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# Detoxification and enhancement of in vitro rumen digestibility of exhausted olive pomace wastes through alkaline hydrogen peroxide treatment

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## Abstract

**Background** Due to the sharp rise in animal feed costs, funding alternatives to substitute high-cost raw materials used in animal feed is a persistent need. This study investigated the effect of alkaline hydrogen peroxide pretreatment as straightforward non-toxic technology to enhance the in vitro rumen digestibility of exhausted olive pomace (EOP), an abundant agricultural waste, to be suitable as animal feedstock. It examined the efficiency to eliminate the toxic phenolic content and minimize lipid oxidation of EOP.

**Results** The pretreatment was first optimized using a central composite experimental design. Under the optimized conditions (1.6% $H_2O_2$, 5% NaOH), the measured phenolic content was 1.51 ± 0.03 mg/100 g dry weight (DW) for treated olive pomace (TOP) versus 4.91 ± 0.06 mg/100 g for the untreated one. The pretreatment showed that approximately 25% of the lignin was removed. Crude proteins, neutral detergent fibers, and acid detergent fibers yields of TOP were, respectively, 3.320 ± 0.05, 75.24 ± 0.23, and 54.05 ± 0.35 g/100 g of DW, significantly more important than those of untreated EOP. The enzymatic hydrolysis with a cellulase-based cocktail (Celluclast15 FPU/gDW), recorded a 48% of reducing sugar yield for TOP against 33% for EOP. When the in vitro organic matter digestibility (IVOMD) was assayed, the potential gas production of TOP (41.371 ml/g DM) was significantly higher than EOP (25.771 ml/g DM). The metabolizable energy of TOP (9.28 kcal/kg DM) was higher than that of EOP (7.78 kcal/kg DM).

**Conclusions** The present study revealed that alkaline hydrogen peroxide (AHP) could be an efficient treatment for the detoxification and enhancement of in vitro rumen digestibility of olive pomace. This straightforward approach demonstrated that treated olive pomace waste may be well valorized as suitable animal feedstock.

**Keywords** Olive pomace, Detoxification, Alkaline hydrogen peroxide, Pretreatment, In vitro digestibility

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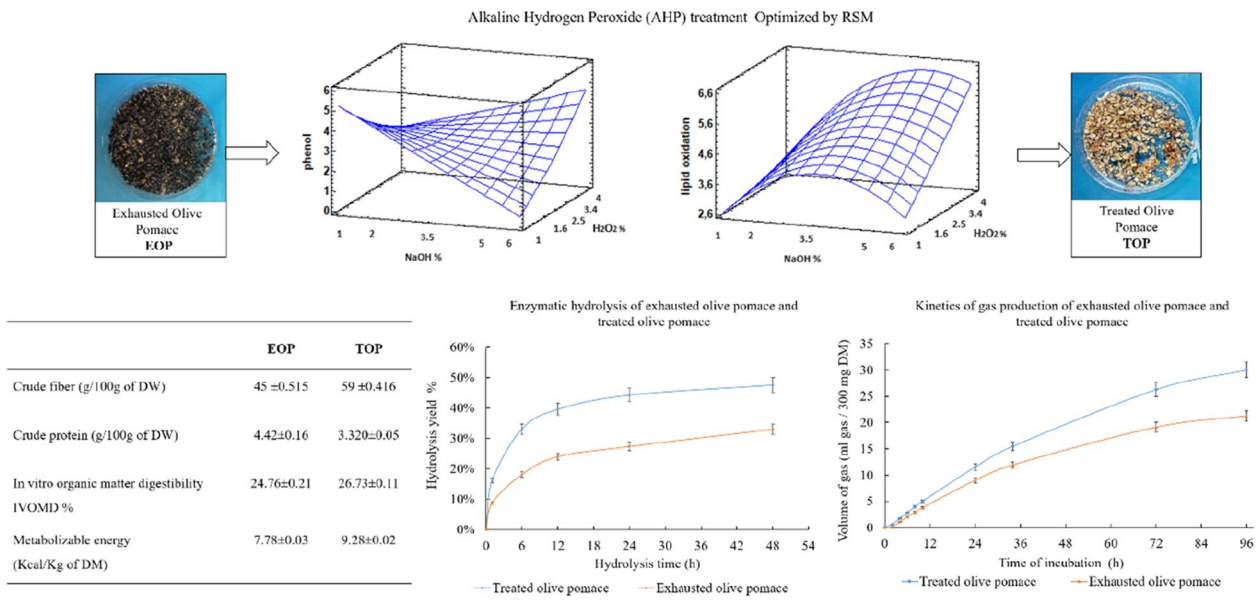
## Graphical Abstract




| | EOP | TOP |
|---|---|---|
| Crude fiber (g/100g of DW) | 45 ±0.515 | 59 ±0.416 |
| Crude protein (g/100g of DW) | 4.42±0.16 | 3.320±0.05 |
| In vitro organic matter digestibility IVOMD % | 24.76±0.21 | 26.73±0.11 |
| Metabolizable energy (Kcal/Kg of DM) | 7.78±0.03 | 9.28±0.02 |

