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Research Article

Bakery Products Enriched with *Eruca Sativa Mill* Defatted Seed Meal Ameliorates Systemic Markers of Inflammation and Glucose and Lipid Metabolism in Adults- A Pilot Study

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Abstract

Introduction: Most patients with metabolic syndrome present non-alcoholic fatty liver disease (NAFD). High consumption of fruit and vegetable has been related to a lower incidence of chronic diseases. Of note, among vegetables, brassicaceae family is at the first place. Therapeutic strategies to treat inflammatory disease as well as cancer are evolving and includes biologically active phytocompounds. A sulfur-containing glucosinolate (GLS) secondary plant compounds exclusively present in Brassicaceae- and their breakdown products including isothiocyanates (NCS) have shown to possess important anti-inflammatory and anti-carcinogenic property. The aim of this work was the evaluation of the effects of daily consumption of E. sativa defatted seed meal enriched bakery products on glucose and lipid metabolism and on systemic markers of inflammation.

Material and Methods: After an 8-week run-in period, patients were asked for 4 weeks to maintain their usual diet replacing 150 gr. of bakery products with the same number of enriched bakery product. Fasting glucose and insulin, HOMA-r, total cholesterol, HDL, LDL, triglyceride, ALT, Gamma-GT, high sensitivity C reactive protein, Interleukin-6, Interleukin-8 and TNF-α were compared before and after the study period.

Results: HOMA-r showed a statistically significant reduction after 4 weeks period as well as cholesterol ratio 3.8 ± 1.0 vs. 3.6 ± 1.0 ($P = 0.03$). Among markers of inflammation, high sensitivity CRP showed a statistically significant reduction (-36.8%) from 1.4 (0.4 - 10.4) to 0.9 (0.4 - 8.7) mg/dl ($P = 0.02$) as well as TNF-α 8.0 ± 3.0 vs. 7.1 ± 2.7 pg/ml (- 11.3%; $P < 0.001$). Despite a mild ALT reduction (- 7.0%), Gamma-GT showed a - 21.0% decrease from 20.0 ± 6.6 to 15.8 ± 6.4 U/L ($P < 0.001$). IL-6 and IL-8 were substantially unmodified.

Conclusion: as cracker can improve glucose and lipids metabolism in parallel with a significant improvement of serum Bakery products enriched with *Eruca sativa Mill* defatted seed meal markers of inflammation and therefore being of interest for patients affected by inflammatory diseases.

Keywords: Bakery Products; *Eruca Sativa Mill*; Glucose and Lipid; Seed Meal

Introduction

Considerable epidemiological evidence suggests that the consumption of brassicaceae is associated with a reduced cancer and cardiovascular diseases risk in humans [1,2]. Brassicaceae enriched diets have also been associated with modifications in plasma lipid and cholesterol profiles [3,4], which may be due, at least in part, to the action of isothiocyanates. These molecules are glucosinolate metabolites which can reduce: *oxidative stress*, principally through the modulation of P450 (CYP) family and the concomitant activation of several antioxidant and phase II enzymes via ARE (antioxidant response element in the promoter of phase II gene, binding site for Nrf2 transcription factor) [1]; *inflammation*, mainly through the direct inhibition of NF-kappaB-binding to DNA and consequent inhibition of cyclo-oxygenase 2 (COX-2) expression or through the modulation of inflammasome activity [5,6]; endothelial dysfunction, likely upregulating Nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent heme oxygenase (HO)-1 and glutamate cysteine ligase [3]; alteration of lipid metabolism with *in vivo* reversion of *non-alcoholic fatty liver disease* (NAFLD) enhancing the phosphorylation of AMPK and acetyl CoA carboxylase [7] or through the control of FAD levels and the modulation of mitochondrial function and citrate release from the Krebs cycle [8]. Sulforaphane (SFN) is the most studied isothiocyanate occurring in Brassicaceae, especially in broccoli and Tuscan black kale sprouts [9,10] Erucin (ERU), an isothiocyanate structurally related with SFN and typically present in the form of its precursor, glucoerucin, in wild rocket (*Eruca sativa* Mill.) seeds, is still less studied in comparison with SFN, even if it was recently recognized as a long-lasting and thiol-dependent H2S-donor, with vasorelaxing effects and antihypertensive activities and with antioxidant and antiinflammation properties in particular in vascular inflammation conditions [11]. ERU is produced from the glucosinolate glucoerucin, by the plant enzyme myrosinase as an endogenous defense mechanism against pathogens, insects and generalist herbivores. Noteworthy, ERU is also produced in the human body after the biotransformation of SFN through the reduction of the sulfur atom [12]. In a previous work, an *E. sativa* ecotype has been selected as the optimal source of glucoerucin to produce food ingredients for the formulation of functional crackers standardized in glucosinolates [13]. The aim of this work was the improvement of crackers recipes and the evaluation of the effects of daily consumption of *E. sativa* defatted seed meal enriched bakery products on glucose and lipid metabolism and on systemic markers of inflammation.

