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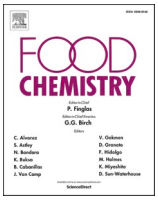

Phenols recovered from olive mill wastewater as natural booster to fortify blood orange juice

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ABSTRACT

In the present study, a tangential membrane filtration system was applied to recover phenols from olive mill wastewater. The obtained concentrates were characterised for physico-chemical traits, antioxidant activity and antimicrobial effects. Results indicated that the highest concentration of hydroxytyrosol (7203.7 mg/L) was detected in the concentrate obtained by reverse osmosis, which also showed the highest antioxidant and antimicrobial activity. Moreover, the same concentrate was added, at different ratio, up to 4:250 v/v, into a commercial blood orange juice. The fortified juice with the addition of the concentrate, up to 2:250 v/v ratio, did not show off-flavour and off-odour compared to the control. Furthermore, after 60 days of refrigerated storage, the fortified juice exhibited a hydroxytyrosol content still complying with the daily intake recommended by EFSA health claim. The obtained results can be industrially useful in producing orange juice added with a natural antioxidant concentrate as a 'clean label' ingredient.

1. Introduction

In the last years, consumers have becoming more and more aware about the ingredients in food and started actively scrutinizing the product labels. At the same time, increasing attention has been paid to the valorisation of agro-industrial waste and to the utilization of by-product, promoting their re-use to develop new functional food. In particular, vegetable by-products are considered valuable sources for the formulation of new natural food additives. Their exploitation represents a low-cost and environmentally friendly strategy that can provide alternatives to synthetic chemical compounds in food industries (Faustino et al., 2019).

Olive oil production represents the main agro-industrial activity in Mediterranean countries, and it is associated with the generation of a large amount of both liquid and solid by-products (Berbel et al., 2018). The olive mill wastewater (OMW), a liquid waste mainly obtained by the 3-phase extraction system, still represents a relevant management problem, above all for small olive oil companies but, at the same time, a high added value resource, being rich in bioactive compounds, such as hydroxytyrosol, tyrosol, oleuropein, flavonoids and others (Romeo, Granuzzo, Foti, Ballistreri, Caggia, & Rapisarda, 2021). The strong antioxidant activity of these compounds turns olive oil by-products into an inexpensive source of natural antioxidants with recognised healthy effects. Hydroxytyrosol has been proven to show anti-inflammatory and antimicrobial activities, to play a role in preventing and combating cardiovascular diseases and metabolic syndromes, with neuroprotective, anticancer and chemomodulatory effects (Robles-Almazan et al., 2018).

The European Food Safety Authority (EFSA), indeed, confirmed the health claim related to olive polyphenols at dose of 5 mg of hydroxytyrosol or its derivatives, corresponding to a daily consumption of 20 g of extra virgin olive oil. Furthermore, a recent study highlighted that the addition of olive by-products to foods exhibited an effect in extending the shelf life and in inhibiting the growth of pathogens (Di Nunzio et al., 2020).

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The recovery of phenols from OMW can be performed through conventional techniques, such as filtration trough membranes, solvent extractions and through emerging non-thermal technologies that reduce sensory alteration and nutritional deprecation of final product (Galanakis et al., 2018a; Barba, Galanakis, Esteve, Frigola, & Vorobiev, 2015). Among them, the membrane extraction technique is one of the most evaluable methods mainly at industrial scale (Caporaso et al., 2019). Furthermore, the membrane filtration technique is characterised by a low energy consumption, good operating conditions and high efficiency in component separation. This technology, based on the capacity of substances to cross the polymeric or inorganic semipermeable membrane at different rates, allows a cost-effective purification of phenolic pool present in OMWs, thanks to the low operating temperature (Cassano, Conidi, Giorno, & Drioli, 2013). The filtration technique involves microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) processes (Mallamaci et al., 2021). Furthermore, the fractions obtained from OMW can be added into food formulations as concentrated stabilised extracts and, in some cases, microencapsulated (Foti et al., 2021a). Therefore, this treatment makes of a by-product an alternative resource of biologically active phenols that can be used to fortify foods and/or beverages (Caporaso et al., 2019; Galanakis, Yücetepe, Kasapoğlu, & Özçelik, 2017).

Orange juice is a natural functional beverage thanks to the high content of vitamin C and flavonoids, the most abundant phenolic compounds present in *Citrus* fruits (Ballistreri et al., 2019). Red orange fruits represent the most important citrus product of Southern Italy. From these fruits, the obtained blood orange juice is characterized by high anthocyanin levels. Thanks to its acidity, orange juice is a suitable matrix to deliver nutraceutical molecules or probiotics and represents a promising candidate for the formulation of new functional beverages (Bonaccorso et al., 2021).

The aim of this study was to select the best concentrate, among the OMW fractions, obtained through ultrafiltration and reverse osmosis at industrial level, for fortification of blood orange juice. For this purpose, physico-chemical, microbiological, antioxidant and antimicrobial properties of different concentrates were evaluated. In addition, the most promising concentrate was added to a commercial blood orange juice, at different amounts, and its effect on physico-chemical, chemical, microbiological and sensory traits was evaluated up to 60 days of refrigerated conditions.

