The Antimicrobial and Antioxidation Activities of Olive Pomace Extract in Pasteurized White Cheese

Hadjer M. Ferguous1, Ghadeer F. Mehyar*1 and Salam A. Ibrahim2

1 Department of Nutrition and Food Technology, School of Agriculture, The University of Jordan, Amman11942, Jordan
2 171 Carver Hall, Food Microbiology and Biotechnology Laboratory, College of Agriculture and Environmental Sciences, North Carolina A & T State University, Greensboro, NC 2711-1064

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ABSTRACT

Olive pomace from an olive mill was collected in December 2021. Proximate analysis revealed that the major constituents of the pomace were fiber (60.85 %) and fat (11.91%). Extraction of the olive pomace by methanol provided a higher yield, phenolic content, and free radical scavenging ability than the ethanol extract, but the latter was used due to its lower toxicity level as the extract is used in foods. The extract contained high concentrations of phenolic compounds, particularly syringic, p-coumaric, vanillin, and caffeic acid which accounted for ~80% of the total phenolic content. When the olive pomace extract (OPE) was used (0.08%) in pasteurized white cheese (PWC), the counts of mesophilic aerobes were significantly reduced to 3.62 log10 CFU/g after 30d of storage at 5°C compared to the control (4.04 log10CFU/g) after the same storage period. The corresponding values for LAB were 3.38 log10 CFU/g and 3.79 log10 CFU/g, respectively. The yeasts and molds were eliminated from the cheese for 14d at all of the tested OPE concentrations (0.01% to 0.08%). Fortification of cheese with OPE alleviated the increase in titrable acidity in cheese during storage, but the fortified cheese exhibited sensory attributes similar to those of the control cheese. It can thus be concluded that fortification of PWC with OPE improves the chemical and microbiobal attributes of the cheese.

Keywords: Olive Pomace, Pasteurized white cheese, Total Polyphenol, antioxidant, antimicrobial.

INTRODUCTION

The olive agri-food chain constitutes the largest agro-industrial sector in the Mediterranean basin and covers over five million hectares in the European Union member states (Gómez-Muñoz et al., 2012). Olive oil consumption increased worldwide surpassing three million tons per year during the last ten years. For example, in Jordan, it is about 20,706 Tons/ year (Department of Statistics, Agriculture; Jordan 2017; Mattas and Tsakiridou, 2017). The olive oil extraction process generates a considerable quantity of by-products that range from 2.75 to 4 tons for each ton of oil (Conterno et al., 2019). About 37,466 tons of olive pomace is produced in Jordan each year and depend on both fruit quality and extraction technology (Department of Statistics, Agriculture; Jordan 2017). The traditional oil
extraction method generates three phases, two of which are by-products (olive cake and wastewater representing 30% and 50%, respectively of the olive fruits) and olive oil about (20% of the olive fruits) (Klen and Vodopivec, 2012). The two-phase extraction system generates olive oil and one by-product which is a combination of liquid and solid (olive cake and wastewater) (Vlyssides et al., 1998). Olive pomace (olive cake or olive husk), is a semi-solid paste, mainly composed of fragments of olive skin, pulp and stone (solid phase), water, and oil (Nunes et al., 2016). It is a phytotoxic and non-biodegradable biomass that is challenging to treat due to its high moisture level (higher energy demand and consequently higher costs during the drying process) and other complex organic compounds (adverse effects on soil) (Salomone and Ioppolo, 2012). Olive pomace is harmful to the environment despite being a heterogeneous mixture and a significant source of bioactive compounds such as fiber, minerals, polyunsaturated fatty acids, and phenolic compounds (Galanakis, 2011). Phenolic compounds are the main antioxidation compounds in olive pomace (≈98%, namely hydroxytyrosol and its derivatives) (Reboredo-Rodriguez et al., 2017). For instance, ~ 5000 kton/year of olive pomace residue is produced in Spain, with an average of 900 g/ton of total phenolic compounds (Panzella et al., 2020). The anti-oxidation activity is based on preventing the formation of free radicals involved in oxidation processes by donating hydrogen atoms or electrons from elements having this activity (Cassano et al., 2017). In addition, the phenolic compounds have been shown to have antiviral, antimicrobial, antioxidant, anti-inflammatory, and anticarcinogenic activities (Chanioti et al., 2017). Recovering a polyphenol-rich extract from olive pomace—a low-cost and widely available by-product—constitutes a key point for the valorization of food additives, particularly antioxidants and antimicrobial extracts from natural sources. This recovery would also support the development of new sustainable bio-economy, reducing environmental problems caused by these wastes and making them suitable for commercialization and development of new food additives (Barba et al., 2016).

Foodborne illnesses related to the consumption of white brined and related cheeses that are contaminated with pathogens and/or their toxins have been recently documented and pose a threat to public health. Moreover, such quality defects in cheese can lead to significant economic losses (Lindqvist et al., 2002). Cheese is among widely consumed foods worldwide (FAO, 2015). A great deal of research has been carried out on the chemical, microbiological, production, and sensory aspects of soft white cheese (Humeid et al., 1986, Haddadin, 2005, Humeid et al., 1990). In this regard, the cheese industry must address the issues of product safety, quality, and shelf-life. Therefore, the main objective of the present study was to investigate the efficiency of using dried olives pomace extract (OPE) as a source of natural antimicrobial and antioxidation components and to investigate the effect of the addition of OPE on the quality and sensory properties of pasteurized white cheese (PWC).

