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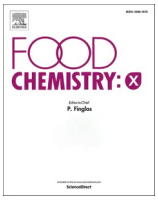

The effect of common duckweed (*Lemna minor* L.) extract on the shelf-life of beef burgers stored in modified atmosphere packs: A metabolomics approach

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A B S T R A C T

The impact of duckweed extracts (DEs) on the shelf-life of packaged beef burgers was evaluated through classical assays and untargeted metabolomics. Beef burgers were formulated with an antioxidants-free control (CON), 1 g/kg sodium ascorbate (ASC), and increasing levels of a DEs, namely 1 (DE1), 5 (DE5), and 10 (DE10) g/kg, packaged under modified atmosphere and stored at 4 °C for 19 days. The DEs, abundant in phytochemicals, determined no issues with the hygienic status of the product. DEs modulated the redox status, being ineffective in preserving linolenic acid from peroxidation. However, the oxidation marker 2-nonenoic acid was down-accumulated in the DE10 sample following 19 days of storage, recording a lower glutathione:glutathione disulfide ratio. The accumulation of adipate semialdehyde revealed the inefficiency of DEs in coping with protein oxidation, while DEs prevented the accumulation of biogenic amines. Therefore, this work suggests a potential pro-oxidant role of the formulated DEs.

1. Introduction

Duckweed is a small, invasive, free-floating aquatic plant widely described in phytoremediation research (Del Buono et al., 2022). Recently, duckweed (*Lemna minor* L.) has been considered as a plant-based ingredient for future foods and as a sustainable alternative source of protein. This plant, included into the family of *Lemnaceae,* is characterized by a wide phytochemical composition, including amino acids, sterols, terpenoids, glucosinolates, polyphenols, and organic acids (Zhang et al., 2023). In the last years, duckweed was exploited mainly as supplement in the animal feed industry and aquaculture, also finding applications as potential biofertilizer and biofuel (Yahaya et al., 2022). This plant contains 20 % to 30 % of protein, with ribulose-1,5-bisphosphate carboxylase as the main protein; additionally, this plant represents a good source of essential amino acids, showing a high *in vitro* digestibility and non-allergenic properties (Yahaya et al., 2022). Duckweed also contains polyunsaturated fatty acids (such as α-linolenic acid) and up to 10 % starch (Yahaya et al., 2022).

Looking at the European scenario, the EFSA Panel on Nutrition, Novel Foods and Food Allergens has delivered a scientific opinion on the safety of water lentil powder as a novel food according to Regulation (EU) 2015/2283 (Turck et al., 2021). Based on the careful assessment of its chemical composition (with a focus on the high protein content), the panel concluded that the proposed novel food was not nutritionally disadvantageous, but additional studies are required when considering a possible safety concern caused by the intake of manganese. A recent application of duckweed as an ingredient in food formulation was reported by Yahaya and co-authors (2022) where 2 % duckweed powder was used in the formulation of ice creams, showing an increase in protein and fibre contents when compared to a negative control. At the same time, this work did not report any issue in terms of metabolite profile, nutritional, and microbiological traits.

Recently, combining high-resolution mass spectrometry with a response surface methodology (RSM) allowed our research group to

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comprehensively evaluate the functional and antioxidant potential of duckweed, thus supporting its further exploitation in food applications (Zhang et al., 2023). In particular, we considered a potential application of duckweed on stored meat, as a food product undergoing several biochemical processes that can affect its final quality before the consumption. Therefore, the utilization of functional additives with antioxidant properties can represent a key advantage. As far as the chemical composition of meat is concerned, this latter strictly depends on the muscle type, and overall, the main components are represented by proteins and lipids (both storage and structural ones). However, as widely reported in scientific literature (Domínguez et al., 2019), these main components are highly prone to oxidation and degradation events, finally determining the generation of several toxic and/or harmful compounds, negatively correlated with both nutritional and sensory quality of the product during its shelf-life period. Overall, both natural and synthetic strategies have been inspected by several research groups to mitigate meat modifications during storage (Munekata et al., 2020). Accordingly, the utilization of plant extracts as natural ingredients represents a valid strategy to increase the shelf-life of meat products, also providing a potential incorporation of bioactive and antioxidant compounds into the final product (Pintado & Delgado-Pando, 2020; Rocchetti et al., 2023a). As reviewed by Munekata and co-authors (2020), the addition of ingredients from plant origin into meat products has already been proven as a successful and consumer-accepted strategy, considering both health- and sustainable-related aspects. Therefore, extenders from plant extracts are optimal candidates to improve both chemical quality and sustainability of the product within its production cycle (Rocchetti et al., 2023a).

Within this innovative scenario, in this work we evaluated the impact of duckweed extracts to extend the shelf-life of packaged beef burgers, by coupling classical microbiological and spectrophotometric assays with untargeted metabolomics. The final aim was to provide new information into the effect of duckweed extracts on meat quality during the shelf-life period.

2. Materials and methods

2.1. Plant material and extract preparation

The accurate details regarding plant collection, harvest, and growth can be found in Zhang et al. (2023). The optimal conditions to obtain the DE were defined according to a RSM study previously published by Zhang et al. (2023). Briefly, 1 g of dried duckweed tissues was extracted in 100 mL of solvent using ultrasound-assisted extraction (UAE; DU-32 ARGOLab, Milan, Italy) for 20 min, setting 50 °C, 120 W, and 80 % ethanol as extraction temperature, ultrasound power and solvent, respectively. After the sonication step, the extracts were centrifuged at 6,000 × *g* for 10 min at 4 °C, and then the supernatants were filtered by means of 0.22 μm syringe filters and finally collected. Following the extraction step, the ethanol was removed using a vacuum rotary evaporator (30 min, 75 °C), and the solution was diluted to the initial volume with water (supplementary Fig. 1). The extract obtained was characterized by the following antioxidant activity-related parameters: 24.9 mg TE/g (DPPH assay), 23.8 mg TE/g (ABTS assay) 34.6 mg TE/g (CUPRAC assay), 40.8 mg EDTAE/g (Metal chelating activity), and 230.1 mmol TE/g (phosphomolybdenum activity) (Zhang et al., 2023).

