming functional foods, such as a functional spreadable cheese, enriched with mountain tea and orange peel, but also a functional refined olive oil, fortified with antioxidants components from orange peel, on postprandial markers of lipemia, glycemia and oxidative stress (Papagianni et al, 2021).

The findings of previous clinical studies-dietary interventions suggest that the consumption of a meal containing cheese can cause a significant increase in the concentration of triglycerides, 2 hours after taking the meal (Drouin-Chartier et al., 2016).

Olive oil, the main characteristic of the Mediterranean Diet, thanks to the bioactive compounds it has in its composition, has been extensively studied in recent years, in vitro and in vivo, highlighting its antioxidant, anti-inflammatory and other actions (Jędrusek-Golińska et al., 2020). In vitro evidence shows that cheese enriched with polyphenols may contribute to maintaining the integrity of polyphenols and antioxidant activity in the gastrointestinal environment (Lamothe et al, 2016). The postprandial effect of consuming functional foods rich in polyphenols (e.g. olive oil, grapes) in combination with meals rich in fats and carbohydrates has been evaluated with promising results, since they seem to have a beneficial effect on metabolic biomarkers and pathways, postprandial (Magrone et al., 2013).

Based on the aforementioned scientific evidence regarding the possible postprandial effect of the bioactive components of olive oil (in which the cheese is extracted for the production of the oil cheese) on specific biomarkers, the present nutritional intervention will be composed.

2.2. Study Objectives / Research Questions

Purpose

The purpose of this study was the investigation of the postprandial responses on serum total, HDL-, LDL- cholesterol, triglycerides, uric acid, glucose and plasma Total Antioxidant Capacity (TAC), after the intake of a traditional, Authentic Ladotyri Mytilinis cheese, compared to corresponding responses after an Italian Parmesan cheese consumption, in healthy participants.

Research Questions

The research questions of this clinical study are the following:

- ✓ Which is the acute effect of ALM-PDO cheese consumption, in the framework of a high-fat and carbohydrates meal, on the postprandial biomarkers of lipemia, glycemia and oxidative stress?
- ✓ Does the postprandial inflammatory and metabolic responses to ALM-PDO cheese differs, compared to Italian Parmesan PDO cheese? LDL- cholesterol and triglycerides determined at 3h after the meal intake, was set as the primary endpoint of the present, pilot study.

3. Summary presentation / study design

3.1. Overview

A nutritional intervention – clinical study of self-comparison design (cross over design: acute study) will take place in a sample of healthy volunteers, where the difference in the effectiveness of the nutritional intervention will be evaluated in the same volunteer. This means that the response of lipemia, glycemia and oxidative stress biomarkers to the intervention meal will be compared with their response to the control meal, in the same volunteer each time. The specific research will be carried out in the specially designed area of

the Human Nutrition unit of the University of the Aegean and in collaboration with the hospital of Lemnos, with which a collaboration protocol has been concluded with the University of the Aegean.

3.2. Study design

In a total sample of approximately 10 healthy volunteers, the number of which was determined through a special statistical program for calculating the required sample of the research to draw safe and reliable conclusions, a nutritional intervention – clinical study of cross-over design will be carried out. The study for each participant will be 7 days long, including the washout period.

Each participant during the first test period and in a state of 12-hour fasting, will be asked to come in the morning hours of 9 am. - 12:30 p.m., the specially designed blood collection room of the Human Nutrition Unit, Department of Food Science and Nutrition, University of the Aegean. In this specially designed area, a blood sample (baseline) will be taken by a doctor or competent staff of the Lemnos Hospital. This will be followed by a discussion with the healthy volunteer, signing of a declaration of participation, filling in a questionnaire and a 24-hour recall of food consumption. The questionnaire that each volunteer will fill out on the first day of the start of the study concerns their medical history and general information about their habits (especially dietary). Afterwards, a meal will be served that will include 2 slices of bread (80g- 40g each) and 2 pieces of cheese (100g- 50g each piece), according to the group each volunteer belongs to, accompanied in each case by 1 glass of water. The consumption of each meal will be completed within the time period of 15 minutes by all participants (controlled). Finally, the following blood samplings will be carried out for the volunteers, which will be conducted 30 minutes, 1.5 and 3 hours after the completion of the meal.

After a seven-day washout period, on the eighth day (2nd test period) the same participants will be asked to come again, in a fasting state, to the specially designed area of the University of the Aegean, at the predetermined morning hours to repeat the procedure nutritional intervention and blood sampling. In other words, a blood sample will be taken by a doctor or competent staff of the Limnos Hospital, followed by a discussion with the volunteer and completion of a 24-hour recall of food consumption questionnaire. The questionnaire that the participants will complete at the start of the second test period is to record their specific eating habits during the washout period (ie the interval between the two periods of the intervention). The meals served to the volunteers are determined by the control or intervention group to which they belong. After each meal is consumed within the 15-min timeperiod, subsequent blood sampling will be done for participants 30, 90, and 180 minutes (min)after the completion of the meal .