## Introduction

In the Mediterranean area, the olive tree is one of the most important oil-producing crops. Tunisia is ranked as the fourth olive oil producer on the world scale with more than 82 million trees planted who is covering about 1.8 million hectares of Tunisian territory [1]. The olive oil industry in Tunisia regenerates a massive quantity of by-products called exhausted olive pomace EOP resulting from drying and subsequent solvent extraction of residual oil from the olive pomace which is the first valorized by-product of olive oil extraction. Inadequate handling of this biomass leads to contamination of soil and water, endangering ecosystems and human health [2, 3]. In fact, in the olive-growing countries of the Mediterranean region, the olive pomace production exceeds 30 million $m^3$/year, resulting in a considerable biomass [4]. Olive pomace is a very highly lignocellulosic biowaste. The latter linked to nitrogen has a very slow degradation, which has some disadvantages in agriculture due to the difficulty of integration into the soil [5]. Its richness in polyphenols generates environmental resistance. These phenolic compounds can act as a phytotoxic agent by inhibiting the growth as well as the germination of plants [6]. They are also the main determinants of the antimicrobial actions of olive pomace which makes them capable of modifying the microbial composition of the soil and only a few microorganisms manage to develop, mainly yeasts and fungi [7].

Moreover, the increase in the price of raw materials used in animal feed has led to a sharp rise in its cost price. Numerous investigations of the use of olive pomace in animal feed have been recently reported in order to reduce feeding cost [8, 9]. Unfortunately, the recalcitrance properties of plant cell wall structure and the high phenolic content both limit the digestibility of olive pomace and reduce therefore its recycling especially in animal feed, hence the necessity of a treatment step [10, 11].

Various pretreatment techniques have been therefore applied. These methods include physical ones, such as high pressure and high temperature, flocculation, adsorption, gasification, and ultrasounds chemical such as pyrolysis, sodium bicarbonate treatment, silage with alkalis, ammonia treatment, and biological treatments with enzyme or with microorganisms in liquid and solid-state fermentation [12–17]. The effects of microwaves, ultrasonic, and alkaline pretreatments on olive pomace for biomethane production were investigated by Elalami et al. [18]. The later study found that alkali treatment effectively removed lignin and increased methane production, while mild microwaves and ultrasonic pretreatments had minimal impact. In another side, Chebaibi et al. [19] explored the biological pretreatment using filamentous fungi on olive pomace for animal feed production. The results demonstrated that this pretreatment method increased

the protein content of the olive pomace and reduced its total phenolic content. Recently, a biorefinery approach was applied to extract antioxidants, lignin, and sugars from exhausted olive pomace using two different pretreatments namely liquid hot water and organosolv where it was shown that these pretreatments improved both delignification efficiency and enzymatic hydrolysis [20]. Nonetheless, it is important to note that the lack of economic feasibility and unsuitability for large-scale industrial use has limited the effects brought upon by olive waste applications. Hence, it is essential to explore research that can integrate transformation processes to convert olive waste into high value-added products, more effectively. This will help to alter the olive oil production's linear economy into a more circular economy [21–23].

Thus, a cost-effective and greener treatment process step that manage huge quantities at an industrial scale is required for the valorization of this biomass in order to lower its resistance to bioconversion. Recent studies have revealed that alkaline hydrogen peroxide (AHP) treatments were highly compatible with enzymatic hydrolysis to yield high amounts of sugars [24, 25]. On top of that, AHP treatment was found to be a mild treatment method that promotes inherently safer and greener processes. In fact, one of the key advantages of using hydrogen peroxide as a solvent in AHP treatment is that it is non-toxic and does not produce harmful by-products during use or disposal. Additionally, it is relatively inexpensive and widely available [26]. These unique features allow AHP treatment to potentially become one of the most promising and intriguing treatment methods used in biorefinery concepts [27].

Our study aims to examine the treatment of olive pomace biomass and its conversion into a valuable product that can be used in the animal feed sector. This conversion process will be achieved by the implementation of a straightforward alkaline hydrogen peroxide (AHP) treatment. Thus, the main objective of this work was the optimization of the AHP treatment using central composite experimental design, as well as evaluating its efficiency in detoxification and digestibility enhancement of olive pomace. Enzymatic hydrolysis and in vitro organic matter digestibility (IVOMD) of EOP and TOP were therefore performed. We hypothesize that this treatment could provide a high-quality animal feedstuff which exhibits superior digestibility and undergoes more efficient enzymatic hydrolysis. It represents sustainable transformation method of this discarded agri-waste.

## Materials and methods

### Biological material

The studied exhausted (known also as torrefied) olive pomace (EOP) was obtained from an olive pomace oil extraction industry (Groupe ZITEX) located in Sfax (south of Tunisia). This EOP is the by-product of olive pomace oil extraction and it is considered as the final waste of the olive oil extraction process.

### Chemical composition

The chemical analysis was performed according to AOAC standard methods to determine the dry matter (DM, method No. 27.005), ash (method No. 27.009), crude fiber (method No. 978.10), crude protein (CP, method No. 990.03), and total lipids (method No. 948.22) [28]. Concerning neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) they were determined using a fiber analyzer (RAYPA, n°32,867) according to Van Soest methods [29], and results are expressed as g/100 g of dry weight (DW). Total sugar content was determined using the phenol–sulfuric acid method [30]. For the GC–MS samples preparation, total fatty acids (TFAs) of total lipids for both exhausted olive pomace and treated olive pomace were transformed into their corresponding methyl esters in order to improve their volatility and stability during GC–MS analysis [31]. GC–MS analyses of EOP and treated olive pomace (TOP) volatile components were carried out on a gas chromatograph HP 5890 (II) coupled to an HP 5972 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) with electron impact ionization (70 eV). An HP-5MSn capillary column (30 m × 0.25 mm, 0.25 µm film thickness; Agilent Technologies, Hewlett-Packard, CA, USA) was used. The column temperature rises from 50 °C to 240 °C at a rate of 5 °C/min. The carrier gas was helium with a flow rate of 1.2 ml/min; the split ratio was 60:1. Scan time and mass range were 1 s and 40–300 m/*z*, respectively [32].