2.2. Preparation of beef burgers

Beef burgers were manufactured by Salumificio Santini (Cremona, Italy), while the storage process was done in the meat pilot plant at the Università Cattolica del Sacro Cuore (Cremona, Italy). For the trial, three independent meat batters of 10.5 kg were prepared in different days, consisting of beef meat 98.8 % (i.e., silver side and top side cut, containing approximately 5 % fat) and 1.2 % of salt (NaCl). The meat was prepared using an industrial meat mincer (Velati, Tribiano, Italy) with a perforated plate of 4 mm and salt was added with an industrial mixer (Velati, Tribiano, Italy). The resulting doughs were subdivided into 5 equal batches following the indications of the study, namely: Control batch [CON], without any additives (only 1.2 % salt), a positive control [ASC], which includes 1 g/kg ascorbic acid, and three additional batches formulated with the duckweed extract at 1 g/kg [DE 1], 5 g/kg [DE 5], and 10 g/kg [DE 10]. Each batch was again individually mixed to obtain optimal uniformity of the mixture, and then a manual burger maker was used to shape burgers into portions of 100 g in weight for the 5 batches described above. Finally, each burger belonging to the 5 batches was packaged in polypropylene thermosealable trays under the following MAP conditions: 66 % oxygen (O_2), 25 % carbon dioxide (CO_2) and 9 % nitrogen (N_2). The packaged samples were stored at 4 °C under light conditions to simulate supermarket conditions. Beef burgers were sampled after 0 (T0), 9 (T9), and 19 (T19) days of storage. All the most important phases dealing with the formulation and packaging of the beef burgers can be found in the flowchart reported as supplementary Fig. 2. Overall, 90 burger samples were produced and analyzed, namely two beef burgers for each treatment *x* five treatments x three sampling points x three different replication (on different days with different raw meat materials and the same ingredients).

2.3. Microbiological, pH and water activity (a_w) analyses

For microbial enumeration of burger samples, 20 g were aseptically removed, ten-fold diluted with 0.9 % NaCl in water, and homogenized (2 min at 260 rpm) in a Stomacher Lab-Blender (400 Circulator; International PBI, Milan, Italy). Decimal dilutions were plated onto the growth media (Oxoid, Milan, Italy) and then incubated. Total viable counts (TVC) of psychrotrophic and mesophilic were determined on Plate Count Agar (Oxoid), considering incubations for 10 days at 7 °C and 30 °C for 72 h, respectively. Besides, yeasts and moulds on Rosa Bengala (Oxoid) added with chloramphenicol (100 mg/L, Sigma, Milan, Italy) were incubated at 25 °C for 5 days. LAB counts were done in MRS Agar for 48 h at 37 °C using the Anaerocult A (Merck, Darmstadt, Germany) (ISO 15214, 1998); Staphylococci on Baird Parker Agar added with egg yolk tellurite emulsion at 37 °C for 48 h in aerobic conditions (ISO 6888–1, 2018). Additionally, a Violet Red Bile Glucose Agar (VRBGA) for 24 h at 37 °C was used for *Enterobacteriaceae* (ISO 21528–2, 2017). To detect *Listeria monocytogenes*, *Salmonella* spp., and *Clostridium perfringens*, the validated procedures, namely ISO 11290-1:2017; ISO 6579-1:2017, and ISO 7937:2005, respectively, were used. After counts, mean and standard deviation values were calculated for each replicate (n = 3) and the results were expressed in Log CFU/g.

The pH was measured by directly inserting the tip of the electrode (pH 127-m; 692 pH/Ion Meter-Metrohm, Laramie, Wyoming, USA) into different portions of the burger samples. The pH meter was calibrated using buffers at pH 4.01 and 7.00 at 22 °C. Furthermore, water activity (a_w) was measured at 25 °C utilizing the a_w-meter AQUALAB Series 3 Model TE (Decagon Devices, Inc., Pullman, WA, USA), following the ISO procedures (ISO 18787:2017). The pH and a_w measurements were done in triplicate at each sampling point (n = 3).

2.4. Colour analysis and TBARS assay for evaluating lipid peroxidation

The colour measurement of the samples was carried out using HunterLab D25 NC (HunterLab, Reston, Virginia). After 30 min bloom time, colour was measured in triplicate (n = 3) for each burger sample, and the results were expressed according to CIEL*a*b* colour space based on a D65 illuminant source, an aperture size of 25.4 mm and a 10° viewing angle. The colour was measured through the three-dimensional colour diagram (L^*, a^* and b^*), where: L^* indicates lightness (L^* = 0 (black), L^* = 100 (white), a^* indicates ($-a^*$ = greenness, $+a^*$ = redness) and b^* chromaticity indicates ($-b^*$ = blueness, $+b^*$ = yellowness) (Biró et al., 2019).

The analysis of lipid stability was done according to the TBARS assay,

following the method published by Vyncke (1975), with few modifications. The results were reported as mg malonaldehyde (MDA)/100 g of burger sample, taking into account three independent replicates (n = 3).

2.5. Extraction of metabolites and untargeted profiling by HRMS metabolomics

The extraction of meat metabolites was done as reported by Rocchetti et al. (2023b). Burger samples (1 g) were ten-fold diluted with a hydro-alcoholic solution (80 % methanol, v/v) acidified with 0.1 % formic acid, and then extracted by means of a homogenizer (Rocchetti et al., 2023b). The extracts were centrifuged (6000 × g for 15 min at 4 °C) and then filtered with 0.2 μm cellulose syringe filters in UHPLC vials. The UHPLC-HRMS analysis was done using a Q-Exactive™ Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) coupled to a Vanquish ultra-high-pressure liquid chromatography (UHPLC) pump, equipped with heated electrospray ionization (HESI)-II probe (Thermo Scientific, USA). All the accurate details regarding the analytical workflow can be found in our previous published works (Rocchetti et al., 2021, 2023b). Water and acetonitrile (both LC-MS grade, from Sigma-Aldrich, Milan, Italy) were selected as mobile phases, following a gradient elution from 6 to 94 % acetonitrile in 35 min, and using 0.1 % formic acid as phase modifier. An ACQUITY UPLC BEH C18 (2.1x100 mm, 1.7 μm) analytical column (at 35 °C) was used for the chromatography separation. The full scan MS analysis (in the *m/z* range 80–1200) consisted in a positive ionization mode (mass resolution: 70,000 at *m/z* 200) with a 200 μL/min flow rate and an injection volume of 6 μL. Additionally, pooled quality control (QC) samples were randomly injected and analysed following a data-dependent (Top N = 3) MS/MS mode, reducing the resolution to 17,500 at *m/z* 200, and reaching the fragmentation of the most abundant ions under stepped Normalized Collisional Energy (i.e., 10, 20, 40 eV).

The collected raw data were processed in the software MS-DIAL (version 4.90) (Tsugawa et al., 2015), considering the automatic peak finding, LOWESS normalization, and annotation via spectral matching. The database used for the annotation step was the comprehensive FooDB (https://www.foodb.ca, accessed: October 2022). All the accurate information regarding both software parameters and identification workflow can be found in our previous works (Rocchetti et al., 2021, 2023b). Overall, the identification step was reached combining mass accuracy, isotopic profile, and spectral matching. Therefore, in this work, a level 2 confidence in annotation (typical of untargeted metabolomics) was achieved, according to the COSMOS standards in metabolomics (Salek et al., 2013).

