

Research Article

By-Products of Olive Oil in the Service of the Deficiency of Food Antioxidants: The Case of Butter

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Further downstream in the olive oil extraction process, the Mediterranean Basin faces a serious environmental threat caused by olive waste. Despite their polluting profile, olive waste is considered to be a very rich source of natural antioxidants, such as polyphenols. In this study, the latter was valued as a source of natural antioxidants and compared with a synthetic antioxidant ascorbic acid. Concentrations of 2, 4, 6, and 8 mg of the olive mill waste water as well as pomace and ascorbic acid are added to butter (commercial butter) and placed under storage conditions in the oven (accelerated test: 60°C) for 3 months. The alteration of the butter used was followed by determination of the peroxide value and acidity and microbiological analysis. The results obtained show that butters containing olive by-products have undergone less marked oxidative deterioration than those of the control (without additives). The best oxidative stability of butter was achieved by adding 80 mg/kg of butter, a result comparable with that obtained by adding ascorbic acid.

1. Introduction

In olive-growing countries, the extraction of olive oil generates a large quantity of by-products (pomace and olive mill waste water (OMWW)) which are very rich in bioactive organic compounds [1–3]. They are generally or often released into the environment, but these by-products are considered toxic. Their toxicity is mainly due to their load of nonbiodegradable organic matter, which makes these by-product compounds recalcitrant to natural degradation. They cause pollution of the soil, atmosphere, and water [2, 4, 5]. Despite their potential pollution, these by-products are a very rich source of phenolic compounds. In addition, as natural antioxidants, these polyphenols are attracting increasing interest in the prevention of many diseases. They are also used as additives for the pharmaceutical, cosmetic,

and food industries, mainly to stabilize vegetable oils. Currently, consumers with a tendency to avoid synthetic antioxidants are suspected of toxic, sensitizing, allergenic, and carcinogenic effects. On the other hand, natural antioxidants are highly sought after in the food industry, are recognized for their effectiveness, and also as a substitute for synthetic additives [6].

Padalino et al. [7] optimized, from a sensory and nutritional point of view, the formulation of durum wheat spaghetti enriched with an industrial by-product of olive oil, indicated as olive pomace and concluded that the appropriate addition of olive pomace flour and transglutaminase for pasta enrichment could provide a starting point for the valorization of olive oil and produce new healthy food products. On the other hand, new types of taralli have been produced by [8] by adding 20% fermented black olive

pomace to the wheat flour. Taralli enriched with fermented pomace showed a low content of saturated fatty acids and high levels of polyphenols, triterpenic acids, tocochromans, and carotenoids, compared with the conventional taralli.

In addition, apart from microbial degradation, lipid degradation by molecular oxygen is the major cause of food deterioration during storage. Lipid oxidation alters fats, oils, and foods that contain it [9]. It can also degrade other lipid components such as vitamins and fat-soluble pigments. This shows that the degradation of a food can range from an acceptable decrease in the intensity of the desirable flavor to total rancidity that makes it inedible, affecting the color as well as the nutritional value; this is on the one hand. On the other hand, oxidation also affects the stability of the food; that is why antioxidants are used [10].

The objective of this work is to highlight the effect of olive oil by-products on the stability of butter. The use of these natural compounds allows us, on the one hand, to adopt an ecodesign approach by eliminating a source of pollution and, on the other hand, to find an alternative source to synthetic antioxidants.

2. Materials and Methods

2.1. Raw Materials Used. Samples of olive pomace (solid waste) and olive mill waste water OMWW (liquid waste) were collected during the winter of 2015 in an olive oil mill in the Fez region, 12 km on the road linking Fez to Séfrou-coordinates 33°93'00"N, 4°90'43"W (Figure 1).

Olive pomace and OMWW are obtained by pressing in a semiautomatic oil mill. These by-products are concentrated at a temperature of 102°C and filtered sterile.

Butter: butter was purchased at a local market. It is a dairy product obtained by churning cream or fresh or fermented milk.

2.2. Preparation of Phenolic Extract

2.2.1. Preparation of Methanol Extract. Ten grams of pomace olive (or 10 mL of olive mill waste water) was extracted by shaking at 150 rpm with 100 ml methanol (80%) and filtered through filter paper no. 1. The filtrate was designated as methanol extract. The experiment was performed at triplicate [6].

2.2.2. Estimation of the Phenolic Content. The total phenolic content was estimated using the Folin–Ciocalteu (FC) assay which is widely used in routine analysis. Briefly, 0.16 ml (three replicates) of the extracts diluted in distilled water was mixed with 0.16 ml of Folin–Ciocalteu reagent. After 5 min, 1.6 ml of 7% sodium carbonate was added. The absorbance of the resulting blue colored solution was measured at 750 nm after 90 min with intermittent shaking. Quantitative measurements were performed, based on a standard calibration curve of point from 0 to 0.5 mg/ml of gallic acid in methanol. The total phenolic content was expressed as gallic acid (GAE) in mg/100 g of extract [6].