### Optimization of alkaline hydrogen peroxide treatment using central composite design

To optimize the alkaline hydrogen peroxide (AHP) treatment of EOP a central composite design (CCD) with two independent variables was performed in a total of 12 experiments carried out in duplicate. The levels of the independent variables were defined based on data from the literature, using our prior knowledge obtained from preliminary experiments, and taking into consideration the particular objectives of the experiment. Thus, the designated low and high levels of the independent variables were set between 1% (*w*/*v*) and 6% (*w*/*v*), and between 1% (*w*/*v*) and 4% (*w*/*v*) for NaOH and $H_2O_2$, respectively (Table 1). NaOH and $H_2O_2$ were prepared in solutions where the concentration of each chemical was expressed as a weight/volume (*w*/*v*) percentage. For the treatment process, it was carried out at room temperature and atmospheric pressure. The treatment solution

**Table 1** Independent variables and their levels in the response surface design

| Independent variables | Symbols | Coded factor levels | | | | |
|---|---|---|---|---|---|---|
| | Coded | − 1.41 | − 1 | 0 | +1 | +1.41 |
| NaOH % | A | 1 | 2 | 3.5 | 5 | 6 |
| $H_2O_2$% | B | 1 | 1.6 | 2.5 | 3.4 | 4 |

was prepared using a mix of an equal volume of NaOH and $H_2O_2$ at different concentration specified by each experiment. Then, EOP was added at a ratio of 1 g of EOP per 10 mL of treatment solution. The reaction mixture was left for 3 h. The solid fraction was filtered, washed with tap water to neutral pH, and finally dried at 50 °C for 48 h. The resulting TOP was then used for further analysis.

The phenol content and lipid oxidation were chosen as model responses to assess the treatment efficiency and the results are summarized in Table 2.

### Chemical analyses

Total phenolic content was determined as descript by Singleton et al. [33] using the Folin–Ciocalteu reagent. The extraction of the phenolic compound from samples was carried out using methanol. The total phenolic content was calculated from a gallic acid standard curve with a linear range (0–0.5 mg Gallic acid/ml) and defined as a gram of gallic acid equivalents per kilogram of DW g (GAE)/kg of DW [33]. Lipid oxidation was determined as descript by Askawa and Matsushita using thiobarbituric acid [34]. 1 g of the sample was homogenized in 16 ml trichloroacetic acid (TCA) (5%) with 0.1 ml of butylated hydroxytoluene (BHT). The homogenate was centrifuged at 15 000 rpm for 10 min. Then, 1 ml of the supernatant was added to 2 ml of thiobarbituric acid (TBA) 0.5%. The mixture was heated at 100 °C for 30 min and then quickly cooled in an ice bath. The absorbance was measured at 532 nm. A linear standard curve of malondialdehyde (MDA) was prepared and lipid oxidation was expressed as mM of MDA equivalents per kilogram of dry weight mM MDA eq/kg of DW calculated using Eq. 1 [34]:

$$\text{MDA equivalents mM/g of DW} = ((\text{A532} - \text{A600}) * \text{total volume of the extract}) / (155 * \text{dry mass of olive pomace}) \quad (1)$$

The extinction coefficient of MDA-TBA abduct at 532 nm is 155 $mM^{-1}$ $cm^{-1}$ with thiobarbituric acid 0.5%.

### Enzymatic hydrolysis

Enzymatic hydrolysis assays of EOP and TOP were performed in 50 mL Erlenmeyer flasks containing a mixture of 5 g dry samples in 28 mL of 0.05 M sodium acetate buffer (pH 4.8) and 15 FPU(filter paper units) g − 1 (Celluclast 1.5 L –cellulase from *Trichoderma reesei*). The hydrolysis assays were carried out in a rotary shaker at 50 °C and 250 rpm for 48 h. Aliquots were taken immediately after enzyme addition (0 h), after 1 h, 3 h, 4 h, 24 h, and after 48 h of reaction. Immediately after sampling,

**Table 2** Phenolic content and lipid oxidation obtained by AHP treatment of EOP at different NaOH concentrations and $H_2O_2$ concentrations according to a rotatable central composite experimental design

| Run | Sodium hydroxide concentrations in % | Hydrogen peroxide concentrations in % | Phenol (g/kg of DW) | Lipid oxidation (mM MDA eq/kg of DW) |
|---|---|---|---|---|
| 1 | 2 | 1.6 | 4.19 ± 0.42 | 3.7 ± 0.04 |
| 2 | 5 | 1.6 | 1.51 ± 0.03 | 4.88 ± 0.03 |
| 3 | 2 | 3.4 | 2.59 ± 0.05 | 3.88 ± 0.02 |
| 4 | 5 | 3.4 | 3.58 ± 0.05 | 6.203 ± 0.07 |
| 5 | 1 | 2.5 | 3.43 ± 0.03 | 3.28 ± 0.05 |
| 6 | 6 | 2.5 | 2.27 ± 0.04 | 3.81 ± 0.07 |
| 7 | 3.5 | 1 | 2.84 ± 0.07 | 3.66 ± 0.07 |
| 8 | 3.5 | 4 | 3.38 ± 0.05 | 6.01 ± 0.16 |
| 9 | 3.5 | 2.5 | 2.75 ± 0.07 | 5.00 ± 0.12 |
| 10 | 3.5 | 2.5 | 2.76 ± 0.07 | 5.01 ± 0.07 |
| 11 | 3.5 | 2.5 | 2.75 ± 0.08 | 5.02 ± 0.04 |
| 12 | 3.5 | 2.5 | 2.74 ± 0.05 | 5.01 ± 0.08 |