2.6. Statistical analysis

The normality and variance homogeneity of the data were evaluated by Shapiro-Wilk test. The data were then submitted to the two-way analysis of variance (ANOVA). The storage time and the treatment were considered as fixed variables, manufacture repetition was taken as random effect and the results of the different analyses (i.e., microbiology, colour parameter, pH, a_w, and MDA values) were considered dependent variables. The difference between the averages was assessed using the Tukey's post hoc test when ANOVA was significant ($P < 0.05$). Furthermore, the interaction between the fixed variables (storage time x treatment) was also considered. The software SPSS (IBM SPSS Statistics, version 26) was used to perform the statistical analysis.

The multivariate analysis of metabolomics data was done using different ad-hoc softwares, namely Mass Profiler Professional B.12.06 (Agilent Technologies), SIMCA 13 (Umetrics, Malmo, Sweden), and MetaboAnalyst 5.0. The raw data generated by UHPLC-HRMS were centered by median value, transformed in Log_2 values, and scaled according to Pareto. The hierarchical cluster analysis (HCA, Euclidean distance) was used as unsupervised tool, while the orthogonal projections to latent structures discriminant analysis (OPLS-DA) was carried out as supervised algorithm. The OPLS-DA models were formerly built considering the impact of storage time as a discriminant factor. More details regarding validation and permutation testing of the prediction models built are reported in Rocchetti et al. (2023b). The variables importance in projection (VIP) selection algorithm was then used to list the discriminant metabolites detected using as threshold values > 1. A Venn diagram was also inspected to evaluate both common and exclusive VIP compounds detected at T9 and T19. Finally, the variation of each discriminant marker compound was evaluated trough a Fold-Change (FC) analysis (cut-off > 1.2) that was performed in the software Mass Profiler Professional B.12.06.

3. Results and discussion

3.1. Phytochemical profiling of the duckweed extract

As the first step, a UHPLC-HRMS approach was used to evaluate the total bioactive content and overall chemical profile of the duckweed extract used as shelf-life extender. The untargeted strategy allowed us to identify 556 compounds, mainly classified into amino acids and peptides, followed by benzenoids, fatty acyls, flavonoids, glycerolipids and glycerophospholipids, organonitrogen and organooxygen compounds, phenolic acids, stilbenes, other phenolics, sulphur compounds (such as glucosinolates), and terpenoids. All the compounds annotated with the corresponding annotation information can be found in supplementary Table 1. Regarding the bioactive content of the duckweed extracts, the semi-quantitative analyses carried out using standard representative compounds for each main class of compounds pointed out a significant abundance of polyphenols (1222.07 μg/g DM), followed by total terpenoids (514.17 μg/g DM) and glucosinolates (18.02 μg/g DM). Regarding the quantification of each main phenolic subclass, the group represented by other phenolics (consisting in lower molecular weight phenolics) recorded high semi-quantitative values, being 729.79 μg/g DM. Regarding flavonoids, the most abundant subclass was represented by flavonols (173.70 μg/g DM), followed by other flavonoids (such as flavone equivalents, being 124.43 μg/g DM), anthocyanins (34.14 μg/g DM), and flavan-3-ols (11.98 μg/g DM). The UHPLC-HRMS findings also outlined a marked distribution of phenolic acids (128.07 μg/g DM) together with a lower abundance of stilbenes (19.96 μg/g DM). Interestingly, the most abundant phytochemicals characterizing the duckweed extract for each subclass were delphinidin 3-(3″-*p*-coumaroylglucoside) (an anthocyanin), gallocatechin 3-gallate (a flavan-3-ol), isoorientin 2″-[*p*-coumaroyl-6-glucoside) and apigenin 6,8-di-*C*-glucoside (flavone equivalents), quercetin 3-rutinoside (a flavonol), 5-(8-pentadecenyl)-1,3-benzenediol (belonging to other phenolics), sinapic acid and isoamyl cinnamate (phenolic acids), lansimide 2 (a stilbene), glucocheirolin (a glucosinolate), and 7,8-dihydrolycopene (a carotenoid).

Our findings on the duckweed composition are coherent with the available scientific literature; in particular, Wahman et al. (2021) provided an untargeted characterization of *L. minor* metabolites structurally confirming the identity of 44 metabolites, mainly belonging to amino acids, flavonols and flavones. On the other hand, Kim et al. (2012) profiled *L. minor* plants cultivated under different concentrations of proline and sucrose, showing presence of 46 compounds, such as secondary metabolites, amino acids, fatty acids, organic acids, phytosterols, and others. As far as the distribution of other chemical classes is concerned, *L. minor* is known to accumulate various essential and non-essential amino acids together with poly-unsaturated fatty acids (PUFA) (Yahaya et al., 2022). Of interest, duckweed's essential amino acid profile can be compared to most plant proteins and more closely resembles animal proteins (Yahaya et al., 2022). Accordingly, our analytical approach enabled the annotation of several amino acids, structurally confirming the identity of five essential amino acids, namely Phe, His, Lys, Val, and Trp, followed by five conditionally essential (Arg, Cys, Gln, Pro, and Tyr) and three remaining non-essential compounds

(Glu, Asn, Ser). Regarding the fatty acid profile outlined by UHPLC-HRMS, it is known that PUFA dominate the fatty acid composition of duckweed, mainly α-linolenic acid (LNA, 18:3n-3; in the range 41–47 %) and linoleic acid (LA, 18:2n-6; in the range 17–18 %) (Yahaya et al., 2022). Coherently, α-linolenic acid and linoleic acid derivatives were annotated and structurally confirmed in the optimized duckweed extract under investigation (supplementary Table 1).

3.2. Microbiological analyses, a_w and pH values

In this study, the beef burgers were analysed for the microbial count following a 19-days storage period and the results obtained are reported in supplementary Table 2. Looking at T0 values, the TVC of mesophilic microorganisms evaluated on PCA at 30 °C reached the value of 4.9 log CFU/g in all samples. After that, following 9 days of storage, the count exceeded the limit of 6.7 log CFU/g (5×10^6 CFU/g) fixed by the European Regulation (Reg. EC 2073, 2005), except for sample DE 10, which reached 6.44 log CFU/g and was significantly ($P < 0.05$) different from the other burger samples. Similar results were obtained for the psychotropic TVC at 7 °C even though, this time also the DE 10 burger showed no significant differences ($P > 0.05$) from the other samples at T9. Similar findings were reported by Ercolini et al. (2009) regarding the mesophilic and psychotropic populations in meat samples stored at 7 °C for 10 days under vacuum-packed. Also, Parafati et al. (2019) found the same results in beef burger patties at the initial time, but after 2 days the mesophilic TVB exceeded the limit of 6.7 log CFU/g. Among mesophilic microorganisms, lactobacilli are the most representative bacteria. Accordingly, the microbial counts revealed initial values around 3.20 – 4.12 Log CFU/g, with DE 10 reporting the lowest ($P < 0.05$) values, then increasing to about 6.58–7.38 after 19 days. Similar trends were outlined for staphylococci and yeasts, although with lower count values, and no statistically significant differences were detected at each single storage time considered among all samples ($P > 0.05$) for these latter. Interestingly, the combination of the fixed variables (i.e., storage time *x* treatment) indicated a higher significant effect on LAB and yeasts ($P < 0.001$), followed by yeasts ($P = 0.004$) and TVC at 30 °C ($P = 0.018$). The combination was not significant for TVC at 7 °C and staphylococci. Additionally, no issues about the hygienic status of the different burgers were detected. The *Enterobacteriaceae* were always above 2 logs CFU/g in each sample (data not shown), thus highlighting the high quality of the meat used for the experimental plan. Conversely, Parafati et al. (2019) found higher *Enterobacteriaceae* values at the initial time (5.4 log CFU/g), underlining the importance of checking the initial quality and hygienic status of the raw material. Moreover, the pathogenic bacteria *Listeria monocytogenes* and *Salmonella* spp. had not been detected in all samples at any time point, indicating no safety issues due to high-quality raw beef meat and good manufacturing practices.