Material and Methods

Glucosinolate enriched crackers, nutraceutical improvement of creacker recipes:

Crackers were produced in the R and D and QA of Colussi-Group (Milan, Italy) by adding *E. sativa* defatted seed meal (DSM) to a standard cracker dough mixed with wheat flour baked at 200 ◦ C in an industrial plant. The operative conditions common to all the tests were: i) 5 minutes, time of mixing; ii) 25°C, temperature of mixing; iii) 4 minutes, time of cooking; iv) 220°C, temperature of cooking; 20 minutes cooling phase, from 140°C to 40 °C. Crackers were made with 85% wheat flour, 10% vegetable oil, 1% malt extract, 0.5% brewer's yeast, 0.5% ammonium bicarbonate. Average nutritional values per 100 g of product were: energy value 439 kcal (1837 kj); protein 11.2 g; Carbohydrates 70 g, of which sugars 3 g; Fats 11.9 gr, of which saturates 5.9 g; 3.6 g dietary fiber. Further informations on the recipes are commercially confidential and their property is of Colussi-Group. *E. sativa* seeds were from CREA-CI Brassicaceae collection [14] and were defatted by an endless screw press. The *E. sativa* DSM full characterization was already reported in Franco., *et al*. Seven recipes were assayed for GSL uptake by using as supplement *E. sativa* DSM in presence or absence of active endogenous myrosinase. Glucosinolates in crackers were determined by HPLC-ES-MS/MS [13]. The presence of free isothiocyanates derived from the hydrolysis of glucosinolates during the step of creacker dough was assayed by extracting 0.25 g crackers in 1 ml dichloromethane under agitation at room temperature for 1 h, adding 72,4 µM benzyl isothiocyanate (BITC) (Sigma Aldrich 89983) as internal standard. After centrifugation (13.000 g for 20 min at 4 °C), the supernatants were filtered through a PVDF membrane (0.2 µm) and injected (1 µl) in a Varian Saturn CP-3800 gas chromatograph coupled with a flame ionization detector (FID), equipped with a J and W HP-5 column (Agilent technologies, 30 m. × 0.25 mm i.d, 0.25 µm film thickness). The analytical conditions were injection temperature 250°C; detector temperature 240°C; flow of the carrier gas (Nitrogen) 1 ml min^{-1}; column oven temperature initially maintained at 60°C for 4 min, followed by a gradient to 220°C, with a rate of 10 °C min^{-1}, finally held for 2 min. The split mode (1:5) was used for injection. Response coefficient 1.62 was calculated by the ratio of the slope of the calibration curves of BITC and of ERU (Santa Cruz sc-204741) dichloromethane solutions in the concentration range of 0.01 ÷ 1 mg/ml.