enzymes were inactivated at 100 °C for 10 min. Aliquots were centrifuged (3 min at 2000 rpm). Reducing sugar content was determined using the dinitrosalicylic acid (DNS) method and sugar yield for each extract was calculated using Eq. 2 [35]:

$$\text{Sugar yield (\%)} = [(\text{Glucose concentration} * \text{volume of reaction} * 0.9)/(\text{Dry mass of olive pomace})] * 100. \quad (2)$$

### In vitro assay

The fermentation kinetics of olive pomace was determined according to the in vitro method of Menke and Steingass (1988). The used rumen fluid was collected from four slaughtered healthy adult Barbarin sheep from the same farm (age and mean body weight averaged 12 months and 36 ± 4 kg, respectively), fed a ration composed of oat hay and barley grain (70/30 on a DM basis). Immediately after slaughter, the rumen content was collected by evisceration into a preheated thermos (39 °C) and taken rapidly to the laboratory, where it was homogenized and filtrated through 4 layers of surgical gauze to eliminate feed particles. Then, the rumen fluid was mixed with artificial saliva (in a 1 to 2 ratio) and prepared according to the procedure described by Menke and Steingass [36]. Approximately 300 ± 5 mg of olive pomace samples were transferred into graded glass syringes (100 ml volume). Syringes were prewarmed to 39 °C before 30 ml of the buffer–rumen fluid mix was poured into each syringe under constant CO2 flow. Syringes were manually agitated 30 min after the start of incubation and then every hour for the first 10 h of incubation. Gas volume was read at incubation (0 h) and after 2, 4, 6, 8, 10, 12, 24, 36, 48, 72, and 96 h of incubation. Two runs were carried out and samples were incubated in triplicate. Three syringes per run with only diluted rumen fluid were incubated as blanks and used to compensate for gas production in the absence of substrate. Cumulative gas was expressed as a milliliter of gas produced per 300 mg of DM and corrected for blanks. Data of the cumulative gas volume produced were fitted using the exponential model proposed by France et al. (Eq. 3) [37]:

$$G = b * \left(1 - e^{-k(t-L)}\right), \quad (3)$$

where $G$ (ml/300 g DM): gas produced at the time ($t$); $b$ (ml): the asymptotic gas production; $K$ ($h^{-1}$): the fractional rate of gas production; and $L$ (h): the lag time between the incubation and the start of fermentation.

The post-incubation parameters such as in vitro organic matter digestibility (IVOMD, %) and metabolizable energy (ME, MJ/kg DM) were estimated by the equations of Menke and Steingass, based on 24 h gas production (ml/300 g) and chemical composition (protein and fat contents) as follows (Eq. 4 and Eq. 5):

$$\text{ME}\left(\text{MJ/kg DM}\right) = 2.20 + 0.136\ \text{GP} + 0.057\ \text{CP}, \quad (4)$$

$$\text{IVOMD (\%)} = 14.88 + 0.889\ \text{GP} + 0.45\ \text{CP} + 0.00651\ \text{XA}, \quad (5)$$

where GP is the net gas production (ml/300 mg DM) at 24 h, CP is crude protein (% of DM), and XA is ash content (% of DM) [38].

### Statistical analysis

All the assays were carried out in triplicate in three different experiments, and the results are expressed as mean values ± standard deviation (SD). Significant differences between the samples were calculated according to the one-way analysis of variance (ANOVA) followed by Tukey tests using SPSS Statistics 20 software with $p < 0.05$ being considered significant.

Optimization of alkaline hydrogen peroxide treatment was carried out using RSM (response surface methodology). The objective is to optimize a response (output variable) this response is influenced by various independent variables (input variables). Statgraphics 19 (Stat-Ease, Inc. Minneapolis, MN, USA) statistical software with a rotatable central composite design was used for the response determination.

## Results and discussion

### Alkaline hydrogen peroxide (AHP) treatment of exhausted olive pomace (EOP)

In order to reduce the natural recalcitrance of the lignocellulose cell wall, the AHP treatment step can significantly decrease lignin and improve cellulose accessibility [39]. The optimization of AHP treatment is a key step to minimize the cost of treatment without increasing both polyphenols content and lipid oxidation.