Regarding the pH value of the different beef burgers (supplementary Table 2), the statistical analyses revealed significant variations of this parameter when considering both the storage days and the treatment tested ($P < 0.01$). In particular, a significant decrease in pH was observed mainly at T19, likely due to the bacteria fermentation, reaching average values of 5.16 for the ASC sample and 5.25—5.26 for DEs-added burger samples (supplementary Table 2). At the end of storage, small differences were detected between DE treatments, recording pH values significantly higher than ASC-based samples (supplementary Table 2). Finally, the a_w values remained quite stable during the entire storage period for all burger samples, being in the range 0.987–0.992 (supplementary Table 2). Overall, no significant differences were recorded when considering each treatment separately ($P > 0.05$). Regarding significant differences at specific time-point, a higher a_w was found at T0 for DE5 sample (supplementary Table 2). Finally, as far as the interactions between the different fixed variables is concerned, the only pH values were significantly affected, recording a *P*-value < 0.001.

3.3. Colour changes and TBARS values during storage

As widely recognized, lipid and protein oxidation are correlated with the presence of volatile compounds potentially involved in off-flavours and deterioration of the product. The most studied lipid oxidation by-products are hydroperoxides, conjugated dienes, isoprostanes, prostaglandins, carbonyls, furans, and MDA. In this work, we coupled a typical spectrophotometric assay (such as TBARS assay) with the colour analysis to preliminary understand DE's impact on beef burgers' oxidative stability during 19 days of storage. A comprehensive overview of these results can be found in Table 1. As can be observed from Table 1, no significant differences were observed for the L^* value during 19-days storage within each treatment, except for the CON sample ($P < 0.01$). However, at the end of the experimental storage time (19 days), significant differences were observed when comparing ASC sample ($L^* = 41.52$) *vs* the remaining samples. Similar results were deduced by inspecting the b^* parameter; in this regard, no significant differences were observed during 19-days storage within each treatment, except for the DE5 sample ($P < 0.05$). Regarding the interactions between the fixed variables tested (i.e., storage time *x* treatment) we found a significant effect only for the parameter L^* ($P < 0.05$), while b^* recorded a *P*-value = 0.154 (thus exceeding the confidence level considered).

On the other hand, interesting results were outlined by looking at the a^* parameter, which resulted similar to the control sample when considering all the duckweed concentrations tested. Generally, a higher ability for ASC to preserve the redness of the burgers was recorded at T9 (Table 1), likely due to a higher antioxidant and preserving effect of ascorbic acid on both lipid and protein oxidation. Accordingly, the TBARS assay confirmed a higher ability of ascorbic acid to cope with oxidative stress, recording a lower MDA production through the entire storage period when compared with the other meat samples under investigation (Table 1). However, it is also important to mention that a significant effect of duckweed extracts was observed at T9 of storage, recording an MDA production lower than the CON sample. Accordingly, as far as the interaction of the fixed variables is concerned, we found that the combination of 'storage time *x* treatment' significantly affected both a^* and MDA parameters, recording *P*-values < 0.001 (data not shown). The MDA (1,3-propanedial) is among the most important and studied aldehydes arising from secondary oxidation of polyunsaturated fatty acids. It is described as directly involved in the generation of rancid aromas at low amounts and represents the major marker of lipid oxidation (Rocchetti et al., 2021). According to scientific literature, MDA values up to 2.5 mg/kg are considered as the acceptable limit in meat products and associated with no rancidity (Zhang et al., 2019). However, it is difficult to clearly correlate TBARS with global acceptability of the meat product, considering the great meat sample variation, the potential methodological errors during TBARS assay, the amount of sample tested, or not well-trained panels/consumers used for sensory analyses (Zhang et al., 2019). Finally, Pearson's correlation coefficients were calculated based on the MDA values and the $L^*a^*b^*$ parameters (not shown). Interestingly, a negative and significant correlation was found between MDA and a^* parameter at both T9 (-0.85; $P < 0.001$) and T19 (-0.87; $P < 0.001$), consistently with scientific literature where it is reported as the redness reduction is a typical marker of long storage periods of meat products (Saluena et al., 2019) because of the advance of lipid and protein oxidation events.

3.4. Discrimination of beef burgers during storage using both unsupervised and supervised statistics

The untargeted metabolomics-based approach enabled the putative annotation of 2612 metabolites, according to a level 2 of confidence (supplementary Table 1). Then, multivariate statistical approaches (both unsupervised and supervised) were used to inspect differences and similarities between the different burger samples during the storage period. The unsupervised statistical approach was used to provide a first

Table 1
Colour parameters and MDA production for the different beef burgers added with ascorbic acid (ASC), and duckweed extracts (i.e., DE 1, DE 5, and DE 10) when compared with control sample (CON).