Verification of the capacity of an exogenous myrosinase of hydrolyzing glucosinolates in crackers

Sinapis alba defatted seeds meal, known for its good content of soluble myrosinase [15], was added to the recipe with maximal uptake of GSLs at different doses (0.01; 0.025; 0.065; 0.08; 0.014; 0.029; and 0.48 g/100 g crackers) and vortexed with 1 g crackers for 10 seconds in 4 ml artificial saliva buffer [Perdigão., *et al.*1998] at 37°C and immediately extracted in 1 ml dichloromethane containing 72,4 µM BITC as internal standard. After centrifugation the supernatants were filtered and injected (1 µl) in a Varian Saturn CP-3800 gas chromatograph coupled with a flame ionization detector (FID) as described in the previous paragraph.

Clinical Study design

All consecutive outpatients who met the inclusion criteria were prospectively enrolled into this pilot study. Study protocol involved an 8-week run-in period, during which the dietary intake of each patient was recorded, and a 4-week period during which patients were asked to maintain the same diet regimen replacing 150 gr carbohydrates with the same amount of crakers enriched with 1% *E. sativa* defatted and myrosinase inactivated defatted seed meal. At the end of the "run-in period" and after the 4-weeks consumption of the enriched bakery products, all patients underwent general clinical evaluation and serum laboratory tests. Serum laboratory tests included fasting glucose and insulin, glycated hemoglobin (HbA1c), total cholesterol, HDL and LDL cholesterol, triglyceride, ALT, Gamma-GT, creatinine, high sensitivity C reactive Protein (hs-CRP), Interleukin 6 (IL-6), Interleukin 8 (IL-8) and Tumor Necrosis Factor alpha (TNF-α). The Homeostasis model assessment for insulin resistance (HOMA-r) was calculated as "fasting insulin x fasting glucose/405". [16]; the Cholesterol ratio was calculated as Total/HDL cholesterol ratio [17]. All the subjects underwent abdominal/liver ultrasound examination of the abdomen at the beginning and at the end of the study. Moreover, all the subjects didn't change lifestyle including exercise and diet habit except for the introduction of the glicosinolate. The study was conducted in accordance with the principles outlined in the Declaration of Helsinki and according to international guidelines; Institution Review Board, namely "Ospedali Riuniti Marche Nord - AORMN Review Board", approved the study protocol (No. AORMN/21425) on September 29, 2014. All participants received information regarding the natural and potential risks of the study and gave written informed consent.

Characteristics of study population

Patients included in the study were patients with age between 18 and 75-year-old, with a BMI greater than 18.5 and lower than 30, euglycemic status (fasting glucose < 100 mg/dl) and normal to mild hypercholesterolemia (LDL cholesterol > 100 and < 160 mg/dl). Exclusion criteria were diagnosis of diabetes mellitus or dyslipidemia requiring any medication acting on glucose, lipid or biliary metabolisms, obesity (BMI ≥ 30), concomitant liver disease, abnormal renal function, uncontrolled arterial hypertension, coronary heart disease, psychiatric disease and pregnancy or planning for pregnancy. Assuming an expected 30% reduction of HOMA-r (expected mean 3.9; standard deviation 0.8) and a 0.05 α-error, 0.20 β-error (power 0.80), sample size analysis estimated the need of enrolling 20 patients.

Statistical analysis

Statistical analysis was performed using MedCalc package (v.11.5 for Windows). Categorical variables are reported as numbers (percentage); continuous variables were reported as mean ± standard deviation (SD) or in median (range) based on their distributions analyzed by the means of Kolmogorov-Smirnov's test. The unpaired Student t test (normally distributed) and Mann-Whitney's test (non-normally distributed) were used for comparison of continuous variables. P values <0.05 were considered statistically significant.

Results

Cracker design

Seven recipes of crackers were analyzed for their glucosinolate and free isothiocyanate content by HPLC-MS/MS (jca2016). As showed in Table 1 only recipes with totally heat deactivated endogenous *E.sativa* DSM myrosinase were found able to preserve good contents of GSLs in the final bakery products with recoveries of added GSLs calculated from total GSLs content in DSMs ranging from 71% to 95% of theoretical GSLs. Recipes 0 and 1 differed for food additives and even if the use of different additives seemed to preserve at least in part glucoerucin and free ERU in recipe 0, the final recovery is very low in the presence of an active myrosinase in the dough phase.