2. Materials and methods

2.1. Olive mill wastewater sampling

The OMW was kindly supplied by the olive oil company "Azienda Olearia Consoli Pasquale & F.lli s.n.c" (Adrano, Sicily). The OMW samples, acidified with 0.6 % of food grade hydrochloric acid, were treated at farm level using the 'Permeaprocess' plant (Permeare s.r.l., Italy). The system consists of a tangential filtration based on selective membranes suitable for purification, fractionation and concentration of compounds. This physical method allows the elimination of water at room temperature by means of a semi-permeable membrane, capable of overcoming osmotic pressure. The technique separates the water present in the samples, concentrating all the present components, including phenols and organic acids. Three concentrates were obtained: the ultrafiltration concentrate (C1 sample), the first osmosis concentrate (*C*2 sample) and the second osmosis concentrate (C3 sample). Moreover, as showed in Fig. 1, the *P*1 sample was obtained from the C1, the *P*2 from the *C*2 and the *P*3 from the C3. All obtained fractions were stored at + 4 °C before analyses.

2.2. Chemical analyses of OMW and obtained fractions

The pH of OMW and obtained fraction samples was measured using a Mettler DL25 pH meter (Mettler-Toledo International Inc., Columbus, OH, USA). In addition, total soluble solid (TSS) value was determined using a refractometer (Atago, RX-5000) and expressed as °Brix. The total phenolic content was detected according to the Folin-Ciocalteu's (FC) colorimetric method. Samples were mixed with 5 mL of FC commercial reagent (Labochimica, Italy) diluted with water 1:10 v/v, added of 4 mL of a 7.5% sodium carbonate solution and left at room temperature away from light. The absorbance of samples was spectrophotometrically measured at 765 nm (Cary 100 Scan UV–Visibile, Agilent, CA, USA). The total phenolic content was expressed as mg of gallic acid equivalents (GAE)/L of sample.

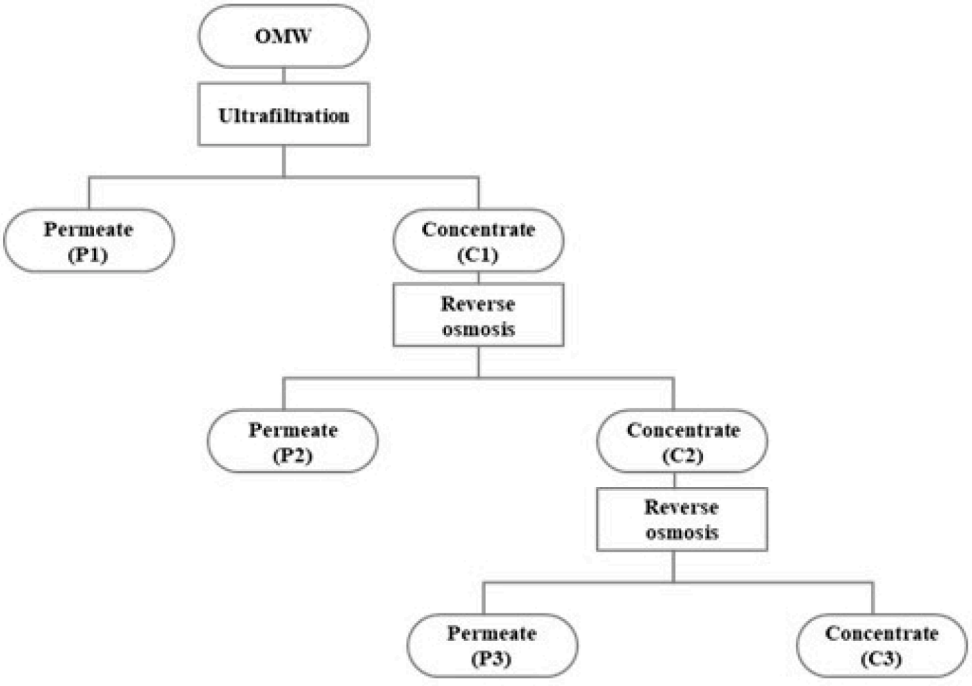

Fig. 1. Flowsheet of OMW filtration process.

2.3. HPLC analyses

2.3.1. Phenol detection

The HPLC analysis of OMW, concentrates, and the *P*1 permeate was performed by directly injecting the filtered samples (0.45 μm PTFE filters, Merck, Germany) into the chromatographic HPLC system. The system consisted of a liquid chromatography Waters Alliance 2695 HPLC equipped with a Waters 996 photodiode array detector (PDA) set at 280 nm and with Waters Empower software (Waters Corporation, MA, USA). The column was a Luna C18 (250 mm X 4.6 mm i.d., 5 m, 100 Å; Phenomenex, Torrence, CA, USA) maintained in an oven at 40 °C. A flow of 1 mL/min was used. The chromatographic separation was performed according to Romeo et al. (2021). The internal standard (I.S.), a 50 mM pure gallic acid (Fluka, Switzerland), was used to quantify the phenolic compounds. The identification of phenolic compounds was obtained by comparing retention time with pure tyrosol (TYR), oleuropein (OLE) and hydroxytyrosol (HT) (Extrasynthese, Genay, France). All the analyses were carried out in triplicate for each sample.

