ITALIAN JOURNAL OF FOOD SCIENCE

Rivista italiana di scienza degli alimenti
(www.itjfs.com)

Volume XXXIII
Number 3
(2021)

ITALIAN JOURNAL OF FOOD SCIENCE

(RIVISTA ITALIANA DI SCIENZA DEGLI ALIMENTI) 2nd series

Founded By Paolo Fantozzi under the aegis of the University of Perugia
Official Journal of the Italian Society of Food Science and Technology
Società Italiana di Scienze e Tecnologie Alimentari (S.I.S.T.Al)
Initially supported in part by the Italian Research Council (CNR) -Rome -Italy
Recognised as a "Journal of High Cultural Level"
by the Ministry of Cultural Heritage -Rome -Italy
(www.itjfs.com))

Publisher:
Codon Publications: email: admin@codonpublications.com, URL: www.codonpublications.com.

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Frequency:

Quarterly -One volume in four issues. Guide for Authors is published in each number and annual indices are published in number 4 of each volume.

Impact Factor:

Impact Factor: 0.736 published in 2018 Journal of Citation Reports, Scopus Cite Score 2020: 1.11. IJFS is abstracted/indexed in: Chemical Abstracts Service (USA); Foods Adlibra Publ. (USA); Gialine - Ensia (F); Institut Information Sci. Acad. Sciences (Russia); Institute for Scientific Information; Current Contents®/ AB & ES; Sci Search® (USA-GB); Int. Food Information Service - IFIS (D); Int. Food Information Service - IFIS (UK); UDL-Edge Citations Index (Malaysia); EBSCO Publishing; Index Copernicus Journal Master List (PL)

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Italian Journal of Food Science, 2021; 33 (3): 1–13

Formulation and optimization of astaxanthin nanoemulsions with marine phospholipids derived from large yellow croaker (*Larimichthys crocea*) roe

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Received: 3 March 2021; Accepted: 2 August 2021; Published: 9 September 2021

PAPER

Abstract

The aim of this work was to investigate the emulsifying capacity of marine phospholipids derived from large yellow croaker roe (LYCRPLs). Initially, conditions for preparing astaxanthin (1% w/w) nanoemulsions with LYCRPLs were optimized based on single-factor experiments, including homogenization pressure, homogenization cycle, emulsifier concentration and corn oil concentration via the response surface methodology. The optimal homogenization pressure was 60 MPa, the optimal number of homogenization cycles was nine, the optimal emulsifier concentration was 4.7%, and the optimal oil concentration was 20%. Under these conditions, the stability, particle size and polydispersity index of nanoemulsions were 0.018 ± 0.0016, 247 ± 4.5 nm and 0.215±0.019, respectively. The droplets of nanoemulsions were characterized by transmission electron microscopy, which revealed that all the droplets were more or less spherical and nonaggregated. In addition, the storage experiments indicated that the nanoemulsions were stable at different temperatures. Therefore, LYCRPLs could be explored as carriers for the delivery of insoluble bioactive compounds in the food industry.

Keywords: astaxanthin, emulsifier, large yellow croaker roe, nanoemulsions, phospholipids

Introduction

An emulsifier is a substance that improves surface tension between various constituent phases in an emulsion and make them form a uniform and stable dispersion system or emulsion (Dickinson, 2009). Depending on the source, two types of emulsifiers exist: natural and synthetic. In recent years, natural emulsifiers have aroused great interest in food and other industries due to their safety, nutritiousness and other advantages, and they have been gradually replacing synthetic emulsifiers (Kim *et al*., 2020). At present, the commonly used food emulsifiers are proteins, polysaccharides, phospholipids and other surfactants (Kralova and Sjöblom, 2009; Xi *et al*., 2018). Many studies have shown that phospholipids possess a significant capacity to serve as emulsifiers. For example, Komaiko *et al*. (2016) found that sunflower phospholipids were an effective natural emulsifier to transport ω-3 polyunsaturated fatty acids to food products. Donsì *et al*. (2011) successfully prepared the emulsion using soybean lecithin. In addition, McClements and Gumus (2016) compared the effects of different natural emulsifiers (soybean lecithin, whey protein and Arabic gum) for preparing corn oil-in-water nanoemulsions. The results showed that phospholipids have good emulsifying capacity.

Large yellow croaker (*Larimichthys crocea*) is one of the major economic marine fish in China, and it is mainly found distributed in Fujian Province. It has been widely consumed due to its delicious taste and nutrition value

(Hui *et al*., 2016). Large yellow croaker roe is a major by-product obtained during the processing of large yellow croaker, which has the advantages of low pricing and easy availability of raw materials. In our previous study (Liang *et al*., 2018), we found that large yellow croaker roe contains large amount of marine phospholipids (MPLs), and their phospholipid species and molecular types have been determined carefully. Recently, MPLs have become the focus of further research. MPLs predominantly contain docosahexaenoic acid (DHA) or eicosa pentaenoic acid (EPA) (Liang *et al*., 2018). However, no findings have been reported on the emulsification capacity of MPLs derived from large yellow croaker roe. Therefore, we assume that large yellow croaker roe phospholipids (LYCRPLs) can be explored as carriers for the delivery of insoluble bioactive compounds in the food industry.

Astaxanthin (AST) is a carotenoid pigment found in numerous organisms (Pan-Utai *et al*., 2021). It is known for its impact on physiological functions, which include anti-oxidative (Pogorzelska *et al*., 2017), anti-tumor (Nagendraprabhu and Sudhandiran, 2011), and anti-inflammatory properties (Ju *et al*., 2017) as well as its ability to improve vision (Li *et al*., 2012), etc. Consequently, AST has the potential for a broad range of applications in functional foods. However, AST is a hydrophobic substance, and its utilization in the food is limited due to its insolubility (Ambati *et al*., 2014). Numerous studies have shown that nanoemulsion technology is an effective method for carrying AST, which can improve its water solubility and bioavailability (Martínez-Delgado *et al*., 2017; Liu *et al.,* 2019). Therefore, we assume that LYCRPLs may emulsify and improve the solubility of AST. Meanwhile, new and more effective means are required to make high-value use of large yellow croaker roe.

The present study aimed to formulate and optimize AST nanoemulsions to prepare AST nanoemulsions with MPL derived from large yellow croaker roe effectively. Homogenization pressure, homogenization cycles, emulsifier concentration and oil concentration were optimized systematically based on the response surface methodology (RSM) for preparing nanoemulsions, and the same were characterized by transmission electron microscopy (TEM). Furthermore, particle size, polydispersity index (PDI) and zeta-potential of nanoemulsions were measured during the 28-day storage period.

Materials and Methods

Materials

Large yellow croaker roe phospholipids containing 76.36% phosphatidylcholine, 12.30% lysophosphatidylcholine, 9.12% phosphatidylethanolamine, 1.09% phosphatidylinositol and 98.87% total phospholipids were obtained from the aquatic food products lab in Fujian Agriculture and Forestry University and prepared and characterized. AST (≥97%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Refined corn oil was purchased from the local supermarket (Fuzhou City, Fujian Province). All chemicals were obtained from Sinopharm Chemical Reagent Co. Ltd (Fujian, China). Double-distilled water (Milli-Q) was used to prepare all solutions and nanoemulsions.

Preparation of nanoemulsions

The nanoemulsions were prepared according to the method provided by Shu *et al.* (2018) with some modifications. An oil phase was prepared by dissolving 1% (w/w) AST in refined corn oil (Figure 1). The mixtures were stirred for 2 h at ambient temperature and subsequently filtered through a 0.45-μm membrane. The aqueous phase was prepared by dispersing LYCRPLs in Milli-Q water. Three homogenization steps were performed to formulate AST nanoemulsions. First, coarse emulsions containing 5–25% (w/w) oil phase and 95– 75% (w/w) aqueous phase were stirred constantly using a magnetic stirrer for 2 h. Next, coarse emulsions were treated via a handheld homogenizer (HN-3K, Hanuo, Shanghai, China) at 20,000 rpm for 5 min. In the last step, the resulting coarse emulsions were passed through an ultra-high-pressure nano-homogenizer (FB-110Q, Litu, Shanghai, China) for 1–7 cycles at a homogenization pressure of 10–80 MPa at ambient temperature. All operating conditions were studied at different levels. The obtained nanoemulsions were stored at 4°C prior to further analysis.

Measurement of particle size, polydispersity index and zeta-potential

The particle size, PDI and zeta-potential of nanoemulsions were measured by dynamic light scattering using a Zeta sizer Nano-ZS90 (Malvern Instruments, Worcestershire, UK). This instrument is able to measure particle sizes ranging from 0.3 to 5,000 nm. The refractive indices of both aqueous and oil phases were set at 1.333 and 1.476, respectively. The samples for measurement were diluted with Milli-Q water at a ratio of 1:500 (v/v) and then placed in the measurement cell. The particle size of nanoemulsions was expressed as *z*-average diameter.

Measurement of stability of nanoemulsions

The stability of nanoemulsions was determined using a stability analyzer LUMFuge (LUMiFuge111, Berlin,

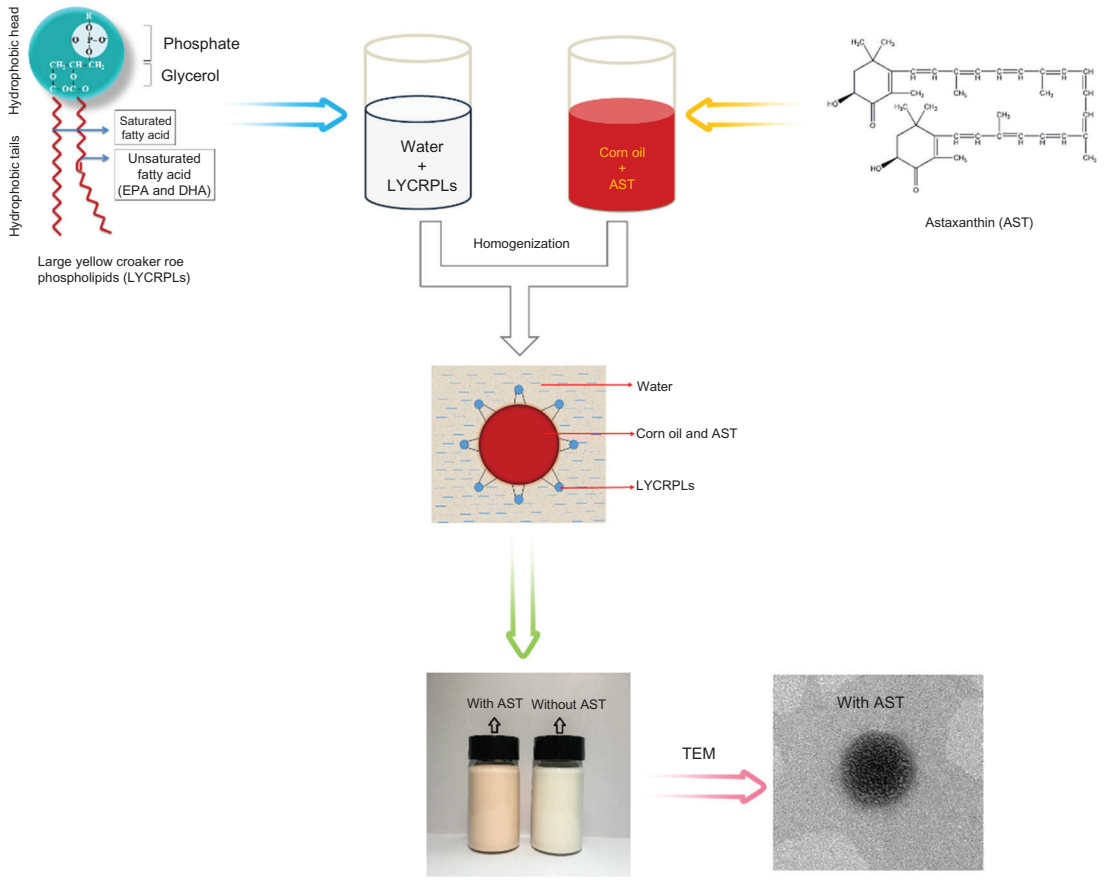

Figure 1. Schematic diagram of preparation of AST nanoemulsions stabilized by LYCRPLs.

Germany). The centrifuge quantifies the stability of the dispersion system by continuously recording dynamic changes in the light transmittance of samples. First, 470-mL nanoemulsion is added in the centrifuge tube, and the tube is subsequently placed in the LUMiFuge111. The operating conditions were 3,000 rpm for 10 min at 20°C. The computer automatically records the light transmittance spectra of the sample.

Microscopic observation of nanoemulsions

The microscopic observation was carried out by using a transmission electron microscope (TEM, HT770, Hitachi Corporation, Japan). Prior to observation by TEM, the nanoemulsions were diluted to acertain concentration with double-distilled water. The appropriate amount of emulsion is absorbed and dripped onto a copper mesh covered with a carbon film. After waiting for 1 min, the remaining liquid was dried using a filter paper. The particles were stained for 1 min with 1% phosphotungstate. The excessive liquid was absorbed by the filter paper and dried naturally, and morphology of the particles was observed by TEM (80 kV).

Experimental design

Single-factor experiments

Single-factor experiments were applied to determine the appropriate range of variables before response surface optimization. Four independent variables were examined in different ranges (Table 1): 10–80 MPa homogenization pressure, 1–7 cycles in a homogenizer, 2–5% concentration of emulsifier, and 5–25% oil concentration. In addition, effects of variables on the particle size and stability of nanoemulsions were determined as described in Sections 2.4 and 2.5.

Response surface experiments

Central Composite Design (CCD) was selected as the experimental RSM design for optimizing AST

Table 1. Processing variables used to run experiments.

Processing variables	Levels				
Homogenization pressure (MPa)	10	20	40	60	80
Homogenization cycles	1	2	3	5	7
Emulsifier concentration (%)	2.0	2.5	3.0	4.0	5.0
Oil concentration (%)	5	10	15	20	25

nanoemulsion formulations. The experimental ranges, −α, −1, 0, +1 and +α, were chosen taking into account the results obtained from single-factor experiments, which are summarized in Table 2. The response variables were stability, particle size and PDI of nanoemulsions. All experiments were executed in a random order. The relationship between coded and uncoded values is given by Equation (1):

$$Y_0 = \frac{(y_0 - y)}{\Delta y_0}, \quad (1)$$

where Y_0 and y_0 represent coded and actual values of independent variables, respectively. Δy_0 indicates step change whereas 'y' is the central value. Specific equations for homogenization pressure (X_1), homogenization cycles (X_2), emulsifier concentration (X_3), and oil concentration (X_4) are as follows:

$$y = \frac{P - 600}{100(2)} \; y_2 = \frac{C - 7}{1}, \quad (2)$$

$$y_3 = \frac{E - 4}{0.5}, \quad (3)$$

$$y_4 = \frac{O - 5}{2.5}, \quad (4)$$

where P, C, E, and O represent homogenization pressure (MPa), homogenization cycles, emulsifier concentration (%), and oil concentration (%), respectively.

A second-order polynomial equation was used to express the stability (Y_1), particle size (Y_2) and PDI (Y_3) of nanoemulsions as a function of independent variables as follows:

$$\begin{aligned} Y_i = {} & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_{21} \\ & + \beta_{22} X_{22} + \beta_{33} X_{23} + \beta_{44} X_{24} + \beta_{12} X_1 X_2 \\ & + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 \\ & + \beta_{34} X_3 X_4, \end{aligned} \quad (5)$$

where Y_i represents the response values, β_0 is a constant, and β_i, β_{ii}, and β_{ij} indicate the linear, quadratic, and interactive coefficients, respectively. The coefficients of the equation were determined using Design expert software version 10.0.7.

Storage stability of nanoemulsions

The LYCRPLs–AST nanoemulsions were prepared according to the optimal conditions obtained from the response surface optimization experiments. The storage temperature was controlled at 5°C, 25°C and 55°C for 28 days. Particle size, PDI and zeta-potential of nanoemulsions were measured at scheduled periods (every 7 days). The methods for determination of particle size, PDI and zeta-potential are described in Section 2.4.

Statistical analysis

The experimental data were analyzed by multiple regression to fit the second-order polynomial equation for independent variables. The significant differences between these independent variables were tested by analysis of variance (ANOVA). The contour plots and response surface plots were created using Design expert software (version 10.0.7) to visualize the influence of independent variables on response variables. Each experiment was performed in triplicate.

Results and Discussion

Single-factor experiments

The effects of homogenization pressure on the particle size and stability of nanoemulsions are shown in Figures 2A and 3A. The particle size of nanoemulsions decreased as the homogenization pressure increased, which is consistent with the stability results. A possible explanation could be that increasing the homogenization pressure results in enhancement of shear force and action strength, which makes nanoemulsions more uniform (Floury *et al.*, 2003). However, the internal temperature of the instrument increases when in operation as the homogenization pressure increases (when

Table 2. Coded and uncoded independent variables used in response surface methodology design.

Independent variable	Symbol	Coded levels				
		−α	−1	0	+1	+α
Homogenization pressure (MPa)	X_1	40	50	60	70	80
Homogenization cycles	X_2	5	6	7	8	9
Emulsifier concentration (%)	X_3	3.0	3.5	4.0	4.5	5.0
Oil concentration (%)	X_4	10.0	12.5	15.0	17.5	20.0

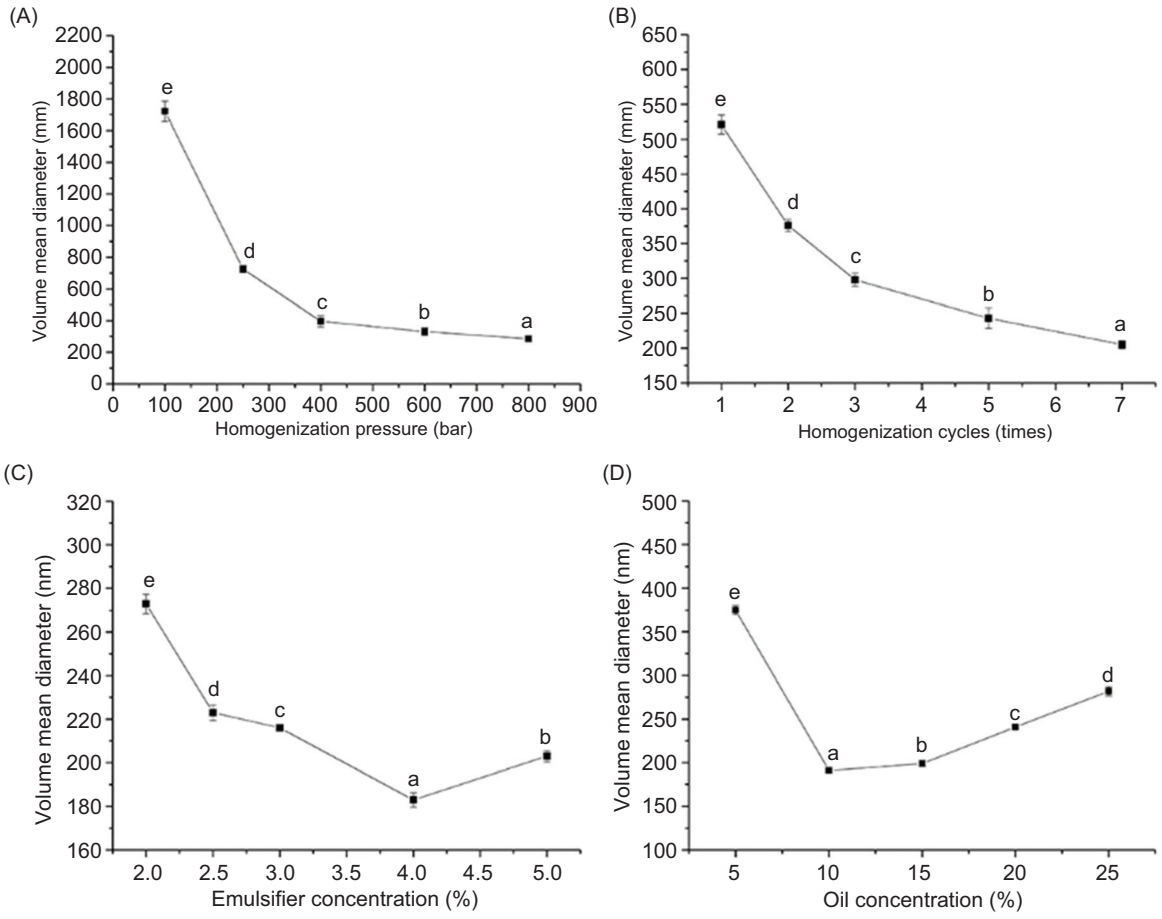

Figure 2. Effects of (A) homogenization pressure, (B) homogenization cycles, (C) emulsifier concentration and (D) oil concentration on the particle size of AST nanoemulsions.

the pressure is higher than 60 MPa), which might result in the deterioration of nanoemulsions. Therefore, a homogenization pressure of 60 MPa was selected as the central point to carry out the response surface optimization test. The fixed variables were five homogenization cycles, 3.0% emulsifier concentration and 10% oil concentration.

Nanoemulsions display lower particle size and better stability as the number of homogenization cycles increases (Figures 2B and 3B), which agrees with the effects of homogenization pressure on nanoemulsions. This phenomenon could be explained as a better combination of water phase with oil phase in emulsions due to the extension of homogenization time. Thus, seven homogenization cycles were selected as the central point in the following optimization experiments. The fixed variables were homogenization pressure: 60 MPa, emulsifier concentration: 3.0% and oil concentration: 10%.

The effects of emulsifier concentration on particle size and stability of nanoemulsions are shown in Figures 2C and 3C. The particle size of nanoemulsions decreased with increase in the concentration of LYCRPLs with emulsifier concentration in the range of 2.0–4.0%. The particle size reached the lowest value if the LYCRPLs' content was 4.0%, and subsequently increased significantly ($P < 0.05$). This trend may be associated with the higher concentrations of emulsifier, which contribute better surface coverage of particles in nanoemulsions (Pola *et al.*, 2019). Meanwhile, there was no significant difference ($P > 0.05$) in the effect of emulsifier concentration on the stability of emulsion when the emulsifier concentration was 4%. This phenomenon has been reported in another study (Llinares *et al.*, 2018). As mentioned previously, an emulsifier concentration of 4% was selected as the central point. The fixed variables were 60 MPa homogenization pressure, five homogenization cycles, and 10% oil concentration.

As the proportion of oil phase increased gradually, the particle size of nanoemulsions at first decreased but increased subsequently, with gradual increase in the stability of nanoemulsions (Figures 2D and 3D). This phenomenon is explained by the fact that increase in oil concentration reduces the surface tension of emulsion

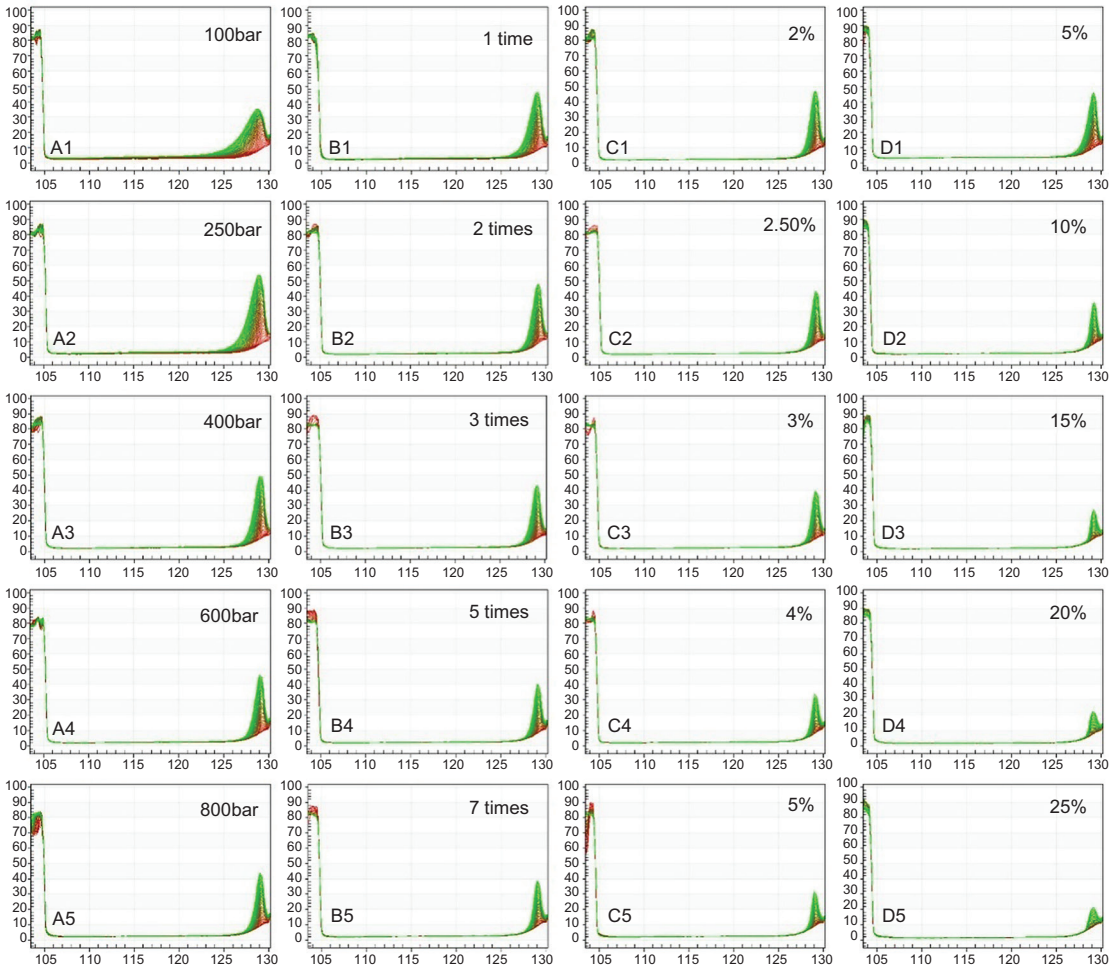

Figure 3. (A1–A5) Effects of homogenization pressure, (B1–B5) homogenization cycles, (C1–C5) emulsifier concentration, and (D1–D5) oil concentration on the stability of AST nanoemulsions.

but enhances its stability (Alba *et al.*, 2021). Nevertheless, higher concentration of oil requires more emulsifier. When the proportion of oil phase increases continuously, the fixed emulsifier concentration is not enough to cover the oil droplet surface, which finally increases the particle size of nanoemulsions (Cha *et al.*, 2019). Therefore, an oil concentration of 15% was selected as the central point for further response to surface experiments. The fixed variables were 60 MPa homogenization pressure, five homogenization cycles, and an emulsifier concentration of 3%.

Fitting the model

The stability, particle size, and PDI values of nanoemulsions obtained from the experiment and their predicted values are given in Table 3. The predicted values agreed well with the data obtained from the response surface design experiment. The experimental data were used to calculate the coefficients of quadratic polynomial equation and to predict the stability, particle size, and PDI values of nanoemulsions.

ANOVA showed that the experimental data could be well expressed by a quadratic polynomial model. The retention coefficients (R^2) of emulsion stability (Y_1), particle size (Y_2) and PDI (Y_3) were 0.9561, 0.9768, and 0.9523, respectively (Table 3). The lack of fit was not significant ($P < 0.05$) compared to the pure error of all variables, which indicates that the experimental model is statistically accurate (Gunst, 2008). In this study, R^2 is close to unity. This implies that the quadratic polynomial model obtained by using the response surface experimental design was sufficient to describe the influence of the independent variables studied on response variables. This shows that the model fits the experimental findings well. The analysis of variance determines the significant level of quadratic polynomial model coefficients. The smaller *P*-value and larger *F*-value show that the independent variable has a higher significant effect on response variables (Mehmood *et al.*, 2018).

Table 3. Predicted and experimental values of nanoemulsion's stability, particle size and polymer dispersity index obtained from the central composite experimental design.

Std	Run	*P* (MPa)	*C* (cycles)	*E* (%)	*O* (%)	Stability of emulsion		Particle size (nm)		PDI	
						Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
1	5	50	6	3.5	12.5	0.102	0.099	301.10	298.12	0.412	0.358
2	25	70	6	3.5	12.5	0.064	0.060	240.50	244.74	0.273	0.276
3	16	50	8	3.5	12.5	0.072	0.078	264.60	269.29	0.438	0.478
4	13	70	8	3.5	12.5	0.058	0.051	239.40	237.74	0.514	0.443
5	11	50	6	4.5	12.5	0.102	0.102	298.00	297.43	0.569	0.543
6	1	70	6	4.5	12.5	0.057	0.054	231.30	228.27	0.413	0.421
7	28	50	8	4.5	12.5	0.100	0.095	296.60	292.27	0.654	0.639
8	26	70	8	4.5	12.5	0.054	0.060	239.90	244.94	0.512	0.564
9	27	50	6	3.5	17.5	0.087	0.080	323.50	322.58	0.907	0.866
10	19	70	6	3.5	17.5	0.066	0.069	313.90	309.92	0.951	0.957
11	12	50	8	3.5	17.5	0.041	0.042	258.70	253.42	0.628	0.611
12	14	70	8	3.5	17.5	0.045	0.045	257.90	262.59	0.712	0.749
13	6	50	6	4.5	17.5	0.057	0.062	306.60	299.95	0.476	0.538
14	21	70	6	4.5	17.5	0.049	0.042	272.10	271.53	0.618	0.588
15	15	50	8	4.5	17.5	0.035	0.039	254.60	254.48	0.251	0.259
16	17	70	8	4.5	17.5	0.032	0.033	253.20	247.87	0.311	0.356
17	23	40	7	4.0	15.0	0.094	0.092	293.50	299.49	0.523	0.546
18	24	80	7	4.0	15.0	0.041	0.046	241.30	239.50	0.586	0.562
19	20	60	5	4.0	15.0	0.060	0.067	294.00	299.14	0.373	0.410
20	22	60	9	4.0	15.0	0.041	0.036	247.60	246.65	0.338	0.299
21	8	60	7	3.0	15.0	0.059	0.063	275.30	273.80	0.720	0.769
22	18	60	7	5.0	15.0	0.055	0.054	252.70	258.39	0.611	0.561
23	3	60	7	4.0	10.0	0.094	0.097	264.40	261.60	0.405	0.438
24	30	60	7	4.0	20.0	0.051	0.050	282.00	288.99	0.773	0.739
25	2	60	7	4.0	15.0	0.055	0.053	247.30	247.50	0.388	0.354
26	10	60	7	4.0	15.0	0.048	0.053	245.00	247.50	0.308	0.354
27	9	60	7	4.0	15.0	0.052	0.053	243.60	247.50	0.373	0.354
28	7	60	7	4.0	15.0	0.054	0.053	251.70	247.50	0.413	0.354
29	4	60	7	4.0	15.0	0.057	0.053	247.70	247.50	0.356	0.354
30	29	60	7	4.0	15.0	0.049	0.053	249.70	247.50	0.284	0.354

P: homogenization pressure; *C*: homogenization cycles; *E*: emulsifier concentration; *O*: oil concentration.

Effect of independent variables on response variables

Effects of independent variables on nanoemulsion's stability, particle size and PDI are summarized in Table 3. Regression coefficients of independent variables are given in Table 4.

Stability of Nanoemulsion

The stability of nanoemulsions was mainly dependent on oil concentration, as it had a significant effect on nanoemulsion's stability at linear ($P < 0.001$), quadratic ($P < 0.001$), and interaction ($P < 0.05$) levels (Table 4). Other factors that contributed significantly to nanoemulsion's stability were the linear terms of homogenization pressure ($P < 0.001$) and homogenization cycles ($P < 0.001$), and quadratic terms ($P < 0.01$) and interaction terms of homogenization pressure ($P < 0.001$) (Table 4).