2.2.3. Evaluation of Antioxidant Activity. Antioxidant activity was evaluated by scavenging radical activity using the 2,2-diphenyl-2-picryl hydrazyl radical method (DPPH) which was adapted from the protocol of Hanato with some modifications. Each sample stock solution (0.2 mg/mL) was diluted to final concentrations of 0.01, 0.02, 0.03, and 0.04 mg/ml, in methanol, and 0.5 mM DPPH methanol solutions. Sample solution of different concentrations was allowed to stand at room temperature in the dark. After 30 min, the decrease in absorbance at 517 nm was measured. The antiradical efficiency is calculated as follows:

$$EA = \frac{1}{IC_{50}} \quad (1)$$

where IC_{50} is the concentration of extract required for obtaining 50% of the reduced form of the DPPH radical.

2.3. Enrichment of Butter by Olive Coproducts. In order to evaluate the antioxidant effect of the various additives, butters used as a carrier for OMWW, pomace, and ascorbic acid are subjected to thermo-oxidative alteration.

First of all, we use an accelerated test: the oven test. Ten sets of test tubes were placed in a thermoregulated oven at 60°C for 90 days to study the stability of the studied butter under storage conditions. Four concentrations of OMWW were prepared (2, 4, 6, and 8 mg/100 g) and four other analogues (2, 4, 6, and 8 mg/100 g) of pomace were added to butters, respectively, in addition to a positive control series of butter plus ascorbic acid (3×10^3 UI/100 g) (synthetic antioxidant) and a negative control series (butter), in order to compare and assess their antioxidant effects.

Then, an additional test was carried out with butter and the same concentrations of OMWW, pomace, and ascorbic acid under ambient thermal conditions of 25°C.

Throughout storage, a periodic 15-day sampling period on all samples in each series was used to continue the alteration of butter as a measure of oxidation, acidity, and microbiology.

2.4. Physicochemical Analyses of the Oxidative Stability of Butter. To evaluate the oxidation evolution of enriched butters, two main parameters (acidity and peroxide value) were determined using standardised methods [11, 12].

2.4.1. Acidity [11]. Acidity is the percentage of free fatty acids in a fatty substance. The determination of the acidity of the oils was carried out according to the AFNOR NF T60-204 standard. The test sample is put in solution in a “solvent” mixture (ethanol/diethyl ether) to trap the fatty acids present. Then, a titration is carried out using a potassium hydroxide solution in the presence of phenolphthalein as a color indicator.

2.4.2. Peroxide Index (PI) [12]. Five grams of butter is dissolved in a mixture of 25 mL of acetic acid/chloroform acid (3/2 v:v) and 1 mL of a saturated solution of potassium iodide. After 5 min reacting in the dark, 75 mL of distilled



FIGURE 1: Location of oil moulin Fez MOROCCO.

water is added and the liberated iodine is titrated with a sodium thiosulfate solution 0.1 N in the presence of starch paste. A control test (without fat) is performed in the same conditions.

2.5. Microbiological Analyses of Butter Stability. Microbiological analyses of butters included the enumeration of total aerobic mesophilic flora (FMAT), total coliforms (TC), yeasts and moulds (L and M), and *Staphylococcus aureus* (*S. aureus*). FMAT was counted on Plate Count Agar (PCA) medium after 48 hours of incubation at 30°C, coliforms on Deoxycholate Lactose agar (DCL) medium after 48 hours of incubation at 37°C, *Staphylococcus aureus* on Chapman agar medium after 48 to 72 hours of incubation at 30°C, and yeasts and molds on sabouraud medium after 48–72 hours of incubation at 28°C.

2.6. Statistical Analysis of Data. The statistical analyses of the data were performed by IBM SPSS Statistics Version 20. The analysis of the comparison of the means was carried out by the Student's *t*-test to compare the evolution profiles of the parameters. All analyses were carried out in triplicate.

To check the validity of all our data, we based the student's *t*-test on the *P* value evaluation, and the results are as follows:

- (i) The determination coefficient of all parameters is close to 1 because it varies between 0.74 and 0.97, which shows the good variation and regression quality of each parameter over time
- (ii) Using the Student's *t*-test, we noticed that the *P* value of each parameter is less than 0.05 (5%), which proves that the variation of each parameter over time is significant at a significance level that is equal to 0.05

3. Results and Discussion

3.1. Characterization of Olive By-Products. OMWW and pomace have an acid pH with values of 4.5 for OMWW and 4.12 for pomace. For pH, the value recorded in our study is within the range cited in the literature (4.5 to 6) [13] (Table 1).

TABLE 1: Physicochemical characterisation of olive waste.