2.3.2. Organic acid detection

For the determination of organic acids, samples were diluted with ultrapure water, at different ratios: the OMW, the C1 and the *P*1 samples at 1:1 v/v; the *C*2 and the C3 samples at 1:10 v/v; while the *P*2 and the *P*3 samples were used as they were. Each sample was then filtered, through a 0.45 μm PTFE syringe filter (Merck, Germany), before being injected into HPLC (the HPLC instruments were described in the previous section) with a DAD detector set at 210 nm (and with spectrum acquisition from 200 to 400 nm). Isocratic elution with 5 mM sulphuric acid was performed on a Rezex ROA Organic Acid H + column (Phenomenex, Torrence, CA, USA). The run time was set at 50 min at 0.6 mL/min. For calibration, pure standards of lactic, citric, acetic, propionic, isobutyric and butyric acids (all purchased from Sigma-Aldrich, Italy) were injected at different concentrations. All the analyses were carried out in triplicate for each sample.

2.4. Microbiological analyses

2.4.1. Microbiological analyses of OMW and concentrate samples

The concentrates were serially diluted and poured into agar plates contained specific media and incubated at specific conditions: de Man, Rogosa, and Sharpe Agar (MRSA, Oxoid, UK) for lactobacilli count, incubated at 32 °C for 48 h under anaerobic conditions; Plate Count Agar (PCA, Oxoid, UK) for mesophilic aerobic bacteria count, incubated at 25 °C for 48 h; Sulphite-Polymyxin-Sulphadiazine Agar (SPS, Oxoid, UK) for the detection of *Clostridium perfringens,* incubated at 35–37 °C for 18–48 h under anaerobic conditions; Sabouraud Dextrose Agar (SAB, Bio-Rad, CA) for yeasts counting, incubated at 25 °C for 48 h. Microbiological analyses were performed in triplicate and results expressed as Log CFU/mL ± standard deviation (SD).

2.4.2. Microbiological analyses of permeate samples

The two permeate samples, *P*2 and *P*3, were microbiologically analysed following the membrane filtration method (Standard Methods for the Examination of Water and Wastewater: APHA, 2012). In detail, for the detection and counting of *Escherichia coli*, 100 mL of sample were filtered on membrane filters (0.45 μm pores, Cellulose, Merck, Germany) and poured in RAPID' *E. coli* 2 Agar plates (Bio-Rad, Italy), incubated at 37 °C for 24 h. For detection of *Clostridium* spores, 1 mL of sample was poured into sterile 50 mL tubes, added with 24 mL of sterile distilled water and tubes heated at 75 ± 5 °C for 10 min. Then, 25 mL of liquid Sulphite Polymyxin Sulphadiazine (SPS) at 45 °C, at double (2X) concentration, were added and tubes incubated at 37 °C for 24 h. For detection of sulphite-reducing bacteria, 1 mL of sample was poured, by inclusion, on SPS plates and plates anaerobically incubated at 37 °C for 24 h. The counting of somatic coliphages was carried out following the "ISO 10705–2:2000(E) - *Water quality- Detection and enumeration of bacteriophages - Part 2: Enumeration of somatic coliphages*" protocol. The detection of intestinal nematodes (helminth eggs) was carried out following the "*Official method suppl. ord. g.u. n. 87*" of 13 April 2000, which foresees a sedimentation phase and a series of centrifugations followed by flotation and observation under microscope. The detection and enumeration of *Legionella* spp. was carried out following the ISO 11731:2017 Water quality - Enumeration of *Legionella* procedure.

2.5. Antioxidant activity of OMW and fractions

Different dilutions of samples were added to the mixture of methanolic solution and 2,2-Diphenyl-1-picrylhydrazyl radical 10^{-4} M (DPPH, Merck, Germany). The absorbance was evaluated at 517 nm and the results expressed as a percentage decrease, compared to the control. Antioxidant activity was expressed with respect to sample volume and the concentration at which 50% radical scavenging occurred (IC_{50}). Stronger radical quenching results at a lower IC_{50} value. Inhibition percentage for each sample was calculated as follows:

$$\%inhibition = \frac{A_0 - A_x}{A_0} 100$$

where A_0 is the absorbance of a DPPH blank and A_x is the sample absorbance.

2.6. Antimicrobial activity of OMW and obtained fractions

The inhibitory activity of OMW and obtained fractions (C1, *P*1, *C*2 and C3) was tested, according to Foti et al. (2021b), against pathogenic strains: *Listeria monocytogenes* ATCC 19114, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10231, *Staphylococcus aureus* ATCC 25213, *Pseudomonas aeuroginosa* ATCC 9027, *Salmonella* Typhimurium ATCC 14028, *Bacillus subtilis* ATCC 19659, *Clostridium sporogenes* ATCC 11437, and *Enterococcus faecalis* ATCC 29212 (American Type Culture Collection). In addition, the same fractions were tested on probiotic strains: *Lacticaseibacillus rhamnosus* CRL1505, *Lacticaseibacillus paracasei* 101/37, *Bifidobacterium animalis* subsp. *paracasei* BLC1 (purchased from Sacco S.r.l., Italy) and *Propionibacterium freudenreichii* DSM 4902 (Leibniz-Institute DSMZ, German collection).

The test was performed on: Potato Dextrose Agar (PDA, Likson, Italy) for *Candida albicans*; MRSA, for lactobacilli; Muller Hinton Agar Base (MHA, Liofichem, Italy) for other bacteria. For the probiotic strains, each individual culture was standardised using Mc Farland 0.5 solution, which corresponded to a cell density of approximately $1.5x10^8$ CFU/mL, while the standardised pathogenic strains were diluted to a cell density of $1x10^6$ CFU/mL. In each plate, containing the selective medium, 1 mL of cell suspension was spatulated, allowed to dry, and then sterile cellulose discs (Ø 6 mm) imbibed with each tested sample, at different dilution rates, were placed. The samples were tested as they were and at different dilution ratio (from 1:2 to 1:16). Distilled water was used as negative control. Plates were incubated at specific temperatures for 48 h and results expressed as diameter of the inhibition halo (mm).