The interactive effects of emulsifier concentration and oil concentration on nanoemulsion's stability are shown in Figure 4B. Both independent variables exert linear effects on nanoemulsion's stability. Up to a certain level, the stability of nanoemulsions increased with increase in both oil and emulsifier concentrations. This trend was observed since the surface tension of the emulsion can be enhanced with increase in oil concentration (Homayoonfal *et al.*, 2014). The effect of oil concentration is to increase the packing of emulsion droplets, reducing their mobility and hence favoring gravitational separation. These results suggest that both emulsifier and oil concentrations had positive effects on stability. The combined effects of homogenization pressure and oil concentration on the stability of nanoemulsions are shown in Figure 4B, which indicates that homogenization pressure exerts a linear effect while oil concentration had a linear and quadratic effects on the stability of nanoemulsion. There was a downregulation of homogenization pressure and oil concentration with decrease in the stability of nanoemulsion. A possible reason for this decrease is that the smaller droplets were made by higher homogenization pressure. This change can more effectively reduce the rate of gravitational separation, and the droplet velocity increases with the square of diameter (Rao and McClements, 2012).

Table 4. Regression coefficient values of different responses for preparation of nanoemulsion by using the response surface methodology.

Variables[a]	Stability of emulsion			Particle size (nm)			PDI		
	Regression coefficient	F-value	P-value	Regression coefficient	F-value	P-value	Regression coefficient	F-value	P-value
a_0	0.053			247.50			0.35		
Linear									
a_1	–0.0115	93.01	<0.0001	–15.00	181.35	<0.0001	0.004	0.12	0.7328
a_2	–0.0077	41.49	<0.0001	–13.12	138.83	<0.0001	–0.028	6.00	0.0270
a_3	–0.0024	3.94	0.0658	–3.85	11.98	0.0035	–0.052	20.92	0.0004
a_4	–0.0118	97.09	<0.0001	6.85	37.79	<0.0001	0.075	43.69	<0.0001
Quadric									
a_{11}	0.0041	13.24	0.0024	5.50	27.87	<0.0001	0.050	22.08	0.0003
a_{22}	–0.0002	0.03	0.8764	6.35	37.15	<0.0001	0.00026	0.0006	0.9808
a_{33}	0.0014	1.67	0.2154	4.65	19.92	0.0005	0.078	53.38	<0.0001
a_{44}	0.0053	22.61	0.0003	6.95	44.50	<0.0001	0.059	30.35	<0.0001
Interaction									
a_{12}	0.0033	5.11	0.0391	5.46	16.01	0.0012	0.012	0.70	0.4148
a_{13}	–0.0021	1.98	0.1798	-3.94	8.36	0.0112	-0.010	0.52	0.4814
a_{14}	0.0072	24.05	0.0002	10.18	55.73	<0.0001	0.043	9.60	0.0073
a_{23}	0.0037	6.33	0.0237	5.92	18.83	0.0006	–0.006	0.19	0.6697
a_{24}	–0.0041	7.68	0.0142	–10.08	54.64	<0.0001	–0.094	45.32	<0.0001
a_{34}	–0.0052	12.53	0.0030	–5.48	16.15	0.0011	–0.128	84.78	<0.0001
R^2	0.9561			0.9768			0.9523		

Note: [a]a_0 is a constant, and a_i, a_{ii}, and a_{ij} are the linear, quadratic, and interactive coefficients of quadratic polynomial equations, respectively.

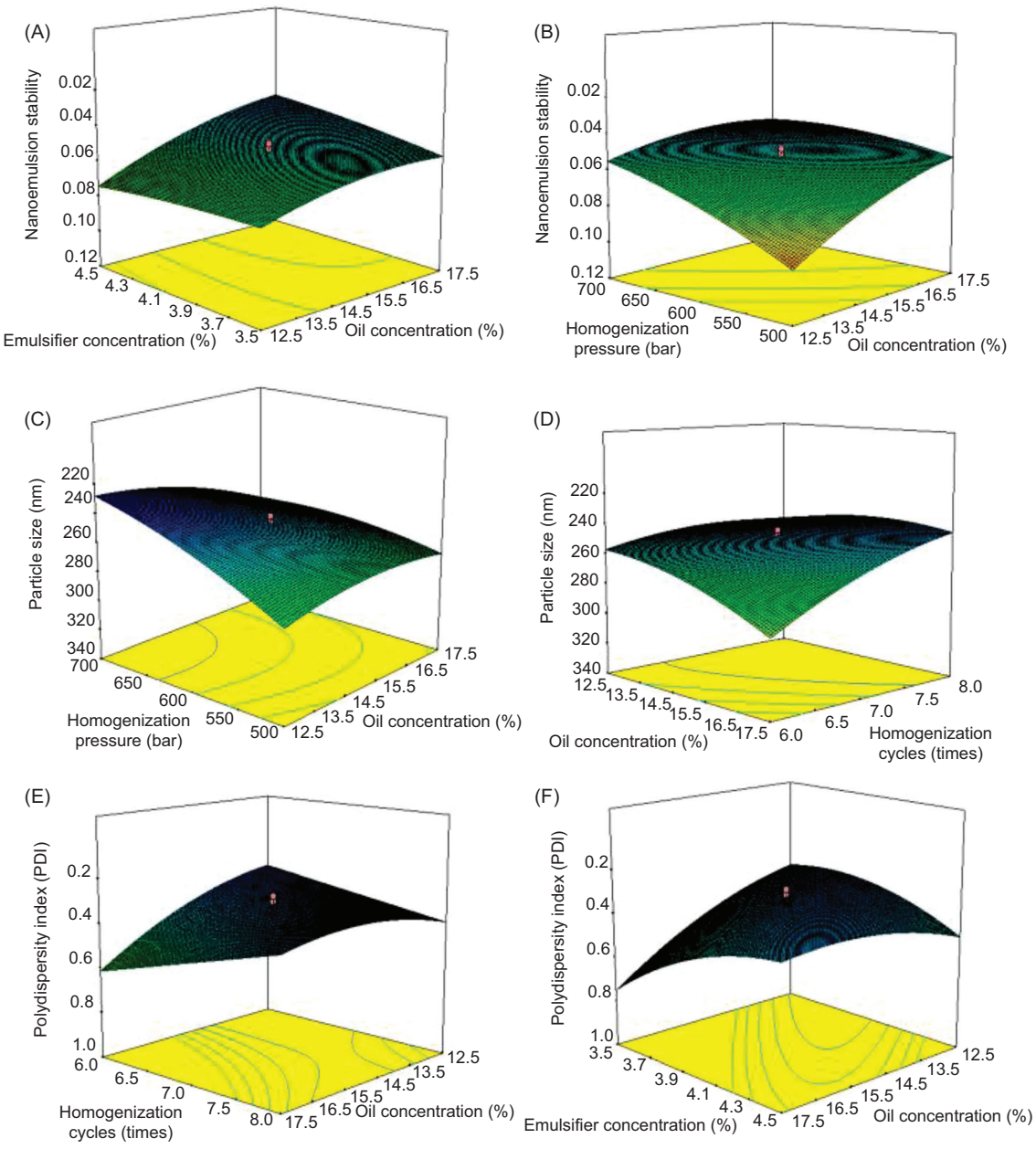

Figure 4. Response surface plots of (A) nanoemulsions stability versus emulsifier concentration (%) and oil concentration (%); (B) nanoemulsions stability versus homogenization pressure (MPa) and oil concentration (%); (C) particle size (nm) versus homogenization pressure (MPa) and oil concentration (%); (d) particle size (nm) versus oil concentration (%) and homogenization cycles; (E) PDI of nanoemulsions versus homogenization cycles and oil concentration (%); and (F) PDI of nanoemulsions versus emulsifier concentration (%) and oil concentration (%).

Particle size

Considering the particle size, homogenization pressure had a significant effect on the particle size of nanoemulsions at linear ($P < 0.001$), quadratic ($P < 0.001$) and interaction ($P < 0.001$) levels (Table 4). Other factors that significantly contribute to particle size were linear effects of homogenization cycles ($P < 0.001$), emulsifier concentration ($P < 0.01$) and oil concentration ($P < 0.001$); quadratic effects of homogenization cycles ($P < 0.001$), emulsifier concentration ($P < 0.001$) and oil concentration ($P < 0.001$)l and interactive effects of homogenization cycles ($P < 0.001$) and oil concentration ($P < 0.001$) (Table 4).

A contour plot in Figure 4C illustrates the particle size as a function of oil concentration and homogenization

pressure. Both of these independent variables had a linear and quadratic effects on particle size. At lower oil concentration, particle size was reduced with increasing homogenization pressure. This decrease was due to effect of higher homogenization pressure on shear force and other fluid-mechanical stresses, resulting in the reduction of droplet size (Floury *et al.*, 2003). Figure 4D describes the interactive effect of homogenization cycles and oil concentration on particle size, which explained the linear effects of both variables on particle size. Particle size of nanoemulsions reduced with the increased cycles of homogenization. Nonetheless, higher concentration of oil resulted in increase in particle size. Increase of homogenization cycles means extension of homogenization time. This trend was consistent with the previous findings that the particle size decreased with strong shear force (Yuan *et al.*, 2008).

Polydispersity index

Polydispersity index is used to measure particle size distribution of emulsion, which evaluates the quality of emulsion (Shi *et al.*, 2021). PDI of nanoemulsions was mainly dependent on oil concentration, which had significant effects on PDI at linear ($P < 0.001$), quadratic ($P < 0.001$) and interaction ($P < 0.01$) levels. Other factors that significantly affected PDI were linear effects of homogenization cycles ($P < 0.05$) and emulsifier concentration ($P < 0.001$); quadratic effects of homogenization pressure ($P < 0.001$) and emulsifier concentration ($P < 0.001$); and interaction effects of homogenization cycles ($P < 0.001$) and emulsifier concentration ($P < 0.001$).

At higher homogenization cycles, a decrease in PDI was observed with increase in oil concentration (as shown in Figure 4e). This decrease in PDI was attributed to strong shear force, which resulted in smaller particle size and higher homogenization cycles, and this way could make both water and oil phase mix thoroughly to form uniform distribution nanoemulsions (Kim *et al.*, 2012). Figure 4F shows the interactive effect of emulsifier and oil concentration on PDI. A decrease in PDI was observed at higher emulsifier and oil concentrations. The reason could be that the emulsifier reduced surface tension in oil–water interface and at the same time improved the bearing capacity of oil phase. This resulted in consistent droplet size of nanoemulsions (Mehmood, 2015).

Optimization of conditions for preparing nanoemulsions

The optimum conditions for preparing nanoemulsions are as follows: homogenization pressure: 60 MPa; nine homogenization cycles; emulsifier concentration: 4.677%; and oil concentration: 20%. Under these conditions, the predicted values of nanoemulsions' stability, particle size and PDI are 0.013, 252.3 nm and 0.170, respectively.

Verification of results

Considering the feasibility of practical operation, the above-mentioned processing conditions were applied in three experiments with some modifications: homogenization pressure: 60 MPa; nine homogenization cycles; emulsifier concentration: 4.7%; and oil concentration: 20%. Under the optimum conditions, the experimental values of nanoemulsions' stability, particle size and PDI were 0.018 ± 0.0016, 247 ± 4.5 nm and 0.215 ± 0.019, respectively. The result revealed that the model was satisfactory and accurate.

Characterization of AST nanoemulsions

Characterization was measured under the optimal preparing conditions of AST nanoemulsions. Figure 5A displays the particle size distribution of nanoemulsions. The PDI value described that AST nanoemulsions had a narrow particle size distribution (Ma and Mu, 2016). Figure 5B shows the TEM observation of the droplets of AST nanoemulsions, and it was observed that nanoemulsion droplets were more or less spherical in shape and nonaggregated, which was consistent with the findings of a previous study (Li *et al.*, 2017).

Storage stability of AST nanoemulsions

During food processing, storage temperature and time are crucial factors for evaluating quality of nanoemulsions; hence, we monitored the particle size, PDI and zeta-potential values of nanoemulsions at scheduled time intervals during 4-week storage period to evaluate their storage stability. As depicted in Figures 6A and 6B, regardless of temperature, the particle size and PDI of nanoemulsions showed an increasing trend. It is considered that this phenomenon is related to the ripening of Ostwald (Reyes *et al.*, 2021). With the extension of storage period, the quality of emulsion changed gradually, with continuous increase in particle size (Young and Nitin, 2019). In general, the system is considered to be relatively stable when the zeta-potential reaches 25 mV in absolute value (Chen *et al.*, 2020). Figure 6C demonstrates that the zeta-potential values increased with the increase of storage days, and the absolute value was above 25 mV at 5°C and 25°C during storage days, which depicted that AST nanoemulsions prepared by LYCRPLs could be stable for 28 days at 5°C and 25°C, and became slightly unstable at higher temperature. This result was inconsistent with particle size and PDI.

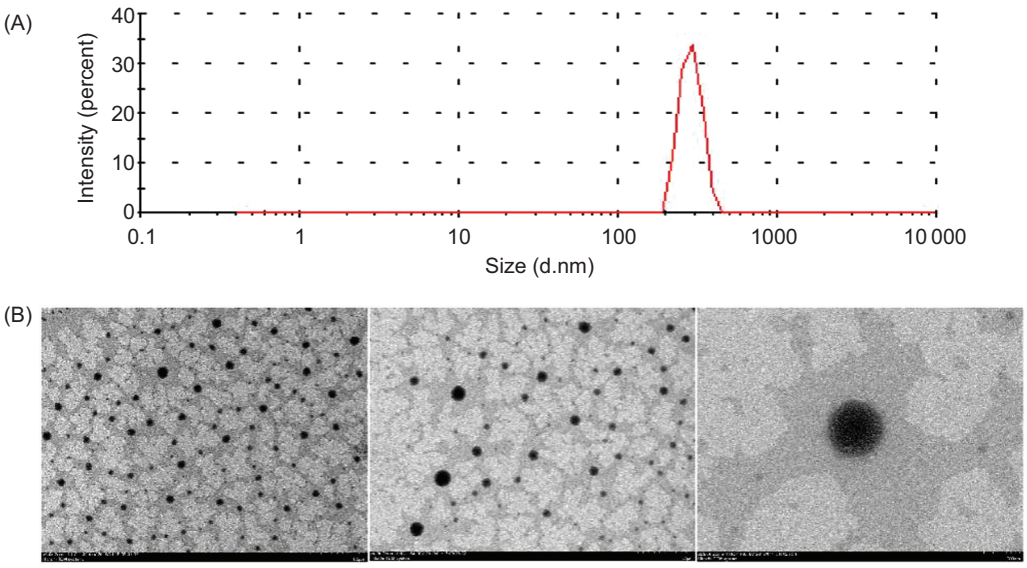

Figure 5. (A) Particle size distribution of AST nanoemulsions with optimal preparing conditions. (B) Transmission electron microscope (TEM) observations of droplets of AST nanoemulsions under optimal preparing conditions.

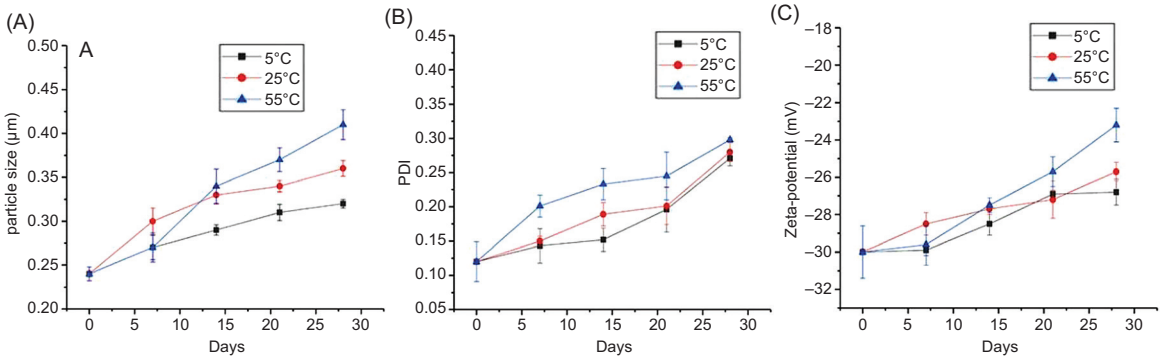

Figure 6. Changes in (A) particle size, (B) PDI and (C) zeta-potential value of AST nanoemulsions during storage.

Conclusion

In this study, AST nanoemulsions were prepared using LYCRPLs as an emulsifier. The four parameters of homogenization pressure, homogenization cycles, emulsifier concentration, and oil concentration for AST nanoemulsions prepared by LYCRPLs were optimized systematically, and droplets of nanoemulsions were characterized by TEM. LYCRPLs have good emulsifying capacity, which could be used as a potential emulsifier. Therefore, differences in the emulsifying properties of LYCRPLs and traditional commercial lecithin need further research.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (31801465). It was also supported by the Outstanding Young Scientific Research Project of Fujian Agriculture and Forestry University (xjq 201808).

Author Contribution

Peng Liang provided the idea of this study and interpreted the results. Luyao Huang designed the study and drafted the manuscript. Lingyun Zhang collected test data, and Ruifen Li helped to revise the manuscript.

Conflict of Interest

The authors declare that they have no conflicts of interest concerning this article. There was no financial support, except those mentioned in the acknowledgments.

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Italian Journal of Food Science, 2021; 33 (3): 14–24

Food purchasing, preservation, and eating behavior during COVID-19 pandemic: A consumer analysis

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Submitted: 7 April 2021; Accepted: 21 August 2021; Published: 11 September 2021

PAPER

Abstract

Due to the highly infectious virus known as COVID-19 impacting the lives of the populace, more than any other event in recent memory, there is a pandemic in the world. In order to determine food purchasing behavior and eating habits, food preservation techniques and source of knowledge about COVID-19, 992 consumers living in İstanbul, the most populous city in Turkey, were surveyed. The questionnaire was disseminated to participants via an online platform. Thirty questions, including the demographics of participants, changes in purchasing behavior, knowledge, and attitudes about food preservation techniques, changes in eating habits, and source of knowledge about COVID-19, were asked. The results of this study surveyed that COVID-19 has changed food purchasing and eating habits of Turkish consumers significantly ($p < 0.05$). During the survey in late March of 2020 and late December of 2020, about 65% of respondents have tried to consume more food that boost the immune system and 58% of the respondents have been more willing to buy fresh products. Consumers have greatly adopted preserving of food stuffs by freezing during quarantine days. This survey revealed that the effective use of media tools could increase awareness and lead to behavioral changes that can reduce the spread of COVID-19, especially in consumers aged over 65 years.

Keywords: COVID-19; eating habits; food purchasing habits; media

Introduction

COVID-19 pandemic is one of the greatest challenges that the world has faced without prior preparation. COVID-19 is much more than a health crisis. It is also an inevitable economic crisis because of the sudden decline in economic activities. The pandemic is giving rise to substantial changes in the social habits of the people throughout the world, which will leave deep scars. Moreover, the COVID-19 pandemic has caused apprehensions about threats to food security (Hirvonen *et al.*, 2021). One of the reasons why COVID-19 pandemic has led to significant changes is the uncertainty about what will happen in the near future, as well as food purchasing, food preservation, and consumption behavior of people because of the lockdown and social isolation directives. During the pandemic, people are compelled to stay at home and to go outside only to meet the most urgent needs such as purchasing food. Hence, COVID-19 has changed consumers' life and spending habits (Criteo Coronavirus Survey, 2020). One of the major problems that COVID-19 has indicated is the debate on food insecurity faced by majority of the population. COVID-19 caused a critical weakness in the US food supply system (Chenarides *et al.*, 2021). It has threatened the accessibility of food by effecting food costs and infrastructure such as public transit access, distribution, shortages of certain products, and changes in food assistance (Niles *et al.*, 2020). This statement has revealed the significance of food security in times of crises and shocks.

ISSN 1120-1770 online, DOI 10.15586/ijfs.v33i3.2048

The COVID-19 pandemic holds several implications for Canadian food supply chains such as maintaining and enhancing supply chain resilience (Hobbs, 2020).

The pandemic is evidently challenging the whole food chain system. One of the major concerns shared by all food companies is preserving the health of workers and the provision of sufficient workforce because of those who do not want to work due to sickness or the fear of coronavirus. Food security is also related to access of consumers to food rather than food availability during lockdown (Gundersen *et al*., 2021). Chenarides *et al*. (2021) reported that food prices are a key determinant of food insecurity. On the other hand, consumers are also expected to take additional measures to protect their health. News about the importance of a strong immune system in the fight against COVID-19 prompted consumers to purchase foods that strengthen the immune system. Food achievement patterns were also substantially altered in comparison to pre-COVID levels (Restrepo *et al*., 2021). Understanding the COVID-19 effect behind restriction policies is also substantial (Aday and Aday, 2020). Changes in the food purchasing behavior varied by age, gender, and education (Ali *et al*., 2021; Wang *et al*., 2020).

Investigating changes in food purchasing, preservation, and consumption habit is critical to both understand how habits of consumers change and adapt during lockdown and to ensure beneficial guidance in emergency management efforts. Creating shock-resistant food systems requires collective action through the entire agri-food chain (Bakalis *et al*., 2020). In particular, at the start of the pandemic crisis, consumer demand for food has soared and some store shelves have been emptied due to over-buying of basic products. Examining for food insecurity and providing resources may decrease short- and long-term results, such as potential long-term effects on health outcomes related to duration of household food insecurity and higher health care expenditures associated with food insecurity. Each country should realize the seriousness of the situation and sometimes tighten or relax measures according to the spread of the pandemic. Therefore, the aim of this survey was to understand how a pandemic such as the COVID-19 influences the food purchasing and preservation behavior. It was hypothesized that food choice, preservation motives, and nutritional quality of diet changed during COVID-19 outbreak. This survey aimed to help address consumer awareness, knowledge and, attitudes about food preservation techniques creating influential food safety and nutrition communications that would help policy makers, educators, communities, health professionals, companies, and others to best understand the most important issues for consumers to adjust their policies and strategies to the current pandemic circumstances. The results of the present study could be a standard in food security, health care, and other service settings during COVID-19 and beyond. Food retailers and distributors may consider increasing their capacity to cope with temporary excess demand by investing in capital and labor resources.

Literature Review

Many studies have been conducted to explain changes in dietary and food purchasing habits during the COVID-19 pandemic. Moreover, several studies have been performed to determine effects of COVID-19 pandemic on food supply chains and consumer panic buying behaviors (Hobbs, 2020). Grashuis *et al*. (2020) investigated the grocery shopping preferences during the COVID-19 pandemic. Their results revealed that COVID-19 caused significant changes in grocery shopping preferences. When COVID-19 is spreading at an increasing rate, consumers are not usually willing to shop at grocery stores. Ben Hassen *et al*. (2020) investigated impact of COVID-19 on food behavior and consumption in Qatar. Their results indicated that consumers adopted healthier diets and increased the consumption of domestic food because of food safety concerns. Chang and Meyerhoefer (2021) surveyed the effects of COVID-19 on online food shopping services. According to their results, COVID-19 pandemic caused a significant increase in online food shopping in Taiwan. Celik and Dane (2020) surveyed the effects of COVID-19 pandemic outbreak on food consumption preferences. Their survey revealed that the first food choice of consumers shifted from meat and bakery to fruits and vegetables. Marty *et al*. (2021) surveyed nutritional value of diet and food choice motives during the COVID-19 pandemic in France. Their results revealed that consumers' awareness of the importance of sustainable food choices significantly increased. However, to the best of the author's knowledge, no data with respect to the real food preservation habits of the population during COVID -19 are available, so far. The present study aimed to analyze both changes in food purchasing and preservation habits which help food authorities to take the necessary precautions during pandemic situations such as COVID-19.

Survey Design

With feedback from key state-level agencies as well as reviews of relevant literature, a survey was developed by observing consumer food behavior to evaluate the changes in food purchasing and preservation behavior of consumers in İstanbul, the most populous city in Turkey. The questionnaire was pilot tested on 20 comparable consumers for clarity and validity, and necessary adjustments were done. Data were collected by a specific

questionnaire. The questionnaire was carefully designed to minimize its influences on consumers and to achieve accurate information that will reflect the consumers' own attitudes. The questionnaire was disseminated to participants via an online platform. The survey lasted from late March to late December of 2020. No direct interactions, such as face-to-face interviews, were carried out. Consumers were asked about their food purchasing, food preservation, and consumption behavior during the COVID-19 pandemic. The data were collected by interviewing 992 randomly selected consumers. Questionnaire design is highlighted in the following sections: (i) demographics of respondents, (ii) changes in purchasing behavior of consumers, (iii) knowledge and attitudes of consumers about food preservation techniques, (iv) changes in eating habits, and (v) source of knowledge about COVID-19 and food. The sections were designed to determine the changes in food purchasing, preservation, and dietary habits as well as to reveal information sources of consumers during the COVID-19 pandemic.

Ethical Aspects

The respondent information required an individual and anonymous completion of the questionnaire. Consumers could quit at any time. It was not possible to link the specific answers to individuals.

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) by the General Linear Model (GLM) procedure of SAS statistical programme (SAS, 1999). All the means were compared by Duncan's multiple range test at the level of $p < 0.05$.

Results

Respondents' characteristics

The demographic properties of consumers are given in Table 1. The age of the youngest consumer was 18, whereas the oldest was 79 years old. Thirty percent of the consumers were under 20 years of age, 39% were between the ages of 20 and 65 years, and 37% were over 65 years. Forty-seven percent of the consumers were male. Thirty percent of the consumers were university graduates, 44% were high school graduates, and 26% were elementary school graduates. Monthly average income of 40% of the participants was between 2400 and 4000 TL (Turkish Lira). In the twelfth month of 2020, 1 dollar was roughly 7.45 TL, and 1 Euro was roughly 8.90 TL. Only 3% of the respondents had their monthly average income above 15,000 TL, which can be considered as a very good salary according to Turkish life standards.

Table 1. Socioeconomic and demographic characteristics of the participants.

Age	
<20	30%
20–65	39%
>65	37%
Gender	
Female	53%
Male	47%
Education	
Elementary	26%
High school	44%
University	30%
Monthly income[a]	
<2400 TL[b]	20%
2400–4000 TL	40%
4000–7500 TL	32%
7500–15,000 TL	21%
>15,000 TL	3%

[a]National average gross income per person was 11,019 dollars in Turkey in 2020.
[b]TL: Turkish Lira, 1 Euro = 8.90 TL (December, 2020).

Changes in the purchasing behavior of consumers

Changes in the purchasing behavior of consumers are given in Table 2. Most of the consumers (83 ± 0.15%) have changed shopping habits since the COVID-19 pandemic started ($p < 0.05$). Forty percent (40 ± 0.08%) of the consumers have adopted online purchasing, whereas 60 ± 0.06% of the consumers have adopted in-store food purchasing. Consumers from all demographics, but especially from the age group below 65 years, have adopted online food purchasing ($p < 0.05$). The citizens aged below 65 years have been restricted to venture out of their homes shortly after the first case has been seen in Turkey. As grocery shopping remains a necessity during the pandemic, many people have questions about how to shop safely. Grocery stores have imposed more and more safety rules such as metering consumers and requiring consumers to wear masks. Due to the news that the virus can live on boxes and cans, consumers have gotten fearful and anxious when shopping at physical grocery stores. It seems that online food purchasing is likely to continue long after the pandemic. Scacchi *et al*. (2021) reported that the COVID-19 pandemic caused adoption of online grocery purchase among Italian consumers, suggesting a modern and low-risk shopping method.

At the beginning of the COVID-19 pandemic, because of market uncertainties, some of the major agricultural producing nations implemented export restrictions. They mainly focused on staple crops that are of high importance for food security including maize, wheat, and rice. Moreover, consumers who believed that they would suffer from food shortages due to COVID-19 stockpiled food in their homes. Shelves were emptied quickly in markets. However, as seen in Table 2, 90 ± 0.07 of the consumers have not experienced food product shortages.

Health professionals are recommending people consider taking some foods for strengthening their immune system daily as the coronavirus lockdown continues. As seen in Table 2, only %10 ± 0.05 of the consumers has purchased food products which they would not have purchased if it was not for COVID-19.

The risk of COVID-19 cross-contamination to food is very low (Gov.uk., 2020). However, since COVID-19 is a respiratory disease (Butler and Barrientos, 2020), the possibility of the respiratory tract becoming infected when chewing contaminated food cannot be completely ruled out. As seen in Table 2, sixty-six percent (66 ± 0.11%) of the consumers purchased more packaged food since the COVID-19 pandemic started ($p < 0.05$). Since people are encouraged to wash foodstuff purchased from supermarkets, which is far more easily done when the product is plastic-wrapped, plastic packaging has played an important role in food safety during lockdown (Packaging Europa, 2020). Consumers from all demographics, but especially those aged more than 65 years ($p < 0.05$), have had a more favorable opinion of the healthfulness of packaged foods. At the same time, 27 ± 0.08% of the respondents said that they have not changed their perceptions on the healthfulness of packaged foods.

Healthy diet has a great importance for health, particularly in times when the immune system might need to fight back (Kau *et al.*, 2011). Sustaining a healthy lifestyle and nutritious diet is extremely important during the COVID-19 pandemic. Avoiding nutrient deficiencies helps ensure that all nutrients required for immune cell triggering, interaction, differentiation, or functional expression are available as needed. Fruits and vegetables are increasingly being consumed thanks to their properties of nutrition and freshness, which are much appreciated. Moreover, freshwater fish and seafood are popular among consumers since their perception is associated with healthy, high-quality products. As seen in Table 2, %58 ± 0.12 of the consumers have been more willing to buy fresh products since the COVID-19 pandemic started ($p < 0.05$). When the results were evaluated in terms of age groups, it was seen that there was no statistically significant difference ($p > 0.05$). Głąbska *et al.* (2020) reported that the COVID-19 pandemic has increased the importance of health and weight control of Polish adolescents.

Hygiene and sanitation are imperatives that must be strictly followed in the food industry. Food handlers should implement hygiene practices accurately to protect the consumers from foodborne diseases. The food safety measures such as cleaning of surfaces and utensils, frequent hand-washing, and cooking food to the right temperature are already implemented to prevent foodborne illnesses. There is every reason to believe that the existing effective hygiene and sanitation measures are as effective on COVID-19 as on other microbiological risks. However, as seen in Table 2, 55 ± 0.09% of the consumers are not sure whether consuming the food they purchase is safe during COVID-19 ($p < 0.05$). When the results were evaluated in terms of age groups, it was seen that there was no statistically significant difference ($p > 0.05$). Food businesses should implement additional hygiene and sanitation measures, based on risk, all the more in the case an employee tests positive for COVID-19.

Ellison *et al.* (2021) surveyed a panel of 1370 U.S. households during the COVID-19 pandemic. The results of their study revealed that The COVID-19 pandemic has substantially changed what is "normal" globally, touching all aspects of life, including food purchasing and acquisition preferences.

Knowledge and attitudes of consumers about food preservation techniques during the COVID-19 pandemic

Appropriate food preservation has allowed for less trips to the grocery store and less time in public places during the COVID-19 pandemic. As seen in Table 3, freezing has been the most preferred food preservation method. Sixty-six percent (66 ± 0.08%) of the consumers have frozen their foods during the COVID-19 pandemic ($p < 0.05$). Freezing is a preferred method since it eliminates the need for purchasing additional equipment and utensils that are required for drying and canning. Most of the respondents (95%) who have adopted freezing to a great extent during the pandemic were aged below 20 years ($p < 0.05$). This may be due to the lack of time for young consumers. However, elderly people have also adopted drying and canning. Turkish people have had refrigerators in their houses for the past 40 years, so the consumers over 65 years are experienced in drying and canning that are ancient food preservation techniques before the advent of refrigerators.

As seen in Table 3, only 20 ± 0.06% of the consumers have felt it necessary to wash or remove packaging before

Table 2. Changes in the purchasing behavior of consumers.