Parameters	Time	ASC	DE 1	DE 5	DE 10	CON	SEM	*Sig.*
L*	T0	42.06^{a}	43.93^{b}	43.93^{b}	43.93^{b}	$42.37^{ab,A}$	0.343	*P < 0.05*
	T9	41.55	45.19	43.96	44.76	41.07^{A}	0.668	ns
	T19	41.52^{a}	47.84^{b}	46.25^{b}	48.40^{b}	$48.20^{b,B}$	0.849	*P < 0.05*
	SEM	0.482	1.068	0.969	1.549	1.826		
	Sig.	ns	ns	ns	ns	*P < 0.01*		
a*	T0	$15.46^{a,B}$	$15.11^{a,C}$	$15.11^{a,C}$	$15.11^{a,C}$	$17.54^{b,C}$	0.281	*P < 0.001*
	T9	$16.12^{b,B}$	$10.79^{a,B}$	$10.91^{a,B}$	$11.09^{a,B}$	$12.11^{a,B}$	0.569	*P < 0.001*
	T19	$11.64^{b,B}$	$4.55^{a,A}$	$4.55^{a,A}$	$4.63^{a,A}$	$4.31^{a,A}$	0.769	*P < 0.001*
	SEM	0.721	1.540	1.542	1.564	1.933		
	Sig.	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*		
b*	T0	13.79^{b}	11.18^{a}	$11.18^{a,A}$	11.18^{a}	13.68^{b}	0.364	*P < 0.001*
	T9	15.08^{b}	10.83^{a}	$11.88^{a,A}$	12.18^{a}	12.85^{ab}	0.493	*P < 0.05*
	T19	15.31	12.58	14.65^{B}	13.91	13.24	0.375	ns
	SEM	0.388	0.346	0.598	0.602	0.397		
	Sig.	ns	ns	*P < 0.05*	ns	ns		
MDA (mg/100 g)	T0	$0.02^{a,A}$	$0.07^{c,A}$	$0.06^{bc,A}$	$0.05^{b,A}$	$0.09^{d,A}$	0.006	*P < 0.001*
	T9	$0.05^{a,B}$	$0.21^{b,B}$	$0.22^{b,B}$	$0.21^{b,B}$	$0.26^{c,B}$	0.019	*P < 0.001*
	T19	$0.23^{a,C}$	$0.31^{bc,C}$	$0.30^{bc,C}$	$0.31^{bc,C}$	$0.32^{c,C}$	0.009	*P < 0.001*
	SEM	0.033	0.035	0.036	0.038	0.035		
	Sig.	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*	*P < 0.001*		

$^{A-C}$ Mean values in the same column (same treatment in different days) with different letters indicate significant differences ($P < 0.05$); $^{a-c}$ Mean values in the same row (different treatments in the same day) with different letters indicate significant differences ($P < 0.05$). SEM: standard error of the mean; ns = not significant ($P > 0.05$); Sig.: Significance.

degree of knowledge on the chemical similarities and differences between the different treatments during the 19-days storage period. The heat map generated with the unsupervised HCA is provided in Fig. 1A and the corresponding PCA score plot showing the total variability explained (Fig. 1B). As far as the HCA heat map is concerned, two main clusters could be found; the cluster on the left side of the heat map was represented by samples at T0, while the second cluster (including two additional sub-clusters, for burgers at T9 and T19) highlighted a clear impact of the storage time. As expected, the heat map clearly indicated that the storage time had a hierarchically higher impact when compared to the treatment considered (i.e., duckweed addition) in determining the observed chemical profiles. Interestingly, the HCA provided a first indication of the chemical differences between ASC and DE burgers; this trend was evident by looking at both storage times (Fig. 1A, second cluster). The principal component analysis (PCA) was then used to plot the overall variability explained by the annotated metabolites. The PCA score plot confirmed the output outlined by HCA, showing a clear effect of the storage time on the chemical modifications, and revealing an exclusive profile following the addition of ASC (Fig. 1B). However, the chemical differences driven by ASC became less evident at T19. Overall, the two principal components (PC1 and PC2) explained the 55 % of the total variability, thus highlighting the suitability of the untargeted metabolomics dataset to study the changes in meat quality and stability during storage.

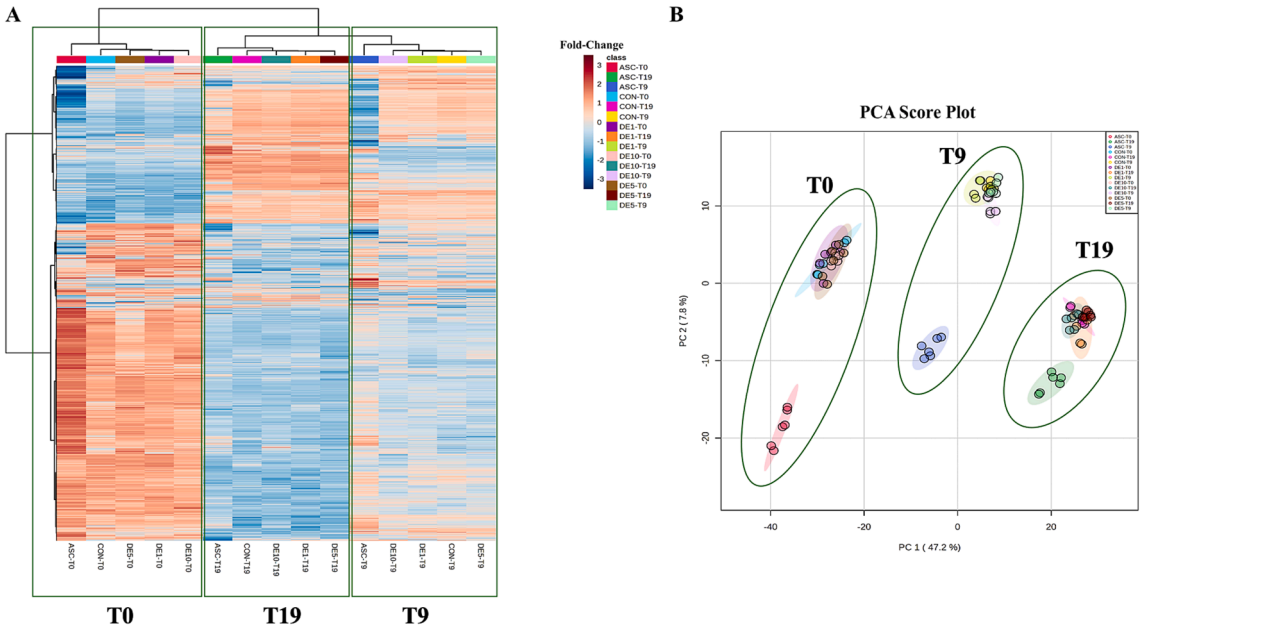

Fig. 1. Unsupervised hierarchical cluster analysis (HCA) (A) and principal component analysis (PCA) score plot (B) built considering the different burger samples at T0, T9, and T19 storage time-points, considering CON, ASC, DE1, DE5, and DE10 treatments.