2.7. Blood orange juice fortification

In the present study, a 100% blood orange juice, without any addition of sugar or preservatives and with an estimated shelf life of 60–65 days, was kindly provided by the Oranfrizer Company (located in Scordia, Sicily). The concentrate *C*2, filtered at 0.22 μm (PTFE filters, Merck, Germany), was added to the blood orange juice at different ratio [1:250 v/v (FBOJ1); 2:250 v/v (FBOJ2); 3:250 v/v (FBOJ3); 4:250 v/v (FBOJ4)] and the obtained fortified blood orange juice (FBOJ) samples were stored at + 4 °C for 60 days. The FBOJ samples were analysed at different times (0, 15 and 60 days) to evaluate chemical, microbiological, and sensory traits compared to the commercial juice as control. All analyses were carried out in triplicate.

2.7.1. Microbiological analysis of FBOJ

The FBOJ samples, obtained by addition of the *C*2 concentrate, were microbiologically analysed at 0, 15 and 60 days of storage on the following media: MRS, for the determination of lactobacilli; PCA, for mesophilic aerobic bacteria count; SAB, for yeasts and moulds. The culture conditions were the same as described in 2.4.1 section.

Microbiological analyses were performed in triplicate and results were expressed as Log CFU/mL ± SD.

2.7.2. Total phenolic content and HPLC assay of FBOJ

The FBOJ samples obtained by addition of the *C*2 concentrate, were tested for total phenolic content as described in 2.2 section and for the quali-quantitative determination of single phenol, by direct injection into HPLC, as described in 2.3.1 section.

2.7.3. Colorimetric assay of FBOJ

The colour of the FBOJ samples, obtained by adding at different ratio the *C*2 concentrate, was determined at different storage times, as previously reported by Timpanaro et al. (2021). The coordinates L* (brightness), a* (green–red component), b* (blue-yellow component), were determined as the average of two transmittance measurements using a spectrophotometer CM-5 (Minolta, Milan, Italy). The parameters L*, a*, b* were determined using the illuminant D65, according to the CIELAB scale.

2.7.4. Sensory analysis of FBOJ

The standard ISO 13299:2016 provides guidelines for developing a sensory profile, which can be obtained for any products that can be evaluated by sight, smell, taste, tact, or hearing. The profile method was performed by a trained panel (EN ISO 8586:2014), and ten panellists (4 males and 6 females, aged between 28 and 45 years) were selected among the staff of CREA - Research Centre for Olive, Fruit and Citrus Crops, located in Acireale, Italy. During the training period, the judges selected the attributes to describe the colour (light orange to deep red), odour of orange, off-odour, acidity, sweetness, flavour, off-flavour, and bitterness using orange fresh juice as control. Judges evaluated the intensity of each attribute by assigning a score between 1 (absence of the sensation) and 9 (extremely intense) on a numerical unipolar scale (ISO 4121:2003). Sensory analyses were performed at the same day of *C*2 addition. All sensory tests were performed at the sensory analysis laboratory devised in accordance with UNI EN ISO 8589: 2014.

2.8. Statistical analyses

All analyses were performed in triplicate. SPSS software (version 21.0, IBM Statistics, Armonk, NY, USA) was used for data processing. Statistical analysis of the results was performed using one-way analysis of variance (ANOVA), and Tukey's HSD post hoc test for means separation at a significance level of $P \leq 0.05$.

3. Results

3.1. Physico-chemical traits of OMW and obtained fractions

The OMW and the obtained fractions were analysed for pH, TSS and total phenolic content. The pH ranged from 3.41 to 3.96. The TSS and total phenols values increased proceeding from ultrafiltration to reverse osmosis, reaching values of 15.17 °Brix and 8523.23 mg/L in the C3 sample (Table 1).

The *P*2 and *P*3 permeates showed the lowest values of both TSS (0.19 and 0.36 °Brix, respectively) and total phenol contents (19.42 mg/L and 55.05 mg/L, respectively).

3.2. Phenols, antioxidant activity and organic acid detection

Zooming on phenolic compounds, except for other phenols present in traces, HT and TYR were the only phenols detected by HPLC analysis, reaching the highest concentration in the *C*2 and C3 samples, with 7203.7 and 6936.2 mg/L (HT) and 1046.6 mg/L and 1613.9 (TYR), respectively (Table 2). It is interesting to point out that the C3 sample, despite the highest total phenolic content, showed a slight lower content of HT.

Table 1
Physico-chemical traits of OMW and obtained fractions.

Samples	pH	TSS (°Brix)	Total phenolic content (mg/L)
OMW	3.92 ± 0.07^{a}	5.40 ± 0.02^{d}	2983.39 ± 0.31^{d}
C1	3.91 ± 0.08^{a}	6.29 ± 0.07^{c}	3244.11 ± 0.21^{c}
P1	3.94 ± 0.06^{a}	5.05 ± 0.07^{d}	2888.02 ± 0.02^{e}
C2	3.96 ± 0.05^{a}	10.35 ± 0.24^{b}	6207.41 ± 0.12^{b}
C3	3.90 ± 0.14^{a}	15.17 ± 0.04^{a}	8523.23 ± 0.03^{a}
P2	3.45 ± 0.01^{b}	0.19 ± 0.04^{e}	19.42 ± 0.01 ^{g}
P3	3.41 ± 0.01^{b}	0.36 ± 0.28^{e}	55.05 ± 0.01^{f}
	**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. **Significance at $P \leq 0.01$.