Parameters	OMWW	Pomace
pH	4.50 ± 0.01	4.12 ± 0.01
EC (mS·cm ⁻¹)	7.00 ± 0.02	5.50 ± 0.01
Density	1.05	0.32 ± 0.02
OM (%)	74.75 ± 5.46	95.18 ± 0.42
OC (%)	43.36 ± 3.16	53.2
FAT (%)	7.67%	72.3%
NTK (mg/L)	0.003 ± 0.0004	0.95

For electrical conductivity, the results obtained during our study are comparable with those found in the literature, which we found for OMWW and pomace, respectively, 7 and 5.5 ms·cm⁻¹. This value gives a general idea of the high salt content present in these effluents, and this is due to the salting practices for the conservation of olives before crushing, in addition to the natural richness of the OMWW in dissolved mineral salts.

The tenures of the fat obtained are almost comparable with those obtained by [16]. It forms a lipid layer on the surface of the OMWW, which could limit natural evaporation. The total nitrogen content is in the order of 0.003 mg/L for OMWW and 0.95 mg/L for pomace (Table 1).

3.2. Characterization of Commercial Butter. The results of the acidity measurements and the peroxide value of the commercial butter used are shown in Table 2.

3.3. Determination of Polyphenols. Spectrophotometric assay using the Folin–Ciocalteu reagent determined the total content of polyphenols in pomace and OMWW. The results obtained from the polyphenols assay are shown in Figure 2.

The polyphenol content in OMWW is higher than that in pomace, which is about 1.15 mg/ml for pomace and 3.65 mg/ml for OMWW. These values obtained are almost equivalent to the one found in the province of Benevento (Italy) [17] using the same extraction protocol.

3.4. Antioxidant Activity. The antioxidant activity of the phenolic extracts of pomace and OMWW and the standard antioxidant (vitamin C and BHT) against the DPPH radical

TABLE 2: Butter characteristics.

Parameter	Acidity % oleic acid (ac.)	Peroxide value meq O ₂ (kg)
Value	0.11	2.9

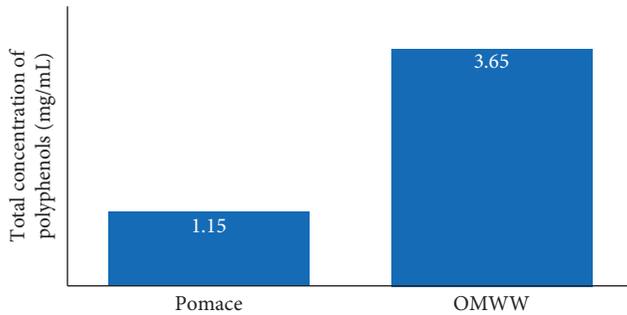


FIGURE 2: Total polyphenol concentration in olive by-products.

is evaluated by the trapping test of the free radical DPPH which is accompanied by its transition from the violet colour to the yellow color measurable at 517 nm.

The results of the antioxidant power of the phenolic extracts of the olive pomace and OMWW tested show that the percentage of inhibition of these extracts is higher than the percentage of inhibition of the standards.

Among the two phenolic extracts, the polyphenol of the OMWW represents the most active extract than the extract of pomace with an IC₅₀ of about 0.15 ± 0.47 mg/mL and an EA of 6.66 ± 0.070, and this activity is more important than the vitamin C used as a control with an IC₅₀ of 200 mg/mL and an EA of 0.005 (Figure 3).

These results of antioxidant activity of phenolic extracts can be explained by the difference in the phenolic composition of these extracts. Indeed, the ability to reduce free radicals is largely influenced by the phenolic composition of the sample [15].

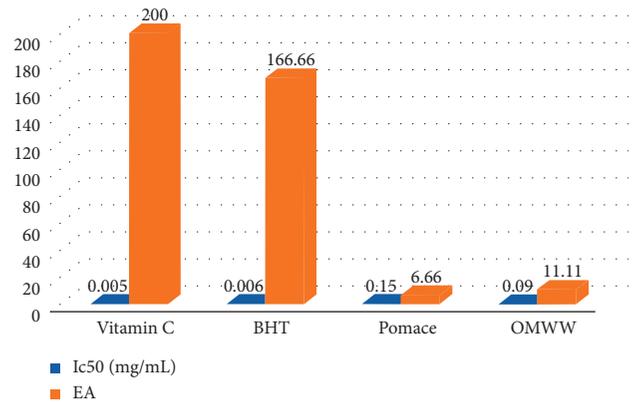
3.5. Study of Oxidative Stability during Storage. The effect of the addition of olive by-products (in the order of 8 mg/100 g) on the stability of butter with respect to oxidation at 60°C and 25°C was studied.

3.5.1. Acidity

(1) *Acidity at 25°C.* Figure 4 shows a small to highly significant difference between the beginning and end of the experiment, which results in a slight variation in acidity for all enriched butters.

At ninety days of storage at 25°C, the acidity of the control butter increased from 0.1% to 3.5%.