3.5. Effect of duckweed extract on oxidation processes during storage

The effect of increasing levels of DEs on both lipid and protein oxidation was then evaluated through supervised multivariate statistics. In particular, two supervised OPLS-DA score plots were built, considering burger samples at both T9 (Fig. 2A) and T19 (Fig. 2B) of storage conditions. Overall, both supervised models were characterized by a high prediction ability, recording Q^2 values of 0.69 (at T9) and 0.74 (at T19), thus confirming the ability of untargeted metabolomics to evaluate the chemical changes of the different meat samples during storage. The OPLS-DA model built on samples at T9 provided a total of 1200 discriminant compounds with a VIP score > 1 (i.e., a high prediction ability), while those built at T19 outlined 1143 discriminant compounds. For both prediction models, a numerically high abundance of amino acids, peptides, lipid derivatives (including fatty esters and amides), aldehydes, ketones, and other bioactive compounds (such as phenolics, glucosinolates, and their metabolites) was detected. The significant metabolites outlined by both prediction models and discussed as related to oxidation phenomena and quality deterioration are reported in Table 2. The highest VIP scores were recorded at T9 for 2 compounds, namely 3-hydroxyhexanoylcarnitine (VIP score = 1.61) and inosine triphosphate (VIP score = 1.60). Medium-chain acylcarnitines (like 3-hydroxyhexanoylcarnitine) are fatty acid metabolites that play important roles in many cellular energy metabolism pathways and have been studied as potential biomarkers of fatty acid oxidation and related disorders (Dambrova et al., 2022). Our data revealed that the accumulation of 3-hydroxyhexanoylcarnitine at T9 was higher in DE1 and CON samples when compared with ASC, DE5, and DE10. In particular, ASC and DE10 showed the lowest accumulation of this metabolite when compared with CON, recording Log_2 FC values of −2.3 and −0.8, respectively. Inosine triphosphate represents an intermediate in the purine metabolism pathway and precursor of inosine 5′-monophosphate that contributes to the umami taste in beef (Uemoto et al., 2017). This compound was significantly up-accumulated in DE10 beef samples when compared with the other treatments, showing a Log_2 FC value of 2.5 when compared with CON sample, thus indicating a potential impact of the extract formulated on the sensory properties of the meat product. As far as the lipid oxidation is concerned, discriminant markers at T9 also included two alkadienals (i.e., reactive aldehydes recognized as secondary lipid oxidation products) potentially affecting meat's taste and odour, namely 2,6-nonadienal (VIP score = 1.01) and 3,6-undecadienal (VIP score = 1.33). Beef burgers added with ASC were the most effective in preserving burgers from lipid oxidation, recording a significant Log_2 down-accumulation of both aldehydes when compared with CON, being −5.1 (for 3,6-undecadienal) and −0.9 (for 2,6-nonadienal). Amid duckweed extracts, DE1 beef samples were the most efficient against forming these reactive aldehydes, recording Log_2 down-accumulation values of −0.6 (for 3,5-undecadienal) and −0.4 (for 2,6-nonadienal). The trends observed for alkadienals are coherent with that measured for 13(S)-hydroperoxylinolenic acid, a hydroperoxide deriving from the peroxidation of linolenic acid. Compared to CON, this metabolite was significantly down-accumulated in ASC burgers, recording a Log_2FC value of −1.7. However, all the duckweed extracts tested (DE1, DE5, and D10) were not effective in preserving linolenic acid from the peroxidation, and this was true mainly by looking at the Log_2FC values recorded for DE1 (0.5) and DE5 (0.6) burger samples. Regarding derivatives of linoleic acid, ASC was again the best in preserving from the accumulation of the hydroperoxide (9S,10E,12Z)-9-hydroperoxy-10,12-octadecadienoate, recording a Log_2FC value of −1.7. However, we also found a significant preserving effect towards this compound as exerted by the duckweed extracts (although lower when compared to ASC), recording the best result for DE1 burgers samples (Log_2FC = -0.6). Another important VIP marker compound detected at T9 of storage is 4-hydroxy-2E-nonenal (VIP score = 1.01), a secondary oxidation product of n-6 fatty acid oxidation, highly reactive and studied in meat science as a final product of lipid peroxidation (Rocchetti et al., 2022). This aldehydic compound has been previously studied for its ability to interact with myoglobin, accelerating oxymyoglobin oxidation (Faustman et al., 2010). Interestingly, at T9, 4-hydroxy-2E-nonenal was down-accumulated in beef burger samples added with DE1 and DE5 when compared with the CON sample, recording Log_2FC values of −1.8 and −1.5, respectively, thus allowing to postulate a potential ability of low doses of duckweed extracts on preserving linoleic acid derivatives from oxidation.

As far as the prediction model at T19 is concerned, the highest VIP scores (>1.65) at T19 were recorded for 2 compounds, being both down-accumulated *vs* the CON sample, namely PE(14:0/18:3(6Z,9Z,12Z)) (a phospholipid) and hovenidulcigenin A (a diterpene lactone). Interestingly, at T19 we measured a significant down accumulation of 13(S)-hydroperoxylinolenic acid in ASC (Log_2FC = -1.97), followed by DE5 (-0.23) and DE10 (-0.17), thus confirming a greater ability of ascorbic acid as an antioxidant to contrast lipid peroxidation on 19 days of storage. Regarding alkadienals, DE10 was the most effective extract to counteract the formation of these marker compounds, such as 2,6-nonadienal and 3,6-undecadienal. Interestingly, the oxidative marker 4-hydroxy-2E-nonenal was not listed among the most important predictors at T19. In contrast, its oxidative derivative, namely 2-nonenoic acid, was listed as highly discriminant (VIP score = 1.25), recording a significant up-accumulation in all samples except beef burgers added with DE10 (Log_2FC = -0.26). Therefore, the absence of 4-hydroxy-2E-nonenal as discriminant marker compounds at T19 is coherent with a dynamic status of lipid oxidation processes during storage time, thus determining further modifications/oxidation of the recognized marker compounds. Also, looking at some typical marker compounds of oxidation events, it is important to mention the trends for glutathione (GSH) and its disulfide oxidized form (GSSG). In living cells, GSSG is reduced into two

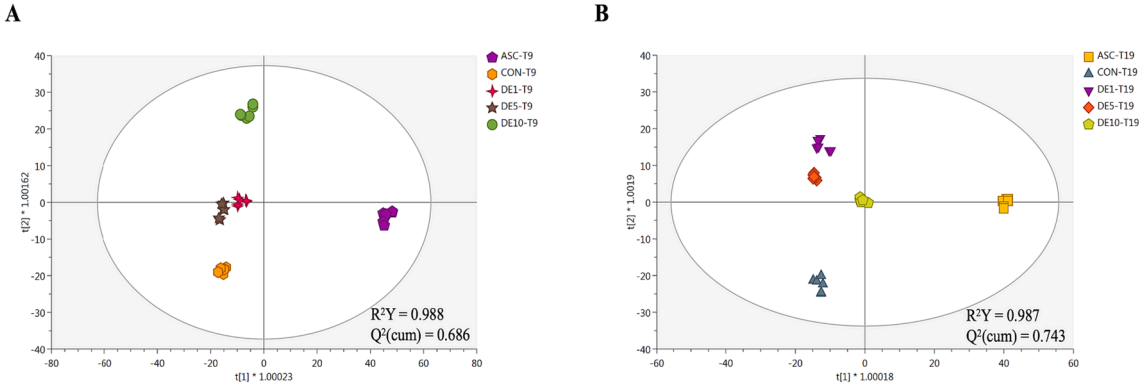

Fig. 2. Supervised orthogonal projections to latent structures discriminant analyses (OPLS-DA) considering the different burger samples at both T9 (A) and T19 (B) storage time-points, considering CON, ASC, DE1, DE5, and DE10 treatments.