Results on antioxidant activity, evaluated by the DPPH method, showed that the proton removal activity of fractions was positively related to the concentration of free phenolic compounds. Lower IC_{50} values are related to a stronger radical quenching activity. As expected, the lowest IC_{50} values were detected for the *C*2 (41.17 IC_{50}) and the C3 samples (50.95 IC_{50}), as reported in Table 2. These results confirmed that the *C*2 concentrate sample, with the highest HT content, exhibited the highest antioxidant activity.

Looking at organic acids, for lactic, acetic and propionic acids a general increase in their concentrations during the filtration process was observed (Table 3), whereas isobutyric acid was detected only in the OMW and C1. The highest concentration of lactic acid was detected in the C3 and *C*2 samples, at 11860.8 and 7953.7 mg/L, respectively, while in the other samples the mean concentration value was 3600 mg/L (in OMW, C1 and *P*1 samples) and 99 and 151 mg/L in *P*2 and *P*3, respectively. The C3 and *C*2 samples showed the highest concentrations of acetic acid, reaching values of 17612.0 and 12137.2 mg/L, respectively, and were the only samples in which propionic acid was found (5393.9 and 2984.4 mg/L, respectively). Citric and butyric acids were never detected in any samples.

3.3. Microbiological analyses of OMW and obtained fractions

Overall, lactobacilli and *Clostridium perfringens* were not detected in OMW, C1, *P*1, *C*2 and C3 samples, whereas a mesophilic aerobic bacteria count, ranging between 4.00 and 4.35 Log CFU/mL, was detected in all samples. Furthermore, yeasts and moulds were not found in the *P*1 and in the *C*2 samples, whereas a mean value of 3.83 Log CFU/mL was counted in the other samples (Table S1, supplementary material).

Regarding the *P*2 and *P*3 permeates, *Escherichia coli* (in 100 mL), *Clostridium* spores, sulphite-reducing bacteria, somatic coliphages, intestinal nematodes (helminth eggs) and *Legionella* spp. were not detected and the results were found to comply with the limits imposed by

Table 2
Phenols and antioxidant activity detected in OMW and in the obtained fractions.

Sample	HT (mg/L)	TYR (mg/L)	IC_{50}
OMW	3321.07 ± 61.73^{c}	508.02 ± 20.40^{c}	87.67 ± 0.17^{a}
C1	3415.15 ± 65.94^{c}	494.37 ± 0.14^{c}	84.00 ± 0.10^{ab}
P1	3327.68 ± 42.58^{c}	499.11 ± 4.07^{c}	80.18 ± 0.18^{b}
C2	7203.67 ± 54.85^{a}	1046.62 ± 2.50^{b}	41.17 ± 0.02^{d}
C3	6936.27 ± 43.82^{b}	1613.97 ± 6.87^{a}	50.95 ± 0.16^{c}
	**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. **Significance at $P \leq 0.01$.

Table 3
Detected organic acids.

Sample	Lactic acid (mg/L)	Acetic acid (mg/L)	Propionic acid (mg/L)	Isobutyric acid (mg/L)
OMW	3583.7 ± 135.80^{c}	6680.9 ± 94.59^{c}	0.00 ± 0.00^{c}	13187.3 ± 507.60^{a}
C1	3554.3 ± 58.78^{c}	6540.6 ± 40.10^{c}	0.00 ± 0.00^{c}	12621.7 ± 374.88^{b}
P1	3733.7 ± 10.35^{c}	6714.7 ± 33.29^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
C2	7953.7 ± 7.93^{b}	12137.2 ± 7.38^{b}	2984.4 ± 89.77^{b}	0.00 ± 0.00^{c}
C3	11860.8 ± 107.20^{a}	17612.0 ± 343.96^{a}	5393.9 ± 500.56^{a}	0.00 ± 0.00^{c}
P2	98.6 ± 4.43^{d}	1177.0 ± 135.83^{e}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
P3	150.8 ± 4.53^{d}	2460.4 ± 49.56^{d}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
	**	**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column are statistically different. **Significance at P ≤ 0.01.

Regulation (EU) 2020/741 on minimum requirements for water reuse (EU, 2020).

3.4. Antimicrobial activity

The antimicrobial activity of the OMW and the obtained fractions on pathogenic and probiotic strains was assessed by evaluation of inhibition zones. Overall, all the tested samples did not show any effect on probiotic tested strains, namely as *L. rhamnosus* CRL1505, *L. paracasei* 101/37, *Bif. animalis* subsp. *paracasei* BLC1 or *P. freudenreichii* DSM 4902. Among the tested samples, the *C*2 and the C3 concentrates showed inhibitory activity against *E. coli* and *P. aeuruginosa,* with an inhibition zone of 12 and 14 mm, respectively (Table S2, supplementary material). Both the *C*2 and the C3 concentrates exhibited a dose-dependent antimicrobial activity against pathogens (Table S2). No inhibitory activity was observed against the other tested pathogens (*L. monocytogenes, C. albicans, St. aureus* or *Salmonella* Typhimurium).