Results obtained for different age groups				
	Overall	**<20**	**20–65**	**>65**
I have changed shopping habits since the COVID-19 pandemic started.				
a) Yes	83 ± 0.15[a]	82 ± 0.08[a]	85 ± 0.12[a]	83 ± 0.09[a]
b) No, never	17 ± 0.12[b]	18 ± 0.07[b]	15 ± 0.12[b]	17 ± 0.07[b]
I have adopted……...food purchasing since the COVID-19 pandemic started (Fill in the blank).				
a) Online	40 ± 0.08[b]	51 ± 0.04[a]	53 ± 0.12[a]	15 ± 0.08[b]
b) In store	60 ± 0.06[a]	49 ± 0.05[a]	47 ± 0.12[b]	85 ± 0.09[a]
I have experienced food product shortages at stores from which I am trying to buy.				
a) Yes	22 ± 0.07[b]	20 ± 0.06[b]	27 ± 0.12[b]	18 ± 0.10[b]
b) No, never	78 ± 0.10[a]	80 ± 0.08[a]	73 ± 0.13[a]	82 ± 0.10[a]
I have purchased food products which I wouldn't have done if it wasn't for COVID-19.				
a) Yes	10 ± 0.05[b]	5 ± 0.06[b]	2 ± 0.08[b]	22 ± 0.11[b]
b) No, never	90 ± 0.07[a]	95 ± 0.06[a]	98 ± 0.07[a]	78 ± 0.14[a]
I have purchased more packaged food since the COVID-19 pandemic started.				
a) Yes	66 ± 0.11[a]	70 ± 0.12[a]	65 ± 0.10[a]	63 ± 0.15[a]
b) No, never	34 ± 0.10[b]	30 ± 0.13[b]	35 ± 0.12[b]	37 ± 0.15[b]
I have had a more favorable opinion of the healthfulness of packaged foods				
a) Yes	73 ± 0.05[a]	63 ± 0.11[a]	72 ± 0.11[a]	85 ± 0.06[a]
b) No, never	27 ± 0.08[b]	37 ± 0.10[b]	28 ± 0.11[b]	15 ± 0.07[b]
I have been more willing to buy fresh products since the COVID-19 pandemic started				
Yes	58 ± 0.12[a]	55 ± 0.05[a]	57 ± 0.10[a]	62 ± 0.09[a]
No	42 ± 0.12[b]	45 ± 0.06[b]	43 ± 0.10[b]	38 ± 0.08[b]
I am very confident that the food I am purchasing is safe to consume.				
Yes	21 ± 0.14[b]	31 ± 0.05[b]	23 ± 0.12[b]	20 ± 0.08[b,c]
No	24 ± 0.10[b]	20 ± 0.04[c]	25 ± 0.11[b]	24 ± 0.07[b]
I am not sure	55 ± 0.09[a]	49 ± 0.04[a]	52 ± 0.12[a]	56 ± 0.14[a]

The values are expressed as the mean ± SD, and different superscript letters show significant differences ($p < 0.05$).

putting food items in the freezer since the start of the COVID-19 pandemic. While it is possible for a person to contact COVID-19 from touching an infected surface or object and then touching his or her face, there is currently no evidence to support the idea that the virus can be spread by food packaging (CSIS, 2020). A majority of the Turkish people (76 ± 0.05%) seem to understand this issue well. When the results were evaluated in terms of age groups, it was seen that there was no statistically significant difference ($p > 0.05$).

Freezing retards the growth of microorganisms and enzymes that cause food spoilage. However, freezing has very little impact on the infectivity of foodborne enteric viruses (Bozkurt *et al.*, 2020; Nasheri *et al.*, 2019). Only a few studies have been performed on the effect of freezing on coronavirus infectivity. Lamarre and Talbot (1989) showed that the infectious titer of human coronavirus did not cause any significant reduction when subjected to 25 cycles of thawing and freezing. As shown in Table 3, 44% of the Turkish consumers think that freezing the foods kills the COVID-19 virus. When the results were evaluated in terms of age groups, majority of all age groups have no idea about this question ($p < 0.05$).

According to the recent study by Pastorino *et al*. (2020), the 92°C and 15 min protocol was able to totally inactivate the virus (>6 Log10 decrease). As given in Table 3, 77% of the Turkish consumers agree that cooked meat is not a great risk for the consumer in terms of COVID-19.

Fifty-five percent (55%) of the Turkish consumers do not agree that COVID-19 can spread through dairy products. However, 20% of them have no idea about this issue.

Table 3. Knowledge and attitudes of consumers about food preservation techniques during the COVID-19 pandemic.

Results obtained for different age groups				
	Overall	**<20**	**20–65**	**>65**
Which food preservation technique have you adopted most during COVID-19 quarantine days?				
a) Drying	21 ± 0.05[b]	3 ± 0.02[b]	27 ± 0.09[b]	33 ± 0.04[b]
b) Freezing	66 ± 0.08[a]	95 ± 0.05[a]	61 ± 0.10[a]	42 ± 0.05[a]
c) Canning	13 ± 0.07[c]	2 ± 0.01[b]	12 ± 0.08[c]	25 ± 0.04[c]
I have needed to wash or remove packaging before putting in the freezer since the COVID-19 pandemic started.				
a) Yes	20 ± 0.06[b]	22 ± 0.09[b]	31 ± 0.07[b]	18 ± 0.04[b]
b) No, never	76 ± 0.05[a]	78 ± 0.10[a]	69 ± 0.08[a]	82 ± 0.05[a]
Freezing the foods kills the COVID-19 virus.				
I absolutely agree	18 ± 0.04[c]	12 ± 0.02[c]	23 ± 0.07[b]	18 ± 0.03[c]
I agree	26 ± 0.03[b]	23 ± 0.04[b]	25 ± 0.06[b]	30 ± 0.03[b]
I have no idea	39 ± 0.05[a]	41 ± 0.04[a]	32 ± 0.06[a]	45 ± 0.04[a]
I do not agree	10 ± 0.06[d]	14 ± 0.02[c]	12 ± 0.03[c]	4 ± 0.08[d]
I absolutely disagree	7 ± 0.05[d]	10 ± 0.03[c]	8 ± 0.09[d]	3 ± 0.06[d]
Cooked meat is not a great risk for the consumer in terms of COVID-19.				
I absolutely agree	42 ± 0.07[a]	41 ± 0.05[a]	45 ± 0.12[a]	40 ± 0.12[a]
I agree	35 ± 0.06[b]	35 ± 0.04[b]	38 ± 0.06[b]	33 ± 0.08[b]
I have no idea	12 ± 0.06[c]	10 ± 0.03[c]	7 ± 0.06[c]	20 ± 0.06[c]
I do not agree	8 ± 0.05[d]	11 ± 0.04[c]	11 ± 0.04[c]	5 ± 0.04[d]
I absolutely disagree	3 ± 0.05[e]	3 ± 0.02[d]	3 ± 0.04[d]	2 ± 0.05[e]
The COVID-19 can spread through dairy products				
I absolutely agree	11 ± 0.05[e]	8 ± 0.07[c,d]	9 ± 0.03[d]	17 ± 0.02[b]
I agree	13 ± 0.04[d]	12 ± 0.07[c]	14 ± 0.04[c]	13 ± 0.04[b,c]
I have no idea	20 ± 0.03[b]	20 ± 0.06[b]	10 ± 0.07[d]	30 ± 0.06[a]
I do not agree	17 ± 0.02[c]	20 ± 0.04[b]	22 ± 0.06[b]	10 ± 0.04[c]
I absolutely disagree	38 ± 0.04[a]	40 ± 0.02[a]	45 ± 0.06[a]	30 ± 0.05[a]

The values are expressed as the mean ± SD, and different superscript letters show significant differences ($p < 0.05$).

The results of this section indicated that much more education is needed on food and COVID-19.

Changes in eating habits of consumers during the covid-19 pandemic

Since many nutritional deficiencies may cause immune dysfunction resulting in increased susceptibility to infectious diseases, special attention should be given to promote immune function to enhance viral resistance among the populace (Khayyatzadeh, 2020). Poor nutritional quality diet is one of the main risk factors for non-communicable diseases (Marty *et al*., 2021). As shown in Table 4, 65 ± 0.15% of the respondents have tried to consume more food that boost the immune system, and 62 ± 0.09% of the consumers have eaten more often since the COVID-19 pandemic started ($p < 0.05$). When the results were evaluated in terms of age groups, it was seen that there was no statistically significant difference ($p > 0.05$).

Antioxidants cause an increase in the number of T-cell subsets, lymphocyte response to mitogen, interleukin-2 production, potentiated natural killer cell activity, and the response to influenza virus vaccine compared with placebo (Chandra, 1992). Many studies have stated that fruits and vegetables that supply micronutrients can support immune function thanks to their high antioxidant activity and their rich vitamin C, vitamin E, and beta-carotene content. Sixty-four percent (64 ± 0.04%) of the respondents have tried to eat more fresh fruit and vegetable since the COVID-19 pandemic started (Table 4). Turkish consumers have had a proper eating habit regarding fruit and vegetable consumption since the COVID-19 pandemic started.

The increase in the consumption of dairy products of consumers was investigated (Table 4). Fifty-two percent (52 ± 0.07%) of the respondents have tried to consume more dairy products since the COVID-19 pandemic started. Dairy products are a great source of energy, protein, and

Table 4. Changes in eating habits.

Results obtained for different age groups	Overall	<20	20–65	>65
I have tried to consume more food that boosts the immune system since the COVID-19 pandemic started.				
a) Yes	65 ± 0.15[a]	60 ± 0.16[a]	70 ± 0.09[a]	65 ± 0.10[a]
b) No	45 ± 0.16[b]	40 ± 0.18[b]	30 ± 0.08[b]	35 ± 0.09[b]
I have eaten……since the COVID- 19 pandemic started. (Fill in the blank)				
a) Less often	38 ± 0.08[b]	46 ± 0.10[b]	33 ± 0.14[b]	36 ± 0.09[b]
b) More often	62 ± 0.09[a]	54 ± 0.07[a]	67 ± 0.12[a]	64 ± 0.08[a]
I have tried to consume more fresh fruit and vegetable since the COVID-19 pandemic started				
a) Yes	64 ± 0.04[a]	50 ± 0.02[a]	71 ± 0.14[a]	70 ± 0.05[a]
b) No	36 ± 0.07[b]	50 ± 0.03[a]	29 ± 0.12[b]	30 ± 0.06[b]
I have tried to consume more dairy products since the COVID-19 pandemic started.				
a) Yes	52 ± 0.07[a]	42 ± 0.06[b]	53 ± 0.05[a]	56 ± 0.04[a]
b) No	48 ± 0.08[b]	48 ± 0.04[a]	47 ± 0.03[b]	44 ± 0.04[b]
I have tried to consume more wholegrain since the COVID-19 pandemic started				
a) Yes	35 ± 0.10[b]	30 ± 0.06[b]	39 ± 0.05[a]	37 ± 0.04[b]
b) No	65 ± 0.09[a]	55 ± 0.07[a]	41 ± 0.05[a]	48 ± 0.04[a]
I have tried to consume less red meat since the COVID-19 pandemic started				
a) Yes	49 ± 0.02[a]	50 ± 0.06[a]	45 ± 0.14[b]	51 ± 0.07[a]
b) No	51 ± 0.03[a]	50 ± 0.06[a]	55 ± 0.12[a]	49 ± 0.07[a]
I have tried to consume more fish and seafood since the COVID-19 pandemic started				
a) Yes	53 ± 0.06[a]	45 ± 0.03[b]	55 ± 0.11[b]	58 ± 0.06[a,b]
b) No	47 ± 0.05[b]	75 ± 0.04[a]	68 ± 0.11[a]	65 ± 0.04[a]
I have tried to consume more home cooked meal since the COVID-19 pandemic started.				
a) Yes	77 ± 0.08[a]	61 ± 0.05[a]	85 ± 0.08[a]	85 ± 0.11[a]
b) No	23 ± 0.07[b]	39 ± 0.06[b]	15 ± 0.07[b]	15 ± 0.12[b]
I have taken vitamin supplement since the COVID-19 pandemic started				
a) Yes	51 ± 0.04[a]	32 ± 0.07[b]	41 ± 0.06[b]	54 ± 0.08[a]
b) No, never	49 ± 0.05[a]	68 ± 0.08[a]	59 ± 0.04[a]	46 ± 0.09[b]

The values are expressed as the mean ± SD, and different superscript letters show significant differences ($p < 0.05$).

micronutrients, including riboflavin, calcium, selenium, magnesium, and vitamins B_5 and B_{12} (Gaucheron, 2011). Therefore, it is very healthy to increase the trend towards the consumption of dairy products.

Whole-grain food intake has a protective effect against oxidative stress, inflammatory and pathology of infectious origin (Jacobs *et al*., 2007). Since micronutrients are generally present in higher concentrations in the outer part of the grain, refined flours which comprise only starchy endosperm are lower in micronutrients than whole grains. As seen in Table 4, only 35 ± 0.10% of the respondents have tried to consume more wholegrain since the COVID-19 pandemic started. In Turkey, the most consumed types of bread are traditional white bread, which comprise only starchy endosperm. This explains why 65% of consumers answered "no" to the question.

Increasing consumption of red meat, especially in its processed forms, has negative health effects (Richi *et al*., 2015). However, 51% of the Turkish consumers have not tried to consume less red meat since the COVID-19 pandemic started. The average red meat consumption in 2019 was 13.6 kg per capita which is less than the developed countries (Özen *et al*., 2019). This rate, which is already low in Turkey, has not changed much with the advent of the COVID-19 pandemic. When the results were evaluated in terms of age groups, it was seen that there was no statistically significant difference ($p > 0.05$).

Fish and other sea foods are a rich source of high protein. They are low in calories, total fat, and saturated fat. They contain numerous vitamins and minerals. As seen in Table 4, 53 ± 0.06% of the respondents have tried to consume more fish and seafood since the COVID-19 pandemic started.

Seventy-seven percent (77 ± 0.08%) of the consumers have tried to consume more home-cooked meal since the COVID-19 pandemic started. In Turkey, during the quarantine days, people started to share their home-made meals and healthy diets through social media. Moreover, people have tried to bake bakery products such as bread, muffin pida. Then, they have shared photographs of them with "stay at home" hashtag in social media.

When the results were evaluated in terms of age groups, consumers over 65 years have paid more attention to eating healthy than other age groups ($p < 0.05$). Older people appear to be at a higher risk of developing more serious complications of COVID-19 disease (CDC, 2020). Although younger people appear generally to be at lower risk (Jordan *et al*., 2020), majority of the consumers aged below 25 years have also paid more attention to eating healthy. It seems that consumers will want to continue maximizing their health in order to boost their immunity and reduce vulnerability to disease long after the pandemic. Marinković and Lazarević (2021) surveyed eating habits and consumer food shopping behavior in Serbia during the COVID-19 pandemic. Their study revealed that eating and purchasing habits of consumers were altered significantly during the Covid-19 pandemic.

Source of knowledge about COVID-19 and food

Consumers have received a variety of information about COVID-19 and food from the websites, televisions, newspapers, scientific journals, university scientists, health professionals, talk shows, and magazines. Many professors and scientists from universities have been invited to TV programs to share their opinions and studies on COVID-19. Moreover, they have given several advices through newspapers. Furthermore, the Minister of Health of the Republic of Turkey have often shared decisions and advises of the scientific committee of COVID-19 through TV and social media.

The source of information is of crucial importance. When people do not trust the source of information, they generally are not willing to change their behaviors (Bolek, 2020). It was investigated whether consumers found media tools reliable during the COVID-19 outbreak.

Fifty-nine percent of the participants have opined that publications and websites of the government give extremely reliable information about the COVID-19 pandemic (Table 5). When the results were evaluated in terms of age groups, this ratio was relatively low (39%) for the participants aged below 25 years ($p < 0.05$). However, majority of the consumers (75%) aged over 65 years rely on websites and government publications for information on COVID-19.

Fifty-nine percent of the consumers think that the daily news on TV is extremely reliable or reliable, whereas 36% of the consumers think that daily news on TV is unreliable or extremely unreliable. Majority of the participants (87%) aged over 65 years rely on daily news on TV ($p < 0.05$). It was revealed that the "age" criterion significantly affected the ratio of trusting the daily news in Turkey ($p < 0.05$).

Newspapers carry information about economy, politics, sports, entertainment, business, trade, industry, and commerce. Many professors and scientists from universities have written articles in newspapers about COVID-19, and they have given advice about hygiene precautions since the COVID-19 pandemic started. As seen in Table 5, majority (81%) of the respondents think that the newspapers are extremely reliable or reliable. Kiousis (2001) showed that newspapers were regarded as more credible than the Internet and television. Hence, newspapers have the very important duty of raising public awareness.

Eighty-four percent of the consumers from all demographics stated that scientific journals are extremely reliable or reliable, whereas only 3% of the consumers think they are unreliable or extremely unreliable. Consumers think that newspapers are as reliable as scientific journals ($p > 0.05$).

Eighty-four percent of the consumers from all demographics indicated that university scientists are extremely reliable or reliable. These results revealed that scientists are one of the primary sources of trusted information and they are very important for public education about COVID-19.

Health professionals have a very important role in improving access and quality of health care for the population. They have been at the front line of the COVID-19 outbreak response and they have been exposed to hazards that put them at risk of infection. As shown in Table 5, 58% of the participants rely on them.

Magazines and talk shows have a wide audience in Turkey and sometimes scientists are invited to these programs. However, only 10 ± 0.07% of the consumers have found them extremely reliable since the COVID-19 pandemic started.

Conclusion

The results of this survey revealed that the COVID-19 pandemic has caused substantial changes in food purchasing behavior and eating habits of Turkish consumers. Consumers from all demographics, but especially from the age group below 65 years, have adopted online food

Table 5. Source of knowledge about COVID-19 and food.

Results obtained for different age groups				
	Overall	**<20**	**20–65**	**>65**
Websites and publications of government				
a) Extremely reliable	26 ± 0.02^{b}	16 ± 0.05^{d,c}	28 ± 0.11^{b}	35 ± 0.04^{b}
b) Reliable	33 ± 0.04^{a}	23 ± 0.02^{b}	36 ± 0.14^{a}	40 ± 0.04^{a}
c) Unreliable	26 ± 0.05^{b}	35 ± 0.03^{a}	25 ± 0.14^{b,c}	18 ± 0.04^{c}
d) Extremely unreliable	11 ± 0.03^{c}	20 ± 0.07^{c}	8 ± 0.12^{c}	5 ± 0.04^{d}
e) No idea	4 ± 0.04^{d}	6 ± 0.02^{d}	3 ± 0.12^{c,d}	2 ± 0.04^{d,e}
Daily news on television				
a) Extremely reliable	30 ± 0.05^{a}	22 ± 0.04^{b,c}	27 ± 0.08^{b}	40 ± 0.09^{a}
b) Reliable	31 ± 0.04^{a}	23 ± 0.07^{b,c}	32 ± 0.04^{a}	37 ± 0.10^{a}
c) Unreliable	22 ± 0.08^{b}	31 ± 0.06^{a}	23 ± 0.05^{c}	12 ± 0.08^{b}
d) Extremely unreliable	14 ± 0.07^{c}	24 ± 0.03^{b}	14 ± 0.04^{d}	5 ± 0.08^{c}
e) No idea	3 ± 0.06^{d}	0 ± 0.04	4 ± 0.03^{e}	6 ± 0.06^{c}
Television and radio programs				
a) Extremely reliable	29 ± 0.04^{a}	25 ± 0.04^{b}	30 ± 0.04^{a}	33 ± 0.04^{a,b}
b) Reliable	27 ± 0.04^{a}	20 ± 0.04^{c}	27 ± 0.04^{a,b}	35 ± 0.04^{a}
c) Unreliable	25 ± 0.04^{a,b}	33 ± 0.04^{a}	22 ± 0.04^{b}	20 ± 0.04^{c}
d) Extremely unreliable	14 ± 0.04^{c}	20 ± 0.04^{c}	16 ± 0.04^{c}	7 ± 0.04^{d}
e) No idea	3 ± 0.04^{d}	2 ± 0.04^{d}	5 ± 0.04^{d}	3 ± 0.04^{d,e}
Newspapers				
a) Extremely reliable	55 ± 0.04^{a}	54 ± 0.04^{a}	50 ± 0.04^{a}	60 ± 0.04^{a}
b) Reliable	26 ± 0.04^{b}	21 ± 0.04^{b}	27 ± 0.04^{b}	29 ± 0.04^{b}
c) Unreliable	8 ± 0.04^{c}	6 ± 0.04^{c,d}	16 ± 0.04^{c}	3 ± 0.04^{c,d}
d) Extremely unreliable	5 ± 0.04^{d}	9 ± 0.04^{c}	4 ± 0.04^{d}	3 ± 0.04^{c,d}
e) No idea	6 ± 0.04^{c,d}	10 ± 0.04^{c}	3 ± 0.04^{d}	5 ± 0.04^{c}
Scientific journals				
a) Extremely reliable	70 ± 0.14^{a}	69 ± 0.05^{a}	72 ± 0.11^{a}	70 ± 0.10^{a}
b) Reliable	14 ± 0.12^{b}	28 ± 0.04^{b}	23 ± 0.12^{b}	20 ± 0.08^{b}
c) Unreliable	2 ± 0.10^{c}	1 ± 0.04^{c}	2 ± 0.08^{c}	3 ± 0.08^{c}
d) Extremely unreliable	1 ± 0.12^{c}	0 ± 0.03	1 ± 0.05^{c}	2 ± 0.06^{c}
e) No idea	3 ± 0.10^{c}	2 ± 0.03^{c}	2 ± 0.05^{c}	5 ± 0.06^{c}
University scientists				
a) Extremely reliable	43 ± 0.14^{a}	38 ± 0.14^{b}	42 ± 0.09^{a}	50 ± 0.12^{a}
b) Reliable	41 ± 0.12^{a,b}	44 ± 0.12^{a}	38 ± 0.10^{b}	40 ± 0.12^{b}
c) Unreliable	10 ± 0.08^{c}	8 ± 0.13^{c}	18 ± 0.09^{c}	4 ± 0.13^{c}
d) Extremely unreliable	3 ± 0.16^{d}	6 ± 0.13^{c,d}	1 ± 0.07^{d}	2 ± 0.13^{c,d}
e) No idea	3 ± 0.16^{d}	4 ± 0.10^{d}	1 ± 0.07^{d}	4 ± 0.14^{c}
Health professionals				
a) Extremely reliable	28 ± 0.07^{a,b}	21 ± 0.07^{b}	28 ± 0.07^{b}	35 ± 0.06^{a}
b) Reliable	30 ± 0.08^{a}	29 ± 0.04^{a}	32 ± 0.08^{a}	30 ± 0.05^{b}
c) Unreliable	18 ± 0.04^{c}	20 ± 0.05^{b}	18 ± 0.05^{c}	15 ± 0.04^{c}
d) Extremely unreliable	11 ± 0.04^{d,e}	15 ± 0.03^{c}	12 ± 0.05^{d}	5 ± 0.03^{d}
e) No idea	13 ± 0.05^{d}	15 ± 0.02^{c}	10 ± 0.06^{d,e}	15 ± 0.03^{c}
Talk shows and magazines				
a) Extremely reliable	10 ± 0.07^{c}	15 ± 0.05^{c}	3 ± 0.06^{e}	12 ± 0.04^{c}
b) Reliable	31 ± 0.06^{a}	29 ± 0.05^{b}	24 ± 0.06^{b,c}	39 ± 0.04^{a}
c) Unreliable	33 ± 0.04^{a}	37 ± 0.04^{a}	35 ± 0.08^{a}	28 ± 0.04^{b}
d) Extremely unreliable	15 ± 0.05^{b}	9 ± 0.04^{d}	26 ± 0.05^{b}	9 ± 0.04^{c,d}
e) No idea	11 ± 0.05^{c}	10 ± 0.02^{d}	12 ± 0.04^{d}	12± 0.04^{c}

The values are expressed as the mean ± SD, and different superscript letters show significant differences ($p < 0.05$).

purchasing. Majority of the respondents have been more than willing to buy fresh products since the COVID-19 pandemic started. Consumers have adopted the practice of preserving their foods by freezing during quarantine days. Moreover, the COVID-19 pandemic has caused many changes in the eating habits of Turkish consumers. Sixty-five percent of the consumers have tried to consume more food that boosts the immune system since the COVID-19 pandemic started. Furthermore, they have had a proper eating habit regarding fruit and vegetable consumption. When the results were evaluated in terms of age groups, consumers over 65 years paid more attention to eating healthy than other age groups ($p < 0.05$). On the other hand, 77% of the consumers have tried to consume more home-cooked meal since the COVID-19 pandemic started. Furthermore, Turkish consumers relied on media tools to a great extent to obtain information about COVID-19. Data obtained from the study indicated that much more education is needed on food and COVID-19. Media tools should be used effectively to communicate accurate information during the COVID-19 pandemic. Future research should investigate how the pandemic affects long-term disparities in food and nutrition for disadvantaged populations and how the evolution of food security impacts, as well as food assistance expansion and health care screenings, may affect food insecurity outcomes as COVID-19 unfolds. Furthermore, it is necessary to carry out studies to compare the effects of media types in conditions such as COVID-19.

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Kaempferol protects rats with severe acute pancreatitis through regulating NF-κB and Keap1–Nrf2 signaling pathway

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Received: 14 July 2021; Accepted: 30 August 2021; Published: 17 September 2021

RESEARCH ARTICLE

Abstract

Kaempferol (KF) is an important natural anti-inflammatory flavonol. Acute pancreatitis (AP) is an inflammatory disorder, which in about 20% cases may develop into severe acute pancreatitis (SAP) with a high mortality rate. This research was to study the effects and mechanism of kaempferol on SAP. SAP was induced by sodium taurocholate. The level of cytokines was analyzed by enzyme-linked-immunosorbent serologic assay. The expression of nuclear factor kappa B (NF-κB) and Kelch-like ECH-associated protein 1–nuclear factor erythroid 2-related factor 2 (Keap1–Nrf2) proteins was analyzed by Western blot assay. Pathological changes in the pancreas were evaluated by hematoxylin and eosin staining. Kaempferol attenuated pancreatic injury in SAP rats, including reduction in inflammatory infiltration and necrosis. The level of serum amylase and lipase was also decreased in kaempferol-treated SAP rats. Kaempferol inhibited the expression of inflammatory mediators (nuclear factor-α, Interlukin-1β, and Interlukin-6), and alleviated the oxidative stress characterized by the decreased malondialdehyde (MDA) and increased superoxide dismutase (SOD) levels. Kaempferol decreased the expression of cleaved caspase 3 and anti-apoptotic protein Bcl-2, which indicated that kaempferol could inhibit apoptosis of pancreatic cells in SAP rats. Kaempferol treatment could decrease the expression of p-p65 and the amount of nuclear Nrf2 (Nu-Nrf2), which demonstrated that kaempferol inhibited the NF-κB activation and enhanced the Keap1–Nrf2 pathway. Our research indicated that kaempferol could attenuate the pancreatic injury of SAP by regulating NF-κB and Keap1–Nrf2 signaling pathway. Kaempferol could serve as a natural candidate for treating SAP.

Keywords: kaempferol; pancreatitis; nuclear factor kappa; inflammatory disorder

Introduction

Acute pancreatitis (AP) is an inflammatory disorder usually initiated by the abnormal activation of digestive enzymes in the pancreas. AP can be classified as mild, moderate, or severe, depending upon the extent of local injury of the pancreas or to systematic and remote organs (Lankisch *et al.*, 2015). According to global estimates, about 20% of AP patients develop moderate to severe acute pancreatitis (SAP), with a higher mortality rate (Lee and Papachristou, 2019). Acinar cell death and local and systemic inflammation are the characteristics of AP (Habtezion *et al.*, 2019). Activation of nuclear factor kappa B (NF-κB) was considered as a fundamental effect during inflammatory

responses in AP (Lawrence, 2009; Montero Vega and de Andrés Martín, 2008). NF-κB activation induces the production of numerous cytokines and chemokines, which then leads to systemic inflammatory responses and multi-system organ failure (Jakkampudi, *et al.*, 2016). Oxidative stress also plays an important role in the pathophysiology of AP and relates with inflammatory responses. The increased free radical activity was found in AP patients (Marek *et al.*, 2018). Oxidative stress in acinar cells triggers inflammatory signaling activation (including NF-κB) and production of various cytokines and chemokines (Hussain, *et al.*, 2016). The nuclear factor erythroid 2-related factor 2 (Nrf2) combined with its inhibitory protein Kelch-like ECH-associated protein 1 (Keap1) is a main pathway involved in antioxidant response and participates in preventing cell damage induced by oxidative stress (Baird and Dinkova-Kostova, 2011). Studies have shown that Keap1–Nrf2 pathway plays an important role in the development of AP. Activating Nrf2 into nucleus could ameliorate injury to the pancreas in AP mice (Liang *et al.*, 2020).

Kaempferol (KF), widely distributed in plant-derived foods or botanical products, is used in traditional medicine isaflavonol, which is known to be an important natural anti-inflammatory compound (Wang *et al.*, 2019). Preclinical and experimental studies showed that kaempferol and its glycosides have various pharmacological activities, including antioxidant, anti-inflammatory, anticancer, cardio-protective, and neuro-protective effects (Calderón-Montaño *et al.*, 2011). Fouzder *et al.* (2021) found that Kaempferol could induce apoptosis of non-small cell lung cancer cells via down-regulation of Nrf2 signaling pathway. In a murine acute liver failure model, kaempferol prolonged the survival time and attenuated liver injury by inhibiting hepatocyte apoptosis via regulating the endoplasmic-reticulum (ER) stress signaling pathway (Wang *et al.*, 2019). In addition, kaempferol was shown to attenuate inflammatory lesions in lipopolysaccharide (LPS)/caerulein-induced AP (Kim *et al.*, 2015). However, a detailed molecular mechanism of kaempferol on SAP needs further investigation. This research studied the mechanism and effects of kaempferol on SAP.

Materials and Methods

Animals and treatment

Male Sprague–Dawley (SD) rats (SPF grade, 280 ± 20 g) were provided by Beijing Vital River Laboratory Animal Technology (Beijing, China), and were housed to acclimate for 1 week. All procedures were in accordance with Guide for the Care and Use of Laboratory Animals (Ref) and approved by the Medical Ethics Committee of Fukuang General Hospital (Approval No. 20180801). These 24 male SD rats were divided into four groups (n = 6): control group (Sham), SAP group, KF low-dosage group (25 mg/kg), and KF high-dosage group (100 mg/kg). SAP was induced by retrograde infusion of 5% sodium taurocholate (Sigma-Aldrich, St. Louis, MO, USA) into the biliopancreatic duct (Fang *et al.*, 2020; Shi *et al.*, 2018). Briefly, rats were anesthetized by pentobarbital sodium, and subjected to sterile laparotomy. AP was inducted into the bile–pancreatic duct by a retrograde infusion of sodium taurocholate. The same procedure was performed for the sham group with no sodium taurocholate injection. Rats in the KF treatment group were given intragastrically 25 mg/kg or 100 mg/kg of kaempferol after surgery. Serum and pancreatic tissues were collected for further experiments.

Biochemical analysis of serum

The activity of serum amylase and lipase was analyzed using automated biochemistry analysis equipment (Hitachi Co., Tokyo, Japan).

Hematoxylin and eosin (H&E) staining

Histopathological changes were evaluated by H&E staining. Pancreatic tissues were fixed in 10% paraformaldehyde for 24 h, dehydrated in graded ethanol series, and embedded in paraffin. Tissues were cut into 4-μm slices and stained with H&E. The histopathological changes in pancreatic tissues were evaluated under light microscope.

Cytokines analysis

Levels of tumor necrosis factor-α (TNF-α), Interlukin (IL)-1β, IL-6, and D-lactic acid were determined by enzyme-linked-immunosorbent serologic assay (ELISA) according the manufacturer's instructions. Absorbance was detected at 450 nm by a microplate reader.