4. Enrollment of participants and case of study withdrawal

The goal is to gather an initial sample of people from the Greek area in order to achieve the collection of a required number of cases for study. The research team will then review the inclusion/exclusion criteria and select the participants who will receive the meals in the two test periods of the study.

4.1. Sample size calculation

In order to note a 40% difference in the postprandial response of triglycerides and/or LDL-cholesterol and in order to have an 80% chance of a statistically significant difference between the studied groups at a significance level of 5%, a necessary sample of 10 subjects was calculated.

4.2. Study inclusion criteria

The criteria that must be met by those interested in conducting the study are the following:

- Age between 18 and 65 years
- Willingness to join and complete the nutritional intervention
- There should be no history of loss of consciousness in previous blood draws as well as personal reasons for not being able to donate blood (e.g. fear)
- Who will sign a written consent to participate in the study

4.3. Exclusion criteria from the study

Participants meeting, at baseline, the following exclusion criteria will be excluded from the study for reasons of safety as well as efficacy of the study:

- ✓ Inability or unwillingness to provide informed consent
- ✓ Age <18 and >30 years
- ✓ Three-month nutritional supplement
- ✓ Chronic diseases history
- ✓ Hemoglobin A1c - HbA1c > 5.7 %
- ✓ Abnormal Body Mass Index (BMI) (> 25 kg / m2)
- ✓ Abnormal hematological or bio-chemical profile (total cholesterol > 240 mg/dL, triglycerides > 250 mg/dL, glucose > 100 mg/dL)
- ✓ Alcohol overdose (> 40 g alcohol / day)
- ✓ Heavy smokers (> 10 cigarettes / day)
- ✓ Concurrent participation in another intervention study or unconsciousness in previous blood draws

4.4. Strategy for participant inclusion and retention

Finding the study participants will be done by the University and/or the wider local community of Lemnos. The volunteers will be either students or members of the teaching staff of the University of the Aegean or residents of the island. One of the members of the research coordinators will undertake the briefing of each healthy volunteer, which will take place in a special area of the University. Those who will meet all the criteria of the study in order to be accepted and at the same time agree in writing to participate in it, on the days that they will come to this special area, they will have the possibility at any time to ask for help either from the main researcher or from anyone else research representative to record any difficulties or questions they may have. The members of the research team will be responsible for timely

informing the participants, in writing or by telephone, of the visits and the biological samples to be collected.

The scientific manager of the study or his representatives will be responsible for the safekeeping of the data and results. The anonymity of the participants will be preserved and the safe keeping of the history of the healthy will be ensured, to which only the personnel who will be employed in this research will have access.

4.5. Participant withdrawal criteria

Participants are free to withdraw from the study at any time. Accordingly the researcher will be able to ask the participant to leave the study if:

o Clinical adverse events, laboratory abnormality, or other medical condition or condition are identified that endanger the participant from remaining in the study

o Participants meet one of the aforementioned exclusion criteria (either newly developed or not previously identified) that precludes further participation in the study

o Participants appear to not adequately adhere to protocol requirements

4.6. Suspension or early termination of the study

This study may be suspended or terminated early only if there is sufficient cause. A written notice documenting the reason for stopping or suspending the study will be provided by the person responsible for this decision (research team, hospital).

5. Study Intervention

5.1. Description of intervention

As part of a meal that will include two slices of bread of 80 grams (g), and a glass of 250 ml of water, the control group will consume two pieces of Italian parmesan cheese (PDO), total weight of 100 grams (g), while the intervention groups will consume corresponding bread meal, where the control cheese will be replaced with 100 grams (two pieces) of Authentic Ladotyri Mytilinis cheese (PDO). The meals will be prepared at the Nutrition and Public Health laboratory of the Human Nutrition Unit, University of the Aegean.

For the selected foods, there does not seem to be any data suggesting that they may have a possible risk or negative impact on health, as on the one hand there is no confirmed adverse effect of its individual components on health based on the scientific literature and on the other hand it appears according to corresponding research in similar foods the potential beneficial role of their biofunctional components in health promotion.

5.2. Assessment of participant compliance for administration

The assessment of the compliance of the participants in the study with regard to the administration of cream cheese, will be achieved following continuous supervision by a member of the research team, of the volunteers who will come to the University site to consume all the meals.

6. Study Procedures

6.1. Questionnaires

A specially designed questionnaire, based on bibliographic data, will be used to collect important data regarding the participants of the study.

The questionnaire will concern the clinical condition and habits (especially nutritional) of the volunteers of the clinical study and will include the following parts:

1st part

General socio-demographic and anthropometric data of the study population (gender, age, anthropometric indicators)

2nd part

Clinical status of each participant (medication, dietary supplements, etc.).

3rd part

Dietary and other habits before the start of the intervention, as well as more specific dietary habits for the washout period.

6.2. Biological samples

The collection of blood samples, by a doctor or competent staff of the Limnos Hospital, of the healthy volunteers of the clinical study will be used to determine indicators of postprandial lipemia, glycemia and oxidative stress in the blood (plasma antioxidant capacity, lipid profile specifically triglycerides, HDL & LDL cholesterol, glucose, etc.) that will be analyzed using a special biochemical analyzer.