Table 2
Discriminant and significant VIP metabolites ($P < 0.05$) associated with meat oxidation and deterioration, considering the comparisons against the CON sample at both T9 and T19 storage times. Each compound is provided with its VIP score and Log_2FC value. * = compounds exclusively associated with a time-point of storage.

Discriminant compounds T9	**VIP score T9**	**Log_2 FC ASC *vs* CON**	**Log_2 FC DE1 *vs* CON**	**Log_2 FC DE5 *vs* CON**	**Log_2 FC DE10 *vs* CON**
L-Ascorbic acid*	1.37 ± 0.58	0.17	0.15	−0.09	−0.26
3-hydroxyhexanoylcarnitine	1.61 ± 0.17	−2.34	0.66	−0.45	−0.79
Inosine triphosphate	1.60 ± 0.27	0.98	1.05	−0.005	2.46
2,6-nonadienal	1.01 ± 0.41	−0.96	−0.44	−0.15	0.28
3,6-undecadienal	1.33 ± 0.08	−5.09	−0.57	−0.25	−0.21
13(S)-hydroperoxylinolenic acid	1.03 ± 0.71	−1.72	0.48	0.65	0.09
(9S,10E,12Z)-9-hydroperoxy-10,12-octadecadienoate	1.02 ± 0.35	−1.74	−0.61	−0.09	−0.15
12,13-Epoxy-9,15-octadecadienoic acid	1.32 ± 0.20	−2.61	−0.31	−0.38	−0.54
4-hydroxy-2E-nonenal*	1.01 ± 0.56	0.13	−1.84	−1.53	0.36
Spermidine	1.50 ± 0.36	0.33	0.24	0.04	0.40
N^1-acetylspermine	1.16 ± 0.29	−4.33	−0.79	−0.75	−0.49
Homogentisic acid*	1.49 ± 0.37	0.28	−0.001	−0.16	−0.09
Discriminant compounds T19	**VIP score T19**	**Log_2 FC ASC *vs* CON**	**Log_2 FC DE1 *vs* CON**	**Log_2 FC DE5 *vs* CON**	**Log_2 FC DE10 *vs* CON**
Glutathione (GSH)*	1.11 ± 0.27	−1.87	−0.18	0.008	0.37
Glutathione disulfide (GSSG)*	1.33 ± 0.51	−0.16	1.06	0.87	−0.07
2,6-nonadienal	1.35 ± 0.21	1.12	1.81	0.31	1.31
3,6-undecadienal	1.31 ± 0.24	−1.32	0.04	−0.04	−0.32
13(S)-hydroperoxylinolenic acid	1.22 ± 0.24	−1.98	−0.08	0.34	−0.170
12,13-Epoxy-9,15-octadecadienoic acid	1.32 ± 0.29	−0.86	0.06	−0.16	−0.07
2-nonenoic acid*	1.25 ± 0.50	0.11	0.18	0.01	−0.26
PE(14:0/18:3(6Z,9Z,12Z))	1.71 ± 0.34	−0.59	−1.39	−1.20	−1.31
Hovenidulcigenin A	1.66 ± 0.44	−1.96	−2.14	−1.81	−1.50
Adipate semialdehyde*	1.01 ± 0.51	−0.07	2.66	0.69	0.51
Tyramine*	1.11 ± 0.59	−0.53	−0.04	−0.84	−0.52
Tryptamine*	1.07 ± 0.38	0.19	−2.16	−3.06	−1.84
Spermidine	1.21 ± 0.46	0.25	0.14	−0.02	0.15
N^1-acetylputrescine*	1.39 ± 0.66	0.90	1.04	0.91	0.74
N^1-acetylspermidine*	1.38 ± 0.14	0.10	0.13	−0.03	−0.32
N^1-acetylspermine	1.09 ± 0.38	−2.14	0.06	−0.37	−0.21
Tryptamine*	1.07 ± 0.38	0.19	−2.16	−3.06	−1.84
Cyanidin-3-*O*-*p*-coumaroyl glycosides*	1.46 ± 0.42	0.17	−1.36	−1.44	−1.29

molecules of glutathione (GSH), a reaction catalysed by the enzyme glutathione reductase. Also, the antioxidant enzymes, namely glutathione peroxidases and peroxiredoxins, can generate GSSG while reducing peroxides and organic hydroperoxides. Other enzymes, e.g., glutaredoxins, can generate GSSG through other mechanisms, including a thiol-disulfide exchange with protein disulfide bonds or other small compounds (i.e., coenzyme A disulfide or dehydroascorbic acid). Therefore, the GSH:GSSG ratio is a very important indicator of cellular health, being a higher ratio associated with less oxidative stress (Wang et al., 2018). In this work, both GSH and GSSG were significant exclusive markers of the OPLS-DA model built at T19, thus likely indicating the activation of the antioxidant machinery involving glutathione and its disulfide to counteract oxidative stress during prolonged storage conditions. In particular, DE1 and DE5 determined a marked activation of the redox system, recording Log_2 Fold-Change values equal to 1.06 and 0.87, respectively, for GSSG. On the other hand, a significant and negative accumulation of GSSG was found when comparing ASC and DE10 with CON sample, recording Log_2 FC values equal to −0.16 and −0.06, respectively, thus reflecting a lower imbalance of the GSH:GSSG ratio. Regarding ascorbic acid as a compound involved in the oxidation impairment of meat, it is known that cattle can synthesize L-ascorbic acid from sugars (such as either D-glucose or D-galactose) by exploiting the glucuronic acid pathway in the liver (Ranjan et al., 2012). The untargeted metabolomics approach revealed a significance of ascorbic acid only at T9, recording a higher accumulation in ASC burger samples at T9 when compared with CON (Log_2 FC = 0.16), followed by DE1 treatment (Log_2 FC = 0.15). On the other hand, this compound was down-accumulated at T9 after applying DE5 and DE10, likely indicating its conversion to its oxidized form to protect against oxidative stress.

Regarding protein oxidation, this phenomenon has not been studied in such detail like for lipid oxidation and peroxidation likely because, despite being proteins susceptible to oxidizing agents, their reaction kinetics are slower compared to lipids (Hadidi et al., 2022). The protein oxidation partially depends on lipid oxidation; in fact, lipid oxidation can generate several radical species reacting with proteins (Nawaz et al., 2022). The protein modification during oxidation events can affect the amino acid composition of protein chains, determining potential polymerization and/or loss of proteolytic activity. In addition, it is known that amino acids, such as Arg, Cys, His, Lys, Met, Phe, Pro, Trp, and Tyr, are prone to oxidation. Interestingly, the compounds alpha-amino adipic semialdehyde (AAS) and gamma-glutaminic semialdehyde (GGS) are considered suitable indicators of protein oxidation, accounting for at least the 60 % of the total carbonyl compounds in foods (Guyon et al., 2016). Under our experimental conditions, we successfully annotated and confirmed the structural identity of the compound adipate semialdehyde; this metabolite resulting from protein oxidation showed a significant prediction ability only at T19, recording a VIP score > 1. Interestingly, this compound was significantly up-accumulated in all DE-added beef burgers compared with CON, particularly for samples prepared with DE1, recording a Log_2FC value of 2.7. Therefore, by inspecting the different metabolites recorded and their variations, it was clear that protein oxidation was hierarchically more pronounced than lipid oxidation in driving the chemical modifications observed by colour analysis and coherently with the matrix analysed (i.e., beef meat).