Recipes 5 was that with the maximum glucoerucin content and for this reason it was chosen for testing the ability of an exogenous myrosinase to hydrolyze GSLs in enriched crackers. Results obtained from a simulated chewing in artificial saliva buffer for 10 sec showed that myrosinase, added as *Sinapis alba* defatted seed meal, was able to release at least 20 µmol of ERU in 10 sec in the range 0.025 ÷ 0.065g/100g crackers, and that at 0.3g/100g crackers a plateu in free ERU release was reached at about 66%. *Sina-*

Recipes	Myrosinase activity	Supplements	Theoretical GSL (µmol/100g)	GRA (µmol/100g)	GER (µmol/100g)	ERU (µmol/10g)	Recovery %*
0	Yes	2,5% *E. sativa* DSM	233	< LOQ	9 ± 1	35 ± 5	18.9
1	Yes	2,5% *E. sativa* DSM**	233	< LOQ	4.0 ± 0.1	n.d.	1.7
2	Yes	2% *E. sativa* DSM	186	< LOQ	4.0 ± 0.1	n.d.	2.2
3	Yes	1,5% *E. sativa* DSM	140	< LOQ	3.0 ± 0.1	n.d.	2.1
4	Yes	1% *E. sativa* DSM	93	< LOQ	3.0 ± 0.1	n.d.	3.2
5	No	2,5% *E. sativa* DSM	187	4.0 ± 0.1	128 ± 5	n.d.	70.6
6	No	1% *E. sativa* DSM	75	3.0 ± 0.1	68 ± 3	n.d.	94.7

Table 1: Glucosinolate and isothiocyanate contents in different crackers recipes and final recoveries of bioactive molecules in new bakery products.

*Calculated as sum of glucosinolates and isothiocyanates detected in the final products; ** different food additives; GSL, glucosinolate; GRA, glucoraphanin; GER, glucoerucin.

pis alba gave the characteristic bitterness and pungent taste yet at 0.1g/100g crackers, so the final recipes were improved by adding 0.025g/100g crackers in the cooling phase.

Baseline analysis

Twenty patients met the inclusion criteria, but one retrieved his consent during the run-in period. Intention-to-treat analysis was conducted on the remaining nineteen patients (5 male; age 48.9 ± 15.7) who fulfilled inclusion criteria, were prospectively enrolled and received at least one dose of enriched bakery product. Of them, two patients (10.5%) presented tobacco smoking habits, 2 (10.5%) mild alcohol consumption and 6 (31.5%) arterial hypertension. At the end of the run-in period, mean height was 1.67 ± 0.09 m, body weight 68.6 ± 14.3 Kg and BMI 24.5 ± 4.2 Kg/m2. Patients' demographic characteristics, comorbidities and baseline laboratory tests were summarized in table 2.

Characteristic	Total (no. 19)
Gender, male, no. (%)	5 (26.3%)
Age, years, mean ± SD	48.9 ± 15.7
Height, m, mean ± SD	1.67 ± 0.09
Weight, Kg, mean ± SD	68.6 ± 14.3
BMI, Kg/m^2, mean ± SD	24.5 ± 4.2
Cigarette smoking, no. (%)	2 (10.5%)
Alcohol consumption, no. (%)	2 (10.5%)
Arterial hypertension, no. (%)	6 (31.6%)
Mean systolic BP, mmHg, mean ± SD	115.4 ± 17.5
Mean diastolic BP, mmHg, mean ± SD	73.9 ± 9.8
Fasting glucose, mg/dl, mean ± SD	84.4 ± 11.5
Fasting Insulin, microUI/ml, mean ± SD	9.5 ± 7.6
HOMA-r, median (range)	1.38 (0.36 - 8.13)
HOMA-r ≥ 3.0, no. (%)	7 (36.8%)
HbA1c, %, mean ± SD	5.3 ± 0.3
HbA1c, mmol/mol, mean ± SD	34.1 ± 2.9
Total cholesterol, mg/dl, mean ± SD	196.0 ± 33.0
HDL cholesterol, mg/dl, mean ± SD	53.1 ± 9.1
LDL cholesterol, mg/dl, mean ± SD	116.4 ± 29.1
Cholesterol ratio*, mean ± SD	3.8 ± 1.0
Triglyceride, mg/dl, mean ± SD	93.7 ± 48.2
ALT, U/l, mean ± SD	24.4 ± 9.7
Gamma-GT, U/l, mean ± SD	20.0 ± 6.6
Creatinine, mg/dl, mean ± SD	0.8 ± 0.1
e-GFR, ml/min/1,73m^2, mean ± SD	85.9 ± 13.9
High sensitivity C reactive Protein, mg/dL	1.4 (0.4 - 10.4)
IL-6, pg/ml, mean ± SD	2.5 ± 0.7
IL-8, pg/ml, median (range)	30.2 (10.0 - 185.0)
Tumor Necrosis Factor alpha, pg/ml, mean ± SD	8.0 ± 3.0