6.3. Statistical analysis

After the above analyzes have been carried out using a special biochemical analyzer, the use of the statistical package SPSS (Statistical package for social sciences: SPSS) will follow in order to record, process and analyze the results of the study. The Kolmogorov–Smirnov test was applied to check normality. The main participants' characteristics will be analyzed by descriptive statistics. Differences in baseline values between groups for each biomarkertested, will be analyzed using a paired sample two tailed t test. Repeated ANOVA measures with Geisser–Greenhouse correction will be conducted, for serum total, HDL-, LDL- cholesterol, triglycerides, glucose, uric acid and plasma Total Antioxidant Capacity (TAC). If time or group was significant, multiple comparison post hoc Bonferroni test will be performed for the comparison of concentrations. To check changes in the metabolic biomarkers from baseline to postprandial responses (within-group variation), the Wilcoxon sign-rank test will be carried out.

7. Maintenance of study records

The study files will remain securely registered for a maximum of 3 years from the date of their registration in special areas of the Human Nutrition Unit, University of the Aegean. Study records should be kept for a maximum of 2 years after the last marketing approval, or longer if required by local regulations.

8. Ethics, Consent and Privacy Statement

8.1. Ethical standards

The members of the research team agree that they will provide their professional expertise and knowledge to conduct the study in accordance with the requirements of the protocol and in accordance with the provisions of the Declaration of Helsinki and the provisions of the General Data Protection Regulation.

8.2. Rules of ethics and research ethics

During the conduct of this clinical study, all fundamental rules of ethical, scientific and research ethics will be observed. Therefore, an application for the approval of the clinical study will be made by the Bioethics and Research Ethics Committee of the University of the Aegean, with the research volunteers also being informed about its purpose, theconfidentiality of the data and the voluntary nature of their participation. Participants will take part in the study after signing a consent form to participate in it.

8.3. Formal consent process

Formal consent in the clinical study is a process that will begin before the enrollment of the interested parties in the study and will continue throughout their participation in it. Those interested will be provided with all the information regarding the benefits and potential risks of the study, as well as a consent form where the necessary procedures will be presented. Every interested party must read it and review it or, in special cases (e.g. blind) be informed by third parties. A copy will be provided to interested parties. The researcher or representative will explain the study and its objectives to each participant and address any questions they may have.

The rights and care of the subjects will be protected by the fact that the quality of their clinical care will not be affected in any way if they refuse to participate in this clinical study. This process will take place at the University where this research will take place.

8.4. Privacy of study participants

Confidentiality will be maintained by the researchers or representatives of the research team. Confidentiality will support the conduct and study of biological samples and genetic tests, as well as any study information concerning participants. The study protocol, documentation, data, as well as other information will be conducted with absolute confidentiality, prohibiting their disclosure to third parties.

9. References

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CONSENT FORM

for the nutritional intervention with the title: " Postprandial Inflammatory and Metabolic Responses Induced by the Authentic Ladotyri Mytilinis PDO Cheese and Italian Parmesan PDO Cheese"

I hereby declare that I agree to participate as a volunteer in the dietary intervention - clinical study with the aim to investigate the postprandial inflammatory and metabolic response induced by the consumption of authentic Mytilini cheese, in biomarkers of healthy volunteers.

The study will last 7 days and I will need to visit the premises of the University of the Aegean two (2) times after consultation with the researchers. During my visits to the premises of the University of the Aegean and in the context of the clinical study-nutritional intervention, I will

receive a meal that will include either Authentic Ladotyri Mytilinis PDO cheese or Italian Parmesan PDO cheese, amounting to 100 grams (g), together with two slices of bread of 80 grams (g) and a glass of 250 ml water.

Before the administration of the above meals, but also 30 minutes (min), 1.5 and 3 hours (h) after their completion, blood will be taken from me by a cooperating doctor, with the aim of using it in measuring biomarkers (antioxidant capacity, lipid profile, glucose, etc.). I will also need to complete questionnaires to assess my clinical condition and nutritional habits.

My participation in the study is voluntary, solely my choice, and no remuneration or other compensation is provided. I can withdraw from the study at any time, without giving any reason and without any consequences.

By signing this document and filling in the boxes below, I agree and give my consent to the following:

1. As a participant I have first been informed about the study, had the opportunity to review the information, decide about my participation, ask questions and receive satisfactory answers. ☐

2. The purposes of the research are fully understood and have been adequately explained to me. ☐

3. I understand what is proposed and the procedures in which I am to participate. ☐

4. I understand that my participation in this study and in particular the data from this research will remain strictly confidential. Only the researchers involved in the study will have access to the data which will not be given to others. ☐

5. I know how the results will be used upon completion of the research program. Personal data will not be included in the results and publications. ☐

For any additional information and assistance, the possibility of a planned visit to a special area of the University of the Aegean, where the study will take place, is provided.

	The volunteer	***The researcher***

Name & Surname		
Signature		
Date		