3.5. Microbiological, chemical and sensory traits of FBOJ

The *C*2 concentrate, the richest fraction in the bioactive compound HY, was included in blood orange juice to fortify the nutraceutical component of the product. The *C*2 concentrate was added to 250 mL commercial orange juice, at a ratio of 1:250 v/v (FBOJ1), 2:250v/v (FBOJ2), 3:250 v/v (FBOJ3), 4:250 v/v (FBOJ4), and the obtained FBOJ analysed at 0, 15 and 60 days of storage, at refrigerated conditions.

Regarding microbiological results, in all samples (both FBOJ and controls) the searched microbial groups (as lactobacilli, mesophilic aerobic bacteria and yeasts and moulds) were not detected at any sampling times, except in controls and FBJO1 which at 60 days of storage showed yeasts and moulds densities of 3.3 and 3.0 Log CFU/mL, respectively (data not shown).

Looking at physico-chemical results, no significant differences in pH and TSS values was found between control and FBOJ samples (Table 4), at any sampling points. Regarding the total phenolic content, data showed that increasing the volume of the *C*2 addition, a higher phenol content was found in the fortified juices. In FBOJ4 sample, where the ratio *C*2/juice was 4:250 v/v, at the same day of fortification (T0), the total phenolic content was 750 mg/L higher than that detected in the control juice. Furthermore, it is interesting to underline that after 60 days of storage, the FBOJ4 sample showed almost the same total phenolic content (3100 mg/L) detected in the control juice at initial time (Table 4).

Zooming at content of bioactive molecules, monitored at different sampling times, the FBOJ samples showed a proportional increase in HT and TYR. It is interesting to highlight that the FBOJ1 sample, obtained by adding the lowest volume of *C*2 extract (1 mL of *C*2 to 250 mL of juice), exhibited an initial concentration of HT and TYR of 26.92 and 43.21 mg/L, and a concentration of 21.67 and 60.10 mg/L of HT and TYR, respectively, after 60 days of storage. This data allows us to state that the FBOJ1 provides, up to the end of estimated shelf life, the recommended health beneficial intake of HT, as recognised by European Foods Safety Authority (EFSA).

Regarding the colour coordinates, the brightness (L*) showed an increasing trend during the shelf life in all tested sample (Table S3, supplementary material). In addition, a significant decrease in the red coordinate (a*), right after 15 days of refrigerated storage, was observed, while the yellow coordinate (b*) remains unchanged over time, reaching the highest values in FBOJ3 and FBOJ4 samples (Table S3, supplementary material).

Table 4
Chemical parameters of FBOJ samples fortified with different additions of *C*2 concentrate.

Samples	Time (days)	pH	TSS (°Brix)	Total phenolic content (mg/L)	HT (mg/L)	TYR (mg/L)
Commercial juice	0	3.38 ± 0.01	11.60 ± 0.08	3142.2 ± 0.54^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}
FBOJ1	0	3.37 ± 0.01	11.55 ± 0.01	3553.2 ± 2.66^{d}	26.92 ± 0.67^{d}	43.21 ± 4.14^{b}
FBOJ2	0	3.38 ± 0.05	11.67 ± 0.01	3643.6 ± 0.54^{c}	67.43 ± 3.77^{c}	52.65 ± 3.81^{ab}
FBOJ3	0	3.39 ± 0.05	11.65 ± 0.01	3715.2 ± 1.63^{b}	82.23 ± 0.75^{b}	58.08 ± 1.62^{a}
FBOJ4	0	3.39 ± 0.00	11.56 ± 0.01	3893.2 ± 1.09^{a}	100.87 ± 1.42^{a}	56.50 ± 0.86^{b}
		n.s.	n.s.	**	**	**
Commercial juice	15	3.3 ± 0.01^{d}	11.36 ± 0.01^{c}	2900.0 ± 0.54^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{b}
FBOJ1	15	3.34 ± 0.01^{c}	11.75 ± 0.01^{a}	3147.9 ± 1.63^{d}	24.28 ± 0.65^{d}	60.20 ± 0.22^{a}
FBOJ2	15	3.35 ± 0.01^{bc}	11.66 ± 0.01^{b}	3174.1 ± 3.26^{c}	48.22 ± 0.38^{c}	58.89 ± 0.97^{a}
FBOJ3	15	3.38 ± 0.01^{a}	11.65 ± 0.00^{b}	3396.8 ± 0.55^{b}	81.01 ± 0.59^{b}	57.75 ± 2.44^{a}
FBOJ4	15	3.36 ± 0.01^{b}	11.67 ± 0.02^{b}	3410.6 ± 0.54^{a}	105.52 ± 1.87^{a}	59.49 ± 0.30^{a}
		**	**	**	**	**
Commercial juice	60	3.31 ± 0.00^{c}	11.70 ± 0.01^{a}	2545.0 ± 0.01^{d}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}
FBOJ1	60	3.32 ± 0.00^{bc}	11.56 ± 0.06^{ab}	2623.1 ± 0.54^{d}	21.67 ± 0.05^{d}	50.11 ± 0.16^{ab}
FBOJ2	60	3.36 ± 0.02^{a}	11.66 ± 0.04^{a}	2851.1 ± 1.09^{c}	46.29 ± 0.56^{c}	53.60 ± 0.85^{a}
FBOJ3	60	3.35 ± 0.00^{ab}	11.29 ± 0.13b	2894.6 ± 0.56^{b}	75.35 ± 2.27^{b}	52.24 ± 0.20^{ab}
FBOJ4	60	3.35 ± 0.01^{abc}	11.26 ± 0.06^{b}	3100.0 ± 1.10^{a}	94.58 ± 2.91^{a}	49.20 ± 2.05^{b}
		*	**	**	**	**

Data are expressed as means ± SD. Mean values with different letters within the same column at the same time interval are statistically different. N.s. not significant; *Significance at P ≤ 0.05; **Significance at P ≤ 0.01.