In this regard, AHP treatment of EOP was investigated using the response surface methodology RSM in which sodium hydroxide (NaOH) varied between 1% (w/v) to 6% ($w/v$) and hydrogen peroxide ($H_2O_2$) varied between 1% ($w/v$) and 4% ($w/v$) (Additional file 1: Fig. S1). The phenol content and lipid oxidation were chosen as model responses to assess the treatment efficiency. ANOVA analysis of the model was performed to evaluate its statistical significance. The proposed linear regression model for phenol and lipid oxidation yield was as follows (Eq. 6 and Eq. 7):

$$\text{Phenol} = 2.75 - 0.363811 * \text{NaOH} + 0.20671 * H_2O_2 + 0.0206245 * \text{NaOH}^2 + 0.8125 * \text{NaOH} * H_2O_2 + 0.150625 * H_2O_2^2 \quad (6)$$

$$\text{Lipid oxidation} = 5.01 + 0.531921 * \text{NaOH} + 0.603301 * H_2O_2 - 0.613188 * \text{NaOH}^2 + 0.28575 * \text{NaOH} * H_2O_2 + 0.0313149 * H_2O_2^2 \quad (7)$$

The results of ANOVA (*F*-test) and the *p*-value were used to check the statistical significance of both phenolic content and lipid oxidation.

For lipid oxidation all parameters were significant ($P \leq 0.05$) with a confidence interval of 95% and the $R^2$ and $R^2$adj values of the fitted model were 0.8096 and 0.749, respectively.

For phenolic content, all parameters were significant ($P \leq 0.05$) with a confidence interval of 95% and the $R^2$ and $R^2$adj values of the fitted model were 0.9888 and 0.9853, respectively.

These results indicated that those models worked well to predict both phenolic content and lipid oxidation.

Figure 1a shows the effects of the $H_2O_2$ concentration and NaOH concentration on the phenolic content of treated olive pomace. Results revealed that the increase in NaOH concentration induces a decrease in phenolic content. However, increases in $H_2O_2$ concentration (%w/v) clearly obstruct the NaOH effect on the phenolic content decrease. Besides, Fig. 1b illustrates the response surface obtained from the mode applied to lipid oxidation of treated olive pomace. An interactive effect between $H_2O_2$ concentration and NaOH concentration is clear. It can be observed that the increase of $H_2O_2$ with the increase of NaOH led to enhanced response. However, even with the increase of $H_2O_2$ at low NaOH concentration, lipid oxidation was low.

According to Table 2, the total phenolic content comprised between 1.51 ± 0.03 g/kg DW (run2) and 4.19 ± 0.42 g/kg DW (run1). Regarding lipid oxidation, it comprised between 3.28 ± 0.05 mM MDA eq/kg of DW (run 5) and 6.203 ± 0.07 mM MDA eq/kg of DW (run 4). Comparing to the control test, the exhausted olive pomace exhibited a value of 4.91 ± 0.06 mg/100 g and 2.52 ± 0.06 mM MDA eq/kg of DW for phenolic content and lipid oxidation, respectively.

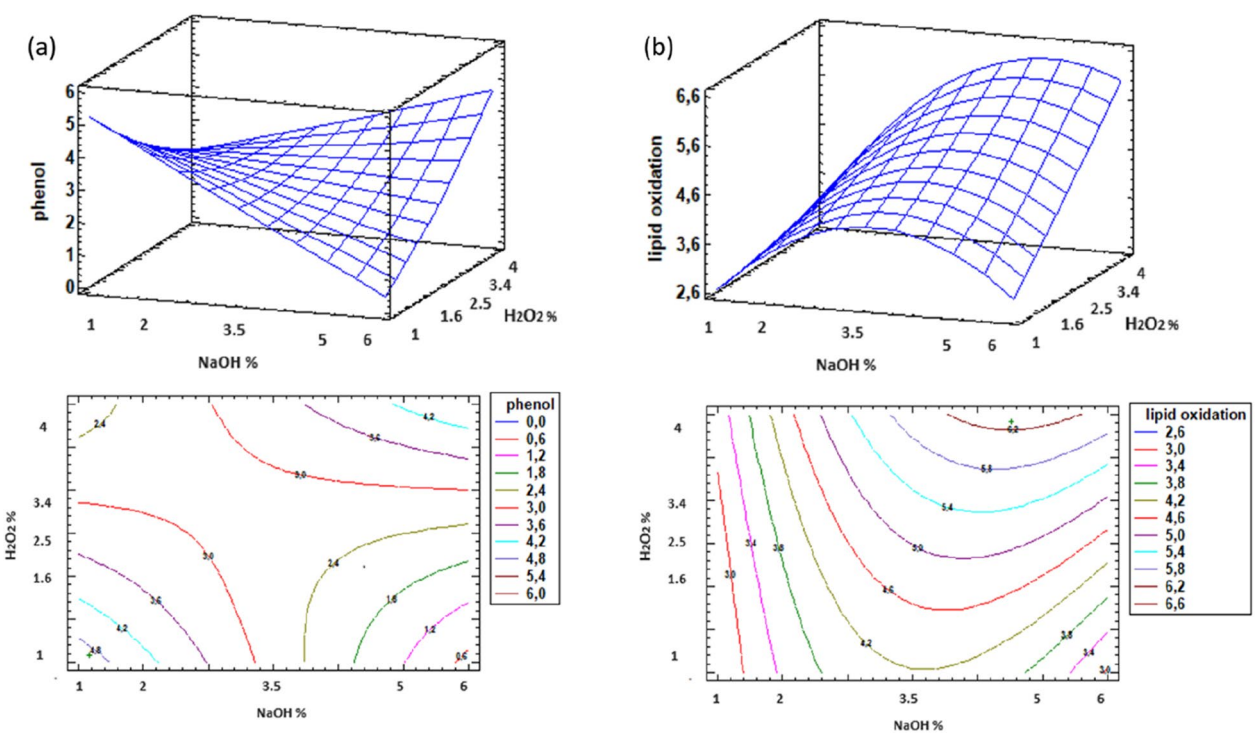



**Fig. 1** **a** Response surface and contour plots for phenolic content (expressed as gallic acid equivalents g/kg DW) as a function of $H_2O_2$ concentration (%*w/v*) and NaOH concentration (%*w/v*); **b** response surface for lipid oxidation and contour plots (expressed as mg malonaldehyde equivalents (MDA)/kg of DW) as a function of $H_2O_2$ concentration (%*w/v*) and NaOH concentration (%*w/v*)

