Olive oil by-product as functional ingredient in bakery products. Influence of processing and evaluation of biological effects

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Nowadays, the strong demand for adequate nutrition is accompanied by concern about environmental pollution and there is a considerable emphasis on the recovery and recycling of food by-products and wastes. In this study, we focused on the exploitation of olive pomace as functional ingredient in biscuits and bread. Standard and enriched bakery products were made using different flours and fermentation protocols. After characterization, they were *in vitro* digested and used for supplementation of intestinal cells (Caco-2), which underwent exogenous inflammation. The enrichment caused a significant increase in the phenolic content in all products, particularly in the sourdough fermented ones. Sourdough fermentation also increased tocol concentration. The increased concentration of bioactive molecules did not reflect the anti-inflammatory effect, which was modulated by the baking procedure. Conventionally fermented bread enriched with 4% pomace and sourdough fermented, not-enriched bread had the greatest anti-inflammatory effect, significantly reducing IL-8 secretion in Caco-2 cells. The cell metabolome was modified only after supplementation with sourdough fermented bread enriched with 4% pomace, probably due to the high concentration of tocopherol that acted synergistically with polyphenols. Our data highlight that changes in chemical composition cannot predict changes in functionality. It is conceivable that matrices (including enrichment) and processing differently modulated bioactive bioaccessibility, and consequently functionality.

Keywords:
Olive oil by-products
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Polyphenols
Inflammation
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Fermentation
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1. Introduction

Olive oil production, an agro-industrial activity of vital economic significance for many Mediterranean countries, is associated with the generation of large quantities of wastes and by products (Berbel & Posadillo, 2018) and there is an increasing interest in using them in food products as functional ingredients. Despite the high concentration of phenolic compounds in the olive fruit, only 2% of the initial concentration is found into virgin olive oil (VOO), while the remaining fraction is found in the olive mill wastewater (OMW) (approximately 53%) and in the olive pomace (OP) (approximately 45%). OMW and OP have a different phenolic profile. Oleuropein is abundant in the OP (up to 0.9%) (Sanchez de Medina, Priego-Capote, & Luque de Castro, 2012) and it is absent in OMW due to enzymatic hydrolysis during the olive oil extraction (Cardinali et al., 2012).

Oleuropein, tyrosol and their derivatives are supposed to be the main culprits of the health benefits related to its consumption (Karkovic Markovic, Toric, Barbaric, & Jakobusic Brala, 2019), this making OP a potential low-cost starting material rich in bioactive phenolics (Antonia Nunes et al., 2018). The use of OP could represent an innovative and low-cost strategy to formulate healthier and value-added foods, and bakery products are good candidates for enrichment. In fact, they are consumed all over the world, and a wide variety of bakery product such as bread, biscuits, crackers, breadsticks, and others can be found on

Abbreviations: Ala, alanine; B, blank; BIS, biscuits; CFB, conventional fermented bread; Cho, choline; d6-DSS, 2,2-dimethyl-2-silapentane-d6-5-sulfonic; DOP, defatted olive pomace; Eth, ethanol; Glu, glucose; GPADHEA, 1-β-D-glucopyranosyl acyclodihydroelenolic acid; IFNγ, interferon gamma; IL-10, interleukin 10; IL-12p70, interleukin 12p70; IL-1α, interleukin 1 alpha; IL-1β, interleukin 1 beta; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; IL-8, interleukin 8; Lac, lactate; LMWF, low molecular weight fraction; LPS, lipopolysaccharide; myI, myo-inositol; MTT, methylthiazolyldiphenyl-tetrazolium bromide; OMW, olive mill wastewater; OP, olive pomace; oPc, o-phosphocholine; q.s., quantum sufficit; SFB, sourdough fermented bread; snG3pc, sn-glycero-3-phosphocholine; TNFα, tumour necrosis factor alpha; US, unsupplemented; VOO, virgin olive oil

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supermarket shelves.

The nutritional value of bakery products can be improved by different strategies including the use of by-products as ingredients (Boubaker, Omri, Blecker, & Bouzouita, 2016). Beyond enrichment, the development of innovative bakery food can be linked to the production process. As example, sourdough fermentation has been shown to modulate the functional features of leavened baked goods (Gobbetti, De Angelis, Di Cagno, Calasso, et al., 2019). Enrichment and modification of the production process could act synergistically and allow the development of functional products.

To verify it, in this study different bakery products (biscuits and breads) were made using different flours and fermentation protocols and eventually enriched with defatted olive pomace (DOP). Since the supposed functionality of the innovative products could not be assessed simply evaluating their chemical composition and changes linked to digestion had to be carefully considered, after chemical characterization products were *in vitro* digested. To test the effects in a biological system, the digested fraction with a molecular weight compatible to absorption was used for supplementation of intestinal cells, which underwent exogenous inflammation. Cultured enterocytes were used as model system since *in vivo* they are in direct contact to the digestion products. Given that in a recent study we evidenced the positive effect of a polyphenols rich extract from olive pomace on inflammation and cell metabolome in intestinal cell culture (Di Nunzio, Picone et al., 2018), the effects of supplementation with the digested bakery products was investigated measuring cytokines secretion and evaluating the modification of cell metabolome by nuclear magnetic resonance (NMR) spectroscopy.

2. Material & methods

The experimental design of the study is depicted in Fig. 1.

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, and Dulbecco's phosphate-buffered saline (DPBS) were purchased from Lonza (Basel, Switzerland). All other chemicals and solvents were of the highest analytical grade from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Fermentation and baking processes

Whole einkorn flour (*Triticum monococcum* L. var. Monlis) and whole wheat flour (*Triticum aestivum* L.) were provided by Prometeo (Urbino, Italy). DOP powder was provided by ISANATUR SPAIN S.L. (Puente La Reina, Spain). DOP powder was obtained from the by-product of olive oil extraction by drying and defatting based on the patent WO2013030426 with further developments to increase the process sustainability.

Six different types of bakery products were prepared and tested:

Table 1
Recipes of bakery products.

Ingredients	BIS0%	BIS2.5%	CFB0%	CFB4%	SFB0%	SFB4%
Whole wheat flour (g)	0.0	0.0	98.6	94.6	97.8	93.9
Whole einkorn flour (g)	36.3	35.4	0.0	0.0	0.0	0.0
Extra-virgin olive oil (g)	8.1	7.9	0.4	0.4	0.5	0.4
Sodium bicarbonate (g)	0.4	0.4	0.0	0.0	0.0	0.0
Potassium hydrogen tartrate (g)	0.5	0.5	0.0	0.0	0.0	0.0
Brown sugar	12.0	11.7	0.0	0.0	0.0	0.0
Water (ml)	42.7	41.6	q.s.	q.s.	q.s.	q.s.
Dough (g)	0.0	0.0	0.0	0.0	1.7	1.7
Yeast beer for CF (g)	0.0	0.0	1.0	0.9	0.0	0.0
DOP (%)	0.0	2.5	0.0	4.0	0.0	4.0

q.s.: quantum sufficit; BIS0%: biscuits made without defatted olive pomace (DOP); BIS2.5%: biscuits made with 2.5% DOP; CFB0%: conventional fermented bread made without DOP; CFB4%: conventional fermented bread made with 4% DOP; SFB0%: sourdough fermented bread made without DOP; SFB4%: sourdough fermented bread made with 4% DOP.

(a) biscuits (BIS) made with whole einkorn flours, with or without 2.5% DOP;
(b) conventional fermented bread (CFB) made with whole wheat flours with or without 4% DOP;
(c) sourdough fermented bread (SFB) made with whole wheat flours with or without 4% DOP.

DOP concentration for enrichment was chosen based on the limit of organoleptic acceptance in consumer preference test (data not shown).

For sourdough preparation, a 10% (fresh weight basis - fwb) mixed-strain containing Lb *plantarum* 98A, Lb. *sanfranciscensis* BB12, Lb. *brevis* 3BHI, and 3% (fwb) of S. *cerevisiae* LBS was added to the dough and fermented at 32 °C for 24 h to obtain a mature sourdough. The microbial load in mature sourdough was approximately 10^9 colony forming units (CFU)/g for LAB and 10^7 CFU/g for S. *cerevisiae*. For conventional fermentation, 14% (fwb) of S. *cerevisiae* LBS was added to the final dough for 1.5 h of leavening at 32 °C.

The recipes of the bakery products are reported in Table 1. Biscuits and breads were cooked in oven at 180 °C for 25 min and 200 °C for 30 min, respectively. After baking, they were cooled at room temperature, cut into small pieces and stored at −18 °C until analysis. Each bakery product was prepared in triplicate on separate days.

2.3. Polyphenol extraction and determination by HPLC-DAD-MS

The phenolic fractions were obtained by solid-liquid extraction as described by Marzocchi et al. (2017) with slight modifications. Briefly, 3 g of powdered sample were defatted by stirring with 30 mL of hexane for 30 min. After removing supernatant, the defatted pellet was extracted with 30 mL of ethanol/water (4:1 v/v) in ultrasonic bath at 40 °C for 15 min. After centrifugation at 3500 rpm for 15 min, the supernatant was removed, and the extraction was repeated.