Detection of oxidative stress injury

Detection of malondialdehyde (MDA) and superoxide dismutase (SOD) was performed using oxidative stress test kits purchased from Nanjing KeyGen (Nanjing, China) according to manufacturer's instructions.

Western blot assay

Pancreatic tissues were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor. The nucleoprotein was extracted using commercial reagents according to the instructions. The concentration

was quantified by BCA method. Equal amount of protein was separated on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with skimmed milk, the membrane was incubated with primary antibodies against Nrf2 (CST #12721, 1:1,000 dilution), HO-1 (CST #43966, 1:1,000 dilution), Lamin B1 (CST #17416, 1:1,500 dilution), Caspase 3 (CST #14220, 1: 1,000 dilution), cleaved caspase 3 (CST #9664, 1: 1,000 dilution), Bcl-2 (CST #3498, 1: 1,000 dilution), GADPH (CST#5174,1:2,000 dilution), and secondary goat anti-mouse or anti-rabbit IgGHRP antibody (1:10,000 dilution). Protein expression was visualized through the enhanced chemiluminescence (ECL) system and analyzed using ImageJ software.

Statistical analysis

All data were presented as mean ± SD. Statistical analysis was performed by SPSS. Statistical differences were analyzed by one-way ANOVA and Student–Newman–Keuls test, and $p < 0.05$ was considered statistically significant.

Results

Kaempferol attenuated pancreatic injury in SAP rats

In order to evaluate pancreatic injury in SAP rats, serum amylase level, lipase level, and histopathological changes in the pancreas were examined. The enzyme activities of both amylase and lipase were significantly increased in SAP rats than sham control ($p < 0.001$). This increase in the enzyme activity of SAP group decreased after KF treatment ($p < 0.001$ vs. sham) on a dose-dependent manner (Figure 1A). H&E staining (Figure 1B) showed that sham group had normal acinar architecture. The pancreatic tissue showed massive edema, sublobular hemorrhages, and necrosis, accompanied with inflammatory infiltration. Treatment with kaempferol significantly ameliorated pancreatic injury characterized by reduction of inflammatory infiltration and necrosis. The effects of 100 mg/kg kaempferol were remarkable on pancreatic injury, and tissue necrosis almost disappeared. These results indicated that kaempferol attenuated pancreatic injury in SAP rats.

Kaempferol inhibited inflammatory response and oxidative stress in SAP rats

As inflammatory response and oxidative stress were important pathological processes during SAP, we evaluated some inflammatory mediators and oxidative stress-related enzymes after KF treatment. As shown in Figure 2A, the pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) were increased in SAP rats ($p < 0.001$ vs. sham), which were obviously inhibited by kaempferol on a dose-dependent manner. Additionally, the MDA level increased and SOD level decreased in SAP rats, which were reversed by kaempferol in a dose-dependent

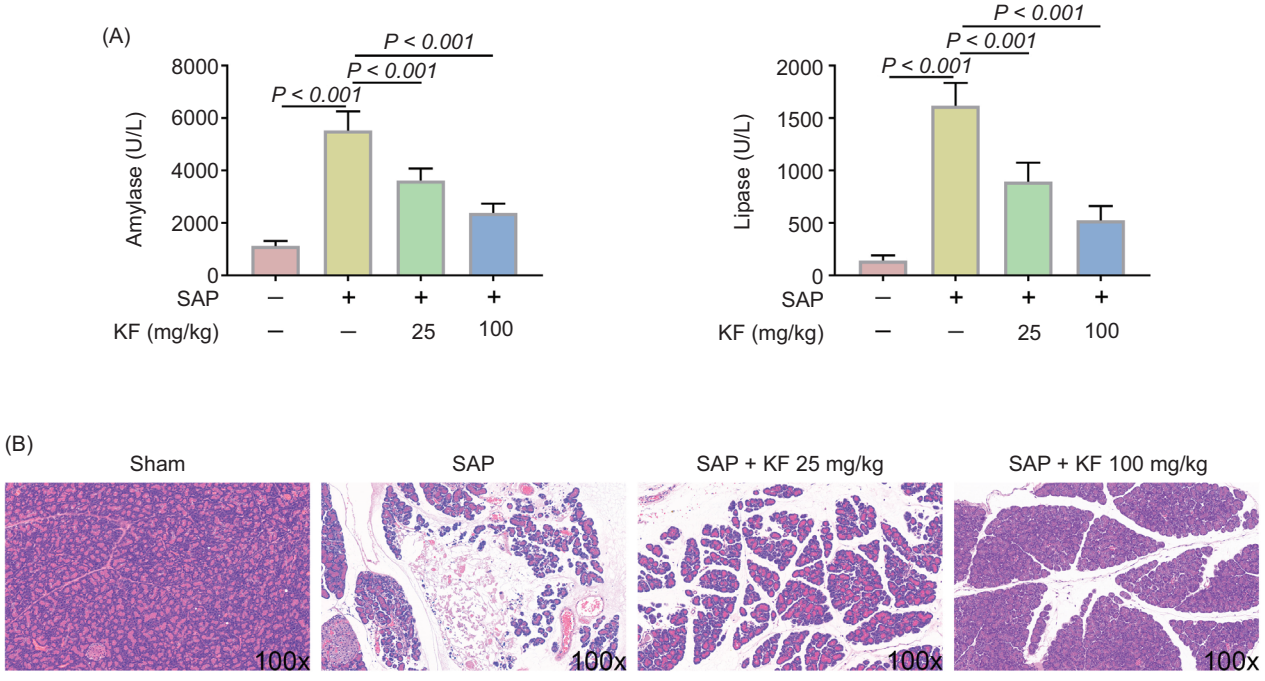

Figure 1. Kaempferol (KF) attenuated pancreatic injury in SAP rats. (A) Serum levels of amylase and lipase. (B) Representative hematoxylin and eosin (H&E) staining of the pancreas. N = 6, ***$p < 0.001$.

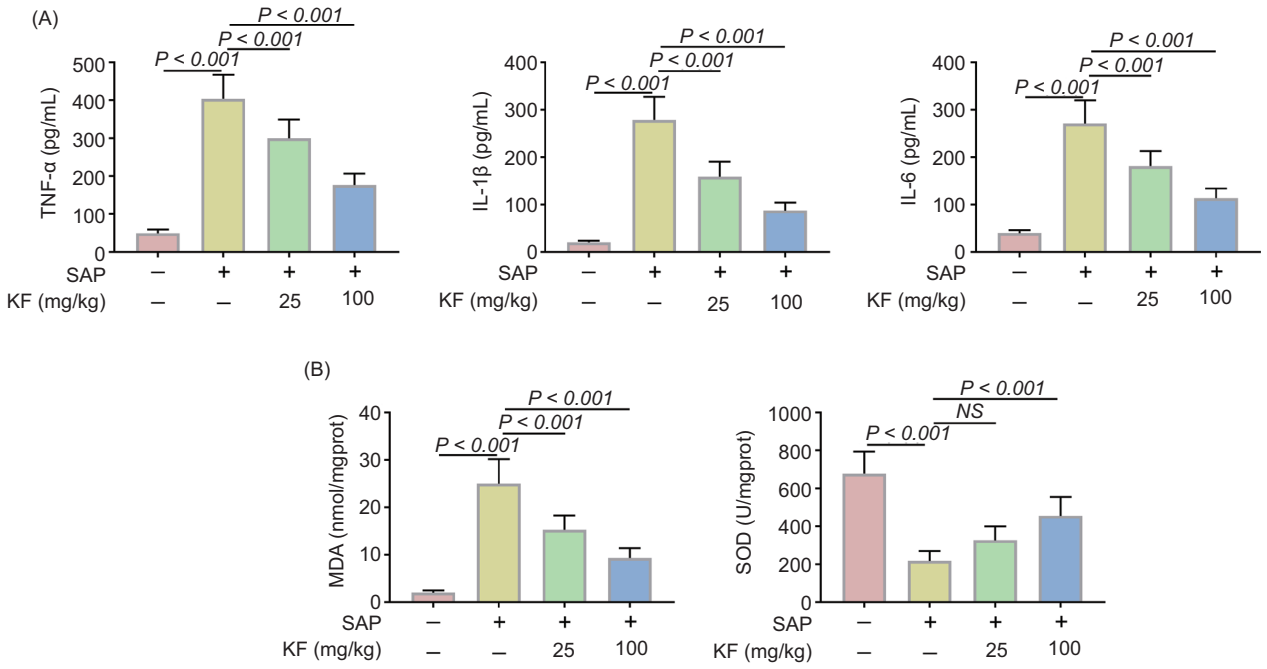

Figure 2. Kaempferol (KF) inhibited inflammatory response and oxidative stress in SAP rats. (A) Levels of pro-inflammatory mediators (TNF-α, IL-1β, and IL-6) of the pancreas analyzed by ELISA. (B) Levels of oxidative stress products (MDA and SOD) of the pancreas. N = 6, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. NS: Not significant.

manner (Figure 2B). All the above inflammatory mediators and oxidative stress enzyme changes were consistent with the H&E results.

Kaempferol inhibited the apoptosis of pancreatic cells in SAP rats

Furthermore, we found the effects of kaempferol on cell apoptosis in pancreatic tissues during SAP. The results (Figure 3) showed that the expression of pro-apoptotic protein Bax and the cleavage of caspase-3 were increased in SAP group. In contrast, the level of anti-apoptotic protein Bcl-2 was decreased in SAP group. All these effects were reversed by KF treatment. These results indicated that kaempferol inhibited the apoptosis of pancreatic cells.

Kaempferol regulated the NF-κB/Keap1–Nrf2 pathway

In order to study the vital roles of NF-κB in inflammatory response and Keap1–Nrf2 pathway in oxidative stress, we further examined the expression of phosphorylated p65 (p-p65), total p65, Keap1 (Nrf2 inhibitory protein), and nuclear Nrf2 (Nu-Nrf2) by Western blot assay (Figure 4). The levels of p-p65 and total p65 increased in SAP group, which indicated the activation of NF-κB signaling pathway, but KF treatment decreased the expression of p-p65. Besides, the level of Nu-Nrf2 increased, and the expression of Keap1 decreased in SAP rats, which demonstrated that the oxidative stress occurred during SAP. The effects were also inhibited by kaempferol. These results indicated that kaempferol had anti-oxidative stress and anti-inflammatory effect via regulating the NF-κB/Keap1–Nrf2 pathway.

Discussion

Kaempferol, a dietary flavonoid which is abundantly present in tea, fruits, vegetables, and beans, has been shown to have anticancer, anti-inflammatory, antidiabetic, antioxidant, cardioprotective, neuroprotective properties (Calderón-Montaño *et al.*, 2011). In various inflammatory injury conditions, kaempferol has a protective effect through regulating of a variety of signaling pathways, including NF-κB, Akt, and Nrf2. For example, Yao *et al.* (2020) found that kaempferol could protect the vascular endothelium by inhibiting oxidative stress inflammatory markers through NF-κB and Nrf2/HO-1 signaling pathway *in vitro* and *in vivo*. In particular, Kim *et al.* (2015) reported that Kaempferol is capable of suppressing *in vivo* inflammatory lesions in gastritis, pancreatitis, and acetic acid-induced writhing. This effect of kaempferol on AP was confirmed in the present results. Kaempferol obviously alleviated pancreatic injury in SAP

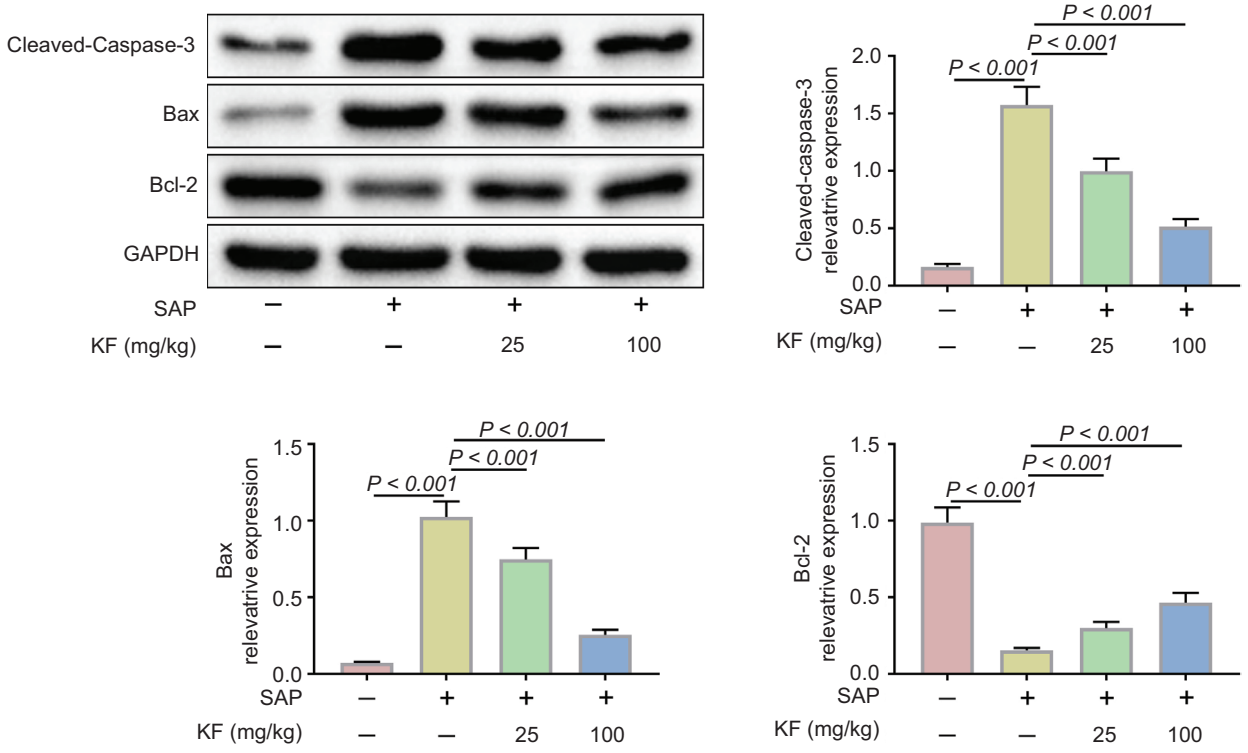

Figure 3. Kaempferol (KF) inhibited the apoptosis of pancreatic cells. Pancreatic Bax, Bcl-2, and cleaved-caspase-3 protein expression analyzed by Western blot assay, and the quantitative results of the blot data. **$p < 0.01$, ***$p < 0.001$.

mice according to the level of serum amylase, lipase, and histological changes in the pancreas.

The main features of AP include vacuolation of pancreatic acinar cells, activation of trypsinogen, apoptosis, necrosis, and infiltration of inflammatory cells (Habtezion *et al.*, 2019). Inflammatory response and oxidative stress have been recognized as two major factors in the pathogenesis of AP (Armstrong *et al.*, 2013). Inflammation is an important causative factor for multiple organ failure. Pancreatic acinar cells in AP patients produced cytokines such as TNFα, IL-6, IL-10, and MCP-1 (Habtezion, 2015). In addition, MCP-1 expressed the initial inflammatory response of leukocytes recruitment to the injured area (Gu *et al.*, 2013). Once immune cells infiltrated the pancreas, the cellular contents released from injured cells activated monocytes and neutrophils and further propagated the inflammation (Habtezion *et al.*, 2019). NF-κB, an ubiquitous transcription factor in cytoplasm, has been confirmed to be a vital factor in inflammatory response. Studies have indicated that activation of NF-κB was an early and central event in inflammatory response in AP (Jakkampudi, *et al.*, 2016). NF-κB contains five subunits, namely p65 (RelA), RelB, c-Rel, NF-κB1, and NF-κB2. Phosphorylation of p65 is one of the main characteristics of NF-κB activation (Oeckinghaus *et al.*, 2011). The present research showed that the levels of TNFα, IL-1β, and IL-6 increased significantly in SAP rats, as well as the phosphorylation of p65, which indicated excessive inflammatory response in SAP rats. Kaempferol could inhibit the production of inflammatory mediators and the activation of NF-κB.

Besides, it is widely reported that oxidative stress response and the generation of reactive oxygen species (ROS) occur in the early phase of AP (Hackert and Werner, 2011). Clinical studies have shown that the level of plasma SOD decreased significantly in SAP patients. Animal experiments have also indicated that plasma MDA could be an early marker for the severity of AP (Ren *et al.*, 2012). In AP models, acinar cell injury induces oxidative stress and changes in MDA and SOD levels (Liu *et al.*, 2018). In this study, the results showed that in sodium taurocholate-induced SAP rats, the activity of SOD decreased and that of MDA increased, which was consistent with the results of previous studies. In addition, the Keap1–Nrf2 signaling pathway was significantly down-regulated in SAP rats. Keap1–Nrf2 is an important signaling antioxidant that exerts protective functioning during SAP (Liang *et al.*, 2020). When cells underwent oxidative stress, Keap1 released Nrf2 into the nucleus and activated downstream genes and antioxidant enzymes (Baird and Dinkova-Kostova, 2011). Previous research has indicated that the activation of

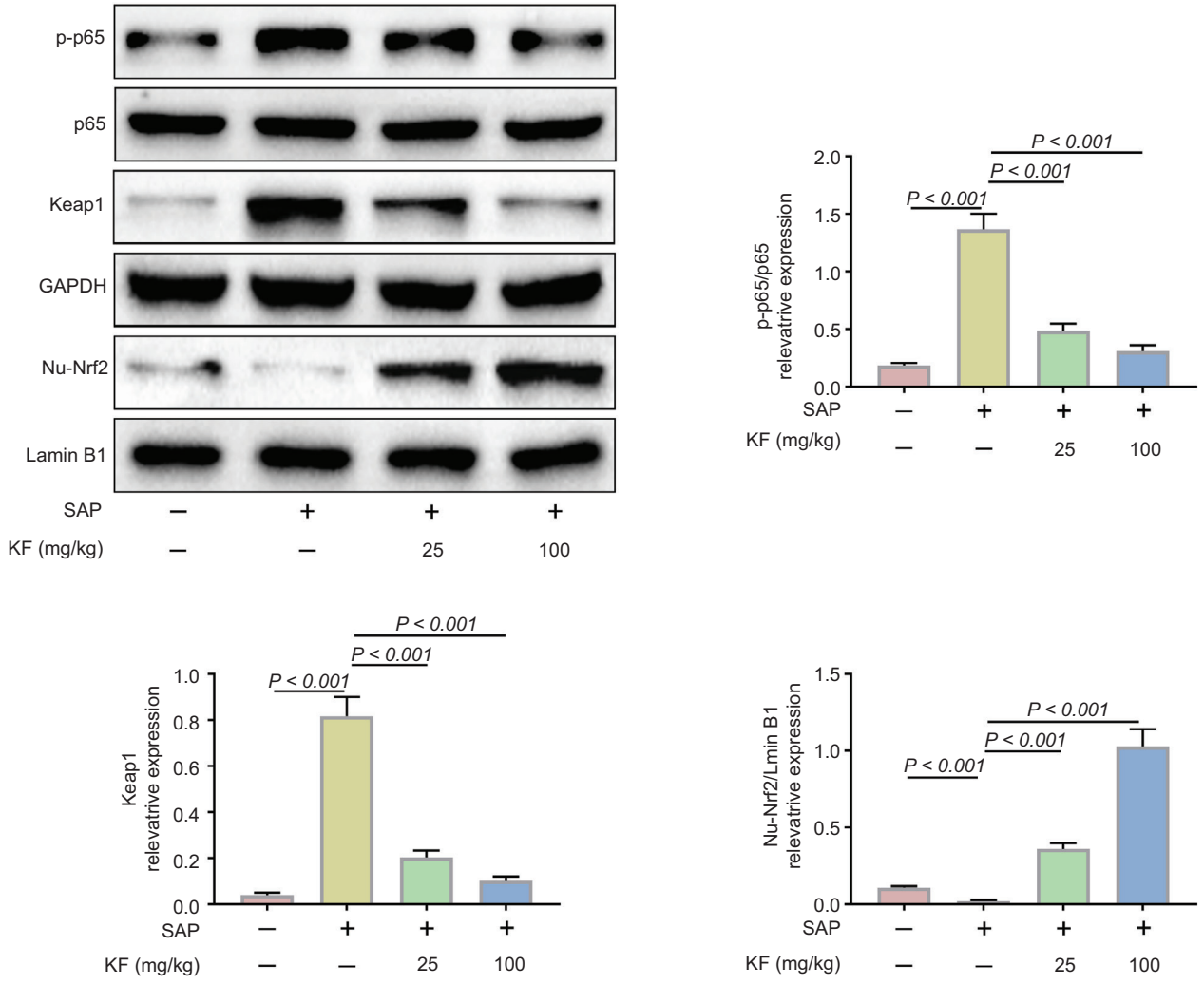

Figure 4. Kaempferol (KF) regulated the NF-κB/Keap1–Nrf2 pathway. Pancreatic p-p65, p-65, Keap-1, and Nrf2 protein expression analyzed by Western blot assay, and the quantitative results of the blot data. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Nrf2 pathway alleviated severity in AP mice. Our results showed that the expression of Keap1 increased remarkably and that of Nu-Nrf2 decreased in SAP rats, which was reversed by kaempferol. Similar antioxidant effects of kaempferol were also found in other pathological conditions. For example, in the case of heart failure in diabetic rats, KF treatment alleviated cardiac injury and apoptosis by regulating Nrf2 and NF-κB signaling pathway (Zhang *et al.*, 2019).

There is a close crosstalk between inflammation and oxidative stress. Pathological calcium signaling, protein kinase C activation, and excessive production of ROS are vital molecular mechanisms that initiate NF-κB-mediated inflammatory response in acinar cells (Reuter *et al.*, 2010). Researchers demonstrated that multiple triggers activate NF-κB, of which ROS contributes the most, causing nuclear translocation (Morgan and Liu, 2011). Besides, the Nrf2 inhibits NF-κB activation by preventing degradation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) (Wardyn *et al.*, 2015). The present study had a limitation that the effect of kaempferol on the crosstalk of NF-κB and Nrf2 was not studied. This could be one of the focuses of the future studies.

Conclusion

To sum up, our research indicated that kaempferol could attenuate the pancreatic injury of SAP by regulating NF-κB and Keap1–Nrf2 signaling pathway. Kaempferol could serve as a natural candidate for treating SAP.

Conflict of Interest

The authors state that there are no conflicts of interest to disclose.

Contribution of Authors

Jun Cai and Suyan Yao designed the study, and supervised data collection. Hao Wang analyzed and interpreted the data. Wei Rong reviewed the draft and prepared the manuscript for publication. All authors read and approved the final manuscript.

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Quality of veiled olive oil: Role of turbidity components

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Received: 28 May 2021; Accepted: 2 September 2021; Published: 18 September 2021

PAPER

Abstract

This study investigated the effects of water and content of solid particles, taken together as well as separately, on stability of veiled olive oil. The following oil samples were obtained through four different separation treatments: veiled, filtered, 'solid-only', and 'water-only'. Changes in chemical, microbial, and sensory characteristics were evaluated during storage (240 days). A significant effect of hydrolysis was shown in veiled and 'water only' oils; in 'solid-only' oils, a slow increase of phenols was observed. A notable microbial activity, with resulting formation of volatile metabolites and sensory defects, was observed in veiled samples. Filtered oils underwent less significant changes.

Keywords: biophenols, hydrolysis, oil-borne microorganism, olive oil quality, volatile compounds

Introduction

Preservation of quality during storage is an important subject for extra-virgin olive oil (EVOO) producers (International Olive Council [IOC], 2018). Good preservation practices are essential to maintain quality of EVOOs up to shelf-life. Moreover, sensory profile and contents of phenolic compounds change during storage, leading to a decrease in hedonic and health characteristics. Filtration is one of the most used stabilization processes for EVOOs (Guerrini *et al*., 2015). Interest in unfiltered oils has increased during last few years (Bimbo *et al*., 2020).

Cloudy aspect of veiled extra-virgin olive oils (VEVOO) is due to the presence of micro-droplets of water and fragments of olive pulp and stone suspended/dispersed in the oil phase (Lercker *et al*., 1994; Koidis *et al*., 2008). Furthermore, different combinations of water and insoluble solids can lead to different 'turbidities' in VEVOOs (Breschi *et al*., 2019). The same degree of turbidity of a VEVOO could be characterized by different water contents, insoluble solid contents, water activity, and/or microbial contamination. Therefore, VEVOO turbidity is not a dichotomous variable, but it is a continuous variable of different proportions of water, insoluble solids, microbial contamination, and water activity (Breschi *et al*., 2019).

The difference between VEVOO and filtered extra-virgin olive oil (FEVOO) during storage is still a controversial and widely studied topic for the quality of olive oil (Cayuela-Sànchez and Caballero-Guerrero, 2019). Some authors have proclaimed that suspended particles play a stabilizing function during storage because most phenolic compounds present in olive oil, having hydrophilic nature, are located in water droplets and insoluble solids (Lonzano-Sànchez *et al*., 2010). Therefore, the presence of suspended particles acts as an antioxidant, providing greater oxidative stability (Lercker *et al*., 1994; Ambrosone *et al*., 2002; Koidis and Boskou, 2006; Migliorini *et al*., 2009). Moreover, the suspended

particles act as buffer against increase in free fatty acid (FFA) and hydrolytic degradation (Frega *et al*., 1999).

On the other hand, in literature, improvement in shelf life because of elimination of sediment by filtration was evidenced. In VEVOO, solid particles and water micro-droplets trap microorganism, mainly yeasts, and constitute a perfect environment for microbial survival (Guerrini *et al*., 2015; Ciafardini and Zullo, 2002a; Ciafardini and Zullo, 2002b; Zullo and Ciafardini, 2020a; Zullo and Ciafardini, 2020b). In veiled oils (VOs), microbial metabolism promoted by a water activity of more than 0.6 (Breschi *et al*., 2019; Bubola *et al*., 2017) was responsible for fast behavior of sensory defects, such as 'fusty' and 'muddy-humidity', and oil debittering phenomena (Zullo and Ciafardini, 2020b ; Zullo *et al*., 2013; Zanoni, 2014; Cayuela *et al*., 2015; Guerrini *et al*., 2020a; Zullo *et al*., 2020). Moreover, the yeast present in VEVOO was responsible for oxidation of phenolic compound and hydrolysis of triacylglycerol (Zullo *et al*., 2013; Romo-Sánchez *et al*., 2010; El Haouhay *et al*., 2018; Ciafardini and Zullo, 2018). Water content also affects the hydrolytic activity of olive oil; hydrolysis is faster at the interface between the two phases of oil and water (Xenakis *et al*., 2010). This effect has been demonstrated with a higher increase of hydroxytyrosol and tyrosol in veiled olive oils than in filtered oils (FO) (Brenes *et al*., 2001; Fregapane *et al*., 2006; Fortini *et al*., 2016; Guerrini *et al*., 2020b).

Given the conflicting results about the role of turbidity on the stability of VEVOOs, in this work, an original research was carried out on the different role of water and insoluble solid particles content during storage of EVOO by testing a wide spectrum of olive oil 'turbidities'.

The present work is a part of wide study on the turbidity and stabilization of olive oil. The first contribution (Breschi *et al*., 2019) allowed defining a set of analyses useful to study turbidities of olive oil based on its physical–chemical and microbiological characterization. Then a specific research (Guerrini *et al*., 2020b) was carried out on the role of water and microorganisms, the two factors that mostly compromise the stability of VEVOOs. Then the dynamics of development of 'fusty' sensory defect and the hydrolysis of phenolic compounds were studied (Guerrini *et al*., 2020a), since these phenomena were always present in the analyzed VEVOOs, with the aim of establishing an adequate filtration schedule.

Finally, the present work aimed (i) to study the contribution of dispersed water droplets or solid particles, which, to different extent, contribute to turbidity in VEVOOs and affect the qualitative characteristics of olive oil during a simulated medium storage period, and (ii) how important the qualification of olive oil turbidity could be to plan separation techniques during crop seasons and storage of olive oil.

Materials and Methods

Olive oil samples

EVOO samples were extracted in October–November 2017 in an industrial continuous plant (TEM, Florence, Italy) in Azienda Agricola La Ranocchiaia (Florence, Italy). The plant was equipped with the following: olive cleaner, blade cutter crusher, sealed vertical malaxer (300 kg), and two-phase horizontal centrifuge (i.e., decanter). The malaxation was carried out at 18°C for 20 min.

Six different 300-kg batches of blend of olive cultivars, harvested in Tuscany, were processed on three different days in 2017: olive oils #1 and #2 were processed on October 31, 2017; olive oils #3 and #4 were processed on November 7, 2017; and olive oils #5 and #6 were processed on November 28, 2017.

Six 20-kg batches of oil from each batch of blended olive cultivars were collected at the end of 'decanter', immediately transferred to the laboratory, and subjected to the following four different water and solid particle separation treatments: (1) first ¼ of oil batches (5 kg of oil) were untreated, forming VO samples for this study (i.e., samples VO#1–VO#6); (2) second ¼ of oil batches (5 kg of oil) were filtered using a portable filter press (Colombo Inox 12, Rover Pompe, Padua, Italy) equipped with five filter sheets (Rover 8, 3-µm cut-off, Rover Pompe, Padua, Italy), forming FO samples for this study (i.e., samples FO#1–FO#6); (3) third ¼ of oil samples (5 kg of oil) were freeze-dried (Modulyo, Edwards, Milan, Italy), forming the 'solid particle-only' (SO) samples for this study, that is, freshly extracted olive oil containing solid particles only without water (i.e., samples SO#1–SO#6); and (4) last ¼ of oil samples (5 kg of oil) were filtered with glass wool using a filter aid to separate solid particles, forming 'water-only' (WO) samples for this study, that is, freshly extracted olive oil containing water only without solid particles (i.e., samples WO#1–WO#6).

All oil samples obtained (4 treatments × 6 different oil batches = 24 oil samples) were bottled in 0.25-L clear glass bottles with a headspace of about 8% of bottle's volume, and immediately analyzed to measure turbidity characterization parameters (i.e., degree of turbidity, water content, water activity, solid particles content, and microbial cell count) as described in Breschi *et al*. (2019). Chemical characteristics (FFA, peroxide value [PV], ultraviolet [UV] spectroscopic indices [K232, K270, and ΔK], and content of phenolic and volatile compounds) and sensory attributes were also measured.

For storage test, all olive oil samples (4 treatments × 6 different oil batches × 4 storage periods = 96 oil samples) were bottled in 0.25-L clear glass bottles with a headspace of about 8% of bottle's volume. These were stored at room temperature (20°C) in a chamber (1.3 × 1.0 × 0.8 m) with internal walls covered with reflective material and a light intensity of 1,900 lux (Master TL-D 90 Graphica lamp, 35 W/390, Philips, Amsterdam, the Netherlands) for 12 h per day. After 45, 120, 180, and 240 days of storage, the olive oil samples were analyzed to measure FFA, PV, K232, K270, ΔK, and phenolic and volatile compounds content and sensory parameters.

Analyses

Turbidity characterization parameters and microbial cell count

The degree of turbidity was measured in nephelometric turbidity unit (NTU) using a Hach Model 2100 turbidimeter (Hach, Loveland, CO, USA). Water content, calculated as percent of water content weight/100-g olive oil sample (% w/w), was analyzed with a Karl Fischer Kit for visual water determination without titrator (37858 HYDRANAL – Moisture Test Kit, Honeywell Fluka, Bucharest, Romania). Water activity (Aw) was measured using a Rotronic Hygroskop DT hygrometer (Michell Italia Srl, Milan, Italy). The solid particles content, calculated as the difference in weight and quantified as percentage of solid particles weight/100-g olive oil sample (% w/w), was measured using the method described in literature (Zullo and Ciafardini, 2018), and calculated by weighing the difference and quantified as % w/w. Microorganisms were enumerated according to the method reported in literature (ZULLO *et al*., 2010): an aliquot of each sample (i.e., ≈20 mL) was taken from each bottle under sterile conditions and filtered through a 0.45-μm sterile nitrocellulose membrane. Then the filtered content was transferred into a 50-mL sterile Falcon tube containing 20-mL sterile physiological solution (0.85% NaCl) and homogenized using UltraTurrax (mod. T25 homogenizer, IKA Milan, Italy). Of each homogenized sample, 200-μL serial dilution was placed on YPD agar medium. Colonies were counted after 48–72 h of incubation at 28°C.