MATERIALS AND METHODS

Pomace collection

Raw olive pomace (about 05 kg) was collected in Balqa-Salt in Jordan from the Aljazzazi Olive Oil Mill in December 2021. Olive pomace was homogenized and collected in polyethylene bags (2.5kg for each bag) and stored in a freezer at -20°C. Before use, the pomace samples were thawed for 24 hr under refrigeration temperature (5°C).

Pomace drying process

One to two kilograms of pomace were dried in an oven at 40°C for 6hr with constant stirring every 15 min until reaching a fixed weight. The dehydrated samples were then packed in polyethylene bags, placed in closed plastic containers, and kept refrigerated at 8°C in a dark place for further analysis.

Biochemical composition of olive pomace

Proximate analyses of dried olive pomace were performed in triplicates and expressed as g/100 g of dry matter (DM). Moisture content was determined using a
direct drying method in a hot air oven (Memmert, Karlklob-West Germany) at 105°C for ~4hr until a constant weight was attained (AOAC,1990). The Ash content level of dried olive pomace was determined by the dry ashing method using a muffle furnace (Carbolite™ CWF 1100; Germany) at 550°C for 6 hr (AOAC, 2011). Soxhlet extractions were performed to determine crude fat content using diethyl ether as a solvent with the Soxhlet extraction apparatus (Electrothermal EME60250/CEBX1; UK) (AOAC, 2000). Protein contents of pomace were determined using the Kjeldahl method with a conversion factor of 6.25 (Protein % = Nitrogen % x 6.25) by a heating digester (VELP Scientifica DK 20, Italy) and behr distillation (behr Labor-Technik, Germany) (AOAC, 2011). The crude fiber content of dried pomace was obtained using an ANKOM200/220 fiber analyser (ANKOM, USA) and filter bags (ANKOM bags F57) according to the Van Soest et al., (1990) method. Total soluble carbohydrates were determined by calculating the percent remaining after all the other components had been measured (Tang, 2003):

\[
% \text{ Soluble carbohydrates} = 100 - \% \text{moisture} - \% \text{protein} - \% \text{l lipid} - \% \text{mineral} - \% \text{fiber}.
\]

**Preparation of olive pomace extracts (OPE)**

The dried olive pomace was extracted using two different extracting solvents including ethanol and water (75:25, soln. A), methanol and water (75:25, soln. B) (Al-Ismail et al., 2006). The weighed dried olive pomace sample (10g) was mixed with 100 ml of each (solutions-A or B) and kept under agitation in a water bath for 1hr at 40°C, sonication in an ultrasonic bath (ISO LAB, Germany) for 30min at 40°C, then filtered with Whatman filter paper No. 4. The filtrated solution was evaporated by a rotary evaporator at temperatures corresponding to each solvent (78 °C for ethanol and 64 °C methanol). The residues (as paste) were then transferred in petri dishes to the oven at 40°C to evaporate any solvent. These residues were weighed (11 g) and then dissolved in distilled water in a volumetric flask and the total volume was made up to 200 ml in order to obtain a 5.5% (g/ml) stock solution. For enhanced purity, the resultant mixture was centrifuged at 4000 RPM for 15 min (Hettich, D-78532, Germany). The supernatant was then collected and stored at refrigeration temperature (~5°C) in dark closed bottles during which it was examined for its antioxidant and antimicrobial activities for further analysis. The average yield content (g/100g DW) of olive pomace extracts (OPE) was determined in triplicate.

**Determination of Antioxidants**

**Total Phenolic content**

The total phenolic compounds were determined according to Al-Ismail et al., (2006). The phenolic compounds present in OPE (ethanol or methanol) were determined by the addition of 0.5ml of the Folin-Ciocalteu Reagent (FCR). The sample (2.5 μl) was left to stand for 60 min and then measured at 760 nm using a spectrophotometer (Biotech Engineering Management Co., UK). Gallic acid was used as the standard for a calibration curve. The total phenolic compound contents (mg/100g) were expressed as Gallic acid equivalent (GAE).

**Antioxidant activity "DPPH Free Radical Scavenging Ability Assays"**

DPPH radical scavenging effect was determined using DPPH (2, 2-Diphenyl-1-picrylhydrazyl) with different concentrations of each OPE, the absorbance of OPE was measured at 517 nm using a spectrophotometer (Biotech Engineering Management Co., UK) after placing the samples in the dark for 30min (Al-Ismail et al., 2006). IC₅₀ (the concentration of extract in mg/ml needed to scavenge 50 % of the DPPH radical) was calculated from the concentration-response curves.

**Determination of functional compounds of olive pomace by the HPLC-MS analysis**

**Pomace extraction process**

The dried olive pomace sample was extracted by methanol with a constant solid–liquid ratio of 1:10 according to Aliakbarian et al. (2011). Testing of the extracts was performed in a high-pressure–high-
temperature reactor (model 4560, PARR Instrument Company, Moline, IL, USA), which contained appropriate valves to allow the introduction and removal of gasses inside the reaction chamber to avoid phenolic oxidation. All tests were carried out under a nitrogen atmosphere by flushing nitrogen through the reactor for 2 minutes. The alcoholic extracts were then centrifuged in a PK131 centrifuge (ALC, Alberta, Canada) at 6000g for 10 min. and supernatants were subjected to quantitative analyses.