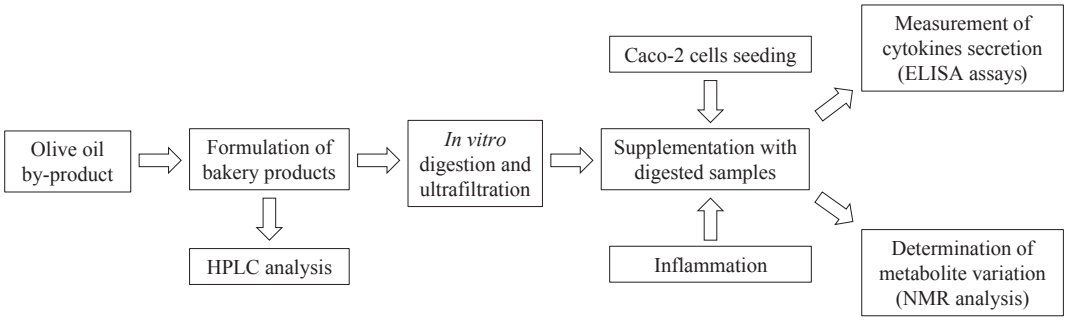

Fig. 1. Experimental design.

Supernatants were pooled, evaporated at 35 °C under vacuum and reconstituted with 6 mL of water/methanol (1:1 v/v). The final extracts were filtered with regenerated 0.2 μm cellulose filters (Millipore, Bedford, MA, USA) and stored at −18 °C until analysis.

Polyphenol profile was determined using an Agilent 1200-LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with vacuum degasser, autosampler, binary pump, HP diode-array, UV–VIS detector and mass spectrometer detector as previously described (Di Nunzio, Toselli, Verardo, Caboni, & Bordoni, 2013). Separations were carried out on a reverse phase column Poroshell 120 SB-C18 (3 × 100 mm, 2.7 μm) from Agilent Technologies (Palo Alto, CA, USA) and the chromatogram was registered at 280 nm. Compounds were identified by analysing their UV and MS spectra and quantified by DAD detection. The individual phenolic compounds were quantified by their UV absorbance against external standards using tyrosol, caffeic acid, ferulic acid, chlorogenic acid, oleuropein, verbascoside and rutin at 280 nm for the different class of phenols. Results are expressed as mg/Kg bakery product.

2.4. Tocol determination by HPLC–FLD

Lipids were extracted from bakery products as previously reported (Boselli, Velazco, Caboni, & Lercker, 2001). One hundred mg of lipids were dissolved in 1 mL hexane and filtered through a 0.2 μm nylon filter, then 2.5 μL were injected in a HPLC 1200 series equipped with a fluorimeter detector (λ_{ex} = 290 nm, λ_{em} = 325 nm) (Agilent Technologies, Palo Alto, CA, USA). Separation of tocopherols was performed by a HILIC Poroshell 120 (3 × 100 mm, 2.7 μm) from Agilent Technologies (Palo Alto, CA, USA) in isocratic conditions using an n-hexane/ethyl acetate/acetic acid (97.3:1.8:0.9 v/v/v) mobile phase. The flow rate was 0.8 mL/min. Tocopherols were identified by co-elution with the corresponding standards. The calibration curve used for quantification was constructed with α- tocopherol standard solutions. Results are expressed as mg/kg bakery product.

2.5. In vitro digestion

The digestion process was performed on 50 g of bakery product or water (blank digestion) for 245 min (5 min of oral, 120 min of gastric and 120 min of intestinal digestion) at 37 °C, according to the INFOGEST standardized protocol (Minekus et al., 2014) as described in Valli, Taccari, Di Nunzio, Danesi, and Bordoni (2018). During *in vitro* digestion, simulated saliva (containing 75 U/mL α-amylase), simulated gastric juice (containing 2000 U/mL pepsin) at acid pH, and simulated pancreatic juice (containing 10 mM bile and 100 U/mL pancreatin) at neutral pH were added. Digested solutions were centrifuged at 50.000*g* for 15 min, and the supernatants filtered with 0.2 μm membranes. To separate compounds which size is small enough to be potentially absorbable through the intestinal mucosa, an aliquot was sequentially ultra-filtered with Amicon Ultra (Millipore, Burlington, MA, USA) at 3 kDa of molecular weight cut-off (low molecular weight fraction, LMWF). Each product was digested three times, and the resulting LMWF were mixed and frozen at −18 °C until use.

2.6. Caco-2 cell culture and supplementation

Caco-2 cells were maintained at 37 °C, 95% air, 5% CO_2 in DMEM supplemented with foetal bovine serum (10% v/v), non-essential amino acids (1% v/v), 100 U/mL penicillin and 100 μg/mL streptomycin. Once a week cells were seeded at 9×10^3 cells/mL into a new 75 cm^2 flask, and medium was refreshed every 48 h (Antognoni et al., 2017).

For experiments, Caco-2 cells were seeded in 24-well at 1×10^5 cells/well concentration (cytotoxicity and inflammation assays) or in 100 mm Petri dishes at 2.7×10^6 cells/dish (NMR assay) and grown for 21 days. Complete differentiation was assessed by measuring the trans epithelial electric resistance of the cell monolayer using the Millicell ERS apparatus (Millipore, Burlington, MA, USA).

In preliminary experiments, to assess cytotoxicity in basal conditions (i.e. without any inflammatory stimulus), cells were supplemented for 24 h with serum-free DMEM containing different concentration of LMWF (2.5, 5, 10, 20 μL/mL medium). Cytotoxicity was assessed as reported below. In further experiments cells were supplemented with the highest LMWF concentration having no cytotoxic effects (5 μL of LMWF/mL medium). To avoid interference due to the vehicle, some cells were supplemented with the same amount of LMWF from blank digestion (B), while other cells received no supplementation (unsupplemented - US).

Concomitant to LMWF supplementation, inflammation was induced exposing cells to interleukin 1 beta (IL-1β) at 10 ng/mL for 24 h.

2.7. Cytotoxicity evaluation

LMWF cytotoxicity was evaluated by assessing cell viability with the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay and by microscopic examination. Cell viability was determined by conversion of the MTT salt to its formazan product detected at 560 nm using a Tecan Infinite M200 microplate reader (Tecan, Männedorf, Switzerland) (Di Nunzio, Bordoni, Aureli, Cubadda, & Gianotti, 2018), and it was expressed as optical density (O.D.). Light microscopy examination of cell morphology and monolayer integrity was performed using an inverted confocal light microscopy model IB (Exacta Optech, Modena, Italy) using 10×, 25× and 40× as magnification.

2.8. Cytokines secretion

Concentration of the pro- and anti-inflammatory cytokines interferon gamma (IFNγ), interleukin 1 alpha (IL-1α), IL-1β, interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin (IL-8), interleukin 10 (IL-10), interleukin 12 p70 (IL-12p70), and tumour necrosis factor alpha (TNFα) was estimated in the cell media by the multiplex sandwich ELISA Ciraplex (Aushon, Billerica, MA, USA) following the manufacturer's instructions. 96-well plates pre-spotted with protein-specific antibodies were used and luminescent signals were detected by Cirascan™ Imaging System.

IL-8 concentration was also estimated in cell media using AlphaLISA kits (Perkin Elmer Inc., Waltham, MA, USA) following the manufacturer's instructions (Valli et al., 2016). We used 96-microwell plates that were read using an EnSpire™ plate reader (Perkin Elmer Inc., Waltham, MA, USA). Results were expressed as pg/mg protein.

2.9. Protein content determination

Cells were washed with cold DPBS, lysed with 500 μL of cold Nonidet P-40 (0.25% v/v in DPBS), incubated on ice with shaking for 30 min and centrifuged at 14.000*g* for 15 min. Supernatants were collected and protein content was determined by Comassie assay (Di Nunzio, Valli, & Bordoni, 2016), using bovine serum albumin as standard.

2.10. HR ^{1}H NMR

Cells were washed with ice-cold DPBS and scraped off. The pellet lysed by sonication and centrifuged at 21.000*g* for 10 min at 4 °C. Five hundred μl of supernatant were added to 10 μL of a D_2O solution of 100 mM 2.2-dimethyl-2-silapentane-d6-5-sulfonic (d6-DSS) with a final concentration in the NMR tube of 9.09 mM. ^{1}H NMR spectra were recorded at 298 K on a Bruker US + Avance III spectrometer operating at 600 MHz, equipped with a BBI-z probe and a B-ACS 60 sampler for automation (Bruker BioSpin, Karlsruhe, Germany). The HOD residual signal was suppressed by applying the Carr–Purcell–Meiboom–Gill spin-echo pulse sequence with a pre-saturation sequence. Each spectrum was acquired using 32 K data points over a 7183.908 Hz spectral width

(12 ppm) and adding 256 transients. A recycle delay of 5 s and a 90° pulse of 11.190 μs were set up. Acquisition time (2.28 s) and recycle delay were adjusted to be 5 times longer than the longitudinal relaxation time of the protons under investigation, which was not longer than 1.4. Data were Fourier transformed and phase and baseline corrections were automatically performed using TopSpin version 3.0 (Bruker BioSpin, Karlsruhe, Germany). Signals were identified by comparing their chemical shift and multiplicity with Chenomx Profiler software data bank (ver. 8.1, Edmonton, Canada) and data in the literature (Picone et al., 2013).