Looking at the sensory parameters, overall, only flavour, off-flavour, bitterness and off-odour showed significant differences (Table 5). The flavour reached the highest score in FBOJ1 sample, but the statistical differences among samples were not related to the concentrate addition. Flavour descriptor decreased in FBOJ3 and FBOJ4 samples, while the perceived bitterness was higher in FBOJ4 sample, compared to other samples. Off-flavour and off-odour descriptors statistically increased in FBOJ3 and FBOJ4 samples (Figure S4, supplementary material).

4. Discussion

Polyphenols from olive fruit, olive mill wastewater or olive oil, *Olea europaea* L. extract and leaf, standardised for their content of HT, possess the health claim approved by EFSA under Article 13 (Health Claims Regulation 1924/2006), in relation to the protection of blood lipids from oxidative damage, which is known to adversely affect cardiovascular health (EFSA, 2011; EC, 2012).

Furthermore, in a recent study, the safety and the effects of HT purified (99.5%) from OMW were assessed by administering HT at a daily dose of 45 mg for 8 weeks to volunteers with mild hyperlipidaemia (Lopez-Huertas & Fonolla, 2017). In particular, the authors demonstrated that the administration of HT did not affect markers of cardiovascular disease, blood lipids, inflammatory status, liver or kidney function and that electrolyte balance with vitamin C increased two-fold at 4 and 8 weeks, compared to baseline levels (Lopez-Huertas & Fonolla, 2017).

In the present study, the tangential membrane filtration technique produced fractions differently concentrated in bioactive compounds. The concentrate *C*2 showed the highest concentration of HT, known for its antioxidant activity and for playing a role as an intracellular and extracellular scavenger of reactive oxygen species (ROS) (Robles-Almanaz et al., 2018). Indeed, in the present study, the higher concentration of HT was positively related to a greater antioxidant activity.

Several studies have reported that the addition of OMW phenolic fraction induces a fortification of the nutraceutical component and increases the shelf life of foods (Mikdame, Kharmach, Mtarfi, Alaoui, Ben Abbou, Rokni, & Rais, 2020; Servili et al., 2011). As a matter of facts, phenolic compounds show wide antimicrobial activity, such as antibacterial, antiviral and antifungal effects (Marković, Torić, Barbarić, & Brala, 2019).

Although HT has been reported to *in vitro* inhibit the growth of several pathogens, included *L. monocytogenes, St. aureus, Salmonella enterica, Yersinia* or beneficial microorganisms, as *L. acidophilus* and *Bifidobacterium bifidum* (Marković et al., 2019), in the present study no inhibitory activity was observed against the probiotic tested strains or against *L. monocytogenes, St. aureus,* and *Salmonella enterica.* In the present study, the *C*2 and C3 concentrates showed inhibitory activity against *P. aeuruginosa* and *E. coli,* otherwise Medina et al. (2016) reported that a MIC value of 400 μg/mL of HT was able to affect the growth of *E. coli,* while MIC values higher than 1000 μg/mL were required to affect the growth of *P. aeuruginosa.*

Among the tested samples, only the *C*2 showed antimicrobial activity against *B. subtilis, Cl. sporogenes* and *E. faecalis*. For *B. subtilis*, the results agreed with those reported by Tafesh et al. (2011) and by Galanakis et al. (2018b) who showed the antimicrobial effect of OMW phenolic extracts, in combination with other antioxidants, against *B. subtilis, E. coli,* and *P. aeruginosa.*

In addition, the two concentrates *C*2 and C3 did not exhibit any antagonist effect against *C. albicans.* This finding could be due to the hydrophilic nature of OMW concentrates, being the more lipophilic constituents partitioned into the olive oil during processing. Diallinas and co-workers (2018) reported that a lower hydrophilic/lipophilic balance could increase the cellular uptake enhancing the antioxidant or antimicrobial activities. However, the exact mechanism by which HT exerts its antimicrobial activity remains not completely understood (Wei et al., 2018) and Reverón and co-workers (2020) suggested an involvement of ROS overproduction as a mechanism of antimicrobial activity. The widest antimicrobial activity of the *C*2 concentrate could be related to the higher antioxidant activity even although the complex chemical composition of concentrates includes wide range of phytochemicals with synergistic effects.

Nevertheless, it is relevant to underline that the controversial results reported in literature could be due to the lack of a standard method or evaluation criteria for screening antimicrobial activity in plant extracts (Nostro, Germano, D'angelo, Marino, & Cannatelli, 2000). Differences in antimicrobial assay, growth media, bacterial strains, inoculum size and cell density of the target microorganism make comparisons of antimicrobial data of plant extracts from different sources very difficult.