Chemical and sensory parameters

The FFA (% oleic acid), PV (meq O_2 kg^{-1}), and UV spectroscopic indices (K232, K270, and ΔK) were measured according to the official EU method (REG. 2016/2095). Extraction, identification, and determination of phenolic compounds was performed in agreement with the official IOC method (IOC/T.20/Doc.29/Rev.1; International Olive Council [IOC], 2017) using an HPLC apparatus comprising Agilent 1200 series system (Agilent technologies, Santa Clara, CA, USA). The system was composed of a quaternary pump equipped with a diode-array detector and autosampler. The analytical conditions were as follows: HPLC column: LiChroCART® 250-4.6 Purospher® STAR RP-18E, 5 μm (250 × 4.6-mm id; Merck KGaA) equipped with a LiChroCART® 4-4 Purospher® STAR RP-18E, 5-μm pre-column (4 × 4 mm). Contents of phenolic compounds in oil samples were studied as total content, content of polyphenols from different family groups (sum of oleuropein and its derivates, sum of ligstroside and its derivates, phenolic acids, flavonoids, and lignans), and content of single representative compounds in EVOO (hydroxytyrosol and tyrosol). Moreover, R-index, which relates the content of the more hydrolysed phenols (hydroxytyrosol and tyrosol) to the less hydrolysed ones (oleuropein and its derivates and ligstroside and its derivates) was calculated as follows (Fiorini *et al*., 2018):

$$\text{R-index}=\frac{\text{hydroxytyrosol + tyrosol}}{\text{oleuropein and its derivates + ligstroside and its derivates}}$$

The content of volatile organic compounds in olive oil was determined using the combination of headspace solid phase microextraction (HS-SPME) and gas chromatography–mass spectrometry (GC–MS) technique as described in literature (Fortini *et al*., 2017). Analyses were carried out by weighing 4.3 g of sample and 0.1 g of internal standard mixture (ISTD MIX) in 20-mL screw-cap vials fitted with a PTFE/silicone septum. After 5 min of equilibrium at 60°C, the SPME fiber (50/30 μm DVB/CAR/PDMS by Supelco, Darmstadt, Germany) was visible in the vial headspace for 20 min while being subjected to orbital shaking (500 rpm). Then the fiber immediately desorbed for 2 min in a gas chromatograph injection port operating in split less mode at 260°C. The identification of volatile compounds was performed by gas chromatography coupled with quadrupole mass spectrometry using a GC-MS scientific trace system (Thermo Fisher, Waltham, MA, USA) equipped with a 30 m × 0.25 mm ID and 0.25-μm DF ZB-FFAP capillary column (Phenomenex, Torrance, CA, USA). The mass detector was operated in scan mode within a mass range of 30–330 Thomson (Th) at 1,500 Th/s, with an ionization energy (IE) of 70 eV. Compounds were identified and quantified (mg/kg) by comparing their mass spectra and retention period with those of ISTD MIX. These consisted of the following 11 compounds: 3,4-dimethyl phenol, 4-methyl-2-pentanol, hexanoic acid-d11, 1-butanol-d10, ethyl acetate-d8, toluene-d8, ethyl hexanoate-d11, acetic acid-2,2,2-d3, 6-chloro-2-hexanone, 3-octanone, trimethylacetaldehyde.

The panel test was carried out according to the official IOC method (IOC/T.20/Doc.15/Rev.10; International Olive Council [IOC], 2018b). Three women and five men, aged 29–58 years, comprised the panel. All panelists were

trained following the official IOC procedure (IOC/T.20/Doc.14/Rev.5; International Olive Council [IOC], 2018a). The panelists worked for the Taste Commission of the Ministerodelle Politiche Agricole Alimentari, Forestali e del Turismo (MIPAAAFT—Italian Ministry of Agri-Food and Forestry Policy and Tourism). For the safety of panelists, WO samples, filtered on glass wool, were not tasted but only smelt out.

Data processing

A linear model that included two tested variables (treatment and storage period) and their interactions were used to fit the experimental data. Data were analyzed with Matlab R2017B software (MathWorks, Natick, MA, USA). A two-way mixed effect ANOVA was performed to assess significant differences ($p \leq 0.05$). Treatment was considered a fixed effect variable, while storage period was taken as a random effect variable.

Six olive oil samples for each treatment were used as replicated for storage study. This choice was done to understand both the behavior of unfiltered oils related to filtered oils, regardless of individual oil turbidity characteristics, and the separated role of water and solid particles during storage of unfiltered olive oils.

Results

Turbidity characterization

Immediately after production, the six VEVOO samples (VO#1–VO#6) used in this study were characterized for different 'turbidities' (Breschi *et al*., 2019). The turbidity grade ranged between 800 and 1,700 NTU, with water content between 0.15 and 0.40% w/w, water activity between 0.60 and 0.85, and insoluble solids content between 0.10 and 0.45% w/w. Microbial cell count was between 2.5 and 4.5 log CFU g^{-1}.

After treatments, turbidity characteristics of olive oil samples changed radically. FEVOO samples (FO#1–FO#6) were characterized by a degree of turbidity grade (10–20 NTU), water (0.04–0.05% w/w), and insoluble solids content (0.00% w/w), water activity (0.30–0.45), and microbial cell count (0.00 log CFU g^{-1}), which were statistically ($p > 0.05$) lower than VO samples. The WO olive oil (WO#1–WO#6) and SO olive oil (SO#1–SO#6) samples were characterized by turbidity characteristics, which were between VEVOO and FEVOO samples. The degree of turbidity grade for WO olive oil samples was between 40 and 90 NTU and that for SO olive oil samples between 150 and 240 NTU. These turbidity grades were characterized by different water content (0.10–0.11% w/w for WO samples; and 0.02–0.04% w/w for SO samples), water activity value (0.45–0.75 for WO samples; and 0.30–0.40 for SO samples), and insoluble solids content (0.00% w/w for WO samples; and 0.15–0.40% w/w for SO samples). The microbial cell counts for WO and SO olive oil samples were 0.5–3.0 log CFU g^{-1} and 0.0–.7 log CFU g^{-1}, respectively.

Chemical parameters and microbial cell count

All olive oil samples resulted from the values of chemical parameters, FFA, PV, K232, K270, and ΔK, in the 'extra-virgin' category during whole storage (Table 1). However, the spectroscopic indices (K232, K270, and ΔK) significantly increased during storage for all treatments ($p \leq 0.01$). VO samples had statistically higher FFA and ΔK values than FO, SO, and WO samples. However, the highest value of K270 was determined in SO olive oil samples.

Microbial cell count was statistically significant for treatment. VO samples had a microbial cell count higher than FO samples; WO olive oil samples had a microbial cell count between VO and FO samples. SO olive oil samples had a microbial cell count between WO and FO samples (i.e., no significant difference than both WO and FO). No statistically significant variation occurred during storage time. However, interactions between time and treatment were statistically significant. In WO and SO olive oil samples, the microbial cell count decreased during storage, in FO samples it did not change, and in VO samples, the microbial contamination increased up to 120 days, then decreased (Figure 1).

Content of phenolic compounds

The content of phenolic compounds of oil samples was studied as total content, content of different family groups of polyphenols, and content of single representative compounds in EVOO, as described in literature (Breschi *et al*., 2019; El Riachy *et al*., 2011) (Table 2).

The total phenolic content was statistically significant ($p \leq 0.001$) for treatment. The content of total phenolic compounds was statistically higher in SO samples than in VO and WO samples, which had a higher content of total phenolic compounds than in FO samples (Table 2). The statistically significant higher content of total phenolic compounds in SO samples was also determined by the sum of oleuropein and its derivates and the sum of ligstroside and its derivates (Table 2). Instead, the content of hydroxytyrosol, tyrosol, and phenolic acids was statistically higher ($p \leq 0.001$) in VO samples than in WO and SO samples, which had higher content of hydroxytyrosol, tyrosol, and phenolic acids than in FO samples (Table 2).

Table 1 Mean values of chemical parameters of all olive oil samples for each separation treatment. Different superscribed letters (^{a,b,c}) in the same row indicate significant differences ($p < 0.05$) for different treatments. Different superscribed letters (^{x,y,z}) in the same column indicate significant differences ($p < 0.05$) for different storage periods. Following are reported in the last five columns: standard error; *p*-value for the storage time (*p*-value t); *p*-value for the treatment (*p*-value T); *p*-value for time–treatment interactions (*p*-value t*T),, and limit value for 'extra-virgin' category (REG. 2016/2095).

	Time (days)	*FO#1–FO#6*	*VO#1–VO#6*	*SO#1–SO#6*	*WO#1–WO#6*	St. Err.	*p*-value t	*p*-value T	*p*-value t*T	R^2	ADJ-R^2	Limit value for 'extra-virgin' category
Acidity (% oleic acid)	0	$0.19^{a,x}$	$0.22^{b,x}$	$0.16^{a,x}$	$0.17^{a,x}$	0.01	n.s.	***	n.s.	0.6490	0.6096	
	45	$0.18^{a,x}$	$0.24^{b,x}$	$0.18^{a,x}$	$0.20^{a,x}$							
	120	$0.17^{a,x}$	$0.25^{b,x}$	$0.20^{a,x}$	$0.18^{a,x}$							≤0.8
	180	$0.17^{a,x}$	$0.24^{b,x}$	$0.21^{a,x}$	$0.19^{a,x}$							
	240	$0.18^{a,x}$	$0.25^{b,x}$	$0.16^{a,x}$	$0.20^{a,x}$							
Peroxide value (meq O_2/kg)	0	$5.4^{a,x}$	$6.3^{a,x}$	$5.9^{a,x}$	$5.8^{a,x}$	0.2	n.s.	n.s.	n.s.	0.1202	0.0216	≤20
	45	$7.6^{a,x}$	$6.4^{a,x}$	$7.5^{a,x}$	$7.2^{a,x}$							
	120	$5.9^{a,x}$	$5.9^{a,x}$	$6.2^{a,x}$	$7.2^{a,x}$							
	180	$7.5^{a,x}$	$5.8^{a,x}$	$5.4^{a,x}$	$6.9^{a,x}$							
	240	$9.2^{a,x}$	$7.5^{a,x}$	$7.2^{a,x}$	$6.3^{a,x}$							
K232	0	$1.69^{a,x}$	$1.68^{a,x}$	$1.77^{a,x}$	$1.70^{a,x}$	0.01	**	n.s.	n.s.	0.5542	0.5042	≤2.50
	45	$1.76^{a,xy}$	$1.74^{a,x}$	$1.80^{a,x}$	$1.79^{a,y}$							
	120	$1.79^{a,y}$	$1.78^{a,y}$	$1.84^{a,xy}$	$1.80^{a,y}$							
	180	$1.81^{a,y}$	$1.78^{a,y}$	$1.82^{a,x}$	$1.81^{a,y}$							
	240	$1.84^{a,y}$	$1.79^{a,y}$	$1.87^{a,y}$	$1.87^{a,y}$							
K270	0	$0.13^{a,x}$	$0.15^{a,x}$	$0.19^{b,x}$	$0.15^{a,x}$	0.01	**	***	n.s.	0.5340	0.4818	≤0.22
	45	$0.15^{a,xy}$	$0.16^{a,x}$	$0.18^{b,x}$	$0.16^{a,x}$							
	120	$0.18^{a,y}$	$0.17^{a,xy}$	$0.21^{b,x}$	$0.17^{a,xy}$							
	180	$0.17^{a,y}$	$0.17^{a,xy}$	$0.20^{b,x}$	$0.17^{a,xy}$							
	240	$0.18^{a,y}$	$0.18^{a,y}$	$0.20^{b,x}$	$0.18^{a,y}$							
ΔK	0	$-0.005^{a,x}$	$-0.004^{a,x}$	$-0.004^{a,x}$	$-0.005^{a,x}$	0.000	***	**	n.s.	0.5215	0.4678	≤0.01
	45	$-0.005^{a,x}$	$-0.002^{b,y}$	$-0.003^{ab,xy}$	$-0.003^{ab,y}$							
	120	$-0.002^{a,y}$	$0.000^{b,z}$	$-0.002^{a,y}$	$-0.001^{ab,z}$							
	180	$-0.002^{a,y}$	$0.000^{b,z}$	$-0.001^{ab,y}$	$-0.001^{ab,z}$							
	240	$-0.002^{a,y}$	$0.000^{b,z}$	$-0.002^{a,y}$	$-0.001^{ab,z}$							

n.s., *, **, and *** indicate significant differences by two-way ANOVA at $p > 0.05$, $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$. Number of replicates = 6. VO: virgin oil; WO: olive oil containing water only; SO: olive oil containing solid particles; FO: filtered oil.

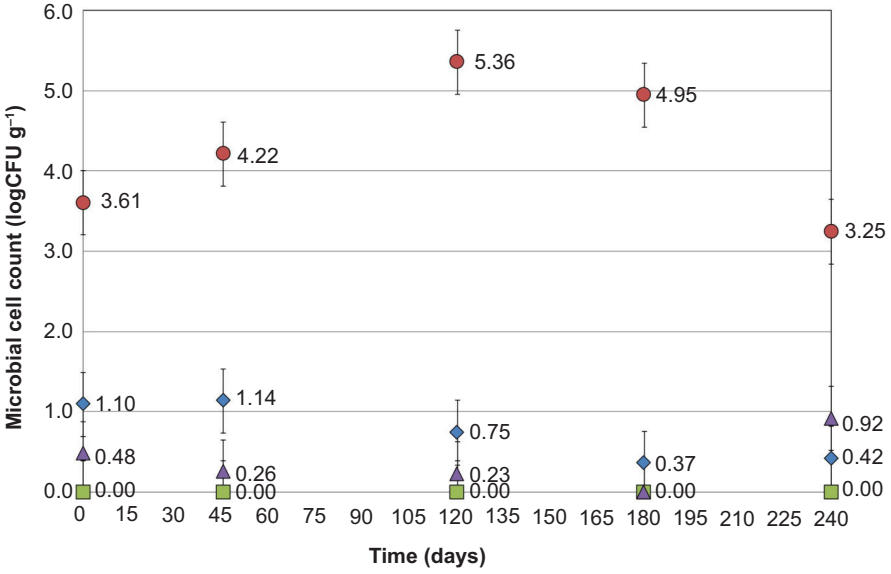

Figure 1 Mean contents and standard error of microbial cell count in samples of virgin oil (VO; red circle), olive oil containing water only (WO; blue diamond), olive oil containing solid particles (SO; purple triangle), and filtered oil (FO; green square) during storage. The R^2 and ADJ-R^2 values of microbial cell count were 0.8522 and 0.8356, respectively.

Significant interactions between storage period and treatment ($p \leq 0.001$) were determined for hydroxytyrosol and tyrosol contents, which statistically increased faster in VO samples than in WO > SO > FO samples during storage (Table 2). Immediately after production (time = 0), the content of both hydroxytyrosol and tyrosol was lower than 10 mg kg^{-1} and 5 mg kg^{-1}, respectively, in all samples. During the 240 days of storage, the contents increased statistically in all samples except FO samples. VO samples had content of hydroxytyrosol and tyrosol statistically ($p \leq 0.001$) higher than in FO samples. Content of both hydroxytyrosol and tyrosol in WO and SO samples was statistically different ($p \leq 0.001$) and was between the content determined in VO and FO samples.

The contents of hydroxytyrosol, tyrosol, oleuropein, and ligstroside and their derivates were used to calculate R-index (R-index = [hydroxytyrosol + tyrosol]/[oleuropein and its derivates + ligstroside and its derivates]), a useful marker of the hydrolysis of secoiridoids (Fiorini *et al.*, 2018). During storage R-index increased significantly ($p \leq 0.001$) in all treatments, demonstrating degradation of phenols (Figure 2). The difference between treatments was statistically significant ($p \leq 0.001$); except at the beginning of storage, the R-index of VO samples was always higher than that of FO and SO samples. WO samples had intermediate value of R-index. Moreover, time–treatment interactions were also statistically significant: in VO samples, the R-index gain was faster than in WO samples, which was faster than SO and FO samples.

The ratio of oxidized form of phenolic compounds to not oxidized form (OX:not OX) during storage period (Figure 3) was determined to observe the effect of oxidation on phenolic compounds. Immediately after production, FO samples showed the OX:not OX ratio value statistically ($p \leq 0.05$) lower than in VO, WO, and SO samples. After 240 days of storage, increase in oxidized forms of phenolic compounds made a statistically significant difference in treatment: the OX:not OX ratio value was higher in FO and SO samples than in WO and VO samples.

Content of volatile compounds

The content of volatile compounds in olive oil samples was studied as described in literature (Guerrini *et al.*, 2020a): pleasant lipoxygenase pathway (LOX pathway) volatile compounds with five (C5) and six (C6) carbon atoms; unpleasant volatile compounds related to 'fusty'/'mouldy'/'vinegary' defects; and unpleasant volatile compounds related to 'rancid' defect.

Some statistically significant differences ($p \leq 0.05$) were identified in C5 and C6 branches of LOX pathway volatile compounds. A statistically significant main effect of filtration was detected in 1-hexanol, E-2-hexenol, Z-3-hexenol, 1-penten-3-one, and E-2-penten-1-ol volatile compounds (Figure 4). The content of all these volatile compounds was higher in VO samples than in FO, WO, and SO samples.

The same statistically significant difference was also determined in 3-methyl-butanal, 2-octanol, and 2-nonanone unpleasant volatile compounds related to 'fusty' defect (Figure 5). Moreover, a statistically significant

Table 2 Mean values of total content, content of groups, and content of single representative phenolic compounds of all oil samples for each separation treatment. Different superscribed letters (^{a,b,c}) in the same row indicate significant differences ($p < 0.05$) for different treatments. Different superscribed letters (^{x,yz}) in the same column indicate significant differences ($p < 0.05$) for different storage periods. In the last four columns are reported standard error; *p*-value for the storage time (*p*-value t); *p*-value for the treatment (*p*-value T); and *p*-value for time–treatment interactions (*p*-value t*T).

	Time (days)	FO#1–FO#6	VO#1–VO#6	SO#1–SO#6	WO#1–WO#6	St. Error	*p*-value t	*p*-value T	*p*-value t*T	R^2	ADJ-R^2
Hydroxy tyrosol (mg/kg)	0	$2.7^{a,x}$	$5.0^{b,x}$	$6.5^{b,x}$	$4.4^{ab,x}$	1.5	***	***	***	0.7985	0.7759
	45	$3.1^{a,x}$	$14.3^{b,y}$	$8.1^{ab,x}$	$8.4^{ab,xy}$						
	120	$4.7^{a,x}$	$20.0^{b,yz}$	$9.4^{ab,x}$	$11.7^{ab,y}$						
	180	$4.7^{a,x}$	$20.0^{b,yz}$	$9.1^{ab,x}$	$13.5^{ab,y}$						
	240	$5.9^{a,x}$	$27.9^{b,z}$	$15.4^{ab,y}$	$17.5^{ab,y}$						
Tyrosol (mg/kg)	0	$2.4^{a,x}$	$2.9^{a,x}$	$3.1^{a,x}$	$3.1^{a,x}$	0.6	***	***	***	0.7504	0.7224
	45	$2.8^{a,x}$	$5.4^{a,x}$	$3.5^{a,x}$	$3.6^{a,x}$						
	120	$3.0^{a,x}$	$7.9^{b,xy}$	$4.2^{ab,xy}$	$4.6^{ab,x}$						
	180	$2.9^{a,x}$	$10.2^{b,y}$	$3.8^{ab,xy}$	$4.1^{a,bx}$						
	240	$4.1^{a,x}$	$11.8^{b,y}$	$5.4^{ab,y}$	$7.1^{ab,y}$						
Sum of oleuropein and its derivates (mg/kg)	0	$290.9^{a,x}$	$369.5^{b,x}$	$437.9^{c,x}$	$384.8^{bc,x}$	13.9	n.s.	***	n.s.	0.6646	0.6270
	45	$248.5^{a,x}$	$307.8^{b,x}$	$427.5^{c,x}$	$346.1^{bc,x}$						
	120	$307.3^{a,x}$	$308.4^{a,x}$	$438.8^{b,x}$	$278.3^{a,x}$						
	180	$298.5^{a,x}$	$282.6^{a,x}$	$425.6^{b,x}$	$326.8^{a,x}$						
	240	$325.9^{b,x}$	$286.6^{a,x}$	$444.9^{c,x}$	$343.5^{b,x}$						
Sum of ligstroside and its derivates (mg/kg)	0	$152.4^{a,x}$	$181.4^{a,x}$	$149.7^{a,x}$	$163.7^{a,x}$	6.7	n.s.	***	n.s.	0.3822	0.3129
	45	$101.4^{a,x}$	$198.6^{b,x}$	$186.4^{b,x}$	$173.5^{b,x}$						
	120	$132.6^{a,x}$	$178.3^{ab,x}$	$206.4^{b,x}$	$149.4^{ab,x}$						
	180	$138.1^{a,x}$	$176.3^{ab,x}$	$229.9^{b,x}$	$161.3^{ab,x}$						
	240	$156.2^{a,x}$	$178.7^{ab,x}$	$214.8^{b,x}$	$178.5^{ab,x}$						
Total contents (mg/kg)	0	$548.4^{a,x}$	$701.2^{c,x}$	$724.6^{c,x}$	$671.5^{b,x}$	20.3	n.s.	***	n.s.	0.6179	0.5751
	45	$445.5^{a,x}$	$655.1^{b,x}$	$732.7^{c,x}$	$644.6^{b,x}$						
	120	$543.6^{a,x}$	$646.6^{b,x}$	$769.6^{c,x}$	$556.2^{a,x}$						
	180	$543.3^{a,x}$	$638.5^{b,x}$	$778.5^{c,x}$	$609.2^{b,x}$						
	240	$597.6^{a,x}$	$637.7^{b,x}$	$798.0^{c,x}$	$676.2^{b,x}$						

n.s., *, **, and *** indicate significant differences by two-way ANOVA at $p > 0.05$, $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$. Number of replicates = 6. VO: virgin oil; WO: olive oil containing water only; SO: olive oil containing solid particles; FO: filtered oil.

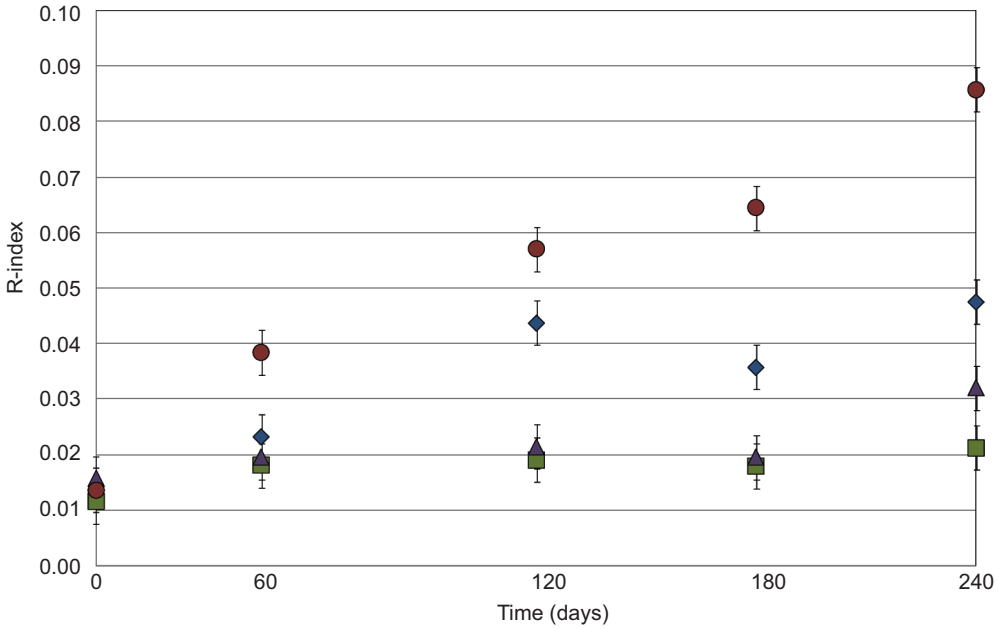

Figure 2. Mean value, standard error of R-index in samples of virgin oil (VO; red circle and line), olive oil containing water only (WO; blue diamond and line), olive oil containing solid particles (SO; purple triangle and line), and filtered oil (FO; green square and line) during storage. The R^2 and ADJ-R^2 values of R-index were 0.8343 and 0.8157, respectively.

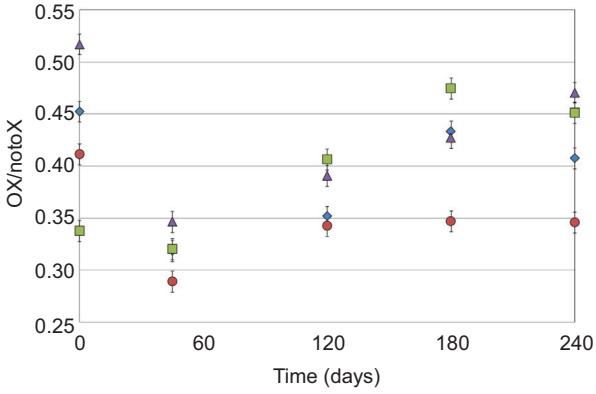

Figure 3. Mean value and standard error of phenolic oxidized–not oxidized form ratio (OX:not OX) in virgin oil VO (red circle), olive oil containing water only WO (blue diamond), olive oil containing solid particles SO (purple triangle), and filtered oil FO (green square) samples during storage. The R^2 and ADJ-R^2 of OX/not OX were 0.1957 and 0.1055, respectively.

effect of treatment was determined in some single and some C5 and C6 LOX pathway volatile compounds, with lower content in SO samples than in FO, WO, and VO samples because of stripping caused by freeze-drying. No statistically significant differences during storage period and no significant interactions between filtration and storage period were determined in all the evaluated volatile compounds of LOX pathway and those related to 'fusty' defect.

The main effect of treatment and storage period and their interactions were not statistically significant for the unpleasant volatile compounds related to 'rancid' defect.

Sensory evaluation

The sensory attributes were evaluated and a significant ($p \leq 0.05$) effect of treatment and storage period was determined. The positive 'fruity' attribute decreased during storage period in all samples. The VO and SO samples were significantly less fruity than FO and WO samples after 120 days of production (Table S1).

The negative 'fusty' and 'winey' defects, both related to microbial activity, and 'rancid' defect, related to oxidation, showed significant ($p \leq 0.001$) increase during storage, and were of higher values in VO samples than in FO, SO, and WO samples after 45 days (Table S1). Furthermore, interactions between filtration and storage period were statistically significant for 'fusty', 'winey' and 'rancid' defects. Indeed, these defects increased faster in VO samples than in FO, WO, and SO samples (Figure 6).

The bitterness and pungency attributes significantly ($p \leq 0.001$) decreased in intensity during storage (Table S1). The VO samples were significantly ($p \leq 0.001$) less bitter and pungent than SO and FO samples after 45 days. WO samples were not tasted due to filtration with glass wool.

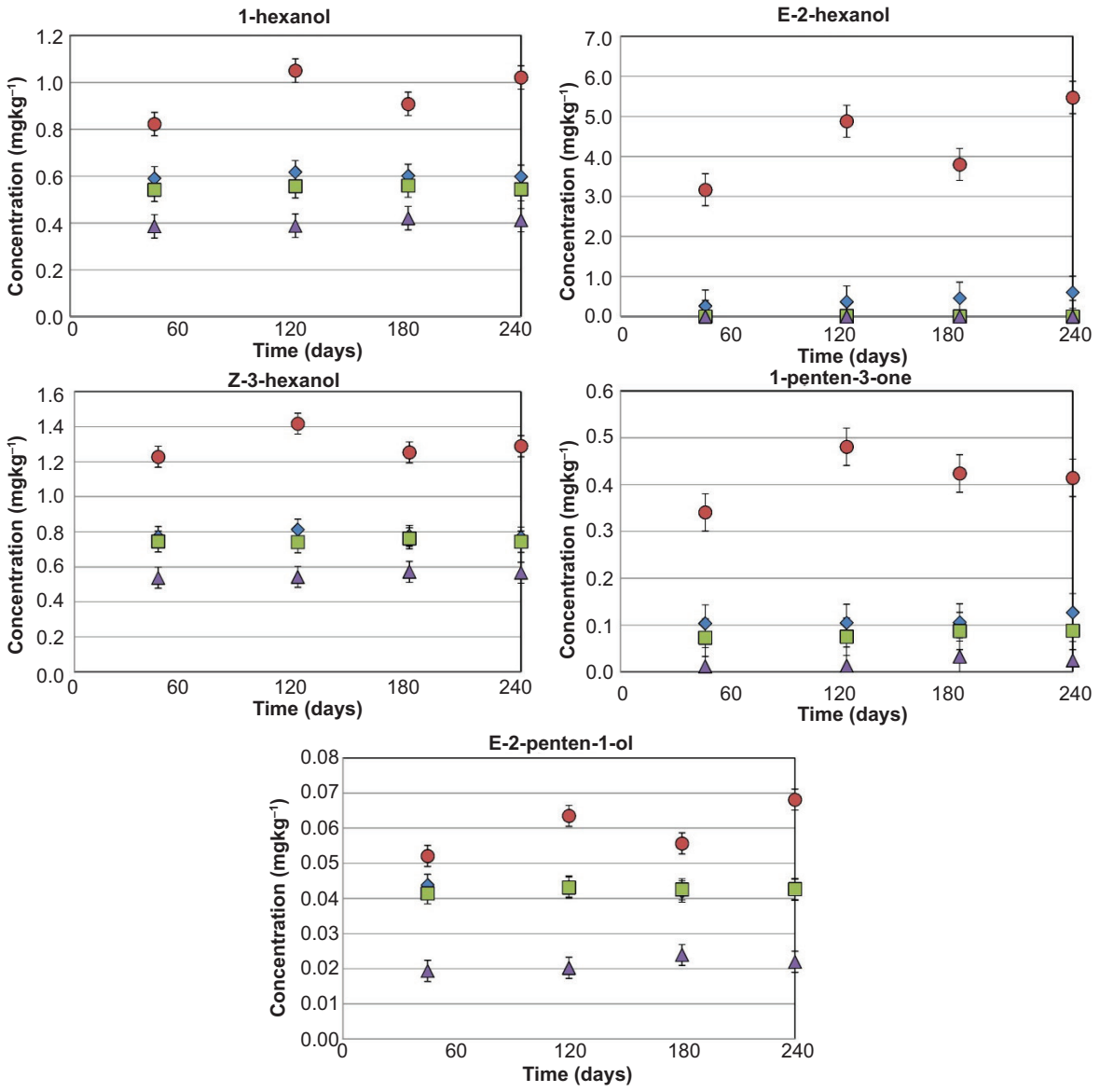

Figure 4. Mean contents and standard error of lipoxygenase (LOX) pathway volatile compounds in virgin oil VO (red circle), olive oil containing water only WO (blue diamond), olive oil containing solid particles SO (purple triangle), and filtered oil FO (green square) samples during storage. Only compounds statistically significant different ($p \leq 0.05$) for time and/or treatment are reported. The R^2 and ADJ-R^2 values for LOX pathway volatile compound are as follows: 1-hexanol, $R^2 = 0.5003$, ADJ-$R^2 = 0.4442$; E-2-hexenol, $R^2 = 0.6473$, ADJ-$R^2 = 0.6077$; Z-3-hexenol, $R^2 = 0.7068$, ADJ-$R^2 = 0.6740$; 1-peten-3-one, $R^2 = 0.5996$, ADJ-$R^2 = 0.5547$; and E-2-penten-1-ol, $R^2 = 0.7460$, ADJ-$R^2 = 0.7175$.