Identification of phenolic compounds in OPE
Free phenolic compound identification was performed according to Aliakbarian et al., (2011) by high-performance liquid chromatography (model Shimadzu ExionLC), using a UV detector (at 280 nm) and a C18 reverse-phase column (model 201TP54, Vyda, Hesperia, CA, USA) with a C18 guard column (Alltech Associates, Inc., Deerfield, IL, USA). Samples were filtered through 0.20 µm membranes (Sartorius Stedim Biotech GmbH, Goettingen, Germany). Separation was achieved using a linear gradient of two solvents: solvent A (0.1% formic acid in water) and solvent B (acetonitrile). The injection volume was 10µl, and the evaluation of each phenolic compound expressed as mg/100g olive pomace, was based on the peak in comparison with standard solutions. Mass spectroscopy was performed in a Turbo Ion Spray model X500 QTOF source in a positive mode according to de la Torre-Carbot et al. (2005) with the following settings: capillary voltage 5000 V, nebulizer gas (N2) 50, curtain gas (N2) 30, collision gas (N2) 7. Drying gas was heated to 550 °C and introduced at a flow rate of 5000 cm³ min⁻¹.

Processing of pasteurized white cheese
Pasteurized white cheese (PWC) was produced in the dairy processing pilot plant of the Department of Nutrition and Food Technology, University of Jordan, using the traditional method for pasteurized cheese production (Humeid and Tukan., 1986).

Fortification and treatment of PWC with OPE
OPE was added to the prepared PWC to achieve levels (0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.06%, 0.07% and 0.08%; w/w%) after the casein was coagulated and drained for 5-8 min. but before the final pressing. The mixtures were mixed with a sterilized spatula under a laminar flow cabinet. A similar sample was prepared but without the OPE addition and stored under the same storage conditions (control). Samples were stored at refrigeration temperature (5°C) for 1 month, and sampled at 24hr, 7d, 14d, 21d, and 30d for physiochemical and microbiological analysis.

Total Phenolic Compound content in PWC treated with OPE
Ten grams of each cheese sample were mixed with 10 ml of methanol solution (75 methanol and 25 ml distilled water) and extracted for 30 min at 50°C in a water bath (Raypa Thermostatic bath; Barcelona, Spain). The mixture was cooled and filtered by cheesecloth, and the residues were washed with 2ml of the same solvent solution. The obtained extracts were immediately analyzed (within 10 min). Total phenolic compounds were determined in duplicate by UV-vis spectrophotometry according to the Folin-Ciocalteu method (Spinelli et al., 2015). The total phenolic content was expressed as mg of gallic acid equivalents (GAEs) per g of dry weight (DW).

Microbiological analysis
Initial suspension and decimal reduction
Decimal dilutions (from 10⁻¹ to 10⁻⁴) from the control and treated PWC samples were prepared using sterilized peptone water (prepared to contain 1.0% peptone and 0.5% NaCl, Oxoid CM0009) at ambient temperature according to ISO 6887-1:1999 method.

Microbial analysis
Mesophilic aerobes, total lactic acid bacteria, yeasts and molds, and Staphylococcus aureus counts were determined according to the ISO methods: EN ISO 4833:1-2013, ISO 15214:1998, ISO 21527-1:2008 and
ISO method 6888-1; 1999, respectively. The media used were plate count agar, MRS agar, potato dextrose agar (supplemented with chloramphenicol and chlortetracycline-HCl), and Baird-Parker agar medium supplemented with potassium tellurite and egg yolk. All media and supplements were from Oxoid Limited (UK, Hampshire).

**Confirmation Tests of S. aureus**

**Coagulase test:**
Representative colonies of *S. aureus* were inoculated into BHI broth (Himedia, India) and incubated for 18-24 hr at 35 ±1°C. An amount of 0.5 ml reconstituted coagulase plasma with EDTA (Liofilchem, Italy) was added to BHI culture (1ml) and mixed thoroughly, incubated at 35 ±1°C, and then examined periodically over 6-h for clot formation. Only firm and complete clots that remained in place when the tube was tilted or inverted were considered positive for *S. aureus*.

**DNase test**
DNase agar plates were prepared, dried, and inoculated with representative colonies of *S. aureus* with a sterile inoculating loop. The plates were then incubated at 35-37°C for 24 hr, flooded with a 1N HCl solution, and the excess acid was topped off. Plates containing a clear zone around the colonies within 5 min were considered positive.

**Chemical analysis of PWC**

**Titrable acidity**
The Titrable acidity of cheese was measured according to the AOAC method (920.124 (2011)). TA expressed was as % of lactic acid.

**Lipid oxidation: thiobarbituric acid (TBA) procedure**
Ten grams of a homogeneous sample were weighed and combined with 25 ml of 20% trichloroacetic acid (TCA) and 20 ml of warm water (Raharjo *et al.*, 1993). The mixture was then homogenized in stomacher bags (Model AES, Labprat) for 2 min, filtered through Whatman filter paper no 1 after which 2 ml of filtrate was combined with 2 ml of 0.02M aqueous 2 thiobarbituric acid (TBA) in a test tube. The tubes were then incubated at 22 ±2°C in a dark room for 20 hr. The absorbance of the resultant solution was measured spectrophotometrically at 532 nm. The TBA number, expressed as mg of malondialdehyde /kg of the sample (ppm), was calculated by measuring the absorbance of a pink-colored mixture of the TBA-reactive substances at 532nm multiplied by a factor of 7.8.