2.11. Statistical analysis

Statistical differences were determined by the one-way analysis of variance (ANOVA) followed by Tukey's post hoc-test considering $p < 0.05$ as significant, and by the Student's *t*-test. NMR spectra processing and PCA analyses were performed using R computational language (ver. 3.5.3). Each ^{1}H NMR spectrum was processed by means of R scripts developed in-house. All other statistical analyses were performed using Prism software ver. 5.0 (GraphPad, San Diego, CA, USA).

3. Results

3.1. Bakery products characterization

The nutritional composition of the bakery products is given in Table 2, according to producer's analysis.

The phenolic profile of bakery products and a representative UV chromatogram of the SFB4% phenolic compounds are reported in Table 3 and Fig. 2, respectively. The total phenol content was greater in all DOP-enriched products than the corresponding controls and was greater in SFB than in other products.

Tocol concentration of bakery products and a representative chromatogram of the BIS2.5% tocol profile are reported in Table 4 and Fig. 3, respectively. The enrichment with DOP caused a significant increase in total tocol content, due mainly to β-tocopherol concentration, only in SFB.

Table 2
Nutritional composition of bakery products.

	BIS0%	BIS2.5%	CFB0%	CFB4%	SFB0%	SFB4%
Humidity	4.4	3.1	5.4	6.8	5.1	5.9
Proteins (g/100 g)	9	9.7	13	12.4	12.9	12.6
Fats (g/100 g)	14.9	15.2	3.2	3.9	3.4	3.7
Fibres (g/100 g)	4.5	5.5	8.1	9.0	7.1	7.6
Ashes (g/100 g)	1.9	2.0	2.0	2.2	2.0	2.3
Carbohydrates (g/100 g)	64.4	65.6	68.3	65.7	69.4	68
Energetic value (Kcal/100 g)	439.0	447.0	370.0	366.0	374.0	371.0
Sodium (mg/Kg)	1843.0	2040.0	3530.0	3720.0	3530.0	3210.0
Saturated fats (%)	13.0	13.1	16.6	15.2	16.5	15.4
Monounsaturated fats (%)	76.2	75.9	58.3	66.6	59.2	64.8
Polyunsaturated fats (%)	11.0	11.1	25.1	18.2	24.3	19.8

Data are mean of results obtained in the three production batches. Standard deviation was lower than 1%. BIS0%: biscuits made without defatted olive pomace (DOP); BIS2.5%: biscuits made with 2.5% DOP; CFB0%: conventional fermented bread made without DOP; CFB4%: conventional fermented bread made with 4% DOP; SFB0%: sourdough fermented bread made without DOP; SFB4%: sourdough fermented bread made with 4% DOP.

3.2. Effects in Caco-2 cells

3.2.1. Experimental set-up in basal condition

In cells supplemented with 10 μL/mL and 20 μL/mL CFB4% and SFB0% light microscopic examination revealed a deep change of cellular morphology and a loss of cell monolayer integrity (Fig. 4). In addition, an increase in MTT conversion was observed (Fig. 5). It was probably related to an enhanced mitochondrial mass and electron transport system activity due to the increased energy requirements under critical condition (Choi, Roche, & Caquet, 2001; Lee, Yin, Chi, & Wei, 2002). Regardless the type of LMWF, the 5 μL/mL concentration had no effect on cell viability, morphology and monolayer integrity and it was therefore used for supplementation in further experiments.

To select the most appropriate inflammatory stimulus, Caco-2 cells were supplemented for 24 h with two different concentrations of lipopolysaccharide (LPS) (100 ng/mL and 500 ng/mL), IL-1β (10 ng/mL and 50 ng/mL), and TNFα (10 ng/mL and 50 ng/mL) alone or in combination (LPS 100 ng/mL + IL-1β 10 ng/mL + TNFα 10 ng/mL and LPS 500 ng/mL + IL-1β 50 ng/mL + TNFα 50 ng/mL). The onset of inflammation was assessed by measuring the secretion of IL-6 and IL-8 using the AlphaLISA kit assay (Fig. 6A and B, respectively). IL-1β supplementation at 10 and 50 ng/mL significantly enhanced IL-6 and IL-8 secretion, without any additive effect due to combination with TNFα and LPS. On this basis, 10 ng/mL IL-1β were used as inflammatory stimulus in further experiments.

3.2.2. Anti-inflammatory effect of bakery products

In basal condition, secretion of most cytokines was below the detection limit. IL-8 was by far the most represented one, and supplementation with B-LMWF did not modify its concentration (Table 5).

Caco-2 cells were stressed by adding 10 ng/mL IL-1β, and the different LMWF were co-supplemented to evaluate their putative anti-inflammatory effect (Table 5). Inflammation caused a significant increase of IL-6 and IL-8 secretion in both US and B supplemented cells compared to basal counterparts ($p < 0.001$). Comparing inflamed cells, IL-8 secretion was significantly lower in CFB4% and SFB0% cells than in US. In CFB4%, it was also lower than in B supplemented enterocytes. All supplementation except BIS2.5% and CFB0% decreased IL-6 secretion.

A relatively high within-group variability was evidenced evaluating cytokine secretion with multiplex sandwich ELISA Ciraplex. Since intra- and inter-assay reproducibility of multiplex assays is sometimes lower than in singleplex assays (Tighe, Ryder, Todd, & Fairclough, 2015), to better investigate the effect of supplementation we measured the secretion of the most expressed cytokine IL-8 also using a singleplex assay, which almost confirmed previous results (Fig. 7). In basal condition, supplementation with LMWF from B digestion did not modify IL-8 secretion, which was strongly increased by the exposure to the inflammatory stimulus in both US and B cells compared to corresponding basal values ($p < 0.001$ in both cases). In inflamed condition, all supplementations except CFB0% reduced IL-8 secretion. In CFB4% supplemented cells, IL-8 secretion was also lower than in B supplemented ones.

3.2.3. Effect on the metabolome

Metabolome analysis was performed on the cell lysate. Before statistics, each ^{1}H NMR spectrum was processed by means of scripts developed in-house in R language. Chemical shift referencing was performed by setting the DSS signal to 0.00 ppm. Moreover, the alignment of the spectra was improved using the *i*Coshift tool (Savorani, Tomasi, & Engelsen, 2010) available at http://www.models.life.ku.dk/algorithms/. The following spectral regions were removed prior to data analysis: the regions including only noise (the spectrum edges between 11.00 and 9.00 ppm and between 0.8 and −1.00 ppm) and the NMR signal which was strongly affected by the residual solvent peak (water between 4.50 and 5.00 ppm). After the Fourier transformation

Table 3
Phenolic profile in bakery products.

#	RT	Phenolic compounds	Mass data ESI$^-$ [M − H]$^-$	BIS0%	BIS2.5%	CFB0%	CFB4%	SFB0%	SFB4%
1	0.8	Quinic acid	191, 111	30.7 ± 0.2^{e}	45 ± 2.0^{e}	164.2 ± 11.2^{a}	128.7 ± 6.0^{b}	71.8 ± 3.5^{d}	87.9 ± 0.1^{c}
2	1.1	Gallic acid der.	305	14.9 ± 0.0^{de}	20.3 ± 0.3 ^{cd}	78.5 ± 7^{a}	67.2 ± 2.9^{b}	11.6 ± 1.1^{e}	26.0 ± 0.1^{c}
3	1.3	Cumaroyl quinic acid	337	14.4 ± 0.3^{c}	15.3 ± 0.2^{b}	26.5 ± 0.6^{a}	15.0 ± 1.0^{c}	12.0 ± 0.8^{d}	8.8 ± 0.7^{e}
4	1.5	Feruloyl quinic acid	367	15.1 ± 0.0^{e}	36.8 ± 0.8^{d}	178.1 ± 9.3^{a}	147.3 ± 1.8^{b}	94.9 ± 1.3^{c}	102.4 ± 4.3^{c}
5	2.5	Hydroxytyrosol /GPADHEA	407	13.3 ± 1.2^{e}	73.6 ± 3.2^{c}	38.1 ± 1.9^{b}	206.8 ± 4.6^{a}	18.7 ± 0.4^{e}	162.1 ± 3.2^{b}
6	3.1	Caffeoyl quinic der.	353	2.5 ± 0.0^{c}	5.9 ± 0.0^{c}	80.5 ± 11.4^{a}	70.4 ± 0.3^{a}	5.3 ± 0.5^{c}	23.0 ± 0.4^{b}
7	3.4	Caffeoyl quinic der.	353	116.0 ± 1.0^{a}	100.5 ± 4.5^{b}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
8	4.5	Caffeoyl quinic der.	353	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	7.0 ± 0.7^{b}	7.1 ± 0.3^{b}	6.8 ± 0.1^{b}	12.2 ± 0.2^{a}
9	5.9	Flavonoid glucoside	481, 449	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	12.8 ± 0.9^{a}	10.2 ± 1.1^{b}	7.3 ± 0.3^{c}	11.9 ± 1.5^{a}
10	6.2	Pinoresinol der.	357	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	66.1 ± 1.3^{b}	76.6 ± 5.4^{a}	37.2 ± 1.5^{c}	61.0 ± 3.3^{b}
11	7.2	Ferulic acid der.	389	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	2.9 ± 0.2^{a}	2.2 ± 0.2^{b}	0.7 ± 0.0^{c}	0.6 ± 0.1^{c}
12	8.3	Hydroxyverbascoside iso.	623	8.4 ± 0.0^{a}	7.3 ± 0.2^{b}	7.5 ± 0.1^{b}	7.1 ± 0.6^{b}	3.4 ± 0.2^{c}	7.1 ± 0.3^{b}
13	8.8	Ferulic acid	193	2.5 ± 0.0^{b}	3.3 ± 0.1^{a}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
14	9.3	Oleuropein der.	391	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	88.9 ± 9.4^{b}	122.9 ± 12.4^{a}	25.8 ± 0.0^{c}	104.5 ± 2.7^{b}
15	9.7	Ferulic acid der.	371, 193	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	1.0 ± 0.0^{b}	1.3 ± 0.2^{a}	1.3 ± 0.0^{a}	1.3 ± 0.2^{a}
16	10.1	Luteolin-7-glucoside	447	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	4.3 ± 0.0^{a}	0.0 ± 0.0^{c}	2.2 ± 0.0^{b}
17	11.2	Verbascoside	623	8.2 ± 0.5^{a}	7.1 ± 0.3^{b}	4.2 ± 0.1^{c}	7.6 ± 0.5^{ab}	2.6 ± 0.0^{d}	4.5 ± 0.2^{c}
18	13.2	Diferulic acid	385, 193	0.0 ± 0.0^{d}	0.9 ± 0.1^{c}	0.0 ± 0.0^{d}	2.1 ± 0.1^{a}	0.0 ± 0.0^{d}	1.5 ± 0.2^{b}
		Total		226.0 ± 2.9^{e}	316.0 ± 2.6^{d}	756.1 ± 53.0^{b}	876.7 ± 33.5^{a}	299.6 ± 5.8^{d}	617.2 ± 6.9^{c}

Data are expressed as mg/Kg bakery product and are mean ± SD of three samples. Statistical analysis was by one-way ANOVA ($p < 0.001$ for each phenol), using Tukey's post-hoc test. Different letters in the same row indicate significant differences (at least $p < 0.05$). GPADHEA: 1-β-D-glucopyranosyl acyclodihydroelenolic acid. RT: retention time expressed in minutes; Der: derivative; Iso: isomers; US: unsupplemented; B: blank; BIS0%: biscuits made without defatted olive pomace (DOP); BIS2.5%: biscuits made with 2.5% DOP; CFB0%: conventional fermented bread made without DOP; CFB4%: conventional fermented bread made with 4% DOP; SFB0%: sourdough fermented bread made without DOP; SFB4%: sourdough fermented bread made with 4% DOP.

and prior to multivariate analysis, data underwent to a pre-statistical improvement. First, spectra were normalized to the unit area to reduce possible dilution effects (Craig, Cloarec, Holmes, Nicholson, & Lindon, 2006). Then, to avoid the effect of peaks misalignments among different spectra due to variations in chemical shift of signals belonging to some titratable acids, a points reduction by the "spectral binning" was performed (Gartland, Beddell, Lindon, & Nicholson, 1991) by subdividing the spectra into 410 bins each integrating 100 data points (0.0183 ppm each). A representative 1H NMR spectrum of SFB4% cell lysate is reported in Fig. 8.

Principal component analysis (PCA) performed on 410 bins is reported in Fig. 9. PC1 vs PC2 accounted for 86% of the total variance, which was mainly located along PC1 (74%). In order to determine variables encompassing most of the discriminative information, bins with a loading value greater than 1% of the overall standard deviation of all loading values were selected (Picone et al., 2018) on the most important bins along PC1 and PC2. The main metabolites involved in the discrimination among groups were glucose (Glu), lactate (Lac), *sn*-glycero-3-phosphocholine (snG3pc), o-phosphocholine (oPc), myo-inositol (myI), choline (Cho), alanine (Ala) and ethanol (Eth).

In basal condition, no differences were detected between US and B supplemented cells. Inflammation caused increased lactate signals in US cells compared to basal counterparts, while no differences were detected in B cells. Supplementation did not cause any modification in the metabolome of inflamed cells, except an increase of Cho and myI and a decrease of snG3pc signals observed in SFB4% cells (Table 6).

4. Discussion

In conventional bakery products, total phenol content reflected the type of flour and fermentation used for baking. The higher total phenol content observed in CFB0% than SFB0% was probably due to the degradation of the cell wall structure by microbial enzymes during yeast fermentation. As reported by Angelino et al. (2017), this could cause the release of the aglycones from their glycoside linked to the fibres, making them more available for hydroalcoholic extraction. The higher total tocol content in conventional biscuits than breads were justified by the use of extra virgin oil for BIS preparation.

Enrichment with DOP increased the concentration of the typical olive oil polyphenols in all bakery products. In enriched products, total

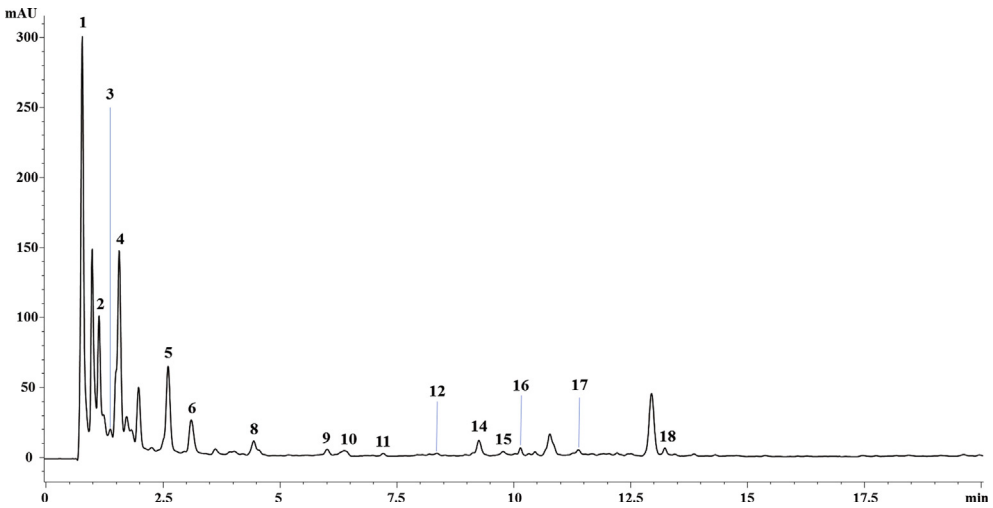

Fig. 2. Chromatogram of the phenolic profile of SFB4%. Peaks: (1) Quinic acid; (2) Gallic acid der.; (3) Cumaroyl quinic acid; (4) Feruloyl quinic acid; (5) Hydroxytyrosol/GPADHEA; (6) Caffeoyl quinic der.; (8) Caffeoyl quinic der.; (9) Flavonoid glucoside; (10) Pinoresinol der.; (11) Ferulic acid der.; (12) Hydroxyverbascoside iso.; (14) Ferulic acid; (15) Ferulic acid der.; (16) Luteolin-7-glucoside; (17) Verbascoside; (18) Diferulic acid.

Table 4
Tocol profile in bakery products.

#	RT	Tocols	BIS0%	BIS2.5%	CFB0%	CFB4%	SFB0%	SFB4%
1	1.5	α-tocopherol	27.5 ± 1.1^{a}	26.9 ± 0.6^{a}	2.1 ± 0.1^{b}	2.9 ± 0.5^{b}	1.5 ± 0.0^{b}	2.8 ± 0.2^{b}
2	1.8	α-tocotrienol	4.7 ± 0.3^{a}	4.1 ± 0.1^{b}	0.6 ± 0.0^{c}	0.4 ± 0.0^{c}	0.5 ± 0.0^{c}	0.4 ± 0.0^{c}
3	2.0	β-tocopherol	19.7 ± 1.1^{a}	21.5 ± 0.8^{a}	4.2 ± 0.2^{d}	4.7 ± 0.3^{d}	8.8 ± 0.2^{c}	15.2 ± 1.0^{b}
4	2.5	β-tocotrienol	7.2 ± 0.7^{a}	6.4 ± 0.2^{a}	3.4 ± 0.1^{b}	3.2 ± 0.2^{b}	2.9 ± 0.1^{b}	3.3 ± 0.2^{b}
		Total	59.1 ± 3.2^{a}	59 ± 1.3^{a}	10.3 ± 0.2^{d}	11.2 ± 1.1 ^{cd}	13.6 ± 0.3 ^{cd}	21.7 ± 1.5^{b}

Data are expressed as mg/Kg bakery product and are mean ± SD of three samples. Statistical analysis was by one-way ANOVA ($p < 0.001$ for each tocol), using Tukey's post-hoc test. Different letters in the same row indicate significant differences (at least $p < 0.05$). RT: retention time expressed in minutes; US: unsupplemented; B: blank; BIS0%: biscuits made without defatted olive pomace (DOP); BIS2.5%: biscuits made with 2.5% DOP; CFB0%: conventional fermented bread made without DOP; CFB4%: conventional fermented bread made with 4% DOP; SFB0%: sourdough fermented bread made without DOP; SFB4%: sourdough fermented bread made with 4% DOP.

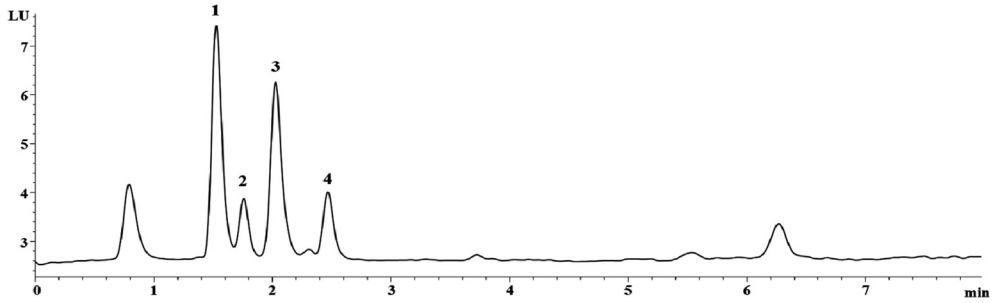

Fig. 3. Chromatogram of the tocol compounds of BIS2.5%. Peaks: (1) α-Tocopherol; (2) α-Tocotrienol; (3) β-Tocopherol; (4) β-Tocotrienol.

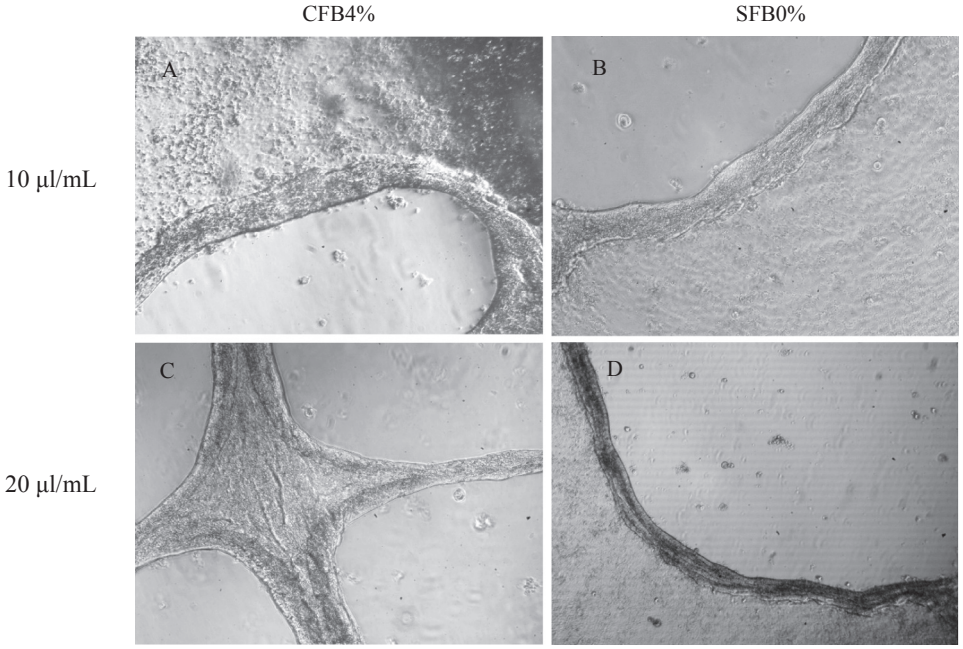

Fig. 4. Microscopic observation of cell morphology and layer integrity after 24 h of supplementation in basal condition. A and B: CFB4% and SFB0% supplemented at 10 µL/mL concentration; C and D: CFB4% and SFB0% supplemented at 20 µL/mL concentration. Each picture is representative of six samples from two independent experiments and is taken at 25× of magnification. CFB4%: conventional fermented bread made with 4% DOP; SFB0%: sourdough fermented bread made without DOP.

phenolics were 140% (BIS), 115% (CFB) and 206% (SFB) compared to not-enriched counterparts. In a recent work (Cedola, Cardinali, D'Antuono, Conte, & Del Nobile, 2020), OMWW and olive paste were used to improve the chemical quality of bread and pasta. Enrichment with OMWW slightly improved phenolic contents and antioxidant activity, which were ameliorated by olive paste addition. It was not possible to compare results by Cedola et al. (2020) to our results since they simply evaluated the theoretical phenolic content and not the actual phenolic content and profile of bakery products. Of note, in their study olive paste negatively influenced the sensory properties due to a very bitter and spicy taste, while in our study DOP concentration for enrichment was chosen based on the limit of organoleptic acceptance in consumer preference test. Fermented olive paste was also used to increase the functionality of Italian bakery products *taralli* (Durante et al., 2019). In that study, the increase of total phenolics was higher than in the present study, and it was justified by a higher addition of fermented olive paste (200 g/kg flour) than of DOP in the present study (70 g/kg flour in BIS and about 40 g/kg flour in CFB and SFB). Organoleptic characteristics of *taralli* were not evaluated.

Enrichment with DOP did not increase tocol concentration in BIS and CFB probably due to defatting, which is known to remove most of vitamin E from olive pomace (Rosello-Soto et al., 2015). It is conceivable that sourdough fermentation caused the increase of tocol concentration in SFB4%, as already reported by Gianotti et al. (2011).

To evaluate their effect in a biological system, bakery products were *in vitro* digested and the LMWFs were used to supplement Caco-2 cells.

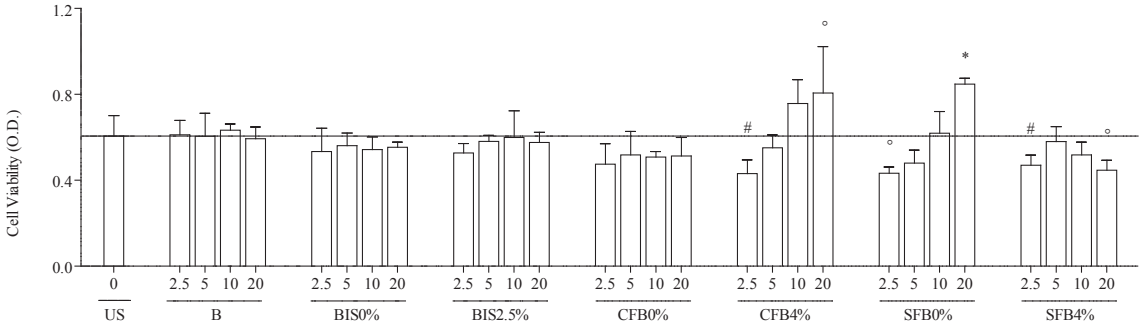

Fig. 5. Cell viability by MTT assay after 24 h of supplementation in basal condition. Cell viability is expressed as optical density (O.D.). Data are mean ± SD of six samples obtained from two independent experiments. Statistical analysis was by the one-way ANOVA ($p < 0.001$) using Dunnett's as post-test to compare supplemented cells to US ones (# $p < 0.05$; ° $p < 0.01$; * $p < 0.001$). US: unsupplemented; B: blank; BIS0%: biscuits made without defatted olive pomace (DOP); BIS2.5%: biscuits made with 2.5% DOP; CFB0%: conventional fermented bread made without DOP; CFB4%: conventional fermented bread made with 4% DOP; SFB0%: sourdough fermented bread made without DOP; SFB4%: sourdough fermented bread made with 4% DOP.

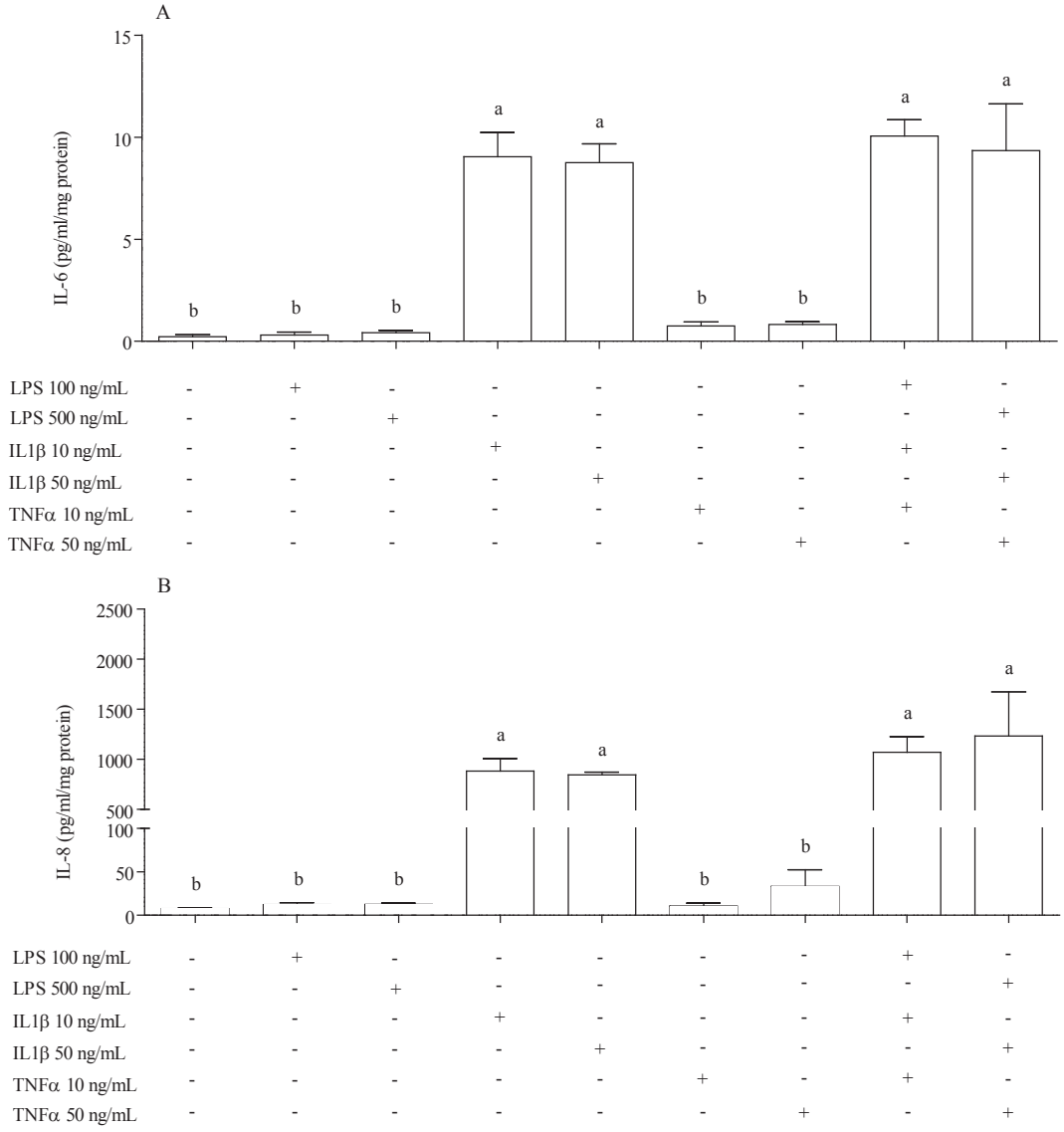

Fig. 6. IL-6 (A) and IL-8 (B) secretion with different inflammatory stimuli. Data are expressed as pg/mg of protein and are mean ± SD of four samples from two independent experiments Statistical analysis was by one-way ANOVA ($p < 0.001$ for each IL), using Tukey's post-hoc test. Different letters in the same row indicate significant differences (at least $p < 0.05$). LPS: lipopolysaccharide; IL-1β: interleukin 1 beta; TNFα: tumour necrosis factor alpha.

Table 5
Cytokine secretion after 24 h of supplementation.

	US	B	US IL-1β (10 ng/mL)	B	BIS0%	BIS2.5%	CFB0%	CFB4%	SFB0%	SFB4%
IFNγ	n.d.	n.d.	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	0.00 ± 0.01[a]	n.d.[a]	0.01 ± 0.01[a]
IL-1α	n.d.	0.34 ± 0.39	n.d.[a]	n.d.[a]	n.d.[a]	0.27 ± 0.44[a]	n.d.[a]	0.37 ± 0.52[a]	n.d.[a]	0.3 ± 0.29[a]
IL-2	n.d.	0.01 ± 0.01	n.d.[a]	n.d.[a]	n.d.[a]	0.02 ± 0.02[a]	n.d.[a]	0.01 ± 0.02[a]	n.d.[a]	0.03 ± 0.03[a]
IL-4	n.d.	n.d.	n.d.[a]	n.d.[a]	n.d.[a]	0.01 ± 0.01[a]	n.d.[a]	n.d.[a]	n.d.[a]	0.01 ± 0.02[a]
IL-6	0.05 ± 0.05	0.15 ± 0.05[†]	0.73 ± 0.32[ab]	0.56 ± 0.17[bcd]	0.61 ± 0.24[bcd]	0.82 ± 0.13[ab]	1.11 ± 0.26[a]	0.14 ± 0.1[d]	0.2 ± 0.22[cd]	0.55 ± 0.12[bcd]
IL-8	22.9 ± 2.3	21 ± 5.8	175.3 ± 44.4[a]	137.1 ± 16.7[ab]	139.9 ± 22.9[ab]	127.2 ± 4.3[abc]	173.6 ± 37.1[a]	62.9 ± 20.7[c]	68.5 ± 52[bc]	127.4 ± 30.4[abc]
IL-10	n.d.	0.01 ± 0.03	n.d.[a]	0.01 ± 0.02[a]	n.d.[a]	0.06 ± 0.09[a]	n.d.[a]	0.02 ± 0.03[a]	n.d.[a]	0.03 ± 0.42[a]
IL-12p70	n.d.	n.d.	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	0.25 ± 0.42[a]
TNFα	n.d.	n.d.	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]

Data are expressed as pg/mg of protein and are mean ± SD of four samples from two independent experiments. Statistical analysis was performed by the Student's *t*-test to compare US and B cells in basal condition († $p < 0.05$), and US and B inflamed cells with the corresponding basal counterparts (IL-6 and IL-8: $p < 0.001$). Inflamed supplemented cells were compared by one-way ANOVA (IL-6 and IL-8: $p < 0.001$) using Tukey's post-hoc test. Different letters in the same row indicate significant differences (at least $p < 0.05$). n.d. = not detectable. The limit of detection was 4.75 fg/ml, 0.177 pg/ml, 40.5 fg/ml, 9.72 fg/ml, 59 fg/ml, 0.25 pg/ml, 0.74 pg/ml for IFNγ, IL-1α, IL-2, IL-4, IL-10, IL-12p70, and TNFα, respectively. US: unsupplemented; B: blank; BIS0%: biscuits made without defatted olive pomace (DOP); BIS2.5%: biscuits made with 2.5% DOP; CFB0%: conventional fermented bread made without DOP; CFB4%: conventional fermented bread made with 4% DOP; SFB0%: sourdough fermented bread without DOP; SFB4%: sourdough fermented bread made with 4% DOP; IFNγ: interferon gamma; IL-1α: interleukin 1 alpha; IL-1β: interleukin 1 beta; IL-2: interleukin 2; IL-4: interleukin 4; IL-6: interleukin 6; IL-8: interleukin 8; IL-10: interleukin 10; IL-12p70: interleukin 12 p70; TNFα: tumour necrosis factor alpha.

To avoid misleading results (Di Nunzio et al., 2017), potential cytotoxicity of LMWF was assessed prior to other experiments. As previously reported (Van De Walle, Hendrickx, Romier, Larondelle, & Schneider, 2010), IL-8 was the main cytokine secreted by Caco-2 cells, and secretion was significantly increased upon the inflammatory stimulus. In inflamed cells, supplementation with blank digesta reduced the secretion of IL-8 secretion to a similar extent to supplementation with digested bakery products. This confirms the anti-inflammatory effects of bile acids (Ward et al., 2017) and indicates that the observed effect was mainly due to the vehicle.

Only LMWF from digested CFB4% and SFB0% actively contributed to the overall anti-inflammatory effect. The effectiveness of CFB4%, which showed the highest phenol concentration, is easily explained. On the contrary, it is difficult to give a motivation to the greater anti-inflammatory activity of SBF0%, which had a lower phenol and tocol content than the ineffective SBF4%. We hypothesize that the interactions between matrices (including DOP enrichment) and processing differently modulated bioactive bioaccessibility, i.e. the percentage that is made available for absorption during digestion, in conventional and experimental breads. This matrix and processing effect were already reported in different food. (Bordoni et al., 2011; Ferranti et al., 2014; Marcolini et al., 2015). Of note, the cytotoxic effect of the highest concentration of CFB4% and SFB0% and not SFB4% confirms that a higher number of active molecules was released from the matrix.

Using an untargeted approach, we evidenced that inflammation had no effect on cell metabolome in our experimental conditions. As well, in inflamed condition cell metabolome was not modified by any supplementation except SFB4%, which caused a decrease in snG3pc and an increase in myI and Cho concentration. In our previous study, olive polyphenols supplementation caused a huge dose-related perturbation of Caco-2 cells metabolome (Di Nunzio, Picone et al., 2018). In that study, the amount of olive polyphenols used for supplementation was 100–1000 times higher than in the present one, and this confirms that polyphenol concentration is a main determinant of metabolome perturbation. Of note, the present study reports the effect of an enriched food, which polyphenol concentration was based on organoleptic acceptance of the product. We speculate that in SFB4% the high concentration of tocopherol acted synergistically with polyphenols. The increase of MyI and Cho, which are important precursors of plasma membrane structured lipids (Tayebati, Marucci, Santinelli, Buccioni, & Amenta, 2015; Thomas, Mills, & Potter, 2016) suggests changes in cell membrane integrity (Ricks et al., 2019; Zeisel, Klatt, & Caudill, 2018) induced by polyphenols and tocopherol. Polyphenols, including olive phenols, incorporate into the lipid bilayer inducing biophysical changes (phospholipid re-packing) and altering the membrane structure (de Granada-Flor, Sousa, Filipe, Santos, & de Almeida, 2019; Saija & Uccella, 2000; Saija et al., 1998; Verstraeten, Fraga, & Oteiza, 2015). As well, tocopherol influences the phase behaviour of lipid bilayer affecting viscosity characteristics and structural transitions of plasmatic membrane (Belov, Mal'tseva, & Pal'mina, 2011; Wang & Quinn, 1999, 2000).

In summary, our results indicate that both the enrichment with a by-product of olive oil production and the baking process influence the functionality of the final products. Several phenolic compounds found in the enriched bakery products possess biological properties (Mateos, Sarria, & Bravo, 2019), so it is not possible to assess which of them was the major determinant of the effects observed at the Caco2 cell level. It is very likely that different phenols acted synergistically. Beside enrichment, the type of fermentation might influence the functional properties of bread, probably by modifying bioaccessibility of phenolic compounds, as previously reported by Katina et al. (2012) and Wang, He, and Chen (2014). Although the recent review by Gobbetti et al. (2019) reported numerous evidences that qualified the sourdough fermentation as the most sustainable and powerful process to exploit the technological, nutritional and functional features of bakery, the present work highlights that it could counteract the effect of specific

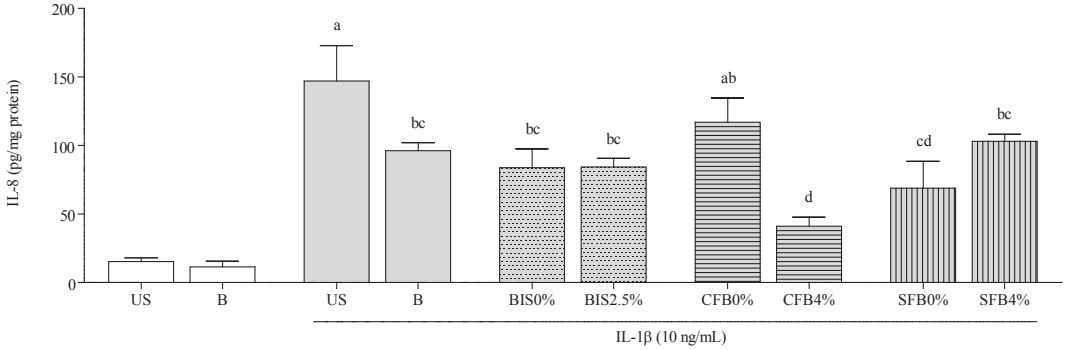

Fig. 7. IL-8 secretion after 24 h of supplementation. Data are expressed as pg/mg of protein and are mean ± SD of four samples from two independent experiments. Statistical analysis was performed by the Student's *t*-test to compare US and B cells in basal condition (n.s.), and US and B inflamed cells with the corresponding basal counterparts ($p < 0.001$ in both cases). Inflamed supplemented cells were compared by one-way ANOVA ($p < 0.001$) using Tukey's post-hoc test. Different letters indicate significant differences (at least $p < 0.05$). US: unsupplemented; B: blank; BIS0%: biscuits made without defatted olive pomace (DOP); BIS2.5%: biscuits made with 2.5% DOP; CFB0%: conventional fermented bread made without DOP; CFB4%: conventional fermented bread made with 4% DOP; SFB0%: sourdough fermented bread made without DOP; SFB4%: sourdough fermented bread made with 4% DOP. IL-1β: interleukin 1 beta.

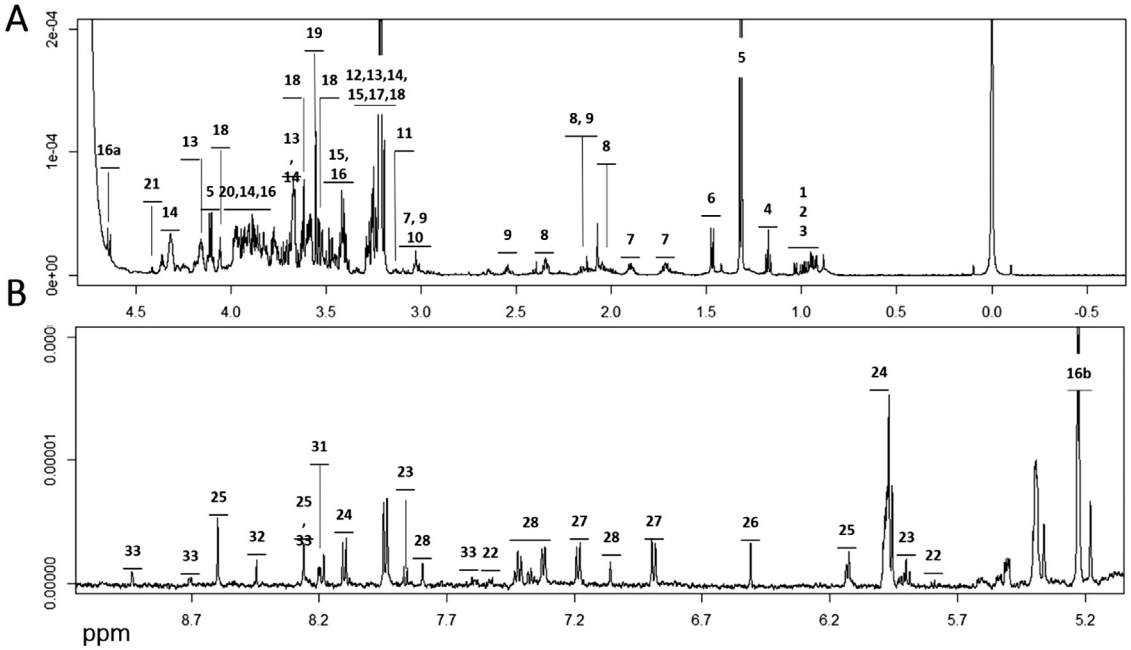

Fig. 8. Representative ^{1}H NMR spectrum in the upfield and midfield region (−0.5:4.60) (panel A) and downfield region (5.00:9.00) (panel B) of SFB4% cell lysate after 24 h of supplementation acquired with 600.13 MHz spectrometer at pH 7.33. (1) Isoleucine (t: 0.929, d: 1.000); (2) Leucine (t: 0.946); (3) Valine (d: 0.979, d: 1.032); (4) Ethanol (t: 1.175); (5) Lactate (d: 1.319, q: 4.106); (6) Alanine (d: 1.469); (7) Lysine (m: 1.716, m: 1.908, t: 3.023); (8) Glutamate (m: 2.042, m: 2.121, m: 2.360); (9) Glutathione (q: 2.167, m: 2.565, m:2.977); (10) Creatine (s: 3.028); (11) Ethanolamine (t: 3.138); (12) Choline (s: 3.193), (13) o-Phosphocholine (s: 3.209, m: 3.582, m: 4.156); (14) *sn*-Glycero-3-phosphocholine (s: 3.218, m: 3.679, m: 4.316), (15) Taurine (t: 3.249, t: 3.416); (16) Glucose (m: 3.395:3.527, m: 3.705:3.894); (16a) β-Glucose (d: 4.640); (16b) α-Glucose (d:5.227); (17) Glucose-6-phosphate (t: 3.274, d: 4.640, d: 5.227); (18) myo-Inositol (t: 3.272, dd:3.528, t:3.615, t: 4.056); (19) Glycine (s: 3.553); (20) O-Phopshoethanoalmine (m: 3.971); (21) 1,3-Dihydroxyacetone (s: 4.413); (22) Uracil (d;5.796, d: 7.528); (23) Uridine (m: 5.905, d: 7.861); (24) UMP (m: 5.975, d:8.101); (25) AMP (d: 6.130, s: 8.260, s: 5.596); (26) Fumarate (s: 6.510); (27) Tyrosine (d: 6.890, d: 7.185); (28) Histamine (s: 7.059, s: 7.794); (29) Phenylalanine (d: 7.322, t: 7.369, t: 7.421); (30) Hypoxanthine (s: 8.181, s: 8.201); (31) Formate (s: 8.445); (32) Nicotinurate (q: 7.590, m: 8.243, dd: 8.705, d:8.931). s: singlet. dd: doublet of doublets. d: doublet. t: triplet. m: multiplet.

enrichments. Therefore, it can be concluded that there are no absolute best techniques and bread-making must be carefully modulated based on ingredients. The effect of processing on phenolics content was clearly evidenced also in pasta enriched with DOP, where cooking significantly decreased the concentration of bioactives (Simonato, Trevisan, Tolve, Favati, & Pasini, 2019).

Results herein reported highlight that the increased concentration of bioactive molecules in the food is not enough to guarantee its functionality in biological systems. Bioaccessibility must be carefully considered, and effectiveness should be demonstrated. To our knowledge, the present study is the first one not limited to evidencing the increase of phenolics and antioxidant potential in enriched bakery products but also evaluating the effect of the digested products in cultured cells. Notably, the contribution of the gut microbiota to polyphenols transformation was not considered in our model system, and we are aware that results obtained *in vitro* do not exactly mirror the *in vivo* effect. Anyway, although clinical intervention studies are the gold standard to verify the health effect of foods, *in vitro* studies can give useful preliminary indications and may represent the first step towards the formulation of effective functional food.

Funding

This study was supported by the EU Project EcoPROLIVE

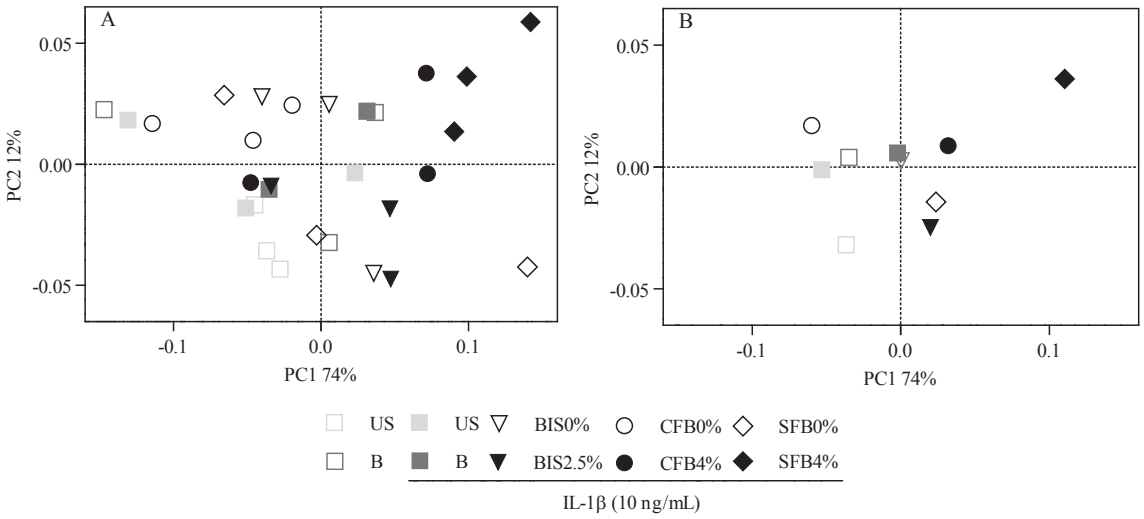

Fig. 9. PCA scores plot of the ^{1}H NMR spectrum for all samples (panel A) and representation of the mean values for each group (panel B). US: unsupplemented; B: blank; BIS0%: biscuits made without defatted olive pomace (DOP); BIS2.5%: biscuits made with 2.5% DOP; CFB0%: conventional fermented bread made without DOP; CFB4%: conventional fermented bread made with 4% DOP; SFB0%: sourdough fermented bread made without DOP; SFB4%: sourdough fermented bread made with 4% DOP; IL-1β: interleukin 1 beta.

Table 6
Integrals of bins from PC loadings.

	US	B	US IL-1β (10 ng/mL)	B	BIS0%	BIS2.5%	CFB0%	CFB4%	SFB0%	SFB4%
Glu	29.5 ± 1.8	30.4 ± 5.9	28.1 ± 0.85^{a}	28.1 ± 3.6^{a}	31.8 ± 4.2^{a}	26.9 ± 5.4^{a}	31.2 ± 2.2^{a}	35.3 ± 0.85^{a}	32.3 ± 3.4^{a}	28.3 ± 0.4^{a}
Lac	57.6 ± 3.2	58.6 ± 4.1	63.1 ± 0.9^{‡a}	58.4 ± 1.6^{a}	57.1 ± 9.5^{a}	63.7 ± 2.1^{a}	59.5 ± 2.3^{a}	62.1 ± 3.1^{a}	63.7 ± 3.8^{a}	54.5 ± 12.2^{a}
snG3pc	47.8 ± 4.74	45.1 ± 6.6	47.1 ± 2.5^{a}	44.5 ± 6.4^{a}	43.2 ± 1.6^{ab}	41.5 ± 3.1^{ab}	46.5 ± 2.5^{a}	40.1 ± 4.9^{ab}	41.3 ± 5.6^{ab}	34.4 ± 3.5^{b}
oPc	47.3 ± 4.1	47.7 ± 5.4	45.3 ± 1.2^{a}	45.4 ± 1.9^{a}	45.5 ± 1.9^{a}	43.8 ± 4.5^{a}	50.1 ± 3.2^{a}	43.6 ± 3.5^{a}	44.1 ± 5.9^{a}	41.8 ± 0.8^{a}
myI	10.7 ± 0.7	12.1 ± 1.7	9.7 ± 1.7^{b}	13.1 ± 2.2^{b}	11.6 ± 1.6^{b}	11.9 ± 2.4^{b}	12.2 ± 1.1^{b}	14.1 ± 3.5^{ab}	11.8 ± 1.7^{b}	19.3 ± 0.8^{a}
Cho	72.2 ± 3.5	88.9 ± 18.5	64.1 ± 10^{b}	94.5 ± 24.9^{b}	83.7 ± 9.2^{b}	89.6 ± 13^{b}	94.1 ± 7^{b}	115.6 ± 35.5^{ab}	90.9 ± 11.7^{b}	166.1 ± 17^{a}
Ala	19.2 ± 3.8	19.8 ± 5.1	22.8 ± 3.1^{a}	21.9 ± 1.4^{a}	21 ± 7.5^{a}	25.5 ± 3.2^{a}	21 ± 1.9^{a}	23 ± 2.4^{a}	24.8 ± 4.3^{a}	25.3 ± 2.4^{a}
Eth	15.3 ± 1.7	16.7 ± 3.1	18.8 ± 7.1^{a}	15.5 ± 5^{a}	15.7 ± 4.8^{a}	15.5 ± 1.8^{a}	16.6 ± 4.3^{a}	13.9 ± 2.5^{a}	15.5 ± 2.9^{a}	14.6 ± 3.3^{a}

Data are mean ± SD of three samples coming from independent experiments. Statistical analysis was performed by the Student's *t*-test to compare US and B cells in basal condition (n.s.), and US and B inflamed cells with the corresponding basal condition (‡ $p < 0.05$). Inflamed supplemented cells were compared by one-way ANOVA (snG3pc: $p < 0.01$; myI and Cho: $p < 0.001$) using Tukey's post-hoc test. Different letters in the same row indicate significant differences (at least $p < 0.05$). US: unsupplemented; B: blank; BIS0%: biscuits made without defatted olive pomace (DOP); BIS2.5%: biscuits made with 2.5% DOP; CFB0%: conventional fermented bread made without DOP; CFB4%: conventional fermented bread made with 4% DOP; SFB0%: sourdough fermented bread made without DOP; SFB4%: sourdough fermented bread made with 4% DOP; Glu: glucose; Lac: lactate; snG3pc: *sn*-glycero-3-phosphocholine; oPc: o-phosphocholine; myI: myo-inositol; Cho: choline; Ala: alanine; Eth: ethanol; IL-1β: interleukin 1 beta.

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CRediT authorship contribution statement

Mattia Di Nunzio: Conceptualization, Formal analysis, Investigation, Methodology, Data curation, Writing - original draft. **Gianfranco Picone:** Formal analysis, Investigation, Methodology, Data curation. **Federica Pasini:** Formal analysis, Investigation, Methodology, Data curation. **Elena Chiarello:** Formal analysis, Investigation, Methodology, Data curation. **Maria Fiorenza Caboni:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing. **Francesco Capozzi:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing. **Andrea Gianotti:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing. **Alessandra Bordoni:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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