The optimization of the AHP treatment stage focused on the minimization of two responses namely the phenolic content and the lipid oxidation in treated olive pomace. In fact, polyphenols such as condensed tannins have a potential anti-nutritional activity due to their inhibitory action on the extracellular enzymes secreted by the ruminal microflora [19, 40]. As for lipid oxidation, resulting from the AHP treatment reaction, it increases the presence of toxic compounds such as free radicals, peroxides and aldehydes which can reduce feed palatability, as well as lead to oxidative stress and less feed intake [41, 42]. From this perspective run 6 (6% NaOH and 2.5% $H_2O_2$) seems to be the best experimental condition. However, hydrogen peroxide can only react with the aliphatic part of lignin without any change or degradation of phenolic compounds while used under alkaline condition hydrogen peroxide become able to attack the phenolic compounds. Also, it should be mentioned that at higher concentrations of $H_2O_2$, the rate of evolution of $O_2$ increases at a rapid rate that reduces the oxygen incorporation at lignin sites, resulting in decreased delignification efficiency [43, 44]. For that reason, the selected optimal conditions for this AHP treatment within the experimental range studied were 5% NaOH and 1.6% $H_2O_2$ (run 2). In those conditions, the consumption of NaOH and $H_2O_2$ was optimized without influencing the treatment efficiency by reducing both phenolic content and lipid oxidation. To validate the optimum RS concentration, an additional experiment with the specified conditions was achieved. It yielded 1.54 ± 0.12 and 4.91 ± 0.07 for the phenolic content and lipid oxidation, respectively, which confirmed that the response model was adequate for the optimization.

The decrease of phenolic content upon alkaline hydrogen peroxide treatment was in agreement with previous literature studies. Thus, G. Jiang et al. 2021 reported up to 37% reduction of phenolic content by AHP treatment of ginseng insoluble dietary fiber [26], while in our case up to 63% reduction of phenolic content under the optimized conditions was obtained. In fact, AHP treatment of biomaterials leads to the formation of oxygen and water as reaction products without causing any secondary pollution. Under the alkaline condition, the decomposition reaction of hydrogen peroxide will generate the hydroperoxide anion HOO- which reacts with hydrogen peroxide to produce highly active hydroxyl radicals $HO^{\cdot}$ and superoxide anion radicals $O_2^{\cdot}$-. Through several reaction pathways, these free radicals and the oxygen molecule can promote the removal of lignin by destroying lactones, ether crosslinks, and cleaving β-O-4 bonds [45]. Furthermore, the application of AHP treatment not only leads to the disruption of the three-dimensional structure of the cell wall, but also results in the liberation and degradation of certain phenolic compounds.

**Table 3** Chemical composition of exhausted olive pomace (EOP) and treated olive pomace (TOP) (g/100 g DW (dry weight))

| Chemical parameters (g/100 g DW) | Exhausted olive pomace (EOP) | Treated olive pomace (TOP) |
|---|---|---|
| Dry matter (DM) | 93.43 ± 0.09* | 91.94 ± 0.05* |
| Ash | 6.22 ± 0.03* | 5.51 ± 0.07* |
| Neutral detergent fiber (NDF) | 66.65 ± 0.33* | 75.24 ± 0.23* |
| Acid detergent fiber (ADF) | 51.99 ± 0.73** | 54.05 ± 0.35** |
| Acid detergent lignin (ADL) | 34.12 ± 0.11* | 25.7 ± 0.18* |
| Crude protein (CP) | 4.42 ± 0.16* | 3.320 ± 0.05* |
| Crude fiber | 45 ± 0.515* | 59 ± 0.416* |
| Total sugar | 2.125 ± 0.23* | 3.845 ± 0.23* |
| Total lipids | 3.47 ± 0.23** | 2.2 ± 0.17* |

Data are expressed as (means ± standard deviation). ($N = 3$); *$p < 0.0001$ **$p < 0.05$

**Table 4** Fatty acid composition (%) of exhausted and treated olive pomace

| Fatty acids composition | Values % | |
|---|---|---|
| | Exhausted olive pomace (EOP) | Treated olive pomace (TOP) |
| C16:0 | 20.09 | 25.97 |
| C18:0 | 31.57 | 30.47 |
| C18:1 | 39.8 | 39.93 |
| C18:2 | 8.54 | 3.62 |
| Total saturated | 51.66 | 56.44 |
| Total unsaturated | 48.34 | 43.55 |

### Chemical composition of exhausted olive pomace (EOP) and treated olive pomace (TOP)

In order to evaluate the effectiveness of alkaline hydrogen peroxide (AHP), the proximate chemical composition of EOP and TOP under optimized conditions was determined. They are summarized in Table 3. Results revealed that NDF, ADF, and crude fiber content in TOP were significantly higher compared to EOP, while a decrease in ADL, crude protein, and dry matter content was reported for TOP. This was somehow expected since AHP treatment caused the destruction of the lignin, which holds the cell wall carbohydrates. Similar results were also reported in a study on wheat straw proving that losses of ADL, and NDF after AHP treatment resulted in increases in ADF and cellulose concentrations [46]. As for the removal rate of lignin from exhausted olive pomace treated by AHP it reached 24.7%. Chang et al. study led

on palm fiber also reported a decrease of lignin from 36% to 21.4% after AHP treatment that broke the rigid structure of palm fiber and removed part of the hemicelluloses and lignin [47]. Actually, within the crude fiber composition, lignin is the mainly constituent that limits the feed value of olive pomace. Thus, the AHP treatment can be an effective solution for the valorization of olive pomace as animal feed [48].

The fatty acids composition analysis (Table 4) highlighted four fatty acids in both EOP and TOP. Two saturated fatty acids which are C16:0 (palmitic acid) and C18:0 (stearic acid), as well as, two unsaturated fatty acids which are C18:1 (oleic acid) and C18:2 (linoleic acid). Both EOP and TOP present a high oleic acid content of 39.8% and 39.93%, respectively. In fact, it has been demonstrated that introducing olive pomace, with a high oleic acid content, in the diet of lactating animals enhances milk fatty acid quality, by increasing monounsaturated fatty acids and decreasing saturated fatty acids [49, 50].

As shown by the chemical composition analysis, the TOP could be a promising feedstock of animal feed. It should be noted that various factors, including edaphoclimatic conditions, agricultural practices, cultivar, and ripening stage, can all have an impact on the proximate composition and bioactive compounds profile of olive pomace [51].

### Enzymatic hydrolysis and in vitro essay

During enzymatic hydrolysis of biomass, the enzyme infiltration into the cell wall depends on the initial lignin content in the raw material. So lignin removal could significantly increase the glucose yield from enzymatic hydrolysis [52]. In order to evaluate the treatment efficiency, enzymatic hydrolysis of the exhausted olive pomace (EOP) and treated olive pomace (TOP) were conducted using Celluclast© (Additional file 1: Fig. S2). The results of reducing sugar yield from enzymatic hydrolysis are shown in Fig. 2. Without alkaline hydrogen peroxide (AHP) treatment, the reducing sugar yield from hydrolysis of EOP after 48 h was 33.0%, while upon AHP treatment, the reducing sugar yield was substantially increased and reached 48.0% for TOP of hydrolyses. Chemical composition analyses showed that during the AHP treatment, the proportion of cellulose, hemicelluloses, and lignin changed. In fact, after treatment and with the removal of partial fractions of lignin, the cellulose and hemicelluloses were more readily available at the active sites of enzymes promoting therefore the contact with the enzyme, and increasing consequently the enzymatic hydrolysis efficiency [53]. Our findings are in accordance with previous studies led on Larch show that AHP treatment increases the enzymatic hydrolysis yield from 20.1% to 28.3% under 2% of $H_2O_2$ charge after 72 h of hydrolysis [54]. Similarly, Tareen et al. study, revealed that the treatment of oil palm trunk with AHP increased glucose concentration from 11.77 (± 0.84) g/L (for untreated biomass) to 46.15 (± 0.32) g/l, resulting in a 59.82% enzyme digestibility after 96 h [39].Since we have obtained promising results with enzymatic hydrolysis, further analysis was carried out using rumen fluid in order to evaluate the possibility of TOP use as animal feed. Table 5 reports the in vitro rumen fermentation parameters, the potential gas production (*b*) value of TOP was significantly higher (41.371 ml/g DM) than EOP (25.771 ml/g DM) ($p \leq 0.0001$). The lag of in vitro fermentation was more important in EOP (1.784) compared

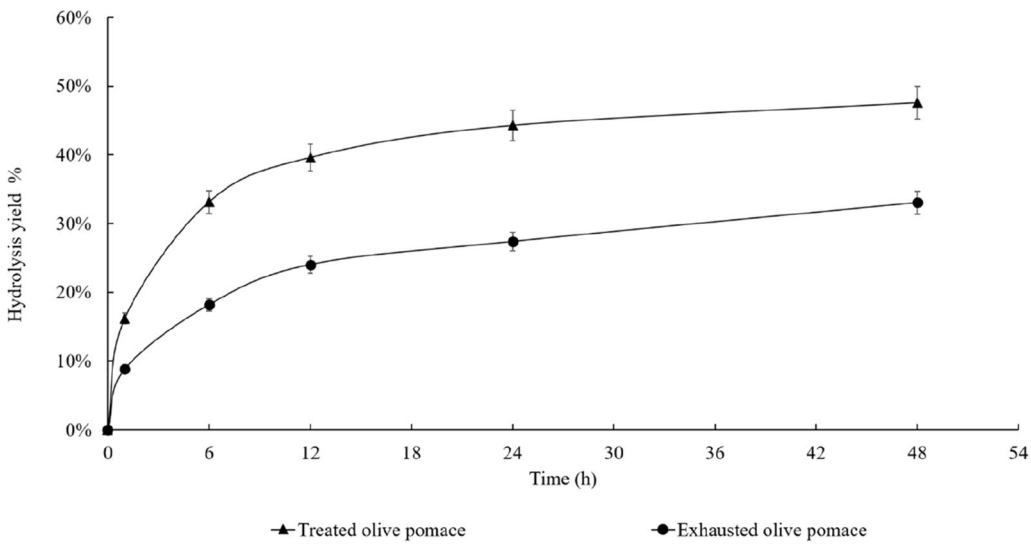



**Fig. 2** Reducing sugar yield from enzymatic hydrolysis of exhausted and treated olive pomace

**Table 5** Kinetic parameters of gas production (model France et al. [37]), in vitro organic matter digestibility, metabolizable energy, and reducing sugar yield from enzymatic hydrolysis of EOP and TOP