**Sensory evaluation**
Sensory analysis of the control and fortified PWC was carried out by 30-panel members (employees and students) after 7d of refrigerated storage (≤ 5°C) (ISO 4121, 2003). Food taste panelists were asked to use the 9-point hedonic scale for overall acceptability, softness, color, taste, and flavor. PWC samples and the control were desalted by soaking in potable tap water overnight in order to reduce the salt content.

**Statistical analysis**
Statistical calculations were performed -Analysis of variance ANOVA- using the SAS program, 2011 (SAS Institute Inc., Cary, NC). Significant differences among means of treatment were determined using the LSD tests. Differences at P ≤ 0.05 were considered to be significant. All treatments were conducted in duplicate or triplicate.

**RESULTS AND DISCUSSION**

**Proximate analysis of olive pomace**
Fiber represented the major constituent (60.85 g/100 g DM) followed by crude fat (11.91g/100 g DM), crude protein, and moisture (6.83 and 8.46 g/100 g DM, respectively) (Table 1). The ash and carbohydrate contents were 5.18 g/100 g DM and 6.77 g/100 g DM, respectively. These values are similar to those reported by other authors (Haddadin *et al.*, 2009; Uribe *et al.*, 2014; Zhao *et al.*, 2022). It was reported that climatic factors can influence the chemical composition of olive pomace as well as other factors such as the agricultural practices, cultivar, the ripening stage, and the process of oil
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extraction (Portarena et al., 2017). Olive pomace contains the remaining olive fat, water, and a large number of bioactive compounds, which can have further valuable and novel applications (Roselló-Soto et al., 2015).

Table 1: Proximate analysis of dried olive pomace (g/100g DM)

<table>
<thead>
<tr>
<th>Component</th>
<th>Mean± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>8.46±0.11</td>
</tr>
<tr>
<td>Ash</td>
<td>5.18±0.31</td>
</tr>
<tr>
<td>Fiber</td>
<td>60.85±0.42</td>
</tr>
<tr>
<td>Fat</td>
<td>11.91±0.67</td>
</tr>
<tr>
<td>Protein</td>
<td>6.83±0.04</td>
</tr>
<tr>
<td>CHO</td>
<td>6.77±0.23</td>
</tr>
</tbody>
</table>

1DM: Dried Matter
2Each value is the mean of three replicates ±SD (Standard Deviation)
3CHO: Carbohydrates

Yield of olive pomace extracts (OPEs)

Methanol extraction had significantly (P≤0.05) higher yield (23.40 g/100g DW) than ethanol extraction (22.18 g/100g DW) (Table 2). These results are similar to those obtained by other authors (Badawy and Smetanska, 2020; Gómez-Cruz et al., 2020; Zhao et al., 2022). The selection of solvents could be a crucial factor in the efficiency of extraction of the bioactive compounds from biomass (De Jesus et al., 2020). Solvents such as ethanol, acetone, and ethyl acetate have been widely used owing to their low toxicity and their being allowed by the European Food Safety Authority (EFSA) for fortification of foods with the extracted functional ingredients (Gullón et al., 2018). It was reported that the use of water with an organic solvent contributes to the creation of a moderately polar medium that ensures the extraction of phenolic compounds, providing better results compared to using a pure organic solvent (Musa et al., 2011). Methanol has high polarity which could produce high extraction yields. However, its toxic characteristic could be a limiting factor when it is used in food and pharmaceutical applications (Hassim et al., 2014). Although the extraction yield of ethanol is significantly lower than ethanol, it has less toxicity and is considered a green, safe, and nontoxic media for extraction compared to other organic solvents such as methanol (Rodriguez-Rojo et al., 2012). Thus, ethanol was chosen to extract the olive pomace to be used in PWC.

Determination of Antioxidants

Total Phenolic content

The methanol extraction contained higher total phenolic content than the ethanol extraction with amounts of 5958.0 mg GAEs/100g DW and 5587.4 mg GAEs/100g DW, respectively (Table 2). These results are comparable to those obtained by Alhamad et al., (2017), Nunes et al., (2021), and Uribe et al., (2014) by using several solvents to extract phenolic compounds from olive or olive pomace. Olive pomace is considered to be an important source of phenolic compounds since only 1–2% of the phenolic compounds in olives go into olive oil through its mechanical extraction process (centrifugation of oil paste), while 53% and 45% of these compounds remain in the liquid waste and the solid by-product (olive pomace), respectively (Chanioti et al., 2017). It was reported that the extraction efficiency of the phenolic compounds from olives and the antioxidant potential is affected by the operation parameters such as the extraction temperature, the extraction time, the liquid-to-solid ratio (L:S), and the type of solvent (Sahin et al., 2013). Polyphenolic yields were found to increase with increasing the polarity of the solvents (from polar to non-polar) as a result of stronger interactions (hydrogen bonds) between the polar sites of the phytochemical compounds and the solvent. The more the hydroxyl groups in the molecule, the higher the polarity (Liu et al., 2007).