Discussion

The experimental data highlighted that water and solid particles had some specific roles to play in the quality evolution of EVOO during storage. The obtained results demonstrated that two degradation phenomena, hydrolysis and microbial activity, were faster in VO samples than in FO, WO, and SO samples.

The presence of water micro-droplets dispersed in oil matrix increased the water/oil exchange surface, and the hydrolysis reaction occurred to a significant extent (XENAKIS *et al.*, 2010). The enzymatic hydrolysis of triglycerides produced, not esterified fatty acids, that increased the FFA value more in VO samples than in FO, SO, and WO samples. Furthermore, the formation of phenolic compounds with low molecular weight, such as hydroxytyrosol and tyrosol (due to chemical hydrolysis of phenolic compounds (Zanoni, 2014; Cinquanta *et al.*, 1997)), was higher in VO samples than in FO, SO, and WO samples. R-index confirmed the above trend in VO samples and established that WO

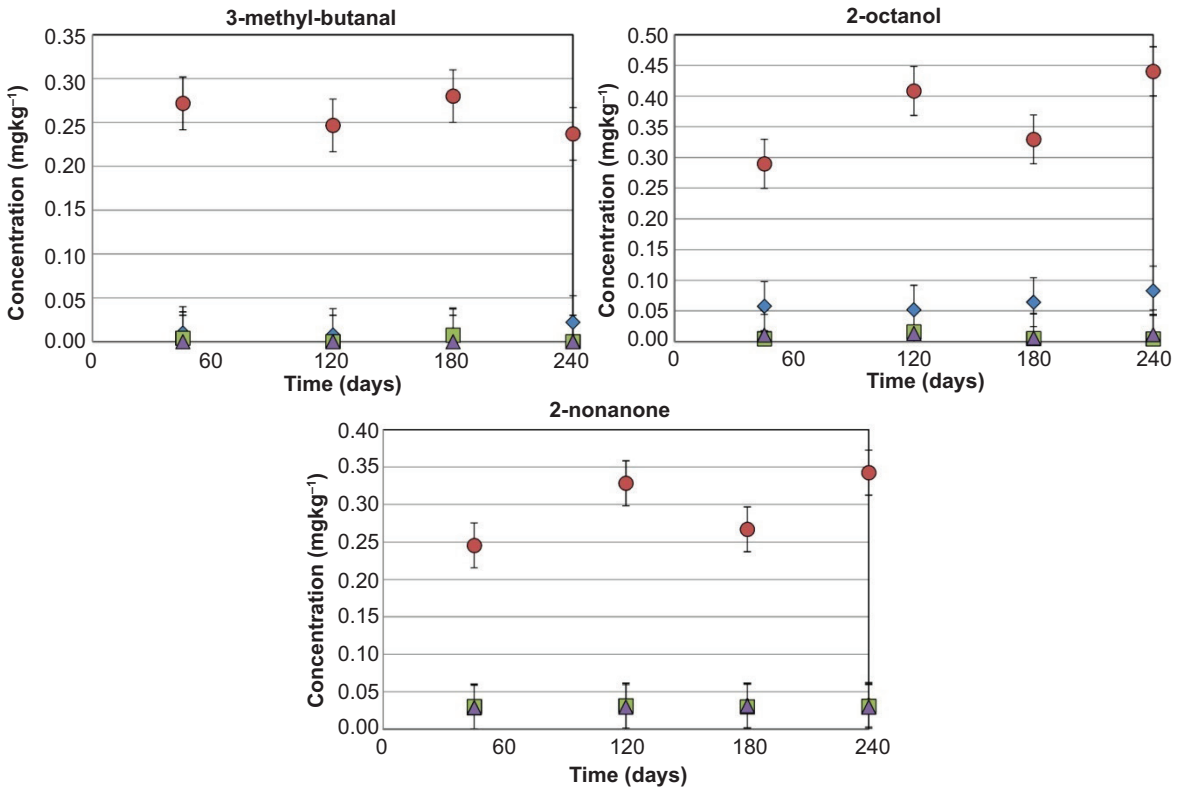

Figure 5. Mean contents and standard error of volatile compounds related to 'fusty' defect in virgin oil VO (red circle), olive oil containing water only WO (blue diamond), olive oil containing solid particles SO (purple triangle), and filtered oil FO (green square) samples during storage. Only compounds statistically significant different ($p \leq 0.05$) for time and/or treatment are reported. The R^2 and ADJ-R^2 values for 'fusty' defect volatile compounds are as follows: 3-methyl-butanal, $R^2 = 0.4201$, ADJ-$R^2 = 0.3551$; 2-octanol, $R^2 = 0.7852$, ADJ-$R^2 = 0.7611$; and 2-nonanone, $R^2 = 0.5197$, ADJ-$R^2 = 0.4659$.

samples, with intermediate water content, had intermediate hydrolytic activity (Figure 2). The cause and effect relationship between the presence of micro-droplets of water in VO samples and the chemical hydrolytic phenomena of phenolic compounds were in accordance with the experimental data given in literature (Guerrini *et al*., 2020b).

The 'fusty' and 'winey' sensory defects and their related volatile compounds were strictly connected to the microbial activity. The microorganism cell count in VO samples was higher than in FO, SO, and WO samples during storage; the microbial survival was due to the favorable environment of VO samples, starting with water activity of >0.6 (Derossi *et al*., 2011), resulting in unpleasant volatile microbial metabolites, such as 3-methyl-butanal, 2-octanol, 2-nonanone (Figure 5).

The microbial activity was also helped by the content of solid particles. Our results highlight that water has to be combined with solid particles for microbial growth. WO and SO samples were not good for microbial survival, and only VO samples had favorable conditions for microbial growth (Figure 1).

The content of solid particles could be involved in promoting the transfer of phenols transfer from solid particles to oil. The SO samples were able to show the above effect, thanks to both absence of water and slow hydrolytic phenomena of phenolic compounds. The significant higher contents of both total phenolic compounds and sum of oleuropein and its derivatives in SO samples (Table 2) could be explained by the mass transfer of phenolic compounds from solid particles to oil. Solid particles consist of olive pulp and core fragments that are rich in high molecular weight phenolic compounds (Jerman Klen *et al*., 2015; Cecchi *et al*., 2018; Morales *et al*., 2005). However, the freeze-drying conditions led to initial oxidation, as shown in OX:not OX ratio values (Figure 3), and stripping of volatile compounds which affected quality parameters, such as K270 (Table 1), and development of 'rancid' defect (Figure 6).

Derived from the experimental results, following are the other functions of water and solid particles in the quality evolution of EVOO during storage, although they had some uncertain aspects.

The water content seemed to promote the LOX enzymatic pathway, which is responsible for 'fruity' positive

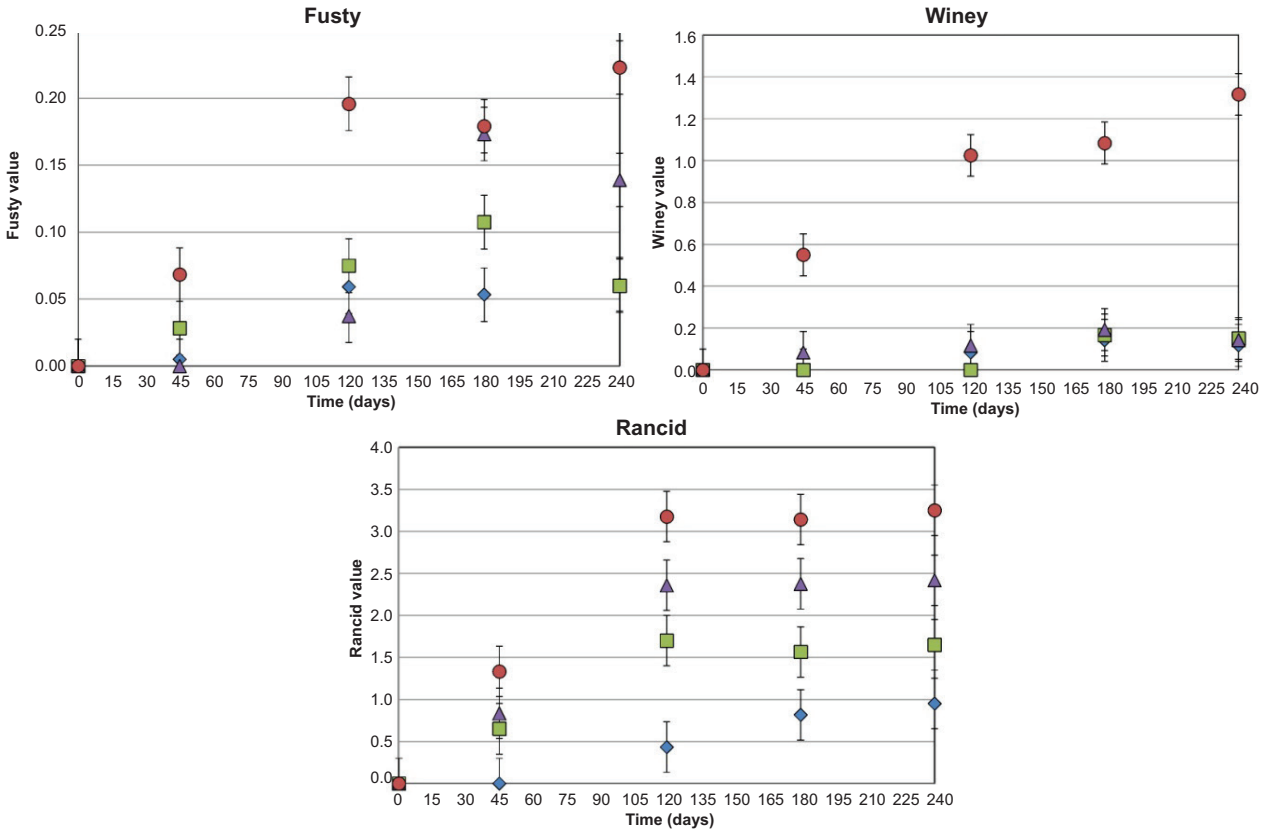

Figure 6. Mean contents and standard error of the 'fusty', 'winey', and 'rancid' defect scores in virgin oil VO (red circle), olive oil containing water only WO (blue diamond), olive oil containing solid particles SO (purple triangle), and filtered oil FO (green square) samples during storage. The R^2 and ADJ-R^2 for each sensory attribute are reported below: 'fusty', R^2 = 0.4908, ADJ-R^2 = 0.4337; 'winey', R^2 = 0.6216, ADJ-R^2 = 0.5792; 'rancid', R^2 = 0.5960, ADJ-R^2 = 0.5507.

sensory attributes. The content of C5 and C6 volatile compounds of LOX pathway was higher in VO samples than in FO and WO samples (Figure 4); however, VO samples had a significant low level of 'fruity' sensory attributes than determined in FO and WO samples. We suppose that significant appearance of 'fusty' defect led panelists to measure decrease in the 'fruity' score of VO samples (Guerrini *et al*., 2020a).

The water content also seemed to protect EVOO against negative oxidative phenomena during storage. The OX:not OX ratio of phenolic compounds (Figure 3) was higher in FO and SO samples than in WO and VO samples because of the stabilizing effect of water on oxidative degradation, as demonstrated in literature (Lercker *et al*., 1994; Ambrosone *et al*., 2002; Koidis and Boskou, 2006; Frega *et al*., 1999). However, the protective effect of water was not shown for chemical parameters, K232, K270, and ΔK, which did not increase significantly during storage as a function of treatments. The effect of treatments was not statistically significant for unpleasant volatile compounds, commonly related to 'rancid' sensory defect. Instead, the 'rancid' sensory defect behavior during storage demonstrated an opposite trend to the above oxidation phenomena: the 'rancid' scores were higher in VO samples than in FO, SO, and WO samples. The significant appearance of 'fusty' defect led panelists to measure an increase in the 'rancid' score of VO samples, since these two defects are characterized by some common volatile compounds (Morales *et al*., 2005).

Conclusions

In this study, an original approach was carried out to understand the significance of VO in terms of preservation of EVOO quality during storage. A clear effect of water content on hydrolytic phenomena and microbial activity was evidenced. Effect of content of solid particles to promote microbial activity was also demonstrated, potentially resulting in the loss of EVOO quality.

The results of the present study asserted that the recommended technique to avoid significant degradation during storage was to quickly filter freshly produced olive oil. However, an immediate filtration is not always possible as veiled olive oil is the product sought for bottling by producers. Therefore, A qualification of oil turbidity,

based on separate measurement of water and insoluble solids contents, is suggested during different processing steps of olive oil chain, such as VO storage in mills, VO supply and storage in oil blenders, and transportation and distribution of veiled EVOO. It follows that, for olive oil producers, the qualification of veiled olive oil in potentially different combinations of water and solid contents (i.e., high–high, high–low, low–high, or low–low) could be useful to plan and control both water/solid separation techniques and storage of oil.

Author Contributions

Lorenzo Guerrini, Alessandro Parenti, and Bruno Zanoni did conceptualization; Carlotta Breschi and Lorenzo Guerrini curated the data. Formal analysis was done by Carlotta Breschi, Ferdinando Corti, and Luca Calamai. Funding acquisition was done by Alessandro Parenti and Bruno Zanoni. Methodology was done by Luca Calamai and Paola Domizio; and software handling was done by Carlotta Breschi and Lorenzo Guerrini. Supervision was carried out by Alessandro Parenti and Bruno Zanoni. Original draft was written by Carlotta Breschi and Lorenzo Guerrini; and the final writing—review and editing—was done by Lorenzo Guerrini, Alessandro Parenti, and Bruno Zanoni.

Funding

This study was supported by the AGER 2 Project, Grant No. 2016-0174, COMPETiTiVE—Claims of Oliveoil to IMProvE The marketValuE of the product.

Conflict of Interest

The authors declare no conflict of interest.

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Table S1 Mean values of sensory attributes and defects of all oil samples for each separation treatment. Superscribed different letters (^{a,b,c}) in the same row indicate significant differences ($p < 0.05$) for different treatments. Superscribed different letters (^{x,y,z}) in the same column indicate significant differences ($p < 0.05$) for different storage periods. Following are reported in the last four columns: standard error; *p*-value for the storage time (*p*-value t); *p*-value for the treatment (*p*-value T); and *p*-value for time–treatment interaction (*p*-value t*T).

	Time (days)	FO#1–FO#6	VO#1–VO#6	SO#1–SO#6	WO#1–WO#6	St. Err.	*p*-value t	*p*-value T	*p*-value t*T	R^2	ADJ-R^2
Fusty	0	$0.00^{a,x}$	$0.00^{a,x}$	$0.00^{a,x}$	$0.00^{a,x}$	0.16	***	***	*	0.4908	0.4337
	45	$0.28^{a,x}$	$0.68^{b,xy}$	$0.00^{a,x}$	$0.05^{a,x}$						
	120	$0.75^{a,xy}$	$1.96^{b,yz}$	$0.38^{a,x}$	$0.59^{a,x}$						
	180	$1.08^{a,y}$	$1.79^{b,y}$	$1.73^{b,y}$	$0.53^{a,x}$						
	240	$0.60^{a,xy}$	$2.23^{c,z}$	$1.39^{b,y}$	$0.61^{a,x}$						
Muddy/Humidity	0	$0.00^{a,x}$	$0.00^{a,x}$	$0.00^{a,x}$	$0.00^{a,x}$	0.06	***	n.s.	n.s.	0.2023	0.1129
	45	$0.00^{a,x}$	$0.00^{a,x}$	$0.00^{a,x}$	$0.00^{a,x}$						
	120	$0.63^{a,y}$	$0.78^{a,y}$	$0.50^{a,y}$	$0.66^{a,y}$						
	180	$0.00^{a,x}$	$0.30^{b,xy}$	$0.08^{a,x}$	$0.00^{a,x}$						
	240	$0.00^{a,x}$	$0.39^{b,xy}$	$0.10^{a,x}$	$0.00^{a,x}$						
Winey	0	$0.00^{a,x}$	$0.00^{a,x}$	$0.00^{a,x}$	$0.00^{a,x}$	0.09	***	***	**	0.6216	0.5792
	45	$0.00^{a,x}$	$0.55^{b,xy}$	$0.08^{a,x}$	$0.00^{a,x}$						
	120	$0.00^{a,x}$	$1.03^{b,y}$	$0.12^{a,xy}$	$0.08^{a,x}$						
	180	$0.17^{a,y}$	$1.08^{b,y}$	$0.19^{a,y}$	$0.14^{a,x}$						
	240	$0.15^{a,y}$	$1.32^{b,y}$	$0.14^{a,xy}$	$0.12^{a,x}$						
Racid	0	$0.00^{a,x}$	$0.00^{a,x}$	$0.00^{a,x}$	$0.00^{a,x}$	0.26	***	***	n.s.	0.5960	0.5507
	45	$0.65^{ab,x}$	$1.33^{b,y}$	$0.83^{ab,x}$	$0.00^{a,x}$						
	120	$1.70^{a,y}$	$3.18^{c,z}$	$2.36^{b,y}$	$0.43^{a,xy}$						
	180	$1.57^{a,xy}$	$3.14^{c,z}$	$2.38^{b,y}$	$0.82^{a,y}$						
	240	$1.65^{a,xy}$	$3.25^{c,z}$	$2.42^{b,y}$	$0.95^{a,y}$						
Fruity	0	$3.40^{a,y}$	$3.37^{a,y}$	$3.12^{a,y}$	$3.57^{a,z}$	0.19	***	***	n.s.	0.5339	0.4816
	45	$2.98^{a,xy}$	$2.33^{a,xy}$	$2.63^{a,xy}$	$3.03^{a,xy}$						
	120	$2.31^{b,x}$	$1.03^{a,x}$	$1.83^{a,x}$	$1.97^{b,x}$						
	180	$2.48^{b,x}$	$1.18^{a,x}$	$1.16^{a,x}$	$2.65^{b,xy}$						
	240	$2.51^{b,x}$	$1.05^{a,x}$	$1.01^{a,x}$	$2.11^{b,x}$						
Bitter	0	$3.42^{a,z}$	$3.30^{a,z}$	$2.80^{a,y}$	n.d.	0.27	-	-	-	0.7410	0.7119
	45	$2.85^{a,y}$	$2.03^{a,y}$	$2.72^{a,y}$	n.d.						
	120	$1.93^{b,x}$	$0.47^{a,x}$	$2.27^{b,xy}$	n.d.						
	180	$2.99^{b,y}$	$1.68^{a,y}$	$1.95^{b,x}$	n.d.						
	240	$2.58^{b,xy}$	$1.12^{a,xy}$	$1.90^{b,x}$	n.d.						
Pungent	0	$4.96^{a,z}$	$4.53^{a,z}$	$4.90^{a,z}$	n.d.	0.39	-	-	-	0.8327	0.8139
	45	$3.53^{b,y}$	$2.73^{a,y}$	$3.93^{b,y}$	n.d.						
	120	$1.78^{b,x}$	$0.63^{a,x}$	$2.63^{b,x}$	n.d.						
	180	$3.40^{b,y}$	$1.21^{a,x}$	$3.08^{b,x}$	n.d.						
	240	$2.75^{b,xy}$	$1.05^{a,x}$	$2.97^{b,x}$	n.d.						

n.s., *, **, and *** indicate significant differences by two-way ANOVA at $p > 0.05$, $p < 0.05$, $p < 0.01$, and $p < 0.001$. n.d. = not detected. Number of replicates = 6. VO: virgin oil; WO: olive oil containing water only; SO: olive oil containing solid particles; FO: filtered oil.

Italian Journal of Food Science, 2021; 33 (3): 47–69

Circular economy in the brewing chain

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Received: 21 September 2021; Accepted: 3 December 2021; Published: 14 December 2021

REVIEW

Abstract

The main aim of this review was to check for the applicability of the concept of circular economy to brewing chain. By analyzing the beer brewing process, it was possible to identify the main brewery wastes formed and packaging materials used as well as their range of composition and yields. In order to reduce the contribution of packaging material to the carbon footprint of beer, it would be necessary to replace one-way containers used nowadays with lighter, reusable, or recycled ones. Even if the contribution of beer consumption phase was taken into account, there was no definitive solution about the less environmentally impacting beer packaging format. The direct management of polyethylene terephthalate (PET) packaging for liquid foodstuffs could make available 100% recycled PET flakes to be reconverted into food-grade bottles with minimum downcycling to other non-food usage. The countless potential uses of brewery wastes in nutritional and biotechnological fields were tested in laboratory by disregarding any cost–benefit or market analysis. This was mainly because the estimated market price of dried brewer's spent grain (BSG) resulted to be about 450% higher than that of conventional lignocellulose residues. All the alternative uses hailed in the literature appeared to be more useful for publishing articles than for defining any economically feasible reusing procedure for all brewery wastes. Owing to their high moisture content, such wastes are so perishable as to prevent their safe usage in the human food chain. Currently, their use as-is in animal feeding is the disposal method not only economically feasible but also able to reduce the greenhouse gas load of beer packed in glass bottles (GB) by about one-third of that associated with packaging materials. Not by chance, it is practiced by most industrial and craft breweries.

Keywords: beer chain; beer packaging formats; brewer's spent grain; brewer's spent yeast; carbon footprint; environmental impact; hot trub; post-consumer packaging waste; disposal methods; spent hops

Introduction

Beer is a globally consumed alcoholic beverage (about 1.91 billion hectoliter (hL) in 2019; Statista, 2021), with its overall market in 2020 amounting to US$623.2 billion (IMARC Group, 2021). In Italy, the overall production of beer in 2020 was about 15.8 million hL, about 71% of which being produced by five major players, such as Heineken Italia with a share of 33.3%, Birra Peroni with 18.3%, Anheuser-Busch InBev with 8.6%, Birra Castello with 5.8%, and Carlsberg Italia with 5.3% share (Associazione dei Birrai e dei Maltatori [Assobirra], 2020). In 2020, 756 craft breweries (i.e., 624 microbreweries and 132 brewpubs) produced 361,000 hL of beer with an average specific gravity of 14° Plato, this being equal to ~3.1% of the Italian beer production (Assobirra, 2020). In Italy, the per capita consumption of beer in 2019 was about 35.2 L. Standard lager is the most popular beer type, representing 84.2% of the overall beer consumption, followed by specialty beers (14.5%)

and low or nonalcoholic ones (1.3%). Owing to decrease in beer consumption outside the home from 45.5% in 1999 to 36.1% in 2019, and conversely the increase in off-sales, the prevailing beer packaging format is dominated by glass bottles (GB; 80.8%), followed by stainless steel kegs (SSK; 11.7%), and finally aluminum cans (AC; 7.5%) (Assobirra, 2020). Most consumers purchase beer in glass bottles (73.0% and 7.8% of which being produced from nonreturnable and returnable GBs, respectively) or aluminum cans, while beer packaged in stainless steel kegs is chiefly for commercial use.

Owing to predictable increase in the global demand of food, this currently representing from 22% to 37% of the world anthropogenic greenhouse gas (GHG) emissions (Rogissart *et al.*, 2019), the economic growth of the food and beverage industry is expected to be greatly hampered by climate-related risks to food security, and water and energy supply (Intergovernmental Panel on Climate Change [IPCC], 2014). As reported by the Beverage Industry Environmental Roundtable (BIER, 2012), the beverage sector has not only started to reduce its impact on the global climate but also to rethink its business models, products, and processes according to the principles of circular economy (Bocconi University *et al.*, 2021).

Over the last 20 years, several business-to-business or business-to-consumer studies (Amienyo and Azapagic, 2016; BIER, 2012; Cimini and Moresi, 2016; Environmental Product Declaration® [EPD], 2011a, 2011b, 2014a, 2014b; Hospido *et al.*, 2005; Koroneos *et al.*, 2005; Muñoz *et al.*, 2012; Narayanaswamy *et al.*, 2005; Shin and Searcy, 2018; Talve, 2001; Williams and Mekonen, 2014) have been conducted to evaluate the environmental impact of beer as packed in different formats, as summarized by Cimini and Moresi (2018c).

Glass bottle or aluminum-can manufacturing and barley cultivation represented the main hot spots of beer life cycle (Amienyo and Azapagic, 2016; Cimini and Moresi, 2016). Only when using reusable steel kegs, barley production was the most impacting step, followed by brewing and distribution (Cimini and Moresi, 2016). As estimated by Mata and Costa (2001) and confirmed by Amienyo and Azapagic (2016), the contribution of returnable glass bottles to the carbon footprint (CF), acidification, photochemical ozone creation, human toxicity, and energy and raw material consumption was smaller than that of nonreturnable bottles after the second reuse, while that to eutrophication, ozone depletion, solid waste, water and auxiliary material consumption was larger even after several reuses.

Holland (2021) described a few means to address the most environmentally altering effects of beer production and distribution (e.g., GHG emissions, and disposal of wastewaters and post-consumer packaging wastes) using circular thinking approaches. The author cited either quite exiguous initiatives (e.g., 100% biodegradable, edible six-pack ring pulling on cans prepared from barley and wheat ribbons, adopted by Saltwater brewery to replace the conventional plastic ones) or more significant ones (e.g., use of waste bread instead of malted barley by Toast Ale in Belgium, wind turbines and solar panels as sources of nonrenewable energy by Heineken in Holland and Italy).

The main aim of this review was to further verify the applicability of circular economy concepts to the brewing chain. To this end, the main steps of the beer brewing process were outlined to point out main brewery wastes in terms of composition and yield factors. Then the real and effective reuses of packaging and biotic wastes were critically reviewed based on their techno-economic feasibility.

Inventory Analysis of the Brewing Process

Figure 1 shows block diagram of the beer production process from its basic raw material, that is, barley. The average chemical composition of barley is provided in Table 1. It differs with barley variety and environmental conditions. The starch, β-glucan, protein, fat, and ash contents vary in the ranges of 65–68%, 4–9%, 10–17%, 2–3%, and 1.5–2.5% (dry basis), respectively (Alijošius *et al.*, 2016; Gupta *et al.*, 2010). An ideal protein content of barley destined to brewing ranges from 9.5% to 12.8% (dry basis), while higher protein contents are suitable for producing malt for distilling (Grains Research & Development Corporation (GRDC), 2018; Paynter, 1996).

Malting is the first step of the brewing process. It consists of three different unit operations: steeping, germination, and drying. During steeping, barley seeds are soaked in water until imbibed with sufficient water to start their sprouting process. The germination phase allows a series of amylases, proteases, and other endogenous hydrolytic enzymes to be produced and/or activated. Final drying stops further growth of germs, reduces water activity, and thus yields a shelf-stable product with active enzymes (i.e., barley malt). The average malt-to-barley ratio ranges from 0.75 (Climate Conservancy, 2008) to 0.79 (Food and Agriculture Organization [FAO], 2009). The main byproduct of malting (i.e., barley rootlets, also known as malt culms, coombes, or sprouts) represents 3–5% (w/w) of the malt produced. It may contain other wastes, such as malt dust, small and broken barley grains, barley dust, acrospires, and husk fractions. As depicted by Neylon *et al.* (2020), its range of composition is provided in Table 1.

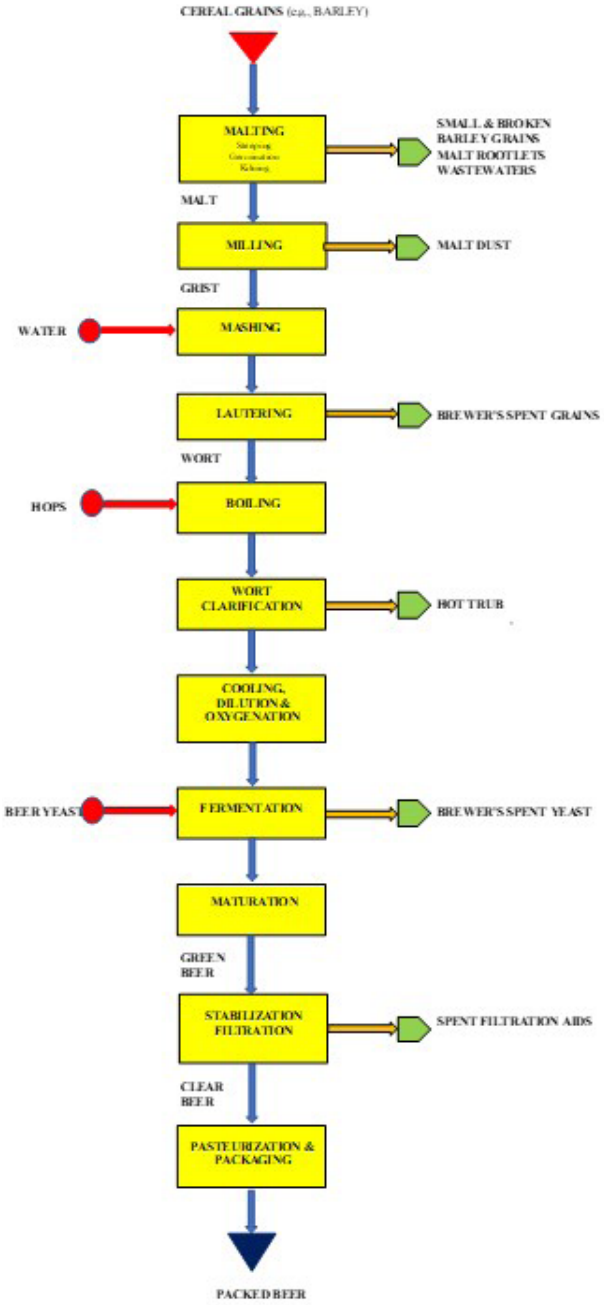

Figure 1. Schematic of the beer production process and its main brewery byproducts.

Malt milling is the second step. It allows the malt to be separated from its chaff, coarsely ground, and sifted into three fractions, namely, the husk, grits, and flour. The smaller the particle size, the greater the extract (and the smaller the filtration rate). Husk is the outer layer of barley kernel undergoing grinding and must be kept intact as possible to allow the formation of porous filter beds and thus minimize filtration time. Larger particles can be reground, thus yielding other byproducts, such as hull fractions and fine malt powders (Crescenzi, 1987; Stubits *et al.*, 1986).

Mashing is the third step. The grit is suspended into hot water to allow starch sugars, proteins, and tannins to be dissolved in the so-called malt extract. In European breweries, malted barley consumption ranges from 15 kg/hL to 18 kg/hL of beer produced (United Nations Environment Program [UNEP], 1996), while in Italian industrial breweries, 1 hL of beer at a specific gravity of 12°Plato (equivalent to an ethanol content of about 5% v/v) needs approximately 12-kg malt and 4-kg unmalted cereals, such as corn grits (Assobirra, 2020). The specific consumption of malted barley appears to be inversely proportional to the brewery size. It is as high as 28–32 kg hL^{-1} in the case of craft breweries with an annual capacity of about 1,000 hL of beer (Beloborodko *et al.*, 2014; Sturm *et al.*, 2012) and as low as 18–20 kg hL^{-1} in the case of industrial breweries with an overall capacity of more than 1 million hL/yr (Cimini and Moresi, 2018c). Referring to the overall volume of abstracted water during malting, brewing, and clearing steps, the specific water consumption was about 4.2 L per liter of beer produced in Italian breweries (Assobirra, 2020), and it can range from 3.5 to 10 L per liter of beer (Olajire, 2020) or to as high as 19 L (Sturm *et al.*, 2013) or 34 L of specific water per liter of beer produced (Pauli, 1997) in old micro-breweries.