For 100 g of butter enriched with 8 mg of OMWW or 8 mg of pomace or 3 × 10³ IU ascorbic acid, the acidity has not changed much. It went from 0.1% to 0.6%. The low evolution of acidity during storage can be explained by the hydrolysis of triglycerides, which is not sufficient to compensate for, or even increase the free fatty acid functions

FIGURE 3: IC₅₀ and EA of the tested extracts.

blocked by polymerization or volatilized during the oxidation phase [6].

(2) *Acidity at 60°C.* Free acidity is an important factor in assessing butter quality and is widely used both as a classic commercial classification criterion. It is also a factor that provides information on the alteration of butters by hydrolysis. Hydrolysis releases fatty acids, the dosage of which gives an idea of the progress of butter degradation [16].

The results of the samples' acidity at 60°C are given in Figure 5. Examination of this figure shows a difference in the temporal evolution (throughout the storage period) of the acidity of both the control sample and the treated samples. It should be noted that during the first storage period, the acidity remains constant for all samples of enriched butter, while there is an increase from the first week for control butter (without additives). Beyond that, there is a stabilization of the enriched butter in terms of their acidity compared with the control butter. This stability is due to the pronounced effect of phenolic compounds contained in olive coproducts. This trend has been mentioned in numerous studies on the oxidative stability of vegetable oils [1, 17, 18].

3.5.2. Peroxide Value

(1) *Peroxide Value (IP) at 25°C.* The variation in the peroxide value as a function of storage time at 25°C is shown in Figure 6. The values of the peroxide value of butters after 90 days of storage are presented in Table 3, and these results remain acceptable. The formation of peroxides from unsaturated fatty acids depends on their release during hydrolysis, which explains its increase.

Based on these results, we can say that butter enriched with olive waste has better resistance to peroxidation than the control and that butter enriched with OMWW is better protected against oxidation than butter enriched with pomace and that are comparable to butter enriched with commercial antioxidant (ascorbic acid).

(2) *Peroxide Value (IP) at 60°C.* The initial value of the peroxide value of the control butter is 2.9 meq O₂/kg. After

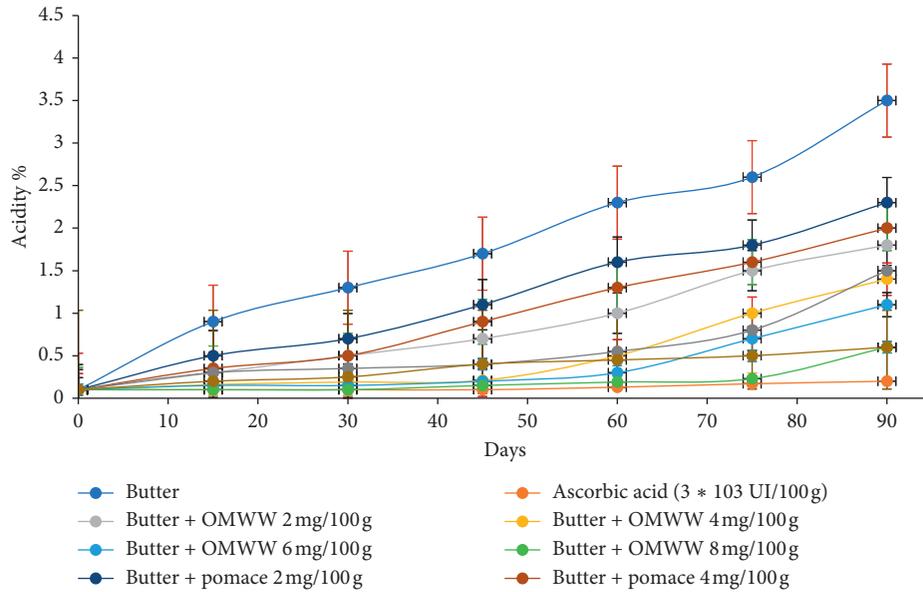


FIGURE 4: Evolution of the acidity as a function of the storage time of butter at 25°C.