Diphenyl-1-Picrylhydrazyl Radical (DPPH•) scavenging ability

DPPH assay was calculated as IC₅₀, the number of extracts that will react with 50% of the added DPPH radicals in the total volume of the assay under the given reaction conditions (Piaxao et al. 2007). From Table 2, the IC₅₀ of OPEs by methanol/water (75/25) was significantly (P ≤ 0.05) higher (1.61 µg/ µg DPPH) than
those obtained by ethanol/water (75/25) (1.56 µg/µg DPPH) (Table 2) so ethanol extracts yielded the highest DPPH radical scavenging activity. The strong scavenging activity of the former could be related to the high concentration of antioxidant compounds in ethanol extract such as hydroxytyrosol, oleuropein, and oleuropein aglycon (Leouifoudi et al., 2014). Furthermore, the ethanolic extract possessing a phenolic compound that contains a higher number of hydroxyl groups has a higher antioxidant activity (Leouifoudi et al., 2014). Our IC$_{50}$ could vary from those stated by other authors (Chanioti et al., 2017 and Badawy and Smetanska, 2020) which could be related to the difference in extraction conditions. The scavenging of the 2, 2-Diphenyl-1-Pierylhydrazyl Radical (DPPH) was used to evaluate the antioxidant activity of the OPEs. This method is based on the capability of an antioxidation compound to donate hydrogen radicals or an electron to DPPH radicals in order to produce a stable free radical with a deep violet color. When the odd electron becomes paired to DPPH radicals, the DPPH is reduced to corresponding hydrazine (DPPH-H) and becomes decolorized from its initial deep violet to a light-yellow color. The degree of fall in the absorbance is measured spectrophotometrically and is proportional to the concentration of the antioxidant (Piaxao et al. 2007). It was reported that the antioxidant capacity of extracts is affected by the solvent system used (Bandoniene et al., 2022).

Table 2: Average extraction yield, total phenolic content (TPC), and IC$_{50}$ values of DPPH inhibition of olive pomace extract by the two solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Extraction yield (g/100g)$^3$</th>
<th>TPC (mg GAEs$^2$/100g DW)$^3$</th>
<th>IC$_{50}$ (µg/µg)$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>22.18$^b±0.05$</td>
<td>5587.4$^b±174.1$</td>
<td>1.56$^b±0.020$</td>
</tr>
<tr>
<td>Methanol</td>
<td>23.40$^a±0.02$</td>
<td>5958.0$^a±174.1$</td>
<td>1.61$^a±0.004$</td>
</tr>
</tbody>
</table>

$^1$IC$_{50}$: The half-maximal inhibitory concentration  
$^2$GAE: Gallic Acid Equivalents  
$^3$Each value is the mean of three replicates ±SD (Standard Deviation)  
Mean within the same column with different subscripts are significantly different at the 5% level of probability (p≤0.05).

Determination of functional compounds in OPE
A detailed analysis of the OPEs by HPLC/MS and the respective chromatograms obtained at 280 nm are presented in Table 3. The UV chromatography demonstrated eight major signals that were tentatively identified by the retention time and according to mass/charge numbers by ion -m/z ion. The chromatograms were counted per second by comparing their relative retention times and spectral data (UV-vis and MS) with those of corresponding analytical standards. It can be concluded from the results summarized in Table 3 that all the compounds isolated were phenolic compounds and those with the highest concentrations were Syringic acid and p-coumaric (peak 4 and 6, respectively) with counts per second (CPS) of 4000 and 2400, respectively. The third and fourth compounds were vanillin and caffeic acid (peaks 5 and 3 respectively) with counts per second of 1900 and 1200, respectively. Then apigenin and tyrosol (peaks 8 and 1) had CPS of 750 and 700, respectively. Vanillic acid and oleuropein (peaks 2 and 7) with CPS of 600 and 240, respectively were the least contained compounds that were found to belong to the secondary metabolic pool of OPE.
Numerous papers in the literature have reported the detailed composition of OPEs from various cultivars/regions/countries. It was described that the total polyphenol contents and its derivatives are considered to represent a type of trademark of this matrix (Difonz et al., 2021C). All compounds identified in the olive pomace belong to the class of polyphenols, as widely reported in the literature. Extraction solvent also plays a pivotal role in the selection of extracted compounds, together with extraction time and temperature (Irakli et al., 2018). Moreover, the European Food Safety Authority (EFSA) has issued a favorable scientific opinion regarding health claims for the dietary consumption of polyphenols and their derivatives (protection of blood lipids from oxidative damage) (EFSA, 2011). Other studies have reported that polyphenol derivative has antioxidant, antimicrobial, anti-inflammatory, and anti-diabetic activity (Salah et al., 2012). These results would indicate that all compounds defined in the current study were found in previous researche (Aliakbarian et al., 2011; Zhao et al., 2022; Ribeiro et al., 2020).

Analysis of fortified pasteurized white cheese (PWC) with OPE

Some researchers have demonstrated the positive effect of OP addition on the properties of different food products (Conterno et al., 2019). However, few studies have reported its incorporation in dairy products such as milk and yogurt (Cho et al., 2020). Our study was thus the first on the fortification of pasteurized white cheese (PWC) with OPEs.

**Total Phenolic Compound content in PWC treated with OPE**

Fig. 1 shows that the addition of OPE (0.02 % to 0.08%) to PWC significantly (P≤0.05) increased the total phenolic content in PWC compared to the control. High amounts of phenolic compounds were found in PWC with high concentrations (0.08%, 0.07%, and 0.06%) of OPE with the amount of total phenol equal to 2836.98, 2538.90 and 2433.13 mg GAEs/100g respectively. It also demonstrates a significant similarity between samples with low concentrations (0.01%, 0.02%, and 0.03%) with amounts of 1077.37, 1086.98, and 1269.67 mg GAEs/100g, respectively, and the control sample (904.29 mg GAEs/100g). These results are similar to those obtained by other authors (Simonato, et al., 2019); (Ribeiro et al., 2021); (Balli et al., 2021); (Cecchi et al., 2019).