Regarding the modifications of proteins, peptides, and amino acids, we found a good prediction ability at T19 of storage for two biogenic amines, namely tyramine and tryptamine, characterized by VIP scores 1.11 and 1.07, respectively. Tyramine (derived from microbial decarboxylase activity on tyrosine), widely reported in the literature as a quality indicator of stored beef samples, together with spermine and spermidine (Triki et al., 2018), was significantly down-accumulated in all burgers added with duckweed extracts, with the lowest value outlined for DE5 burger samples (Log_2FC = −0.84). In this regard, beef samples added with ASC showed a Log_2FC down accumulation of −0.52. Tryptamine (derived from microbial decarboxylase activity on tryptophan) was significantly down-accumulated only in beef burgers added with duckweed extracts, recording the maximum effect for the DE5 samples (Log_2FC = −3.06), followed by DE1 and DE10. Regarding other

typical polyamines, such as putrescine, spermidine and spermine, the untargeted metabolomics approach showed a higher prediction ability of these metabolites after 19 days of storage, annotating several intermediates typical of their turnover, namely N^1-acetylputrescine, N^1-acetylspermidine, and N^1-acetylspermine (Table 2). The interconversion of polyamines represents an important cyclic process, directly involved in their turnover and regulating intracellular homeostasis (Schirone et al., 2022). Interestingly, our findings suggested that duckweed extracts were particularly effective against the accumulation of biogenic amines arising from aromatic amino acids, such as tyrosine and tryptophan. This technological effect could be crucial in defining the overall quality of beef burger samples within shelf-life and could be linked to the recognized antimicrobial activity of duckweed extracts (Gülçin et al., 2010). As the final step, a Venn diagram was built to compare VIP discriminant compounds at T9 and T19, to point out the metabolites most contributing to the different behaviour observed in terms of protein and lipid oxidation phenomena. As a first consideration, a higher number of exclusive discriminant metabolites was found at T19 (i.e., 19 compounds), with a large abundance of polyphenols (13 compounds, including anthocyanins, flavanols, flavones, isoflavonoids, stilbenes, and lower-molecular-weight phenolics), followed by 5 terpenoids and 1 glucosinolate. Indeed, the Venn diagram highlighted only 5 exclusive and discriminant compounds potentially associated with the duckweed extracts at T9, with the highest prediction ability owned by homogentisic acid (VIP score = 1.49).

The results obtained in this work agree with the complexity of oxidative events when considering the storage of a perishable food product such as meat. Regarding using duckweed extract as shelf-life extender, our data suggested that duckweed phytochemicals characterizing the formulated extracts (i.e., DE1, DE5, and DE10) presented low chemical stability or limited biological activity in the meat environment. The next plausible strategy could probably be based on encapsulating the extracts within *ad-hoc* designed colloidal particles thus providing the best possible dispersibility, chemical stability and matrix compatibility. As recently reviewed by Hadidi et al. (2022), encapsulation methods (e.g., freeze drying, spray-drying, and coacervation) can enhance the stability of meat products through the addition of antioxidants rom plant extracts; therefore, further studies on the encapsulation of *L. minor* phytochemicals appear of both great interest and relevance for meat science area. Besides the application of duckweed extract, it is also important to report that lipid oxidation in meat is strictly linked to myoglobin oxidation; in particular, as reviewed by Faustman et al. (2010), these processes are strongly intercorrelated. Meat discoloration is basically due to the conversion of oxymyoglobin to metmyoglobin and mainly based on the iron redox status in myoglobin. Overall, muscles containing higher relative proportions of red fibers, and thus more lipid and oxygen consumption rates, are reported to discolour more quickly (Faustman al., 2010). Furthermore, many factors can affect oxymyoglobin oxidation, such as temperature, pH, metmyoglobin reducing activity, partial oxygen pressure and lipid oxidation. Also, oxymyoglobin oxidation is favoured by higher temperatures, lower pH values and the presence of non-heme iron. Some of these factors (such as lipid oxidation, packaging conditions, and non-heme iron provided by duckweed extract) may have been involved in the results obtained in this work on packaged beef burgers, looking at meat discoloration and lipid oxidation.

4. Conclusions

In this work, a duckweed extract (at three concentration levels) was tested to evaluate its ability as a shelf-life extender during 19-days of storage under modified atmosphere packaging conditions. The duckweed extracts added to beef burgers changed meat metabolome, likely due to the abundance of different bioactive compounds, such as polyphenols, terpenoids, and glucosinolates. Metabolomics revealed that duckweed extracts were ineffective in preserving linolenic acid from peroxidation, while a higher antioxidant potential was observed towards linoleic acid derivatives following 9 days of storage. The redox status of the meat matrix was greatly affected by duckweed inclusion, recording a lower imbalance of the GSH:GSSG ratio at 19 days of storage for meat samples added with ascorbic acid and 10 g/kg of duckweed extract. Also, this work showed a hierarchically higher impact of protein oxidation to drive the oxidation of the product by looking at the accumulation of the marker gamma-glutaminic. However, the different duckweed extracts were greatly effective against the accumulation of biogenic amines. Taken together, our findings suggest that duckweed, as a source of several antioxidant compounds, could be exploited to extend the shelf-life of meat-based products, considering its preserving activity towards the oxidation of linoleic acid derivatives. However, more studies are needed to better formulate the extracts (e.g., using different encapsulation-based technologies) and to better understand the mechanisms of action of duckweed phytochemicals (as antioxidant and/or pro-oxidant agents) when considering both lipid and protein oxidation events. Finally, our findings confirmed that untargeted metabolomics can provide new and robust biomarkers in meat science, thus representing a novel approach to assess the ability of natural extracts as extenders during storage.

CRediT authorship contribution statement

Gabriele Rocchetti: Methodology, Formal analysis, Data curation, Software, Investigation, Writing – original draft, Writing – review & editing, Visualization, Validation. **Annalisa Rebecchi:** Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Leilei Zhang:** Methodology, Formal analysis. **Michele Dallolio:** Methodology, Writing – original draft. **Daniele Del Buono:** Conceptualization, Writing – review & editing. **Giorgio Freschi:** Writing – review & editing. **Lugi Lucini:** Conceptualization, Supervision, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.101013.

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