| Kinetic parameters | Exhausted olive pomace (EOP) | Treated olive pomace (TOP) |
|---|---|---|
| *b* | 25.771[b] | 41.371[a] |
| *k* | 0.019[a] | 0.014[b] |
| *L* | 1.784[a] | 1.005[b] |
| G24 (ml/300 mg of DM) | 7.917[b] | 11.007[a] |
| IVOMD (%) | 24.76 ± 0.21[a] | 26.73 ± 0.11[b] |
| ME (Kcal/kg of DM) | 7.78 ± 0.03[a] | 9.28 ± 0.02[b] |
| RS24 (%) | 27.4[a] | 44.3[b] |

*b*, asymptotic gas production; *k*, the fractional rate of gas production; *L*, lag time; G24, 24 h net gas production; ME, metabolizable energy; IVOMD, in vitro organic matter digestibility; RS24, reducing sugar yield after 24 h of enzymatic hydrolysis. The data marked with different letters (a, b) in the same line indicate significant differences at $P \leq 0.0001$

to TOP (1.005) this could be explained by the fact that the high content of lignin in EOP makes from this feedstuff difficult to be metabolized by rumen microorganisms. This was somehow expected since AHP treatment of EOP can effectively remove lignin the main physical barrier that limited the access of ruminal glycosyl hydrolases to its substrates [55]. Interestingly, a high in vitro organic matter digestibly (IVOMD) of 26.73% was observed in TOP compared to 24.76% in EOP ($p \leq 0.0001$). Also, the metabolizable energy of TOP (9.28 kcal/kg DM) was more important than the metabolizable energy of EOP (7.78 kcal/kg DM). The kinetics of gas production of EOP and TOP showed that gas production gradually increases to reach a maximum of 21.66 ± 2.02(ml/300 mg of DM) and of 31.67 ± 2.57(ml/300 mg of DM) after 96 h of fermentation for EOP and TOP, respectively (Additional file 1: Fig. S3). The phenolic content decrease upon AHP treatment of EOP can be related to the improvement of the in vitro gas production from rumen fermentation. In a previous study, it was found that plant phenolic compounds such as tannin can decrease up to 50% of in vitro gas production from rumen fermentation. Moreover, the degradability of EOP was probably affected by the oil content of this by-product, which reduces bacterial attachment to fiber particles [56]. The in vitro digestibility evaluation of control and fermented olive cake (OC) in the study of Neifar et al. [57] revealed that gas production rises to 25.35 ml/g dry substrate (ds) greater than the control OC (9.31 ml/ g ds). Concerning in vitro gas production promoted by olive pomace degradation, the only information has been reported by Al-Masri (2003) who found values ranging from 18.8 to 36.4 ml gas/g of olive pomace [58].

This study revealed that AHP treatment enhances the quality of olive pomace as animal feedstock. However, for an effective scale-up of the process, the optimization of the water consumption and the temperature during washing and drying stages seems to be necessary.

## Conclusion

Olive pomace is considered as one of the most abundant agricultural by-products in the Mediterranean area which caused pollution problems due to its high content of phenolic compounds. In this study, we investigate the detoxification of this waste and its conversion to a value-added product exploited in the animal feed field based on a straightforward alkaline hydrogen peroxide (AHP) treatment. Actually, AHP treatment of exhausted olive pomace significantly improved the quality of olive pomace by enhancing its in vitro rumen digestibility and decreasing its anti-nutritional compounds content such as polyphenols and lignin. This study presents a proof of using AHP as an effective mild treatment that offer high enzymatic hydrolysis and in vitro rumen digestibility with low cost and limited environmental damages. Performing AHP with green solvents facilitates therefore the design and scale-up of eco-friendly transformation processes of olive pomace biomass and its application as substitute of the high-cost ingredients in animal feed sector. This study could inspire further investigations into a detailed economic analysis of this processing approach.

### Abbreviations

| | |
|---|---|
| RSM | Response surface methodology |
| AHP | Alkaline hydrogen peroxide |
| EOP | Exhausted olive pomace |
| TOP | Treated olive pomace |
| NDF | Neutral detergent fiber |
| ADF | Acid detergent fiber |
| ADL | Acid detergent lignin |
| ME | Metabolizable energy |
| IVOMD | In vitro organic matter digestibility |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-024-00533-9.

**Additional file 1: Fig. S1.** Olive pomace before AHP treatment (EOP) and after AHP treatment (TOP) in the optimized condition. **Fig. S2.** Enzymatic hydrolysis of treated olive pomace **a** before hydrolysis and **b** after hydrolysis. **Fig. S3.** Kinetics of gas production (ml per 300 mg DM) of studied Exhausted Olive Pomace (EOP) and Treated Olive Pomace (TOP)

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**Author contributions**
MR carried out most of the experiments, the interpretation of data, and the redaction-correction of the manuscript. BYN participated in the interpretation of results and the correction of the manuscript. DC participated in the interpretation of results. MN supervised the in vitro essay experiments. SI conceptualized and supervised the work and participated in the correction of the manuscript.

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## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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## Publisher's Note