<table>
<thead>
<tr>
<th>Analyte Peak Number</th>
<th>Analyte Peak Name</th>
<th>Count Per Second</th>
<th>Retention time (Min.)</th>
<th>Molecular weight (Da)</th>
<th>Chemical Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>04</td>
<td>Syringic</td>
<td>4000</td>
<td>0.57</td>
<td>199.0601</td>
<td>C_{9}H_{10}O_{5}</td>
</tr>
<tr>
<td>06</td>
<td>p-Coumaric</td>
<td>2400</td>
<td>3.17</td>
<td>165.05462</td>
<td>C_{9}H_{8}O_{3}</td>
</tr>
<tr>
<td>05</td>
<td>Vanillin</td>
<td>1900</td>
<td>13.65</td>
<td>153.05462</td>
<td>C_{8}C_{8}O_{3}</td>
</tr>
<tr>
<td>03</td>
<td>Caffeic acid</td>
<td>1200</td>
<td>0.93</td>
<td>181.04954</td>
<td>C_{9}H_{8}O_{4}</td>
</tr>
<tr>
<td>08</td>
<td>Apigenin</td>
<td>750</td>
<td>18.23</td>
<td>271.0601</td>
<td>C_{15}H_{10}O_{5}</td>
</tr>
<tr>
<td>01</td>
<td>Tyrosol</td>
<td>700</td>
<td>10.61</td>
<td>139.07536</td>
<td>C_{8}H_{10}O_{2}</td>
</tr>
<tr>
<td>02</td>
<td>Vanillic acid</td>
<td>600</td>
<td>8.40</td>
<td>169.04954</td>
<td>C_{8}H_{8}O_{4}</td>
</tr>
<tr>
<td>07</td>
<td>Oleuropein</td>
<td>240</td>
<td>15.90</td>
<td>558.21812</td>
<td>C_{25}H_{32}O_{13}</td>
</tr>
</tbody>
</table>

Table 3: Identified compounds by the HPLC/MS in the olive pomace extract
The presence of relatively low concentrations of total phenolic compounds in the control (PWC without OPE) could possibly be due to the presence of low molecular weight antioxidant compounds in milk components such as polyphenols that are affected by animal feeding and heat treatment of the milk (Chouchouli et al., 2013). The low levels of total phenolic compound values in the PWC fortified with low concentrations of OPEs (from 0.01% to 0.03%) may diminish during the storage period. This is due to interactions of these phenolic compounds with the small amounts of monounsaturated fatty acid (25%) and polyunsaturated fatty acid (2.5%) in cheese which can act to protect lipid peroxidation by retarding reaction with hydrophilic radicals (Lindmark, 2008). This could lead to the eventual loss of the ability of phenolic compounds to prevent oxidation of the unsaturated fatty acid (UFAs') in the PWC, which makes the product more vulnerable to rancidity. This could explain the lower amount of TPC in PWC treated with low concentrations of OPE (Gorelik et al., 2013; Jakobek, 2019). Abdel-Aal and Rabalski (2013) suggested that the decrease in bioactive compounds in a food product with storage depends on several factors including -the type of product, the recipe, processing conditions, and the phenolic compound content of the food product.

**Microbiological analysis**

**Mesophilic aerobes**

It should be noted that immediately after the cheese production (up to 1d), the control and the fortified PWC with a low concentration of OPE (from 0.01% to 0.05%) had higher total mesophilic aerobes with log mean counts of 3.44 log_{10}CFU/g and 3.23 log_{10}CFU/g, respectively. The mesophilic aerobes count was significantly (P<0.05) lower (2.97 log_{10}CFU/g to 2.72 log_{10}CFU/g) as a result of adding a high concentration of OPE (0.06% to 0.08%), respectively. After 7d of storage, there was no significant increase in the mesophilic counts in both the control and treated samples. However, the counts began to increase again at higher rates especially at lower concentrations of OPE (0.01% to 0.05%) and the control until a month of refrigeration storage (4.04 log_{10}CFU/g). Samples with high concentrations of OPE (0.06% to 0.08%) maintained low levels of mesophilic aerobes counts (3.74 log_{10}CFU/g to 3.61 log_{10}CFU/g) after 30d of refrigerated storage (Fig. 2). The mesophilic count development in the control samples was a result of the normal manifested microbial growth in dairy product due to poor hygiene conditions and a lack of refrigeration (Haddad et al., 2017). It was reported that the inhibitory activity of the OPE in the fortified PWC with a high concentration of OPE was greater against Gram-positive bacteria (L. innocua and S. aureus) than Gram-negative bacteria (Gómez-Cruz et al., 2013).

**Figure 1**: Effect of fortification of pasteurized white cheese with different concentrations of olive pomace extract on the content of total phenolic compounds

![Graph showing the effect of olive pomace extract concentration on total phenolic compounds in pasteurized white cheese.](image-url)
This would be due to the fact that the polyphenol structure (hydroxyl group –OH interacts with the microbial cell membrane resulting in cell death –membrane disruption-) (Xue and Zhong, 2013). However, the Gram-negative bacteria (E. coli and Salmonella sp) are rich in lipopolysaccharides that restrict the penetration of foreign molecules and increase the resistance of the bacteria to these antimicrobial agents (Harkat-Madouri et al., 2015).