Table 2: Baseline demographic characteristics and serum biochemistry of the entire study population.

Abbreviation: BP: Blood Pressure; HbA1c: Glycated Hemoglobin; e-GFR: Estimated Glomerular Filtration Rate. * Cardiac Risk Index Calculated as Total/HDL Cholesterol Ratio.

Glucose metabolism. Baseline fasting glucose was 84.4 ± 11.5 mg/dL and fasting insulin 9.5 ± 7.6 microUI/ml; median HOMA-r was 1.38 (0.36 - 8.13). Seven (36.8%) patients presented euglycemic insulin resistance (HOMA-r ≥ 3.0). Mean glycated hemoglobin was 5.3 ± 0.3% (34.1 ± 2.9 mmol/mol).

Lipid metabolism. Baseline total cholesterol was 196.0 ± 33.0 mg/dl, HDL cholesterol 53.1 ± 9.1 mg/dl and LDL cholesterol 116.4 ± 29.1 mg/dl; cholesterol ratio was 3.8 ± 1.0. Fasting triglyceride 93.7 ± 48.2 mg/dl.

Systemic inflammation markers. The evaluation of systemic microinflammation markers showed hs-CRP levels of 1.4 (0.4 - 10.4) mg/dl, IL-6 2.5 ± 0.7 ng/ml, IL-8 30.2 (10.0 - 185.0) ng/ml and TNF-α 8.0 ± 3.0 ng/ml.

13 outof 19 patients presented hepatomegaly at the first abdomen ultrasound.

Metabolic effects of enriched bakery products consumption

The effects of 4-week consumption of the enriched dietary products were reported in table 3.

Glucose metabolism. HOMA-r showed a mean - 8.7% reduction from 1.38 (0.36 - 8.13) to 1.26 (0.34 - 5.70) (P = 0.12), reflecting a - 12.7% reduction of fasting insulin concentration 9.5 ± 7.6 vs. 8.3 ± 5.9 microUI/ml (P = 0.15) despite unmodified fasting glycaemia. If we consider only patients with euglycemic insulin resistance (no. 7), HOMA-r showed a statistically significant reduction from 3.82 (3.15 - 8.13) to 3.48 (2.02 - 5.70) (P = 0.03).

Lipid metabolism. Total cholesterol showed only a mild (- 1.6%) reduction; however, HDL cholesterol showed a 5.0% increase from 53.1 ± 9.1 to 55.8 ± 11.2 mg/dl (P = 0.02) and LDL cholesterol decreased from 116.4 ± 29.1 to 111.5 ± 27.2 mg/dl (P = 0.03). These alterations led to a significant reduction of the cholesterol ratio 3.8 ± 1.0 vs. 3.6 ± 1.0 (P = 0.03). Fasting triglyceride concentrations were substantially unmodified (- 2.0%), reflecting unchanged dietary habits before and during the study period.