Lautering is the fourth step of the brewing process. It is carried out in the lauter tun to fractionate wort from the so-called wet brewer's spent grains (BSGs), these being the chief byproduct of this process with an average amount of 16 kg hL^{-1} of beer produced (Assobirra, 2020). Table 1 provides their range of composition and amount, as described by several authors along with the International Finance Corporation (IFC, 2007) and UNEP (1996).

Wort boiling or *hopping* is the fifth step, which comprises the addition of different types and amounts of hops to boiling wort according to the style of beer to be produced. As boiling proceeds, the malt enzymatic pool is deactivated, and as water is evaporated, malt proteins and hop tannins tend to precipitate at the bottom of the concentrated hopped wort. The average amount of hop pellets used in the Italian breweries is around 260 g hL^{-1} (Assobirra, 2020), even if it is quite lower (~92 g hL^{-1}) in the case of lager (Cimini and Moresi, 2016).

Wort clarification is the sixth step. By feeding tangentially the hopped wort in a whirlpool separator, the resulting moderate centrifugal action allows the *hot trub* and spent hops to be separated from clear wort. Table 1 provides the range of composition of such a proteinaceous residue, as described by Rachwał *et al.* (2020). Moreover, its overall quantity varies from 0.2 to 0.4 kg hL^{-1} depending on the amount and type of protein present in the used barley, which, in turn, is dependent on crop location, seasonal factors, and genetics (Barchet, 2019).

Table 1. Range of chemical composition of barley and its main brewery byproducts, namely, malt barley rootlets (MBR), brewer's spent grain (BSG), spent hops/hot trub (HT), and brewer's spent yeast (BSY).

Component	Barley	MBR	BSG	HT	BSY	Unit
Moisture	12.8	8.2–12.9	75–90	80–90	74–86	g/100 g
Carbohydrates	0.624	0.51–0.60	0.45–0.61	0.20	0.4	g/g DM
Protein	0.113	0.203–0.387	0.142–0.300	0.40–0.70	0.15–0.49	g/g DM
Fat	0.019	0.017–0.044	0.06–0.13	0.045	0.04–0.10	g/g DM
Ash	0.03	0.028–0.087	0.011–0.050	0.06–0.25	0.02–0.085	g/g DM
Total fiber	0.215	0.43	0.44–0.84	0.23–0.26	0.25–0.53	g/g DM
Specific amount		0.03–0.05 kg kg^{-1} malt	14–19	0.2–0.4	2–4	kg/hL beer
References	Alijošius *et al.*, 2016	Neylon *et al.*, 2020	Jackowski *et al.*, 2020; Karlović *et al.*, 2020; Rachwał *et al.*, 2020			

DM: dry matter; hL: hectoliter.

Wort cooling is the seventh step. It allows the clear wort to be cooled to a temperature depending on the yeast used and the style of beer being produced. It usually ranges from 16 to 20°C and from 10 to 13°C in the case of ale and lager production, respectively. If the wort coming out of the whirlpool separator has a higher strength than that required for fermentation, it is diluted with water to obtain its correct specific gravity. Moreover, to allow yeast replication in the early stages of fermentation and guarantee adequate fermentation, an appropriate level (7–18 mg/L) of dissolved oxygen in the wort is provided by aerating the wort on hot or cold side of wort heat exchanger. The oxygen consumption was 1.43 g/hL of lager produced (Cimini and Moresi, 2016).

Wort fermentation is the eighth step of the brewing process by which fermentable sugars are converted into ethanol, carbon dioxide, and several other metabolic byproducts by brewer's yeast. These have a significant effect on the taste, aroma, and other characteristic properties of the style of beer under production. On stoichiometric basis, 1-g maltose must be theoretically converted into 0.538-g ethanol. The average inoculation rate in Italian breweries is 0.8 kg hL^{-1} of beer (Assobirra, 2020). Such a phase is usually accomplished in cylindroconical fermenters. Their angle at the bottom of tanks allows the yeast to settle in the bottom of conic vessel at the end of primary fermentation before being collected without exposure to air. Thus, a rough beer relatively free of yeast could be discharged. After harvesting, the yeast is stored with its own liquid under gentle agitation at 0°C for next fermentation. In this manner, brewer's yeast could be used sequentially for four to six times (Karlović *et al.*, 2020). The range of composition of brewer's surplus yeast (BSY) is provided in Table 1, as described by different studies. Its amount ranges from 2 to 4 kg hL^{-1} of the beer produced (IFC, 2007; UNEP, 1996); its mean value in Italy was 1.6 kg hL^{-1} by assuming a 10% dry matter (DM) content (Assobirra, 2020).

Maturation is the ninth step of the process. The freshly brewed liquid undergoes the second fermentation, providing beer its characteristic color in as long as 3 weeks to 3 months depending on the type of beer being produced.

Beer clarification and stabilization is the tenth step. Proteins, yeast particles, and resins from the hop left in the beer after the first and second fermentation phases are generally removed by filtration in the presence of filter aids (i.e., kieselguhr slurry or diatomaceous earth [DE]). Then, to avoid permanent or chill haze (Siebert *et al.*, 1996), haze active polyphenols are removed by using polyvinylpolypyrrolidone (PVPP), while haze active proteins are removed by means of silica hydrogel or tannic acid. In European breweries, the specific consumption of DE ranges from 80 to 570 g/hL of beer (IFC, 2007; UNEP, 1996), while that of non-regenerable PVPP ranges from 20 to 40 g (Gopal and Rehmanji, 2000) and that of regenerable PVPP by around 0.1 g hL^{-1} (Cimini and Moresi, 2015).

Filling is the final step of the beer brewing process. If the finished beer is to be kept in glass bottles, it is first bottled and then batch-pasteurized to prolong its shelf life. When using cans or kegs, clear beer undergoes flash-pasteurization and is packed aseptically. By referring to main packaging formats in use (Cimini and Moresi, 2016, 2018c), 1 hL of lager (weighing ~100.5 kg) would require as much as 43.9 or 56.1 kg of 66-cL or 33-cL amber glass bottles, just 4.4 kg of 66-cL polyethylene terephthalate (PET) bottles, 4.9 kg of 33-cL aluminum cans, and as much as 32.0 kg of 30-L stainless steel kegs (Table 2). This clearly the great contribution of packaging materials per unit volume of beer delivered, especially in the case of glass bottles and stainless steel kegs.

For further details of the brewing process, refer to Eßlinger (2009).

Table 2. Mass of the packaging materials used to pack 1 hL of beer in different formats (66-cL and 33-cL amber glass bottles [GB]; 66-cL PET bottles [PB]; 33-cL aluminum cans [AC]; 30-L stainless steel kegs [SSK]), as described by Cimini and Moresi (2016, 2018c).

Packaging format Packaging materials	66-cL GB	33-cL GB	66-cL PB	33-cL AC	30-L SSK	Unit
Glass	43.9	56.1	0.0	0.0	0.0	kg/hL
Paper & cardboard	3.0	3.4	3.0	1.2	0.0	kg/hL
Plastic	0.1	0.1	4.4	0.3	0.0	kg/hL
Steel	0.3	0.6	0.0	0.0	32.0	kg/hL
Aluminum	0.0	0.0	0.0	4.9	0.0	kg/hL
Wood	2.8	3.2	3.0	1.9	2.5	kg/hL
Adhesive materials	0.2	0.2	0.2	0.2	0.0	kg/hL
Overall	50.3	63.6	10.6	8.5	34.5	kg/hL

Application of the Concept of Circular Economy to the Beer Brewing Process

The *linear economy* model reflects man-made ecosystems for food production, which requires not only a continuous supply of energy and mass from outside, as nutrients are not recycled at the crop cultivation site, but also the treatment of wastes. On the contrary, the so-called *circular economy* model refers to natural ecosystems, which are capable of self-regeneration. As sketched in Figure 2, its priority area is aimed at eliminating waste and pollution, keeping products and materials in use, and regenerating natural systems (Bocconi University *et al.*, 2021).

According to the waste hierarchy set out in Article 4 of the revised waste framework (Directive 2008/98/EC; European Union [EU], 2008), any waste must be handled in a manner that does not have a negative impact on the environment or human health. First, its formation must be prevented using, for instance, less material in design and manufacture. When the waste has been formed, its entire apparatus or replacement parts must be refurnished to be reused, recycled, submitted to other recovery options, and, as the least preferred option, disposed of via landfilling or incineration with no recovery of energy. Such a waste hierarchy was further detailed by Garcia-Garcia *et al.* (2015), as shown in Figure 3.

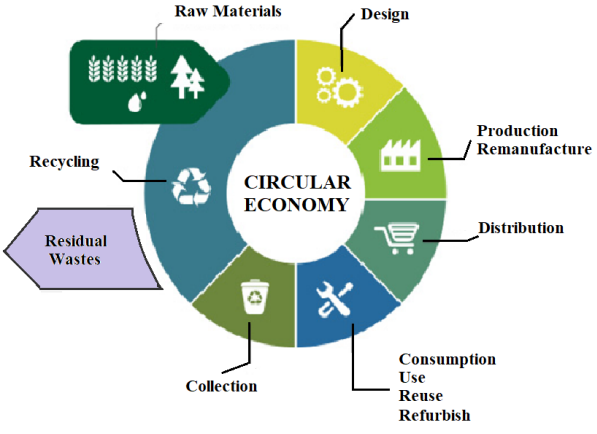

Figure 2. Schematic of the circular economy concept.

Prevention of wastage of food is the alternative preferred mostly, followed by food redistribution to people in need, and then to animals, unless it is composed of products of animal origin or is a catering waste. If such options are not applied, then food waste can be regarded as a source from which several valuable products (e.g., fats, proteins, polysaccharides, polyphenols, etc.) could be extracted selectively. Then it may be submitted for anaerobic digestion, composting, thermal valorization, or spread for land fertilization or improvement. Waste burning with no energy recovery and landfilling are regarded as the least preferable management options to use (Garcia-Garcia *et al.*, 2015).

By referring to the brewing chain, application of the concept of circular economy essentially refers to the following two aspects:

1. The reuse of abiotic materials, such as packaging materials, and spent kieselguhr, their overall weight being mainly made of glass bottles, which are, for instance, used to pack about 81% of the entire beer produced in Italy (Assobirra, 2020).
2. The reuse of biotic materials, namely, the main byproducts of the beer brewing process.

End of Life of Abiotic Materials

Packaging materials

All abiotic materials arising from beer packaging and pallet management at distribution centers (DC) generally undergo separate waste collection to allow recycling of

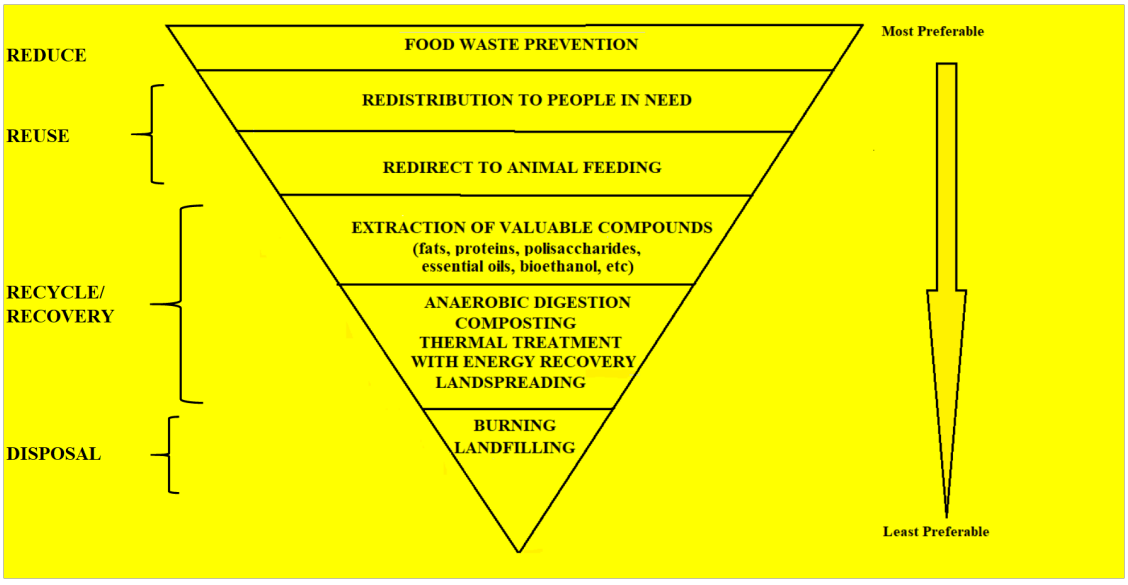

Figure 3. Food waste hierarchy used to select different waste management alternatives according to their environmental preference, as reworked by Garcia-Garcia *et al.* (2015).

glass, plastic, aluminum, steel, paper, or wood for their recycling.

The discarded fraction of packaging components varies from 0.4% in the case of glass bottles to 3.5% in the case of the stretch wrap film, as detected in an industrial brewery (Cimini and Moresi, 2016).

The disposal scenarios of all packaging wastes, resulting from beer processing and post-consumption, generally coincide with those of municipal solid waste. For instance, Table 3 refers to the overall Italian management scenarios in 2019 (Mariotta and Tuscano, 2020). It is noted that the minimum objective (65%) of recycling, in terms of weight, for all packaging wastes, to be met by 31 December 2025 according to Directive 2018/852/EU (EU, 2018), has been achieved nationwide since 2019, although there are differences in some districts of South Italy and plastic recycling rate is still lower than the target value of 50% (Mariotta and Tuscano, 2020).

In order to measure the environmental impact of main packaging materials used to pack beer, we referred to the only effect of beer on climate change, since this impact category was found to be highly correlated to the fossil *cumulative energy demand*, which, in turn, affected several other categories, such as acidification, eutrophication, and photochemical ozone formation (Huijbregts *et al.*, 2006) as well as the nitrogen and phosphorous emissions resulting from synthetically fertilized soils used for agri-food cultivation (Huijbregts *et al.*, 2010). Under these circumstances, by accounting for the overall weight factors attributed to the effects of such impact categories in the product environmental footprint methodology,

Table 3. Overall Italian waste management scenarios for packaging wastes in 2019, as described by Mariotta and Tuscano (2020).

Waste management scenarios	Landfill	Recycling	Incineration
Waste	(%)	(%)	(%)
Glass	22.7	77.3	0
Paper and cardboard	11.6	80.8	7.6
Iron	17.8	82.2	0
Plastic	10.1	45.5	44.4
Aluminum	23.9	70.0	6.1
Wood	34.8	63.1	2.1
Overall	**19.2**	**70.0**	**10.8**

the contribution of climate change and its related impact categories would represent 50% or 53% of the overall weighted endpoint score when including or excluding the toxicity-related impact categories (Sala *et al.*, 2018). Owing to the diverse contribution of packaging materials and transportation, the beer packaging format exerted a diverse influence on the GHGs emitted to produce and distribute industrial beer from the factory gate to the distribution centers, as assessed previously (Cimini and Moresi, 2016). By disregarding the GHG credits derived from the use of brewery wastes as cattle feed, these being about 12 kg of carbon dioxide equivalent (CO_{2e}) per hL of beer (Cimini and Moresi, 2016), the business-to-business product carbon footprint (CF) of beer was found to be of the order of 69 or 78 kg CO_{2e} hL^{-1}, or 81 or 37 kg CO_{2e} hL^{-1} if the beer was packed in 66- or 33-cL glass bottles, or 33-cL aluminum cans or 30-L stainless steel kegs, respectively (Table 4). Since kegs, on average, are

Table 4. Contribution of different life cycle phases to the GHG emitted to produce and distribute 1 hL of lager as packed in containers of different volumes and masses, as described by Cimini and Moresi (2016).

Beer primary packaging type	GB		AC	SSK
Volume (L)/Mass (kg)	0.66/0.290	0.33/0.185	0.33/00123	30.00/9.6
Life cycle phases	**GHG emissions (kg CO_{2e} hL^{-1})**			
Raw materials & processing aids	16.88	16.88	16.88	16.88
Brewing processing & packaging	8.41	8.4	8.33	8.41
Packaging materials	33.33	42.19	47.55	1.86
Transportation	9.71	10.67	8.09	9.26
Waste disposal	0.58	0.58	0.57	0.61
Beer production and distribution	**68.91**	**78.71**	**81.42**	**37.02**

GHG: greenhouse gas; GB: glass bottles; AC: aluminum cans; SSK: stainless steel kegs.

used for 72 times, the contribution of packaging materials was just 5% of the overall GHG burden, while that of glass bottles and aluminum cans was from 5 to 6 times higher, respectively. On the contrary, the contribution of transportation increased to 25% in the case of kegs in consequence of their tare (9.6 kg), while it was, respectively, 14% or 10% if glass bottles or aluminum cans are used (Table 4).

Thus, to reduce the contribution of packaging materials to the carbon footprint of beer, it would be necessary to resort to:

1. lighter bottles or kegs,
2. bottles, including greater percentage of recycled materials,
3. containers reusable as many times as possible.

In the case of beer packed in glass bottles, 10% decrease in the mass of glass bottles would reduce the carbon footprint of beer by 2.4–2.6% (Cimini and Moresi, 2016) to a maximum of 5% (Amienyo and Azapagic, 2016) due to lower impact for their manufacture and transportation. Approximately 70% less GHG emissions were estimated when the Tuborg® beer was packed in 20-L plastic drums weighing 290 g each (EPD, 2011a).

Further savings are expected by the replacement of glass bottles or aluminum cans with nanoclay-enriched polyethylene terephthalate (PET) bottles, their empty bottle weight being ~26 g, and their carbon footprint near to one-third of that (~9 kg CO_{2e} kg^{-1}) of 50% recycled aluminum cans (Cimini and Moresi, 2016, 2018c). As provided in Table 5, when beer was packed in PET bottles instead of glass bottles, the GHG contribution of packaging materials reduced by about 29%, while that of transportation by 25–57%, as the brewery capacity was reduced from 2 million to 500 hL per year.

Nevertheless, as expected, the use of such a lighter packaging material was not so effective, since the cradle-to-grave GHG burden of beer decreased by as low as ~1.7–3.3%. This was a direct consequence of different average recycling rates of glass (70.3%) and plastic (37.9%) wastes registered in Italy in 2014 (Cimini and Moresi, 2018c). In fact, the GHG load of brewery and post-consumer packaging waste disposal was negative in the case of glass bottles (–11 kg CO_{2e} hL^{-1}) but positive in the case of PET bottles (+4.6 kg CO_{2e} hL^{-1}). By referring to the current higher recycling rates of such packaging wastes (Table 3), the glass and plastic recycling rates, respectively, increased by circa +10% and +20% with respect to the aforementioned basic values. This involved a reduction in the cradle-to-grave carbon footprint of 0.9–1.3% and 0.6–0.8% with respect to the basic cases, as the brewery capacity increased from 500 to 2 million hL per year, as estimated by using the same life-cycle assessment (LCA) model of beer production developed previously (Cimini and Moresi, 2018c).

Moreover, the use of steel cans would give rise to lower effect than usage of glass bottles and aluminum cans not only on GHG emissions (BIER, 2012) but also on other environmental impact categories, such as eutrophication, creation of photochemical oxidants, and freshwater aquatic ecotoxicity potentials (Amienyo and Azapagic, 2016).

Any increase in glass or PET recycled content would improve the environmental sustainability of resulting bottles either for the lower energy needed for their manufacture or the minor quantity of post-consumption packaging waste to be landfilled. In fact, since the bottle emission factor linearly decreases as the recycled material content increases, 10% increase in the recycled glass or PET content reduced the carbon footprint of beer by 2.2–2.5% depending on the size of brewery (Cimini

Table 5. Contribution of main beer life cycle phases to cradle-to-grave (C2G) carbon footprint (CF_{C2G}) of 1-hL beer packed in 66-cL glass or PET bottles by breweries of different annual capacity, as described by Cimini and Moresi (2018c).

Brewery capacity (hL/year)	2×10^6		5×10^5		5×10^4		5×10^2	
Beer primary packaging type	GB	PB	GB	PB	GB	PB	GB	PB
Life cycle phases	GHG emissions (kg CO_{2e} hL^{-1})							
Raw materials and processing aids	23.9		27.0		30.6		41.7	
Brewing and packaging processing	12.1		13.7		16.8		49.5	
Packaging materials	34.0	24.0	34.3	24.3	33.6	23.7	33.6	23.7
Waste and effluent disposal	1.9	2.0	3.1	3.1	0.6	0.6	0.6	0.6
CO_{2e} credits from byproduct use as feed	–2.2	–2.2	–2.5	–2.5	–2.9	–2.9	–3.8	–3.8
Transportation to DCs	19.8	15.1	20.5	13.5	22.5	12.6	15.6	5.9
Transportation from DCs to retailers	9.7	7.1	7.3	5.3	2.4	1.8	2.4	1.8
Retailer refrigeration	0.3		0.3		0.3		0.3	
Consumer phase	18.1		18.1		18.1		18.1	
Post-consumer waste disposal	–11.0	4.6	–11.0	4.6	–11.0	4.6	–11.0	4.6
CF_{C2G}	**106.7**	**104.9**	**110.8**	**107.5**	**111.0**	**106.1**	**147.1**	**142.3**

GHG: Greenhouse gases; DCs: distribution center; GB: glass bottles; PB: PET bottles.

and Moresi, 2018c) or by approximately 3% in the case of glass bottles as estimated by Amienyo and Azapagic (2016). Except for water demand, all other environmental impact categories reduced by 0.5% in the case of eutrophication potential, and 2% in the case of abiotic depletion potential (Amienyo and Azapagic, 2016).

Thus, the idea of increasing recycling rate has proliferated in several countries. For instance, in France, mineral water in bottles made of 100% recycled PET (R-PET) has been commercialized since 2019. In Italy, usage of R-PET for producing bottles and trays for food was approved by the 2021 Budget Law on 31 December 2020 with the condition that the material derived from other bottles would be used for food purposes only. In Germany, thanks to the container deposit legislation operating since 1 January 2003 and despite the opposition of the German bottling industry and retailers, any empty plastic or glass bottle returned to a grocery store receives a credit ranging from €8 to 25 cents to be discounted at the cash desk. To avoid any contamination problem, recycling companies currently submit plastic bottles to the following procedure. First, such items are automatically collected using the barcode system, sorted by type and color, and aggregated in bales. Once foreign materials, labels and caps are removed by infrared-ray sorting and pre-washing, the resulting material is shredded into flakes, which are then dried at 150–180°C. The food reuse of such R-PET flakes involves a decontamination process of thermal or chemical type at 280°C or with a caustic detergent. This procedure has been also adopted by CORIPET (https://coripet.it/), a voluntary nonprofit consortium of producers, converters, and recyclers of PET bottles. The procedure was recognized as an autonomous system for the direct management of PET packaging of liquid foodstuffs by the Italian Ministry of Environment (see Decree No. 44 of 28 July 2021). Thanks to the deposit system adopted in Germany, 99% of refillable bottles and 97% of one-way bottles are returned to supermarkets and grocery stores (ANON, 2017). Thus, such a packaging waste recycling system must theoretically give rise to PET recycling rates near to 100%. To this end, CORIPET is intended to reach 25% PET recycling by 2025. In Germany, 96–98% R-PET recovery in 2015 supported the formation of new PET bottles from about 34% of total recovery, the remainder being directed to non-food uses, such as plastic sheets and films (27%), textile fibers (22.6), and so forth (Deutsche Welle [DW], n.d.).

The German container deposit legislation appears to be an appropriate incentive for transiting toward a circular economy, even if it has so far given rise to a greater aliquot for downcycling (66%) than for effective recycling and reuse, which indeed would strictly require the conversion of empty bottles into new useable bottles for food purposes. Strictly speaking, in the case of beverage packaging, a sustainable waste management must make use of refillable bottles only (ANON, 2017).

Since it is useless to reinvent the wheel, it is worth remembering that up to the early 1960s, the Italian customers used to pay a deposit on each glass bottle bought, which they reclaimed by returning the empty bottle. Obviously, such a system could become valid not only for glass bottles but also for plastic ones, even if the latter are refilled for 20–25 times and the former for up to 50 times (DW, n.d.). Of course, the reintroduction of reusable bottles demands not only for new infrastructure,

financial incentives, and behavioral changes (Amienyo and Azapagic, 2016) but also for several other factors such as the distance empty bottles travel by road for cleaning and refilling, and the water and detergents used for the cleaning process. Such factors were accounted for the carbon footprint of beer in reusable 30-L stainless steel kegs was half of that of beer packed in 66-cL glass bottles (Table 4). Nevertheless, this cannot be decisive for reducing the environmental impact of beer, since 80.8% of beer sales are in glass bottles and just 11.7% in stainless steel kegs (Assobirra, 2020).

The environmental impact of returnable glass bottles was assessed either for beer in Portugal (Mata and COSTA, 2001) or for carbonated soft drinks in the United Kingdom (Amienyo *et al.*, 2013). In both studies, the environmental load depended on the percentage of bottles returned and the number of times each bottle was reused. In the case of 50% reuse and up to six reuse cycles, returnable bottles reduced several impact categories, except eutrophication and ozone layer depletion (Mata and Costa, 2001). Global warming was reduced by ~40%, as the glass bottles were reused just once, its minimum asymptotic value being achieved for eight reuses (Amienyo *et al.*, 2013).

Before deciding which is really less environmentally -impacting among one-way, recycled, and or reusable beer packaging, the contribution of beer consumption phase (embracing not only beer refrigeration, dispensing, and losses but also consumer displacement and treatment of the wastewater formed) should be assessed, as specified by the beer product category rules (EPD, 2019; Technical Secretariat for the Beer Pilot [TSBP], 2016).

Normand *et al.* (2012) and Watson (2008) recommended the consumption of beer in kegs directly in pub, within a walking distance to avoid car use, especially if the pub was supplied directly from the neighboring brewery via pipelines. In addition, keg distribution was found to affect negatively the local traffic at historic sites, such as Bruges in Belgium (AFP, 2014), or during beer festivals, such as the October fest in Munich, Germany (Becker, 2014).

Since 64% of the Italian beer consumption is internal (Assobirra, 2020), it would probably be useful to attempt reducing the environmental impact of beer consumption by favoring the diffusion of returnable 10- to 30-L keykegs, made from 100% R-PET (https://www.keykeg.com), for summer time get-together parties at the expense of present day most popular beer formats available in the market (i.e., glass bottles and cans). In this manner, it could be possible to emulate the current success of 3- to 15-L bag-in-boxes for red and white wines available at both physical stores and online shops.

Spent filtration aids

Generally, spent filtration aids resulting from rough beer filtration are in the form of DE or Kieselguhr slurry, which is rich in suspended solids (e.g., diatom frustules, yeast, and hop and malt residues) and thus highly pollutant (Olajire, 2020). World Health Organization has classified such slurry as hazardous waste; moreover, its disposal costs in agriculture are as high as €170 per metric tons (Mg) (Fillaudeau *et al.*, 2006). Nevertheless, owing to their high filtration rate and efficiency, DE dead-end filters are still largely used by the majority of breweries. In a large-size brewery, the specific consumption of DE is around 112 g hL^{-1} of beer, giving rise to ~336 kg hL^{-1} of spent DE sludge (Cimini and Moresi, 2016).

After beer filtration, sludge is not recycled and generally landfilled. However, thanks to its high contents of available phosphorus (0.37–0.42 g kg^{-1}), potassium (0.9–3.3 g kg^{-1}), organic carbon (0.3 kg kg^{-1}), and total nitrogen (0.02 kg kg^{-1}), it is used in agriculture to improve soil fertility with no significant risk to the environment (Dessalew *et al.*, 2017). The main potential reuse opportunities of this material include: (1) its recycling as additive to construction masonry materials, such as concrete, cement, and brick (Ferraz *et al.*, 2011); and (2) its regeneration via chemical, physical, or biological methods. The latter up to now is unable to replace totally virgin DE (Olajire, 2020). Moreover, the thermal and acid or alkaline agent regeneration methods cannot be regarded as sustainable recycling methods because of their low efficiency and serious secondary pollution to the environment as well as high processing costs (Li *et al.*, 2015). On the contrary, the biological methods appeared to be not only almost zero cost-effective but also capable of improving the adsorption capability of brewery-spent diatomite toward dyes and heavy metals from polluted wastewaters (Gong *et al.*, 2019).

In order to decrease DE consumption and thus reduce DE sludge formation, it would be possible to resort to a cleaner filtration technology, such as cross flow microfiltration, which has been applied successfully in other food sectors over a long period of time (Cheryan, 1998). Unfortunately, so far, its application to beer clarification has been penalized by average permeation fluxes (50–100 L m^{-2} h^{-1}) of about one-fifth of that obtainable (250–500 L m^{-2} h^{-1}) with conventional kieselguhr filters (Buttrick, 2010), which are also dependent on the initial turbidity of rough beer (Cimini and Moresi, 2014). Only by submitting pre-centrifuged rough beer to a specific enzymatic (i.e., Brewers Clarex®) treatment and then to clarification at 30°C using ceramic 1.4-µm hollow-fiber membrane modules, it was possible to limit the in-bottle chill-haze formation more effectively than with the PVPP treatment, and what is more to enhance the average permeation flux up to 2,000 L m^{-2} h^{-1} with no filtration residues

to be disposed of (Cimini and Moresi, 2018a,b, 2020). Final polishing of the resulting beer permeates through 0.45-µm cartridge filter, resulting in a brilliant, colloidally stable, and microbiologically safe beer ready to be packed aseptically without any thermal pasteurization (Cimini and Moresi, 2020).

End of Life of Brewing Biotic Materials

In large-size breweries, wet BSGs, as well as hot trub and BSY, are generally used as feed supplement for both ruminants and nonruminants (Cimini and Moresi, 2016; Kerby and Vriesekoop, 2017). How craft breweries generally deal with the disposal of their byproducts is practically unknown, especially in Italy. By resorting to the information provided by 90 British craft brewers interviewed by Kerby and Vriesekoop (2017), BSGs were destined to feed formulation by about 94% of the rural craft breweries, while the remainder was nearly equally directed to composting or landspreading. The urban counterparts exhibited almost the same disposal scenario, although in smaller craft breweries the percentage of BSGs used as feed ingredient reduced to ~76% at the expense of that converted into compost (20%). It was also noted that a large rural brewery worked in partnership with a local pig farmer to breed pigs with BSG and serve the resulting pork meat in its own tap house. Altogether, animal feed was the primary route of BSG disposal, this mirroring the practices of industrial-size breweries.

Regarding spent hops/hot trub, their residual bitterness prevents them from being used as an animal feed. Nevertheless, owing to its minimum amount (0.2–0.4 kg hL^{-1} beer; Table 1), such a byproduct can be appropriately added to BSG to formulate feed that is not rejected by cattle. Nevertheless, the UK craft breweries appeared to reuse it as fertilizer (~40%) or compost (~40%) or dispose it of in the landfill (7–10%), as reported by Kerby and Vriesekoop (2017).

Even if the majority of breweries reuses yeast to inoculate the next batch of wort, 2–4 kg of surplus yeast per hectoliter of beer (Table 1) is disposed of as BSY. Among the smaller rural and urban craft breweries, the primary disposal method (55–60% of the overall amount) was through municipal sewage system, although such percentage decreased with increase in the size of brewery. Altogether, 10% of small urban craft breweries, as well as 20% of medium- and larger-size rural craft breweries, got rid of BSY as animal feed. Similar proportions were used for composting and fertilizing purposes. Other uses, such as mixed substrate for anaerobic digestion or inoculum for the fermentation step of a distillery, were additionally pointed out by Kerby and Vriesekoop (2017).