Figure 2: Effect of fortification of pasteurized white cheese with different concentrations of olive pomace extract on its mesophilic aerobic count (log10CFU/g) during refrigerated storage (5°C)

Lactic Acid Bacteria (LAB)

Immediately after PWC production (up to 1d), the control PWC and the fortified PWC with a low concentration of OPE (from 0.01% to 0.05%) had higher total LAB with mean counts of 3.02 log10 CFU/g and 2.86 log10 CFU/g, respectively. However, the counts decreased significantly (P<0.05) to 2.48 log10 CFU/g by adding a high concentration of OPE (0.06% to 0.08%). After 14d of storage, there was a significant increase in the LAB in both the control and treated samples with low concentrations of OPE (0.01% to 0.06%) up until the end of the refrigerated storage, where the control had the highest count (3.79 log10CFU/g). Samples treated with higher concentrations of OPE (0.06% to 0.08%) elicited lower levels of LAB counts (3.38 log10CFU/g) during the 30 d storage period (Figure 3). OPEs inhibited the growth of LAB (antimicrobial effect against Gram-positive bacteria) (Gómez-Cruz et al., 2020; Nunes et al., 2021). The antimicrobial effectiveness of oleuropein and other phenolic compounds found in OPE has been proven mostly against various bacteria including the lactic acid bacteria, namely: Lactobacillus plantarum, L. brevis, and Leuconostoc mesenteroides, and against bacteria associated with infections of the human intestinal or respiratory tracts (Soler-Rivas et al., 2000; Fleming et al., 1973).

Figure 3: Effect of fortification of pasteurized white cheese with different concentrations of olive pomace extract on its lactic acid bacteria count (log10CFU/g) during refrigerated storage (5°C)
Yeasts and Molds counts

Untreated PWC (control) and the fortified PWC with all concentrations of OPE (from 0.01% to 0.08%) had undetectable counts of yeasts and molds for up to 14d of storage (Figure 4). After 21 and 30d of refrigerated storage, there was a significant increase in the yeasts and molds counts in both the control and treated samples with low concentrations of OPE (0.01% to 0.05%, respectively). The counts were kept below 2.4 log\(_{10}\)CFU/g and 2.7 log\(_{10}\)CFU/g in treated samples and in the control after 30d of storage, respectively. Winkelhausen et al. (2005) demonstrated that the phenolic compounds derived from olive pomace have good potential as a natural fungicide against common pathogens, especially against Alternaria solani, Botrytis cinerea, and Fusarium culmorum. The authors attributed the antifungal activity of the OPE to the aldehyde moiety in the vanillin. Additionally, vanillin was reported to pose a remarkable antimicrobial activity against (Saccharomyces cerevisiae, Zygosaccharomyces bailii, and Zygosaccharomyces rouxii (Fitzgerald, 2003).

![Graph showing yeast and mold counts](image)

**Figure 4:** Effect of fortification of pasteurized white cheese with different concentrations of olive pomace extract on its yeast and molds counts (log10CFU/g) during refrigerated storage (5°C)

**Staphylococcus aureus count**

All of the treated samples and control PWC did not contain detected S. aureus (data is not shown) as all the grown colonies gave negative coagulase and DNase tests. However, it was demonstrated that OPE components have an inhibitory effect OPE especially against Gram-positive bacteria (L. innocua and S. aureus) (Gómez-Cruz et al., 2020 Nunes et al., 2021). Yakhlef et al. (2018) demonstrated the bactericidal effect of olive mill wastewater, which contained high concentration of phenolic (10.828 mg/L) with minimal bactericidal concentration (MBC) for all target microorganisms (Pseudomonas fluorescens (CECT 378), Staphylococcus aureus (CECT 239), Escherichia coli (CECT 434), and Enterococcus faecalis (CECT 481) was 2.5%. The absence of S. aureus in the PWC could be due to cheese pasteurization that eliminates all known milk-borne pathogens (Alegbeleye et al., 2018).

**Chemical analysis of cheese**

**Titrable acidity**

In general, there was a significant (P≤0.05) decrease in titrable acidity of fortified PWC with increasing OPE concentration throughout the 30d of refrigerated storage. The control samples maintained significantly (p ≤0.05) higher titrable acidity compared to all treated samples at each particular sampling time (Figure 5). In addition, there was a significant (P≤0.05) increase in titrable acidity within each PWC treatment and the control during the 30d storage period to a maximum of 0.183% (Figure 5) due to lactose fermentation and the formation of lactic acid and other organic acids in the cheese. Aini et al. (2020) and
Elewa et al. (2009) observed the development of acidity in different kinds of cheese during the refrigeration period as a direct response to converting the residual lactose into lactic acid by the available micro-flora. These results are concomitant with the results of lactic acid bacteria count (Figure 3), where LAB counts decreased with an increase in OPE concentration, thereby producing less lactic acid (Kim et al., 2017).