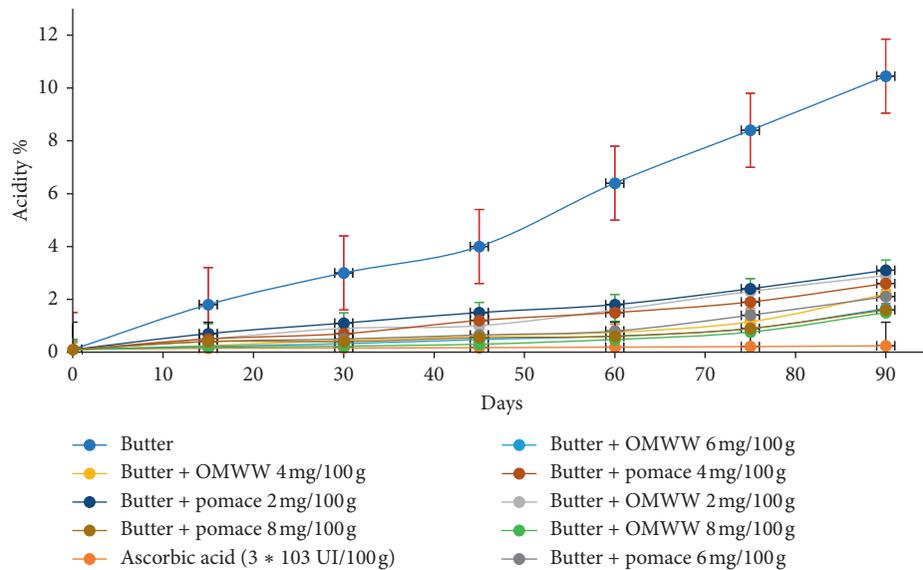


FIGURE 5: Evolution of the acidity as a function of the storage time of butter at 60°C.

15 days of storage, it reached 5 meq O₂/kg, and at the end of the experiment (after 90 days of storage), this value reached 73 meq O₂/kg (Figure 7). For butter enriched with OMWW and pomace and ascorbic acid, the peroxide value increases slightly (Table 4). Indeed, under the same operating conditions, the evolution curve of the butter IP enriched either by olive by-products or by ascorbic acid remains low compared with that of the control. In the light of the results of the PI, it can be seen that the impact of olive oil co-products is significant with regard to the oxidation of butter. Indeed, the lower the PI, the better the oxidative stability of the butter during storage. Phenolic compounds in olive by-products have a significant effect on the trapping of active O₂ by reducing the primary and secondary compounds generated during oxidation. The results

obtained are comparable or even lower than the results reported by Bouhadjra [19] and Gharby et al. [20] who found that the stability of the oils is well correlated with the total phenol content and the presence of a high level of natural antioxidants, the most important of which are tocopherols. Other studies on the effect of phenolic extracts from olive leaves and fruits on the oxidative stability of olive oil samples of different varieties have shown the same trend [1, 2, 20, 21].

3.6. Microbiological Analyses. The variations in the populations of the main microbial groups searched for during the process of storage of control butters and enriched by olive by-products are shown in Figures 8–10.

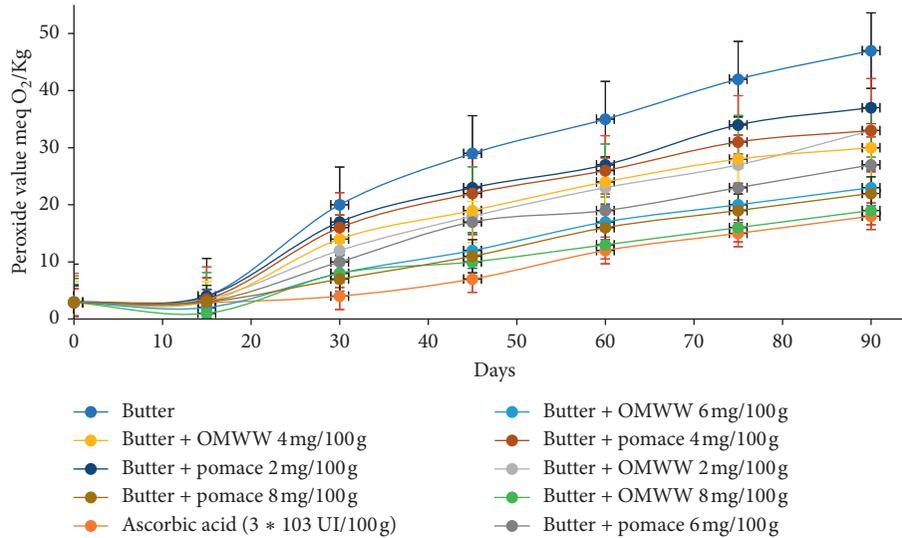


FIGURE 6: Evolution of the peroxide value as a function of the storage time of butter at 25°C.

TABLE 3: Butter peroxide value at 90 days storage at 25°C.

Butter	Control	+8 mg of OMWW	+8 mg of pomace	+3 × 10 ³ IU ascorbic acid
IP (meq of O ₂ /kg)	47	19	22	18

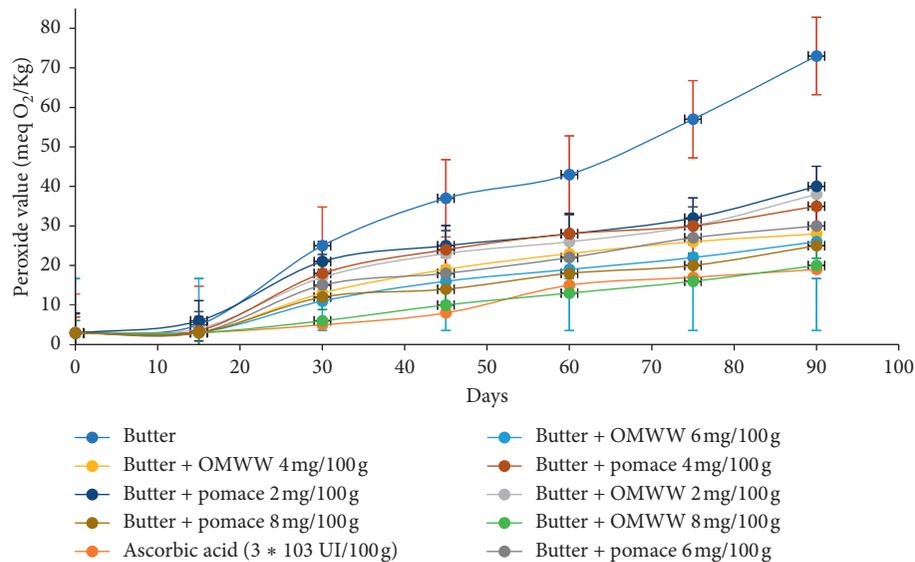


FIGURE 7: Evolution of the peroxide value as a function of the storage time of butter at 60°C.

TABLE 4: Butter peroxide value at 90 days storage at 60°C.

Butter	Control	+8 mg of OMWW	+8 mg of pomace	+3 × 10 ³ IU ascorbic acid
IP (meq of O ₂ /kg)	73	20	25	19

3.6.1. *Total Mesophilic Aerobic Flora.* The results of the total mesophilic aerobic flora count are presented in Figure 8. The study of microbial activity shows that the level of microorganisms is higher for butter without any additives (extreme number of germs is 0 to 80 (CFU/mL) from 1 to 90 days) than for butter enriched with 2 mg/100 g by OMWW

(1 to 27 (CFU/mL) from 45 to 90 days) and by 2 mg/100 g pomace (2 to 30 (CFU/mL), by day 45 to 90 days) and by increasing the concentration of olive by-products in butters, microbial activity tends towards zero until the absence of total mesophilic aerobic flora in butters enriched with 8 mg of OMWW, 8 mg of pomace, and 3 × 10³ IU ascorbic acid.

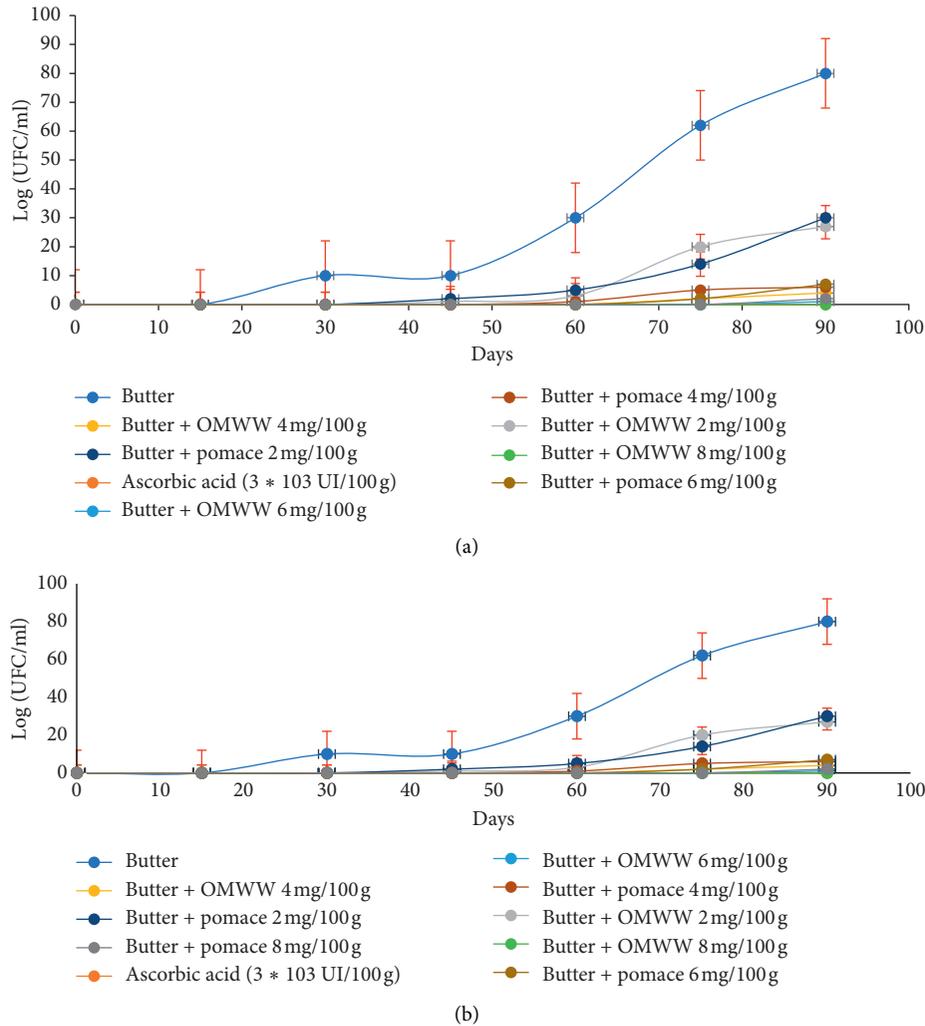


FIGURE 8: Evolution of FTAM butter populations as a function of time. (a) Samples stored at 25°C. (b) Samples stored at 60°C.

The result of the total aerobic mesophilic flora of the enriched butter with the different concentrations of olive oil by-products is in compliance with standard $<10^2$ (CFU/mL). This shows a good microbiological quality which is explained by a respect and a vigorous application of manufacturing and hygiene practices for all types of butter with and without additives [22].

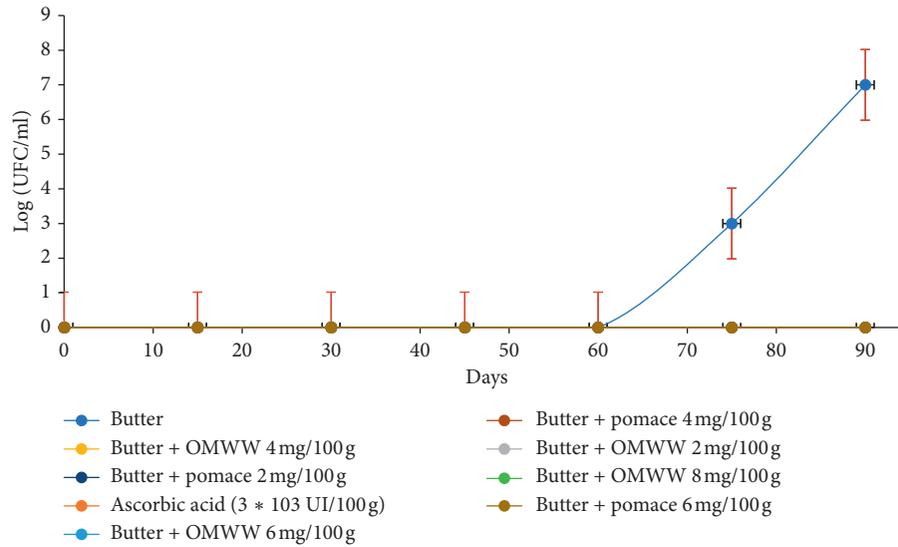
It is frequently accepted that antioxidants could destroy germs. Then, the addition of by-products, containing polyphenols, to butter ensures better preservation and extends the deadline for butter [23].

3.6.2. Total Coliforms. Coliform count results are presented in Figure 9, and coliforms disappear in most samples of enriched butter at the end of storage at both temperatures. The number of coliforms is too low during storage. This suggests an environment unfavorable to any microbial growth and/or even survival of the initial butter flora (0 to 10 (CFU/mL)). Indeed, the absence of coliforms in the final product indicates good hygiene practice throughout the manufacturing process [24].

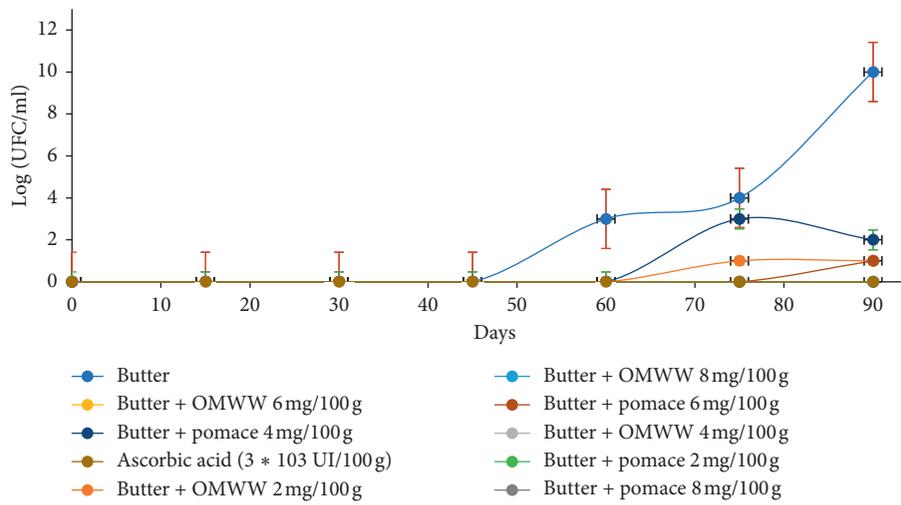
3.6.3. Yeasts and Molds. The fungal flora count results are shown in Figure 10 for the different types of butter. Molds and yeasts were not counted, but their presence is fairly constant in all samples.

According to the results obtained, the absence of yeasts and molds in the product means that it is of satisfactory quality compared with the required standards. This is due to the application of effective heat treatment (pasteurization) and the presence of polyphenols in olive oil by-products that have fungistatic and bacteriostatic properties (replaced in the butter industries by ascorbic acid and salts) [24].

3.6.4. Staphylococcus aureus. Pathogens such as *Staphylococcus aureus* are not tolerable in butter. This germ is a major pathogen; it could cause breast infections. These are accompanied by an increase in permeability between the blood compartment and the milk, which results in changes in the composition of the milk [25]. The results obtained show that the *Staphylococcus* contamination threshold in all butter samples from the first to the last day

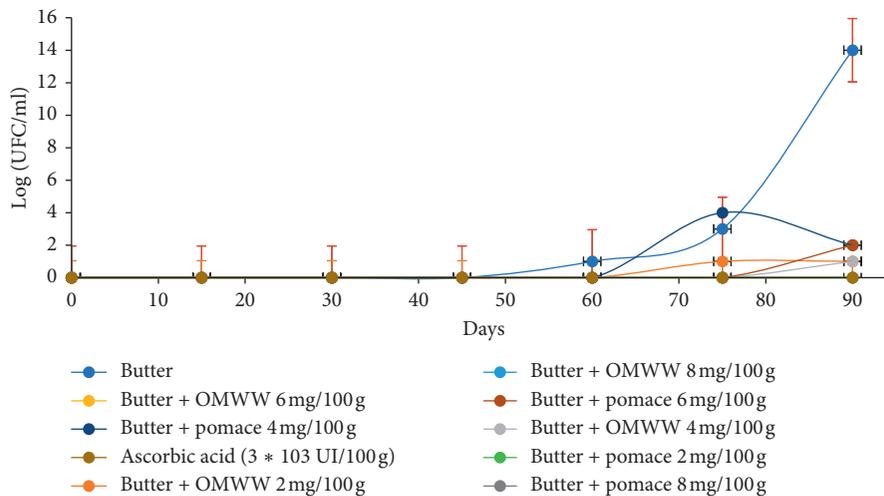


(a)



(b)

FIGURE 9: Evolution of total butter coliform populations as a function of time. (a) Samples stored at 25°C. (b) Samples stored at 60°C.



(a)

FIGURE 10: Continued.

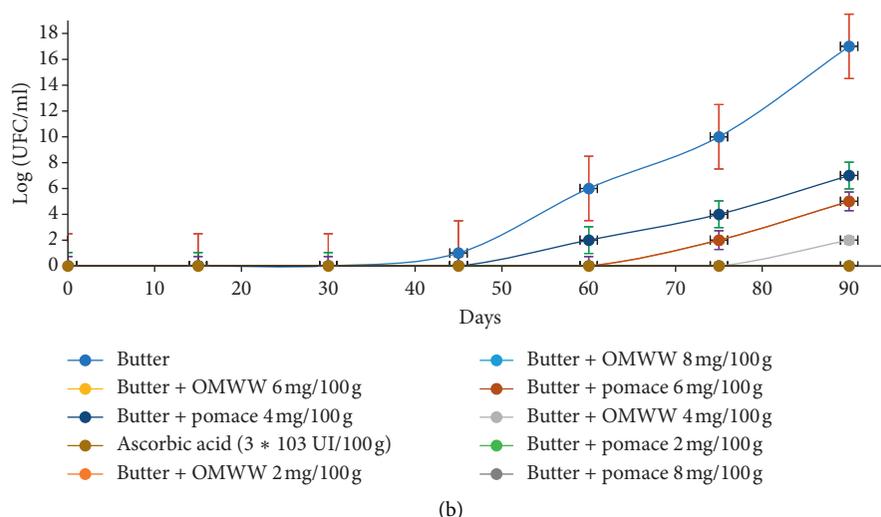


FIGURE 10: Evolution of yeast and mold populations of butters as a function of time. (a) Samples stored at 25°C. (b) Samples stored at 60°C.

of sampling (a total absence of this germ) is in compliance with the standards [26].

4. Conclusion

The study conducted on the stability of butter by olive oil by-products, OMWW or pomace, reports that the latter is rich in polyphenols, which gives them the characteristic of antioxidants. These by-products were tested at different concentrations (2, 4, 6, and 8 mg/100 g of butter) and under two different storage conditions in terms of temperature (25°C and 60°C). Stability was assessed by chemical (acidity and peroxide index) and microbiological (FMAT, *Staphylococcus aureus*, total coliforms, and yeasts and molds) tests.

The results recorded reveal that the addition of 80 mg of OMWW or pomace to 1 kg of butter confers resistance against oxidative stress during storage at 25°C or in the oven at 60°C, for 3 months. This is reflected in the low values of the acidity and peroxide values of the treated samples compared with the control.

In conclusion, the use of coproducts from the olive oil sector has a double advantage. On the one hand, being natural compounds can substitute synthetic additives that are involved in health risks. On the other hand, the valorization of these coproducts is mainly part of the fight against pollution and also the contribution to a green approach in olive oil-producing countries and to benefit from this raw material on an industrial scale.

Data Availability

All tables and figures mentioned and referred to are included in the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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