The addition of the *C*2 concentrate in blood orange juice has been here proposed to obtain a functional beverage with a high content of both flavonoids and HT. Indeed, orange juice is a natural source of vitamin C, and a 200 mL dose provides up to 80% of recommended daily intake (Klimczak, Małecka, Szlachta, & Gliszczyńska-Świgło, 2007).

Zooming on the effect of the *C*2 addition on chemical composition of juice, the results here reported confirmed that the nutraceutical value of the juice was increased and the HT was still revealed up to 60 days of storage at refrigerated conditions. Furthermore, no microbiological differences were observed in samples at any *C*2 additions, although the yeast and mould growth were detected after 60 days in control and in fortified juice with the lowest addition of *C*2 (FBOJ1 sample).

The addition of any compound to food could have a detrimental effect on sensory and technological properties (Marinelli, Padalino, Nardiello, Del Nobile, & Conte, 2015). The colour of orange juice influences consumers' choice, above all for blood orange juices that are rich in anthocyanins, responsible of the dark red colour. In this study, the colour of FBOJ was monitored at different times, revealing significant statistical differences on L*, a* and b* parameters. Sensory analyses were carried out at the same day of fortification in order to assess the perception threshold of the *C*2 addition to the juice. This parameter is an important preliminary step to investigate the proper concentration of a fortifying agent in designing a new functional food or beverage.

Moreover, results of present study confirmed that membrane filtration techniques produce permeate fractions (the *P*2 and *P*3) suitable for irrigation, being compliant with limits imposed by Regulation (EU) 2020/741 on minimum requirements for water reuse (EU, 2020) and with the legal limits for releasing into the aquatic system (Cassano et al., 2013; Paraskeva, Papadakis, Tsarouchi, Kanellopoulou, & Koutsoukos, 2007; Russo, 2007).

Table 5
Sensory traits of FBOJ samples fortified with different additions of *C*2 concentrate.

	Commercial juice	FBOJ1	FBOJ2	FBOJ3	FBOJ4	
Colour	5.62 ± 0.44	5.65 ± 0.44	5.75 ± 0.38	5.68 ± 0.37	5.75 ± 0.38	n. s.
Odour of orange	5.62 ± 0.44	5.42 ± 0.57	5.18 ± 0.59	5.00 ± 0.46	5.06 ± 0.68	n. s.
Acidity	5.00 ± 0.27	5.14 ± 0.20	5.12 ± 0.23	5.06 ± 0.32	5.31 ± 0.37	n. s.
Sweetness	4.50 ± 0.38	4.40 ± 0.25	4.69 ± 0.53	4.19 ± 0.26	4.31 ± 0.37	n. s.
Flavour	5.69 ± 0.37^{ab}	5.85 ± 0.62^{a}	5.50 ± 0.38^{ab}	5.12 ± 0.64^{b}	5.25 ± 0.38^{ab}	*
Off-flavour	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	2.62 ± 0.52^{a}	2.69 ± 0.46^{a}	**
Bitterness	1.31 ± 0.26^{b}	1.37 ± 0.23^{ab}	1.38 ± 0.23^{ab}	1.44 ± 0.18^{ab}	1.69 ± 0.26^{a}	*
Off-odour	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	1.81 ± 0.59^{a}	2.06 ± 0.42^{a}	**

Data are expressed as means ± SD. Mean values with different letters within the same row are statistically different: ** significance at $p \leq 0.01$; * significance at $p \leq 0.05$; n.s., not significant.

5. Conclusions

Many consumers currently require supplement with vitamins, minerals and other nutrients, and as never before research-based evidence are required to correctly inform consumer, mainly on natural compounds. At the same time, an increasing interest has been posed on the ingredients used in food products, with a major challenge for 'clean label' ingredient. The present study confirmed that the tangential membrane filtration, an eco-friendly technique, represents a suitable valorisation strategy of OMW. This extraction technology on an industrial scale can effectively represent an income-generating solution for the olive oil industry by creating a collaboration with local food and beverage companies for the formulation of new products with high nutraceutical value. In order to overcome the seasonality of olive oil production, a crucial point could be the adaptation of the extraction process to several matrices with the aim to create a continuous production and obtain a supply cycle of phenolic concentrates for food industry. The concentrates rich in HT and TYR, obtained by reverse osmosis, exhibited antimicrobial and antioxidant activity, whereas the last two permeates, thanks to the low chemical load and for complying with the limits fixed by Regulation (EU) 2020/741 on minimum requirements for water reuse (EU, 2020), are suitable for the context of a circular economy. The addition of these concentrates in orange juice formulation implies an increase of phenolic content and provides the suitable amount of molecules with healthy effect on consumer. In detail, the FBOJ samples obtained by adding 2 mL of concentrate into 250 mL of juice showed a higher nutraceutical content without any sensory change. The OMW phenol concentrate and blood orange juice combined in a new functional beverage highlight the beneficial effect of the Mediterranean diet.

CRediT authorship contribution statement

Paola Foti: Investigation, Methodology, Writing – original draft. **Paride S. Occhipinti:** Methodology. **Flora V. Romeo:** Investigation, Methodology, Writing – original draft, Software, Resources, Methodology. **Nicolina Timpanaro:** Methodology, Software. **Teresa Musumeci:** Conceptualization, Writing – review & editing. **Cinzia L. Randazzo:** Conceptualization, Supervision, Writing – review & editing. **Cinzia Caggia:** Methodology, Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Paola Foti reports financial support, administrative support, and equipment, drugs, or supplies were provided by PON RI 2014-2020.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133428.

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