Systemic inflammation markers and liver biochemistry. High sensitivity CRP showed a statistically significant reduction (-36.8%) from 1.4 (0.4 - 10.4) to 0.9 (0.4 - 8.7) mg/dl (P = 0.02) as well as TNF-α 8.0 ± 3.0 vs. 7.1 ± 2.7 pg/ml (- 11.3%; P < 0.001). Despite a mild ALT reduction (- 7.0%), Gamma-GT showed a - 21.0% decrease from 20.0 ± 6.6 to 15.8 ± 6.4 U/L (P < 0.001). IL-6 and IL-8 were substantially unmodified.

	Basal (no.19)	Post-treatment (no.19)	Change	P*
Fasting glucose, mg/dl	84.4 ± 11.5	85.2 ± 12.0	---	---
Fasting Insulin, microUI/ml	9.5 ± 7.6	8.3 ± 5.9	- 12.7%	0.15
HOMA-r	1.38 (0.36 - 8.13)	1.26 (0.34 - 5.70)	- 8.7%	0.12
HbA1c, %	5.3 ± 0.3	5.3 ± 0.3	---	---
HbA1c, mmol/mol	34.1 ± 2.9	34.2 ± 3.1	---	---
Total cholesterol, mg/dl	196.0 ± 33.0	192.9 ± 33.7	- 1.6%	0.12
HDL cholesterol, mg/dl	53.1 ± 9.1	55.8 ± 11.2	+ 5.0%	0.02
LDL cholesterol, mg/dl	116.4 ± 29.1	111.5 ± 27.2	- 4.3%	0.03
Cholesterol ratio	3.8 ± 1.0	3.6 ± 1.0	- 5.3%	0.03
Triglyceride, mg/dl	93.7 ± 48.2	91.9 ± 48	- 2.0%	---
ALT, U/L	24.4 ± 9.7	22.7 ± 9.7	- 7.0%	0.25
Gamma-GT, U/L	20.0 ± 6.6	15.8 ± 6.4	- 21.0%	<0.001
Creatinine, mg/dl	0.8 ± 0.1	0.8 ± 0.1	---	---
e-GFR, ml/min/1,73m^2	85.9 ± 13.9	88.4 ± 14.5	+ 2.9%	0.09
High sensitivity CRP, mg/dl	1.4 (0.4 - 10.4)	0.9 (0.4 - 8.7)	- 36.8%	0.02
IL-6, pg/ml	2.5 ± 0.7	2.3 ± 0.6	- 8.0%	0.23
IL-8, pg/ml	30.2 (10.0 - 185.0)	30.1 (9.3 - 130.0)	---	---
TNF-α, pg/ml	8.0 ± 3.0	7.1 ± 2.7	- 11.3%	<0.001

Table 3: Metabolic effects of enriched bakery products consumption.

* P value for the comparison between basal and post-treatment values.

Ultrasound examination showed a reduction in hepatomegaly in all the patients.

Discussion and Conclusion

This preliminary study provides with a possible new natural actor that may be useful in the prevention and treatment of fatty liver disease. E. sativa defatted seed meal enriched bakery products were produced and assembled with the aim to improve glucose and lipid metabolism and systemic markers of inflammation based on the evidences of the role of brassicaceae on cancer and cardiovascular diseases risk.

Notably, in our pilot study the main markers of chronic inflammation improved after a short period of treatment (4 weeks), Of note, among these markers C-reactive protein and TNF alpha improved as well as glucose and lipid metabolism with particular regard to the significant drop in LDL cholesterol. Of interest, markers of liver's function and on particular GGT which is activated in the fatty liver, has undergone a statistically significant decrease together with the clinical data of a reduction in hepatomegaly and an improvement in the ultrasound evaluation.

These data support the effect of the product on the glucose and liver metabolism and therefore the hepatoprotective effect that might be related to the anti-inflammatory capacities of the product itself.

Moreover, E. sativa defatted seed meal enriched bakery products, can improve liver biochemistry in parallel with a significant improvement of serum markers of inflammation and therefore being of interest for patients affected by inflammatory conditions such as fatty liver diseases as well as cardiovascular conditions.

In conclusion, this small pilot study offer a diet product option to be adopted by patients with altered glucose and lipid metabolism to prevent chronic conditions. This small-controlled study will have to be confirmed on a larger number of patients and for a longer period.

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