The techno-scientific literature is full of proposals about the potential uses of brewery wastes (Aliyu and Bala, 2011; Cook, 2011; Huige, 2006; Jackowski *et al.*, 2020; Karlović *et al.*, 2020; Kusch-Brandt *et al.*, 2019; Mussatto, 2009; Neylon *et al.*, 2020; Rachwal *et al.*, 2020). Despite, they are acclaimed as a panacea for most of the world's problems, their high moisture content (Table 1) makes them perishable very quickly and *de facto* unreusable, especially in the human food chain. By accounting for the waste hierarchy set out by Directive 2008/98/EC (EU, 2008) and Garcia-Garcia *et al.* (2015), Figure 4 shows a ranking of their potential upgrading proposals.

Malt barley rootlets

Malt barley rootlets (MBR) are removed from malted barley, since they impart a bitter aftertaste to beer (Karlović *et al.* 2020). Table 6 classifies their potential applications in accordance with the food waste hierarchy illustrated in Figure 4.

If their mycotoxin content is low, MBRs should be first used as a food ingredient, thanks to their high protein and fiber contents (Table 1). Some of their applications are summarized in Table 6. Regardless of representing the second priority choice (Figure 4), MBRs are nowadays quite exclusively utilized by the animal feed industry (Table 6). As the third priority choice, MBRs could be used as substrate for extracting several valuable products, such as enzymes and antioxidants, or for microbial cultivation and fermentation. The Achilles' heel of their extraction processes is the need for complex and expensive purification steps to fractionate the enzyme of choice from quite numerous other unsought enzymes. In fact, it was found to be more effective and easier to obtain nucleotide extracts from the autolysis of selected high-ribonucleic acid containing baker's yeasts than from MBRs (Sombutyanuchit *et al.*, 2001). In addition, the commercial interest for using such extracts in food and cosmetics is limited due to high operating costs of their extraction processes (Bonnely *et al.*, 2000). Concerning their use as an economic alternative to the conventional de Mann, Rogosa, Sharpe growth media (Laitila *et al.*, 2004), it must be remarked that such investigations were performed in laboratories only and did not account for the market size of such a growth medium and thus for its real processing costs, as well as the impact of MBR market price on the final product. Finally, the so-called optimized lactic acid production from BSG and MBR hydrolysate by Radosavljević *et al.* (2020) did not account for the problematic recovery of lactate from such an exhausted production medium. This phase would be by far more complex than the traditional one (Moresi and Parente, 1999), which relies on the purer carbon sources (e.g., raw sugar extracted from sugar beet or sugarcane, and corn starch hydrolysates) used by world's largest

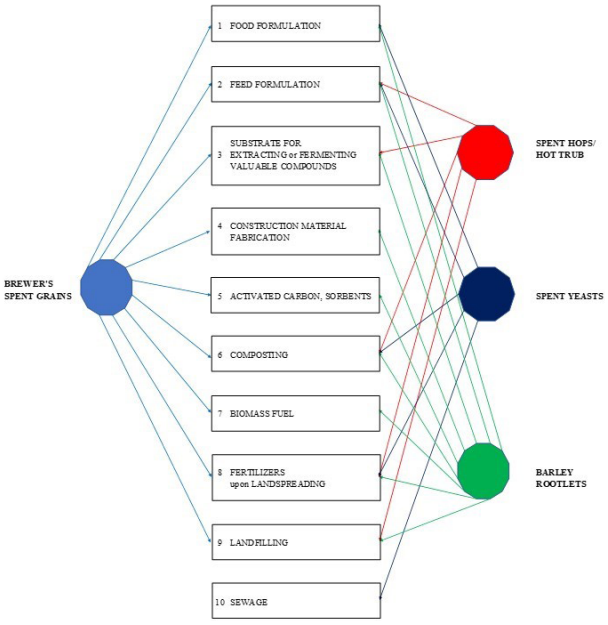

Figure 4. Potential uses of main brewery wastes (e.g., barley rootlets, brewer's spent grain, spent hops/hot trub, brewer's spent yeast) as described in the literature and ranked according to the food waste hierarchy set out by Directive 2008/98/EC (EU, 2008) and Garcia-Garcia *et al.* (2015).

lactate manufacturers such as BASF, Galactic, Musashino Chemical, and Dow (Grand View Research [GVR], 2021). As the fifth priority choice being reported in Table 6, MBRs are converted into biochar, its use for soil amendment (Chan *et al.*, 2007), as a sorbent for several water contaminants (Grilla *et al.*, 2020; Neylon *et al.*, 2020), or as a catalyst in the transesterification step of biodiesel production (Tsavatopoulou *et al.*, 2020).

Finally, as the least preferable waste management options, MBRs might in all probability be used for composting and landfilling (Table 6).

Brewer's spent grains

Figure 4 shows how the food waste hierarchy specified by Directive 2008/98/EC (EU, 2008) could be applied to manage the disposal of BSG according to circular economy template.

In the first place, because of their high protein and fiber contents (Table 1), such spent grains could be used in the food industry. The technical literature reports quite an innumerable list of food applications (Aliyu and Bala,

Table 6. Main potential uses of malt barley rootlets (MBR) as classified according to the food waste hierarchy shown in Figure 3.

Food waste hierarchy	Main MBR reuses	Remarks and references
1	Food formulation	Neylon *et al.* (2020) listed a series of food products, such as bread, biscuits, and sausages, enriched with different aliquots of MBRs as such (Chiş *et al.*, 2020) or fermented with *Lactobacillus plantarum* sp. (Waters *et al.*, 2013) to improve their nutritional properties.
2	Feed additive	MBRs are generally blended with other malting byproducts (e.g., barley dust, malt dust, and small-size barley grains) and compressed to obtain the so-called *malt residual pellets* with a bulk density and a protein content of about 600 kg m^{-3} and 18% (w/w), respectively (The Maltsters Association of Great Britain [MAGB], n.d.). Owing to the potential high risk of being contaminated by mycotoxins, such pellets must be appropriately dosed before feeding, for instance, weaner piglets, which are as sensitive to zearalenone as humans (MAGB, n.d.).
3	Source of enzymes	MBRs are a source of invertase, superoxide dismutase, nucleases, phosphotransferase, phosphomonoesterase, and especially 50-phosphodiesterase (Neylon *et al.*, 2020). In particular, the latter is used commercially to make nucleotides (Sakaguchi *et al.*, 1963; Sakaguchi and Kuninaka, 1965) for enhancing the flavor of broths and soups (Yamaguchi, 1998).
	Source of multicomponent extracts	MBRs are also a source of natural antioxidants, including ascorbic acid and glutathione, potentially useable in food and cosmetics (Bonnely *et al.*, 2000).
	Microbial growth substrate	MBRs were used as a cheap growth and storage medium for lactic acid bacteria (Neylon *et al.*, 2020). It had an estimated 20% lower price with respect to that of the conventional de Mann, Rogosa, Sharpe growth media (Laitila *et al.*, 2004).
	Bioproduct substrate	Hydrolysates of MBRs and brewers' spent grains were used as substrate for lactic acid production (Radosavljević *et al.*, 2020).
5	Activated carbon	MBRs were converted into biochar upon heating at ~450°C in a pyrolysis plant (Chan *et al.*, 2007). Its application at rates more than 50 Mg/ha in conjunction with N fertilizer (100 kg N/ha) improved not only the fertilizer effectiveness but also soil quality (Chan *et al.*, 2007). The biochar sorbent properties for several water contaminants (e.g., chlorine, chloroform, chromium, mercury, methylene blue, phenanthrene, trimethoprim, and uranium) were reported by Grilla *et al.* (2020) and Neylon *et al.* (2020). Untreated MBR biochar was also used as a catalyst in the transesterification step of biodiesel production (Tsavatopoulou *et al.*, 2020).
6	Composting	
9	Landfilling	

2011; Cook, 2011; Huige, 2006; Jackowski *et al.*, 2020; Karlović *et al.*, 2020; Mussatto, 2009; Rachwal *et al.*, 2020). Table 7 lists such applications in accordance with the food waste hierarchy shown in Figure 4.

In particular, the BSG fortification of food products had no effect on the taste, smell, and consistency of the final product of choice, as well as on its appreciation by the end consumer on condition that it was not greater than 10% (w/w) in dry pasta (Nocente *et al.*, 2019) or 25–30% (w/w) in bread (Stojceska *et al.*, 2008) and snacks (Petrovic *et al.*, 2017). Of course, such fortified foods had greater fiber content and a lower glycemic index (Kirjoranta *et al.*, 2016). When 15 parts of BSG were homogenized with a pre-emulsion made of 5 parts of carboxymethyl cellulose and 80 parts of ice, such addition to chicken meat batters at 20–25% level resulted in reduced-fat chicken sausages with an overall sensory acceptability not statistically different from that of a reference product prepared with 15% pork back fat (Choi *et al.*, 2014). On the contrary, conventional chicken patties exhibited net improvement in their cooking loss, consistency, color, and sensory properties if they included no more than 3% BSG (Kim *et al.*, 2013). BSG was also used to prepare a probiotic drink (Tan *et al.*, 2020). However, since experimental trials have been so far performed in Erlenmeyer flasks with no sensory tests and no cost–benefit analysis, such alternative use of BSG as a novel nutritional beverage appears to be very premature. Even other food applications listed in Table 7 have been tested in laboratory only. Nevertheless, as Anheuser-Busch InBev, a leading brewer of the United States, realized that it had to dispose of about 1.4 million Mg of BSG annually, it started a new company called EverGrain to convert BSG into a low-starch, proteinaceous, and fibrous material at a pilot-scale plant at Newark (New Jersey, USA). Owing to its successful use in the preparation of several foods and beverages (e.g., plant-based milk, bread, pizza crust, pasta, granola bars, meat alternatives, and smoothies), EverGrain decided to

Table 7. Main potential uses of brewer's spent grain (BSG) as classified according to the food waste hierarchy shown in Figure 3.

Food waste hierarchy	Main BSG reuses	Remarks and references
1	Partially exhausted raw material	It can be recovered from the uppermost layers of BSG discharged after lautering. Since it contains undigested starch, it might be integrated with appropriate doses of fresh malt and reused in the subsequent wort batch to produce low-alcohol or alcohol-free beers (Zürcher and Gruss, 1990).
	High-protein and high-fiber containing ingredient	It was used to: (a) Enrich soft wheat flour and formulate: (i) breads (Steinmacher *et al.*, 2012), (ii) breadsticks (Ktenioudaki *et al.*, 2012), (iii) cookies (Kissell *et al.*, 1979; Petrovic *et al.*, 2017), and (iv) baked snacks (Kirjoranta *et al.*, 2016; Ktenioudaki *et al.*, 2013). (b) Enrich hard wheat semolina to prepare several dry pastas (Cappa and Alamprese, 2017; Nocente *et al.*, 2019). (c) Reduce fat content in some meat products: (i) frankfurters (Özvural *et al.*, 2009), (ii) smoked sausages (Nagy *et al.*, 2017), (iii) chicken sausages (Choi *et al.*, 2014), and (iv) chicken patties (Kim *et al.*, 2013).
	Main substrate for probiotic beverages	Upon suspension of 200 g L^{-1} of pre-ground BSG in sterile water, and fermentation of the resulting medium with *Bacillus subtilis* WX-17 (i.e., rod-shaped, Gram-positive bacteria generally recognized as key health promoter), it was recovered as a liquor rich in viable cells (7.2×10^9 CFU mL^{-1}), several essential amino acids, and citric acid cycle intermediate metabolites, and with a high antioxidant activity (Tan *et al.*, 2020).
2	Feed additive	BSG can be used to feed (i) cattle (Cimini and Moresi, 2016), (ii) pigs (Kerby and Vriesekoop, 2017), (iii) aquaculture fish (Nazzaro *et al.*, 2021), (iv) poultry (Rachwał *et al.*, 2020), and (v) edible insects (Mancini *et al.*, 2019).
3	Source of proteins	The recovery of proteins, as such or hydrolyzed to formulate vegan foods, asks for quite complex extraction and purification processes using alkaline (Du *et al.*, 2020) and/or acid solutions (Qin *et al.*, 2018), subcritical water at 200°C and 40 bar (Du *et al.*, 2020) or 185°C and 50 bar (Alonso-Riaño *et al.*, 2021), hydrothermal pretreatment at 60°C, ultrasound-assisted enzymatic pretreatment (Yu *et al.*, 2020), or steam explosion (Rommi *et al.*, 2018).
	Source of polyphenolics	Recovery of polyphenolics was performed using quite different processes, namely alkaline hydrolysis, enzymatic hydrolysis, acetone–water, or ethanol–water extraction as such or assisted by ultrasound or microwave, or supercritical carbon dioxide extraction (Jackowski *et al.*, 2020; Karlović *et al.*, 2020; Rachwal *et al.*, 2020; Stefanello *et al.*, 2018.

Food waste hierarchy	Main BSG reuses	Remarks and references
	Source of arabinoxylan (AX)	Such polysaccharide consists of two monomers (xylose and arabinose) and may be recovered from BSG using the integrated process as set up by Vleira *et al.* (2014) where increasing concentrations of KOH or NaOH allowed ~83% of total proteins and ~70% of total arabinoxylan to be extracted sequentially. The efficiency of such a process was further improved with the help of ultrasound (Reis *et al.*, 2015) or microwaves (Coelho *et al.*, 2014).
	Source of multicomponent extracts	These were recovered by submitting BSG or other brewery wastes to water leaching under moderate conditions (Almendinger *et al.*, 2020). Their carbohydrate or amino acid concentration was generally smaller than 10 mg per g DM or 2 mg per g DM, respectively. Thus, their biological activity should be significantly enhanced to be properly utilized in cosmetic products (Almendinger *et al.*, 2020).
	Source of cellulose nanofibers	Such nanofibers could be used as emulsion or dispersion agents in food preparations (Rachwal *et al.*, 2020). Their recovery from dried BSG required quite a complex procedure consisting of the following steps: primary alkaline treatment with 0.1-M NaOH at 60°C for 2 h to get rid of proteinaceous matter; bleaching of the lignocellulose residue with 0.7% (w/v) sodium chlorite at a boiling point for 2 h; filtering and residue resuspension in 5% (w/v) sodium bisulfite at room temperature for 1 h; filtering and washing with distilled water; secondary alkaline treatment with 17.5% NaOH at room temperature for 8 h; washing and dispersion in water at 1.5% (w/v); and final homogenization at 700–800 bar for 20 cycles (Mishra *et al.*, 2017). However, no information about their processing costs is available.
	Microbial growth substrate	It was used as a growth substrate for several microorganisms, such as *Escherichia coli*, actinobacteria, *Bifidobacterium adolescentis*, *Lactobacillus* spp., and yeasts in alternative to expensive nitrogen sources, such as yeast extract and peptone (Cooray *et al.*, 2017; Rachwał *et al.*, 2020).
	Mushroom substrate	It was used to cultivate mushrooms, such as *Pleurotus ostreatus*, *Lentinula edodes*, and *Hericium erinaceus*. The trials carried out at the Mycoterra Farm (Westhampton, MA, USA) suggested not only that BSG should be handled with care to avoid cross-contamination of laboratory environment but also that grain savings from BSG substitution were not so significant to support such a use financially, especially in spawn stages (Mycoterra Farm, 2015).
	Bioproduct substrate	BSG was used as substrate for several bioproducts (Rachwał *et al.*, 2020), such as succinic acid (Cooray *et al.*, 2017), microbial oil (Saenge *et al.*, 2011), fatty acids and carotenoids (Zalynthios and Varzakas, 2016), xylitol (Mussatto and Roberto, 2008), pullulan (Singh and Saini, 2012), or citric acid (Femi-ola and Atere, 2013).
	Microbe-immobilizing carrier	It was used to immobilize yeasts (Bányik *et al.*, 2001).
4	Additive for bio-composites	BSG was used as an environment-friendly reinforcement or filler component in: 1. polyurethane foam composites, even if the foam matrix was found to be less compatible than that using ground tire rubber (Formela *et al.*, 2017); 2. food packaging trays made of BSG, potato starch, glycerol, and chitosan or glyoxal in replacement of expanded polystyrene, even if their flexural strength (~3.8 MPa) decreased to 0.4 MPa after contact with water (Ferreira *et al.*, 2019); 3. clay bricks as substitute for sawdust at 5–15% of dried BSG in brick making (Ferraz *et al.*, 2013); addition of just 3.5% (w/w) of BSG yielded stronger, more porous, and less dense bricks than standard ones in large-scale tests (Russ *et al.*, 2005); 4. wood polymer composites by twin-screw extrusion of pre-dried BSG at 120–180°C, this lowering the specific mechanical energy consumption by 20% and improving their thermal stability (Hejna *et al.*, 2021).
5	Activated carbon	BSG, as such or pelletized, was converted into biochar via pyrolysis and micro-gasification under high-temperature (400–500°C) and low-oxygen conditions with an average yield of 18.6% (w/w) (Sperandio *et al.*, 2017). Activated carbon from BSG exhibited adsorption capacity for metallic ions, phenolic compounds, and color quite similar or even effective than that of their commercial counterparts (Mussatto *et al.*, 2010).
6	Composting	A proper dosage of wet BSG with a lignocellulosic bulking agent (e.g., wheat straw) and sheep or pig manure favored its appropriate composting (Assandri *et al.*, 2021).
7	Biomass fuel	BSG could be used as a: (i) solid biomass having a lower calorific value (LCV) of 13.7 ± 0.7 MJ kg^{-1} at ~8% (w/w) moisture content, and a positive economic return, its estimated production cost and its market price being €110–140 kg^{-1} and €230–270 kg^{-1}, respectively (Sperandio *et al.*, 2017); (ii) hydrochar, a coal-like product obtained by hydrothermal carbonization in a closed reactor at 180–280°C and 2–6 MPa for 5– 240 min (Jackowski *et al.*, 2019); (iii) substrate for production of bioethanol upon acid pretreatment and inoculation of single or mixed microbial cultures, such as *Pichia stipitis* and *Kluyveromyces marxianus* (White *et al.*, 2008), *Saccharomyces cerevisiae* and *Aspergillus oryzae* (Wilkinson *et al.*, 2017), and *Fusarium oxysporum* (Xiros *et al.*, 2008); (iv) substrate for BSG anaerobic digestion in continuously stirred bioreactors yielding from 0.56 g (Wang *et al.*, 2015) to 0.81 g (Vitanza *et al.*, 2016) of biomethane per gram of total organic matter, even if both yields and kinetics were implemented by resorting to microwave-assisted alkaline pre-treatment (Kan *et al.*, 2018) or by supplementing 5% biochar (Dudek *et al.*, 2019) or trace elements (Bougrier *et al.*, 2018).

(*continued*)

Table 7. (Continued)

Food waste hierarchy	Main BSG reuses	Remarks and references
8	Organic fertilizer	BSG might be used as: (i) organic fertilizer because of its P, K, protein, cellulose, lignin, and hemicellulose contents; the mixture of BSG (5 Mg ha^{-1}) and NPK fertilizer (200 kg ha^{-1}) affecting positively the growth of maize and increasing soil aggregation (Nsoanya and Nweke, 2015); (ii) biofertilizer useful against soil-born insects; once BSG is inoculated with the spores of entomopathogenic fungi *Beauveria bassiana* the, accumulation of 10 metabolic compounds in the fermented biomass is found to be effective against *Galleria mellonella* larvae (Qiu *et al.*, 2019).
9	Landfilling	Wet BSG is landfilled by 7–10% of the UK craft breweries (Kerby and Vriesekoop, 2017).

build its first full-scale production facility at Anheuser-Busch's historic headquarter in St. Louis, Missouri (EverGrain, 2021). Thus, it is highly probable that more amounts of BSG would be utilized in the food sector and no more diverted to the second option of food waste hierarchy depicted in Figure 4.

The second waste disposal prospect (Figure 4) is used by most of the industrial and craft breweries to dispose of fresh BSG as feed additive in animal and insect nutrition (Table 7). To this end, under the EU Regulation No. 183/2005 (EU, 2005), food companies (including breweries) willing to sell their byproducts as feed materials are to register with their local authorities and develop a specific hazard analysis and critical control point (HACCP) plan to comply with traceability requirement and keep the risk of biological, chemical, and physical contamination of food wastes as low as practically attainable. Additionally, because of high moisture content (Table 1), BSG is so perishable that it must be fed within 2 or 3 days of manufacture unless it is stored at 5°C, dried or pickled (Jackowski *et al.*, 2020). Whereas its drying is hardly practiced, since the high operating costs are not rewarded by the final feed use, pickling is a low-cost operation capable of extending the shelf life of BSG with no counter effects on its quality (Jackowski *et al.*, 2020).

The third waste disposal prospect (Figure 4) involved the use of BSG as substrate for extracting proteins, polyphenolics, arabinoxylan (AX), and cellulose nanofibers, or for microbial growth and fermentation, as summarized in Table 7.

The recovery of proteins from BSG is not an easy task. For instance, upon suspending about 100-g BSG in 1 L of aqueous NaOH (pH > 11) at 40°C for 2 h, and centrifuging, it was possible to recover a protein-rich precipitate with an extraction yield of about 21% and purity of 60% (DU *et al.*, 2020). Further extraction of such a residue with subcritical water at 200°C and 40 bar for 20 min enhanced protein extraction yield by an extra 7% (DU *et al.*, 2020). Subsequent alkaline and dilute acid pretreatments boosted the extraction yield to ~95% with the counter effects of greater solubilization of carbohydrates and lignin and a lower purity of proteins—this making by far more difficult the protein separation and purification steps (Qin *et al.*, 2018). Similar problems affect the recovery of polyphenolics and arabinoxylans from BSG (Jackowski *et al.*, 2020; Karlović *et al.*, 2020; Rachwal *et al.*, 2020). Moreover, the resulting extracts rich in ferulic and p-coumaric acids had to be micro-encapsulated not only to mask their pungent odor and bitter taste conveyed to fortified fish burger but also to prevent their degradation (Spinelli *et al.*, 2016).

Finally, the suggested use of BSG as inexpensive substrate for several bioproducts (Rachwał *et al.*, 2020) relies on tests carried out in laboratory with no account for their feasibility and processing costs in pilot and/or industrial plant. The use of BSG as a remunerative substrate per citric acid production was, for instance, just puerile, since it was drawn in the absence of any comparison among the citric acid yield factors and production rates in laboratory and industrial plant. Moreover, it is not known which bioproduct recovery and purification steps are to be used when dealing with a multicomponent matrix such as BSG instead of glucose syrups currently utilized (Moresi and Parente, 1999) by the world's largest citric acid manufacturers such as Anhui, Archer Daniels Midland, Cargill, Huangshi Xinghua Biochemical, Jungbunzlauer, Tate & Lyle, etc. (GR-Store, 2021).

In accordance with the fourth and fifth waste management options (Figure 4), BSG could be used to reinforce different biocomposites or produce activated carbon and biochar, as provided in Table 7. Even if the adsorption capability of biochar might help to improve water and chemical fertilizer retention in agricultural soils, as well as limit their nitrate leaching and N_2O and CH_4 emissions, no cost–benefit analysis has been carried out so far.

Concerning the sixth waste management option (Figure 4), BSG may be composted on condition that it is mixed with wheat straw and sheep or pig manure to adjust its initial moisture content (60–65% w/w), carbon/

nitrogen (C/N) ratio (20:30), and pH (5.5–7.5) (Assandri *et al.*, 2021).

The seventh waste disposal option shown in Figure 4 refers to the utilization of dried BSG as a solid biomass—its lower heating value ranging from 76 to 90% of 15–18 MJ kg^{-1} of the majority of solid biomasses with ~10% moisture content (Paládi, 2013).

Regarding hydrothermal carbonization of wet BSG, the resulting hydrochar could be used as biofuel, feedstock for gasification, soil additive for nutrient enrichment, adsorbent, or precursor of activated carbon (Jackowski *et al.*, 2019).

Although no information is currently available about the economic practicability of BSG conversion into hydrobiochar or bioethanol, production of biogas from BSG was regarded as economically unviable unless the process included coproduction of other more profitable products (González-García *et al.*, 2018). The enzymatic hydrolysis of cellulose, hemicellulose, and lignin would facilitate the release of fermentable sugars and thus improve the conversion yield into bioethanol or biomethane per unit mass of the lignocellulosic material consumed. However, it is still unknown, how the market price of commercial cellulolytic enzymes would affect the biofuel production costs.

As given in Table 7, BSG might be disposed of as an organic fertilizer per se or pre-fermented to be effective against soil-born insects (Qiu *et al.*, 2019).

Finally, BSG could be landfilled as the least preferable waste management alternative as shown in Figure 4.

Most of the above-mentioned potential uses of BSG are also elucidated by a recent bibliometric analysis carried out by Sganzerla *et al.* (2021), where up to 510 papers over the last 30 years were retrieved from the Web of Science© database. Globally, 65 countries have been involved in studies linked to BSG, and Brazil has been the most productive nation with up to 70 papers. It was demonstrated that a great interest existed in the valorization of BSG, even if no feasibility study was identified to sustain any of such research activities on an industrial scale. Although a bibliometric study pointed out the possibility of developing a biorefinery using BSG as raw material, it underlined the difficulty of identifying the most appropriate and economically viable chemico-physical or enzymatic processes applicable to upgrade the product yield of choice as well as to lower their environmental effects.

Spent Hops/Hot Trub

Figure 4 shows the main disposal methods of spent hops/hot trub, these being ranked according to the above-mentioned food waste hierarchy. Beyond the methods reviewed by Kerby and Vriesekoop (2017), it is worthy pointing out the possibility of fractionating about 0.11 g of a series of mono- and sesqui-terpenes from 100 g of dry HT by hydrodistillation—these essential oils acting as natural repellents against two Coleoptera (i.e., *Rhyzopertha dominica* and *Sitophilus granaries*) that cause big economic loss to stored foods (Bedini *et al.*, 2015). Obviously, even in this case no cost–benefit analysis was carried out to measure the real applicability for such eco-friendly repellents.

Brewer's Spent Yeast

Figure 4 illustrates the real and potential disposal methods of BSY as classified according to the above-mentioned food waste hierarchy.

Although BSY has long been used to produce a dark-brown food spread named *Marmite* (this being invented by the German scientist, Justus Freiherr von Liebig, and nowadays produced by Unilever in the United Kingdom), none of the 90 craft breweries interviewed by Kerby and Vriesekoop (2017) supplied BSY to any Marmite factory, probably because of the small amounts available. Marmite is a rich source of vitamin B complex, which is spread on bread, toast, or crackers and imparts the so-called umami taste, typical of the amino acid L-glutamate and 5'-ribonucleotides. Its main analogues are the Australian Vegemite, Swiss Cenovis, Brazilian Cenovit, and German Vitam-R (Wikipedia, 2021).

Owing to its high protein content (Table 1), BSY can be converted into protein concentrates and isolates (Karlović *et al.*, 2020). It is directly added to energy bars in the proportion of 10–30% (w/w) to significantly increase their protein and phytic acid contents as well as density (Stojceska *et al.*, 2008). BSY is used as a source of food-grade yeast extracts. NaCl- or saponin-induced autolysis of cell yeast gives rise to yeast extracts containing different free amino acid contents as well as peptides of diverse molecular masses. In this manner, these can be used to tailor-make novel functional foods having peculiar taste profiles, or more conventionally to enhance the flavor of food products by dosing appropriately specific components, namely, nucleotides, peptides, and amino acids (mainly glutamic acid; Podpora *et al.*, 2016).

Notwithstanding the fact that it is used to formulate animal feed as the second option of the above-mentioned food waste hierarchy (Figure 4), wet BSY combined with BSG and hot trub is primarily sold to farmers as a low-cost feed additive, especially by large-size breweries (Cimini and Moresi, 2016).

Owing to its high moisture content and chemical composition (Table 1), BSY degrades easily. Thus, before being administrated to animals, BSY must be dried or stabilized by adding organic acids to avoid its fermentation in the gastrointestinal tract of animals, especially pigs, which are highly sensitive to such disorders (Crawshaw, 2004). Wet BSY is mainly used to feed cattle, but its high digestibility has to be checked for other animals, such as fish, horses, turkeys, hens, and swine (Crawshaw, 2004).

The third waste management option in Figure 4 suggests using BSY as a source of several useful compounds, such as enzymes (Ferreira *et al.*, 2010) and especially invertase (De León-González *et al.*, 2016), polyphenolics (Vieira *et al.*, 2016), ergocalciferol used in vitamin D deficiency (Metzger *et al.*, 2012), saccharides such as β-glucans (Thammakiti *et al.*, 2004), and trehalose (Mahmud *et al.*, 2010). In particular, β-glucans extracted from BSY are used to replace partially fat in mayonnaise to lower its energy value and improve its storage stability (Worrasinchai *et al.*, 2006). BSY are also used as a growth medium for *Lactobacilli* and *Pediococci* (Champagne *et al.*, 2003).

All the above-mentioned BSY applications are experimented in laboratory without any cost–benefit analysis to assess their real feasibility.

Finally, BSY can be used as a fertilizer by composting or land spreading, although these waste management options are the least preferred ones to be applied (Figure 4).

Concluding remarks

To conclude, this analysis was about the potential utilization of brewery wastes. It is worth summarizing the results of an economic market analysis carried out by Buffington (2014) on the assumption of feeding a biorefinery located centrally with respect to two large-size beer manufacturers in the United States with their BSGs shipped "as-is," that is, with an average moisture content of 70% (w/w). Since the current acquisition cost of other alternative agroforestry wastes, such as stalks and straws, at an average moisture content of ~10% (w/w) is around US$40 Mg^{-1}, the effective acquisition cost for BSG would be US$133.30 per dry Mg. Moreover, accounting for the depreciation costs of bio-refinery, drying and storage processing costs of BSG, logistic costs, and a 5% net profit, the processed BSG market price would increase up to US$179 Mg^{-1}. In this scenario, using BSG, as well as other brewery wastes, as a biomass feedstock appears to be in no manner market-justifiable. Moreover, direct disposal of malting and brewery byproducts as animal feed is the primary option for small-, medium-, and large-size breweries, since it represents an avoided production of feed and gives rise to quite a significant CO_{2e} credits equaling to about one-third of the contribution of packaging materials (Table 5).

Conclusions

In the light of the concept of circular economy, the brewing chain is expected to deal with the reuse of abiotic (e.g., packaging materials and spent kieselguhr sludge) and biotic (e.g., brewery wastes) materials to approach the zero-waste objective.

The pros and cons of different packaging alternatives (e.g., one-way, lighter, reusable, or recycled containers) were analyzed. Even if the contribution of beer consumption phase was taken into account, there was no definitive result about the less environment-impacting beer packaging format. Autonomous system for the direct management of PET packaging for liquid foodstuffs, recently recognized by the Italian Ministry of the Environment, might help to make available 100% R-PET flakes ready to be reconverted into food-grade bottles with minimum downcycling to other non-food uses.

Concerning numerous studies suggesting alternative utilization of brewery wastes in nutritional as well as biotechnological fields, it was pointed out that the majority of these was just tested in laboratory and included no cost–benefit or market analysis. Even when the concept of biorefinery was stressed upon as an interesting strategy to upgrade brewery wastes, none of the final bioproducts obtained seems to be market-justifiable, mainly because the estimated market price of dried BSG was about 450% higher than that of conventional lignocellulose residues. Except for the Anheuser-Busch's initiative of quickly converting wet brewer's grains into a low-starch and high-protein and high-fiber containing ingredient for foods and beverages, the high moisture content of all brewery wastes makes them perishable to prevent their safe usage in the human food chain. Their prompt use as an animal feed appears to be the only disposable method not only economically feasible but also able to lower by about one-third the GHG load of packaging materials. Not by chance, it is currently practiced by both industrial and craft breweries. All other alternative uses, hailed in the literature as a panacea for most of global problems, appear to be more useful for publishing articles than for defining any economically feasible reusing procedure for all the brewery wastes of concern. Under these circumstances, to support its transition toward a circular economy, the beer industry must primarily reduce, reuse, recycle, and recover as much as possible the beer packaging materials.

Acknowledgments

This research was supported by the Italian Ministry of Instruction, University and Research, with special grant PRIN 2015 – prot. 2015MFP4RC_002.

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