**Figure 5:** Effect of fortification of pasteurized white cheese with different concentrations of olive pomace extract on its titrable acidity during refrigerated storage (5°C)

**Lipid oxidation of cheese**

In general, there was a significant (P ≤ 0.05) decrease in the lipid oxidation (as the TBA value) of fortified PWC with increasing OPE concentration throughout the refrigerated storage of 30d. The control samples maintained significantly (p ≤ 0.05) higher lipid oxidation levels compared to the treated samples at each particular sampling time. In addition, there was a significant (P ≤ 0.05) increase in lipid oxidation within each PWC treatment during the 30d storage period to a maximum of 0.746% (Figure 6). Table 5 shows that the OPE consisted of a number of aromatic compounds such as hydroxytyrosol, tyrosol, caffeic acid, oleuropein, and oleuropein aglycon which may have antioxidation activity (Leouifoudi et al., 2014). Phenolic compounds are secondary plant metabolites that have the ability to act as antioxidants by neutralizing reactive oxygen and nitrogen species by their hydroxyl groups, thereby making these compounds good hydrogen donors. Moreover, phenolic compounds can moderate free radical generation by chelating metal ions (Gómez-Cruz et al., 2020). Gülçin, (2005) found that caffeic acid at concentrations of 10 and 30g/mL showed 68.2 and 75.8% inhibition of lipid peroxidation in linoleic acid emulsion, respectively. The significant increase of TBAs in the control sample and samples with low concentrations of OPE was the result of oxidation of the product by several factors such as light on fat and the level of oxygen in the heavily brined solution (Al-Ismail et al., 2003, Mehyar et al., 2012). Moreover, the low surface/volume ratio of the cheese pieces and the low diffusion rate of the dissolved oxygen through these pieces could have led to minimum contact of the fat with the dissolved oxygen, thus preventing excessive oxidation (Al-Ismail et al., 2003). Our results were similar to those obtained by other authors (Vitali Cepo et al., 2018) and (Innosa et al., 2020).
Figure 6: Effect of fortification of pasteurized white cheese with different concentrations of olive pomace extract on lipid oxidation (expressed as mg/kg of malondialdehyde, MDA) in PWC as determined by thiobarbituric acid assay

Sensory evaluation

In general, there were no significant (P>0.05) differences in sensory properties between the OPE-treated samples and the control. However, the PWC color in the OPE-treated samples was significantly better compared to the control. In addition, although there were no significant (P>0.05) differences within each PWC treatment during the 30d storage period, significant improvement in softness was observed at a higher concentration of OPE (0.08%). The significant difference in color of the PWC-treated samples at concentrations of 0.01%, 0.04%, and 0.05% compared to the control could be attributed to the oxidation of fats. In the PWC control, this resulted in a transition from white to a more yellowish color. The significant increase in softness on most fortified pasteurized PWC compared to the control sample could be due to the decomposition of the cheese protein as a result of the increase in bacterial activity at the beginning of the second week, especially in the absence of OPE (the control). Despite the slight changes in color and softness, the fortified PWC attained approximately the same overall acceptability scores as the untreated PWC sample. It can thus be concluded that the addition of OPE did not result in significant sensory changes to the product from the standpoint of consumer acceptance (Figure 7). These results are compatible with those provided by other authors (Lin et al., 2017); (Badawy and Smetanska, 2020); (Cecchi et al., 2019) and (Simsek et al., 2021).

Figure 7: Sensory evaluation of PWC with OPE at day 7 during refrigerated storage (5°C)
Conclusions
Olive pomace is rich in phenolic compounds that have high antioxidation and antimicrobial activities. Incorporation of olive pomace extract in pasteurized white cheese enriched the cheese with phenolic compounds and slightly improved the microbial quality of PWC. Treatments of PWC with OPE retarded lipid oxidation and improved the sensory properties of the cheese. This study thus demonstrated the effectiveness of incorporating olive pomace in the PWC. Additional research is warranted in order to explore the antimicrobial activity of OPE and their minimum inhibitory concentration in PWC against various common foodborne pathogens.

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الأنشطة المضادة للميكروبات والأكسدة لمصطلح جفت الزيتون في الجينة البيضاء المستورة

هاجر م. فرقوس، غدير فوزي مهيار، إسلام أ. إبراهيم

قسم التغذية والتصنيع الغذائي، كلية الزراعة، الجامعة الأردنية، الأردن.
جامعة ولاية كارولينا الشمالية الزراعية والتقنية، الولايات المتحدة الأمريكية


ملخص

تم جمع ثفل الزيتون من معاصر الزيتون في ديسمبر 2021. أظهر التحليل التقريبي أن المكونات الرئيسية للثفل هي الألياف (60.85٪) والدهن (11.91٪). أعطى مستخلص ثفل الزيتون بواسطة الميثانول إنتاجية أعلى من محتوى الفينول وقدرة أعلى على إزالة الجذور الحرة من مستخلص الالكانتيل، ولكن تم استخدام الأخير نظرًا لانخفاض سمنته عند استخدامه في الاغذية.

يتضمن المستخلص تركيزات عالية من المركبات الفينولية وخاصة caffeic acid، vanillin، p-coumaric، syringic acid واللقاحيات القادرة على تحبب الكائنات الحية المجهرية الهوائية والمحبة للحرارة إلى 3.62 لوغ وحدة مكونة للمستعمرة/ غم بعد 30 يومًا من التخزين عند 5 °C، و3.38 لوغ وحدة مكونة للمستعمرة/ غم بعد 30 يومًا من التخزين عند 10 °C.

الكلمات الدالة: ثفل الزيتون، الجبن البيضاء المبستر، إجمالي البوليفينول، مضادات الأكسدة، مضادات الميكروبات

g.mehyar@ju.edu.jo

* الباحث المعتمد للمراسلة: