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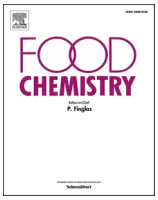

Mango (*Mangifera indica* L.) young leaf extract as brine additive to improve the functional properties of mozzarella cheese

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ABSTRACT

Mango (*Mangifera indica* L.) has been an important plant in traditional medicine for over 4000 years, probably because of its remarkable antioxidant activity. In this study, an aqueous extract from mango red leaves (M-RLE) was evaluated for its polyphenol profile and antioxidant activity. The extract was used as brine replacement (at 5%, 10% and 20% v/v) in fresh mozzarella cheese for improving its functional properties. During storage (12 d at 4 ± °C), compositional analysis performed on mozzarella has shown a progressive increase of iriflophenone 3-*C*-glucoside and mangiferin, the compounds most present in the extract, with a noticeable preference for the benzophenone. At the same time, the antioxidant activity of mozzarella peaked at 12 d of storage, suggesting a binding action of that matrix for the M-RLE bioactive compounds. Moreover, the use of the M-RLE has not negatively influenced the *Lactobacillus* spp. population of mozzarella, even at the highest concentration.

1. Introduction

The traditional mozzarella cheese commercialized in Italy is generally produced from local raw milk and characterized by a very short shelf-life, ranging from 1 to 2 weeks at the most (Gammariello, Conte, & Del Nobile, 2010; Ricciardi et al., 2015), which drastically limits its export despite the high worldwide demand. The shelf-life of mozzarella can be affected by the liquid preservation called "brine" in which the product is immersed, that is not a selective substrate for microbial growth and that can harbor psychrotrophic bacteria associated with its spoilage (Andreani et al., 2014; Baruzzi, Lagonigro, Quintieri, Morea, & Caputo, 2012; Guidone et al., 2016). Several studies available in literature evaluated how to prolong mozzarella's shelf life through the modification of brine composition and pH with novel solutions (Faccia, Gambacorta, Natrella, & Caponio, 2019; Zappia, Branca, Piscopo, & Poiana, 2020), while others researches (Dai, Jiang, Corke, & Shah, 2018; Martinez-Martínez & Velez-Ruiz, 2019; Urgu, Unluturk, & Koca, 2018) are more focused on the improvement of its nutritional and functional features. With reference to the microbiological aspects of the product, Ariza et al. (2021) studied the antilisterial effects of a formulation based on citrus extract containing, among others, flavonoids and limonene from bitter orange on a series of ready-to-eat foods, including mozzarella. The authors found out a significant improvement in the hygienic quality of the tested foods, but without measuring the impact of the extract on their organoleptic properties (Ariza et al., 2021).

To the best of our knowledge, no scientific work has been focused on the modification of brine composition with a natural extract in order to improve the functional features of mozzarella throughout the shelf life (Shah, Patel, Patel, & Parmar, 2010), taking advantage of the bioactivity of extract constituents (mainly small compounds). Small bioactive compounds are part of the secondary ("specialized") metabolism of plants and therefore present in variable amounts in all vegetable matrices (Siracusa & Ruberto, 2014). To humans, these compounds are of considerable importance for their properties, such as antimicrobial, anti-inflammatory, antioxidant and generally health-promoting ones (Napoli, Siracusa & Ruberto, 2020). Mango (*Mangifera indica* L.) is a tropical fruit whose cultivation over the years has gained importance also in the Mediterranean area, Italy and especially Sicily (Shah et al., 2010). Many research studies confirm that the leaves of mango tree are an important source of compounds possessing free radical scavenging and antimicrobial activities, such as gallic acid derivatives, benzophenones and xanthones (Ediriweera, Tennekoon, & Samarakoon, 2017).

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Mangiferin, a *C*-glycoside xanthone, is considered characteristic of this species (Luo et al., 2012; Pan et al., 2018; Ribeiro, Barbosa, Queiroz, Knödler, & Schieber, 2008; Ribeiro & Schieber, 2010; Sultana, Hussain, Asif, & Munir, 2012; Zhang et al., 2011). Ramírez et al. (2016) reported that the mango leaf tea contains 0.72 ± 0.08 mg/mL of mangiferin, 1.59 ± 0.11 mg GAE/mL of total phenolics and 80.33% ± 0.18 of radical scavenging activity determined by DPPH method, from which probably derive many benefit properties, such as the decrease of the visceral fat accumulation, the regulation of glucose metabolism, the induction of anti-inflammatory markers and the improvement of adipocytes hypertrophy possessing an anti-obesity effects (Medina Ramírez et al., 2018; Ramírez et al., 2016, 2017). Moreover, a recent study of Rambabu, Bharath, Banat, Show, & Cocoletzi (2019) evidenced as the use of a mango leaf extract incorporated in chitosan film can be considered as a suitable alternative for active packaging films for food preservation. To the best of our knowledge, no study is present in literature dealing with the use of this vegetable matrix in order to improve the functional properties of a food product.

In this context, the present study aims at evaluating the profile and content of polyphenols in an aqueous extract from red young mango leaves. The same extract was also evaluated for its antioxidant activity. As mango leaf extract showed a high content of polyphenols, it was then employed as additive in mozzarella brine, in order to increase the functional properties of this product throughout the refrigerated storage. Moreover, since mozzarella cheese is strongly affected by microbial growth and mass transfer between cheese and brine that determined appearance and texture change, mozzarella samples were evaluated up to 12 d of refrigerated storage for physico-chemical parameters, texture and microbial population in order to verify the effect of extract addition. The overall workflow of our study is displayed in Fig. 1.

2. Materials and methods

2.1. Preparation of mango red leaf extracts (M-RLE)

Leaves of mango tree (*Mangifera indica* L.) "Kensington pride" cultivar characterized by a red color were collected in July from a farm company located in Sicily (Italy), immediately transported to the laboratories of the Department of Agriculture, Food and Environment (Di3A - University of Catania) and dried at 35 ± 2 °C until constant weight. After dehydration, leaves were crushed with a pestle and mortar in order to obtain a homogeneous sample that was subjected to an aqueous extraction following the method reported by Palmeri et al. (2016). In brief, 5 g of powdered dry leaves were soaked in hot water (90 °C) for 30 min in the dark. The resulting suspension was then centrifuged at 15,000 rpm at 4 °C for 10 min (centrifuge ALC 4239R, ALC, Winchester, VA, USA), the supernatant solution recovered by filtration (0.45 μm pore size membrane filter, Millipore®, Burlington, MA, USA) and stored in the dark at −20 °C until use.

2.2. Compositional features, antioxidant activity and physico-chemical characteristics of M-RLE

2.2.1. Compositional analysis (HPLC/DAD and HPLC/ESI-MS)

A small aliquot (5 mL) of the above-mentioned M-RLE was transferred into 2 mL HPLC amber vials and directly subjected to analytical determinations. The aqueous extract, object of this study, was evaluated for its polyphenol profile and content through qualitative and quantitative chromatographic analyses performed on a Ultimate3000 UHPLC focussed instrument equipped with a binary high-pressure pump, a Photodiode Array detector, a Thermostated Column Compartment and an Automated Sample Injector (Thermo Fisher Scientific, Inc., Milan,

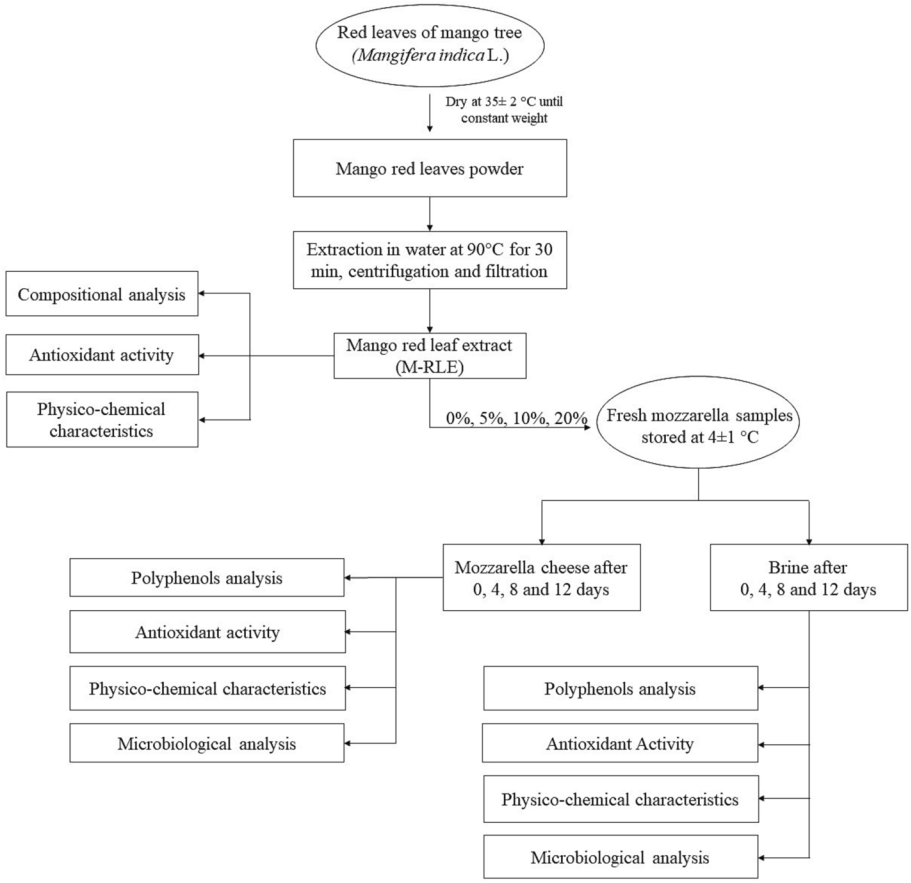

Fig. 1. Workflow of the study.

Italy). The elution conditions used (column, guard column, elution program) as well as quantification parameters are reported in supplementary material. A series of HPLC/ESI-MS analyses were also performed to corroborate the assignments (see Sferrazzo et al. 2022 for analysis and acquisition parameters). All the analyses were carried out in triplicate.

2.2.2. Antioxidant activity, pH and color of M-RLE

Antioxidant activity of M-RLE was evaluated through two methods: the DPPH (1,1-Diphenyl-2-picryl-hydrazyl) assay reported by Brand-Williams, Cuvelier, & Berset (1995) and the FRAP (Ferric Reducing Antioxidant Power) assay reported by Perna, Intaglietta, Simonetti & Gambacorta (2014) with slight modifications.

In brief, the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity assay was conducted by mixing 3 mL of methanol DPPH (2,2-diphenyl-1-picrylhydrazyl) solution (100 μM) with 50 μL of extract, homogenized and incubated in the dark for 1 h at 25 °C. The Control sample (blank) was prepared in the same way but the same amount of extract was replaced with methanol. At the end of the reaction period the absorbance of each sample was read at 515 nm using a Perkin Elmer lambda 25 UV–Vis spectrometer (PerkinElmer Inc., Waltham, MA, USA). Trolox was used as a standard to create an eight-point standard curve (0–75 mg/L) and for each extract the antioxidant capacity was expressed as mg/L of trolox equivalents. FRAP assay was conducted mixing the extract with 2.9 mL of FRAP reagent, daily prepared by adding 10 mL of 300 mM acetate buffer (pH 3.6), 1 mL of 10 mM 2,4,6-tripyridal-s-triazine (TPTZ) in 40 mM HCl, and 1 mL of 20 mM $FeCl_3$ (in the ratio 10:1:1 vol/vol/vol). The mixture was incubated at 37 °C for 30 min and at the end of the reaction period the absorbance of each sample was read, against acetate buffer (pH 3.6), at 593 nm using Perkin Elmer lambda 25 UV–Vis spectrometer (PerkinElmer Inc., Waltham, MA, USA). The antioxidant capacity was expressed as mg/L of sample of trolox equivalents.

The M-RLE prepared as reported above was analyzed for pH and color parameters. The pH was measured using a Eutech pH 700 Meter (Thermo Fisher Scientific Inc., Waltham, MA, USA) by placing a pH probe (Eutech pH 700 Meter) directly in the extract. The color was measured using a portable colorimeter Konica Minolta CM-2500d (Bremen, Germany) equipped with a Light Protection Tube with plate 40 mm (CR-A33b) and a sample cell made of optical glass (Glass Cell CR-A504), using as illuminant D65. CIE L*a*b* space parameters were expressed as Lightness (L*), redness (a*) yellowness (b*).

2.3. Preparation of mozzarella samples with M-RLE as brine ingredient

Fresh mozzarella cheese was bought in a small dairy located in the Catania province (Italy) at the beginning of the shelf life and immediately transported, under refrigerated condition, to the laboratories of Di3A. Samples were divided under sterile conditions into four different trays with a lid, previously exposed to UV radiation, in order to obtain six pieces of mozzarella of about 50 g for each tray. Mozzarella samples were then covered with their own brine solutions (about 150 mL for each tray), in which the M-RLE was added to reach a concentration equal to 5, 10 and 20% (vol/vol). A Control sample was made with brine without any extract addition. Each tray was stored under refrigerated condition (4 ± 1 °C) up to 12 days.

2.4. Determination of bioactive compounds of mozzarella cheese added with M-RLE

In order to monitor the chemical composition and functional features of the mozzarella portions added with M-RLE along time, a series of analyses were carried out on all samples at different time intervals: 0 (immediately after preparation), 4, 8, and 12 d of refrigerated storage (4 ± 1 °C), for a total of 4 samples × 4 sampling times. All the analyses were performed both on mozzarella portions (M) immersed in M-RLE brine solutions and on the corresponding brines (BS), plus the Control sample. For each determination a minimum of three repetitions were performed and results were expressed as the mean values ± standard deviation.

2.4.1. M-RLE polyphenols in experimental mozzarella and brine samples

The presence and amount of M-RLE polyphenols in mozzarella and corresponding brines, object of the study, were assessed by treating aliquots (5 g) of cheese samples with a mixture of methanol-formic acid–water (80:1:19) as reported in Parafati, Pesce, Siracusa, Fallico, Restuccia, & Palmeri (2021). The corresponding brine samples were centrifuged twice at 15 °C and 10000 rpm (Sorvall RC-5B Refrigerated Super Speed centrifuge, Fisher Scientific Italia, Rodano (MI), Italy), filtered (0.45 μm pore size membrane filter, Millipore®, Burlington, MA, USA) transferred in 2 mL HPLC amber vials and sent to analytical determinations following the same protocol reported in paragraph 2.2.1.

2.4.2. Antioxidant activity

The antioxidant activity of mozzarella cheese immersed in brine solution containing M-RLE at different concentrations (5%, 10% 15% and 20%) was evaluated after 4, 8 and 12 d of storage at 4 ± 1 °C. Antioxidant activity of treated samples was compared with that of Control sample made without any extract addition; this latest was also evaluated at the beginning of the storage period (time 0) in order to establish the initial antioxidant activity of the mozzarella cheese.

The bioactive compounds from mozzarella cheese were recovered by using a mixture of methanol:formic acid:water (80:1:19) and treated as already reported by Parafati et al. (2021). In brief, an aliquot (5 g) of each sample under study was extracted with the mentioned mixture, centrifuged twice at 15 °C and 9000 rpm (Sorvall RC-5B Refrigerated Super Speed centrifuge, Fisher Scientific Italia, Rodano (MI), Italy) and filtered through a 0.45 μm pore size membrane filter (Millipore®, Burlington, MA, USA). The clear supernatants were analyzed using the DPPH and FRAP methods as previously described in section 2.2.2. With the same protocol, after centrifugation and filtration, all the corresponding preserving brine samples were also analyzed. Each analysis was repeated at least three times.

2.4.3. Physicochemical properties of mozzarella and color evaluation

The Relative Humidity (RH%) of each mozzarella sample was determined using a gravimetric method by drying the sample at 105 °C until constant weight.

Textural properties of mozzarella cheese samples were analyzed by using the Texture Analyzer Zwick/Roell model Z010 (Zwick Roell Italia S.r.L., Genova, Italy) equipped with a spherical probe (P/1S). Texture analysis was conducted following the method reported by Dai et al. (2019) with slight modifications. In brief, each sample maintained at 4 ± 1 °C was cut into approximately 40 mm in diameter and 25 mm in height and penetrated with the probe up to of 5 mm of depth. Test conditions were: pre-load of 0.01 N, cell load of 50 N, and a cross head speed constant of 1 mm/s and turned to its original position at the constant speed of 10.0 mm/s. The parameters firmness (N), representing the force at the maximum distance of a penetration, were monitored up to 12 d of storage.

The pH of mozzarella cheese was evaluated by homogenizing 10 g of each sample in 100 mL of distilled water through the Ultra Turrax T18 equipment (IKA ULTRA-TURRAX®, Wilmington, NC, USA). Immediately after the sample homogenization, pH was measured by using a Eutech pH 700 Meter (Thermo Fisher Scientific Inc., Waltham, MA, USA). The pH was evaluated also on brine by placing the pH probe directly into the solution.

Final values of RH%, firmness and pH were expressed as mean ± standard deviation of three independent replicates.

The color of mozzarella surface and brine solution was described in terms of Lightness (L*), redness (a*), yellowness (b*) space values and chroma (C*), representing the color intensity (CIE L*a*b*), by using a

Konica Minolta CM-2500d (Konica Minolta sensing Europe B.V., Bremen, Germany) equipped with a Light Protection Tube with plate 40 mm (CR-A33b) and a sample cell made of optical glass (Glass Cell CR-A504), using as illuminant D65. Color parameters were checked up to 12 d of refrigerated storage and expressed as mean value ± standard deviation of 6 random readings.

2.4.4. Microbiological analysis

The effect on microbiological counts of M-RLE addition at different concentrations to the brine solution of mozzarella cheese was evaluated by monitoring the microbial population development after 0, 4, 8, and 12 d of storage at 4 ± 1 °C. Mozzarella cheese (each of approximately 25 g) was weighed under sterile condition and aseptically transferred into a stomacher filter bag containing proportional amount of sterile Ringers solution (Oxoid, Basingstoke, UK) and homogenized for 5 min with a stomacher. Serial dilutions of each mozzarella sample and serial dilutions of the respective brines, prepared with the same diluent, were plated in Petri dishes containing: Plate Count Agar (PCA; Oxoid, Basingstoke, UK) with cycloheximide 0.1% solution (Oxoid), to monitor the growth of Total Mesophilic Bacteria (TMB); Mannitol Salt Agar (MSA; Oxoid, Basingstoke, UK) for the count of *Staphylococcus* spp.; Violet Red Bile Glucose Agar (VRBGA; Oxoid), to evaluate the growth of total *Enterobacteriaceae*; Pseudomonas Agar Base (PAB, CM0559, Oxoid), supplemented with Pseudomonas CFC selective agar supplement (SR0103, Oxoid) for the count of total *Pseudomonas* spp; De Man, Rogosa and Sharpe agar (MRS; Oxoid) for the enumeration of *Lactobacillus* spp. The plates were incubated for 24–48 h at 32 or 25 °C (for the *Pseudomonas* spp. count). Bacterial colonies were counted from 3 replicates, and the mean was expressed as log CFU (colony forming unit)/g of mozzarella ± standard deviation and log CFU/mL of brine ± standard deviation.

2.5. Statistical analysis

Data from *in vitro* and *in vivo* experiments were analyzed separately by using the Statistical package software Minitab ™ version 20.0.

One-way analysis of variance (ANOVA) was performed on mean values and Fisher's test was carried out for the comparison of difference among treatments. Differences between sample means were considered significant at $p \leq 0.05$. Standard deviation was obtained from the statistical model and is shown as bars in the figures.

3. Results and discussion

3.1. Physico-chemical evaluation of mango leaf extracts

3.1.1. Polyphenol profile and content of M. indica red leaves aqueous extract (M-RLE)

We have recently evaluated the compositional features and biological activities of a hydroalcoholic extract from mango red leaves (Sferrazzo et al., 2022). In this paper, an eco-compatible extraction method was applied involving the use of pure distilled water as the sole solvent, in order to better suit its use in a food matrix. The corresponding HPLC-DAD chromatogram, visualized at 280 nm, is shown in Fig. S1 (supplementary material). Similarly to what previously reported, benzophenones are the dominant polyphenols in M-RLE, followed by the xanthone mangiferin. Unlike other classes of polyphenols, xanthones are present in nature only in 20 families of higher plants including Clusiaceae and Anacardiaceae that respectively count the *Garcinia mangostana* (mangosteen) and *Mangifera indica* species, where the presence of the *C*-glycosilated xanthone mangiferin can be considered as a peculiarity (Remali, Sahidin, & Aizat, 2022). In aqueous M-RLE nine peaks were identified and quantified; results are reported in Table 1. Apart from mangiferin (peak 5) and peaks 1 and 9, corresponding to gallic acid and galloyl hexose, respectively, all the other chromatographic signals belong to the class of benzophenones, as already mentioned. In contrast with the compositional results reported in Sferrazzo et al. (2022), no flavonoids were detected. It therefore appears that, relatively to this specific matrix, water shows a clear preference towards molecules having a benzophenone/xanthone structure. Iriflophenone 3-*C*-glucoside is undoubtedly the dominant compound; generally, derivatives of iriflophenone, an acetate/shikimate mixed biosynthesis benzophenone (see Fig. S2 in supplementary material) are particularly abundant, covering together>66% of the entire extract (Table 1).

Table 1
Peak list and quantitative data (expressed in mg compound/g dried vegetable material) of metabolites from *M. indica* red leaves aqueous extract (M-RLE) object of this study.

Peak	Rt, min[a]	compound tentative identification	biochemical class	mg/g dried leaves
1	4,537	gallic acid [b]	organic acid	13,24
2	6,339	maclurin 3-*C*- glucoside	benzophenones	41,53
3	8,818	iriflophenone 3-*C*-glucoside	benzophenones	167,73
4	11,211	iriflophenone 3-*C*- (2-*O*-galloyl) glucoside	benzophenones	46,57
5	13,126	mangiferin [b]	xanthones	84,52
6	14,555	iriflophenone derivative isomer 1 [c]	benzophenones	14,36
7	16,68	iriflophenone 3-*C*- (2,6 di-*O*-galloyl) glucoside	benzophenones	36,02
8	19,951	iriflophenone derivative isomer 2 [c]	benzophenones	41,08
9	21,531	galloyl hexose [c]	organic acid	14,92
		Total polyphenols		***459,97***

[a] as mean of three replicates; [b] co-injection with pure analytical standard; [c] correct isomer not identified.

3.1.2. Antioxidant activity, pH and color of mango leaf extract

Extract obtained through water extraction evidenced a really high antioxidant activity equal to 191.23 ± 0.26 and 23616.86 ± 0.27 mg/L of trolox equivalents, respectively evaluated trough DPPH and FRAP assays and expressed with different magnitude in relation to the used assay. The extract obtained recorded a pH equal to 5.13 ± 0.02 and an intense color tending to brick red, as can be derived from the CIE L*a*b* coordinates registered the values of 21.86 ± 0.29, 15.83 ± 0.20 and 34.96 ± 0.35, respectively for L*, a* and b*.

Our results agree with that reported by Ramírez et al. (2016) who identified the decoction as the optimal method for the extraction of mangiferin and, using 5% of leaves, obtained an extract with a pH value equal to 5.140 ± 0.04 and antioxidant activity of 80.331 ± 0.18% when evaluated by DPPH test.

3.2. Effect of mango leaf extract enrichment on mozzarella and brine samples

3.2.1. M-RLE polyphenols in mozzarella and brine samples

Fig. 2 (a and b) and Table S1 and S2 (supplementary material) report the results obtained when analyzing mozzarella samples whose brines have been enriched with M-RLE with different M-RLE/brine ratios (5, 10 and 20%, see also experimental section). There is certainly a retention of phenolic compounds exerted by the cheese matrix, and this retention is concentration and time dependent, as evidenced by the bar graphic in Fig. 2 (a and b). In fact, for the 5% M-RLE the amount of polyphenols found in mozzarella samples has a peak at 8 d of refrigerated storage, at the same time reaching the significantly ($p < 0.0.5$) lowest value in brine (Fig. 2 a and b). Is possible to note that this trend decreases with increasing the amount % of M-RLE in brine (that is, in the order 5% > 10% > 20%). For the 5% and 10% brine solutions we obtained comparable results, far from the double values expected. When considering the amount of total phenolics present in brines, a specular trend to respect to that of cheese samples can be observed, thus confirming a dynamic equilibrium and a mass transfer phenomenon occurring in the

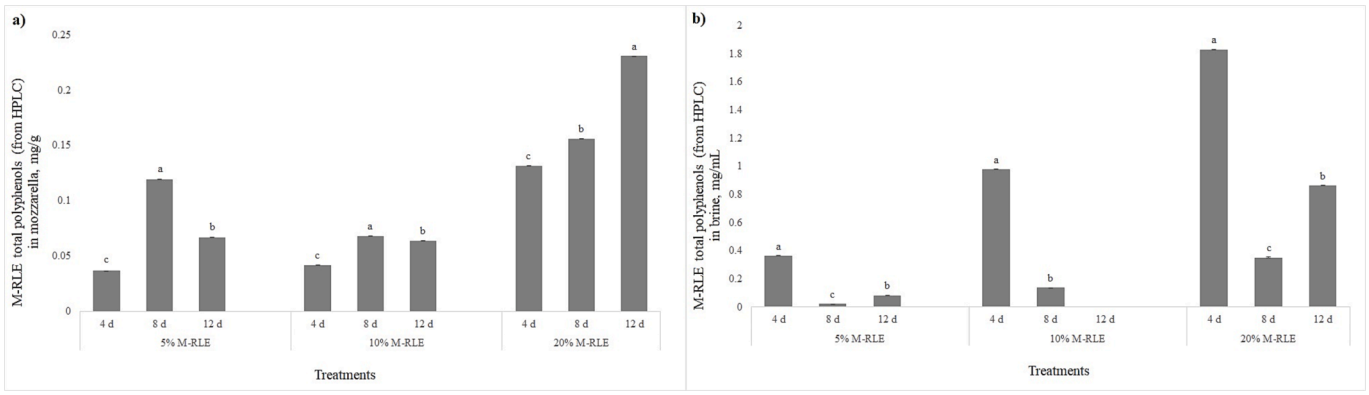

Fig. 2. Total polyphenols (as sum of individual contributions from HPLC analyses) in mozzarella (a) and brine (b) samples object of this study evaluated after 4, 8 and 12 d of refrigerated storage (4 ± 1 °C). See Tables S1, S2 and text for further details. Within each extract concentration (5%, 10% and 20%) column chart with different letter are significantly different according to Fisher's least significant difference test ($p < 0.05$). Vertical bars indicate the standard deviation of the mean.

system mozzarella/brine, as reported in literature (Faccia et al., 2019; Luo, Pan, Guo, & Ren, 2013). Interestingly, when considering the ratio between the two main compounds of the original M-RLE, that is, iriflophenone 3-C-glucoside and mangiferin, a preferential retention by the cheese matrix can be observed, as depicted in Fig. 3. This preference towards the benzophenone is definitely time dependent and can be found for all the solutions (5, 10 and 20%) studied (Fig. 2). In fact, among each treatment (5, 10 and 20% of M-RLE), is possible to note the significantly ($p < 0.05$) highest retention in mozzarella after 12 d and at the same time the significantly ($p < 0.05$) lowest value in brine (Fig. 2). We could hypothesize it is related to the even minimal structural differences between the two molecules, and the corresponding differences in solubility in brine as well as interactions with the mozzarella matrix. In fact, several examples in literature report the ability of milk proteins, including caseins, to bind a wide range of polyphenols due to their self-association into micelles in aqueous solutions (Yildirim-Elikoglu & Erdem, 2018). Nevertheless, further studies with pure analytical standards are compulsory to better clarify the mechanisms underlying this preferential retention.

3.2.2. *Antioxidant properties of mozzarella cheese enriched with M-RLE*

Fig. 4 (a and b) displays the antioxidant activity of mozzarella cheese immersed in brine enriched with different amounts of M-RLE and stored up to 12 d of refrigerated storage at 4 ± 1 °C.

Data obtained using the two different assays, DPPH and FRAP, show the same antioxidant activity trend, albeit with different magnitudes which are specific for each assay.

Analyzing the data obtained from the DPPH assay, it is possible to affirm that mozzarella during storage evidenced increasing antioxidant activity values when immersed in the preserving liquid containing higher quantities of M-RLE. In particular, the DPPH assay (Fig. 4a) evidenced the significantly ($p < 0.05$) higher value of antioxidant activity for the sample immersed for 12 d in brine containing 20% of M-RLE, followed by that immersed for 8 and 4 d in the same brine. Lowering the brine extract concentration (5 and 10% M-RLE), the highest antioxidant activity is obtained after 8 d of refrigerated storage (Fig. 4a).

With reference to the FRAP assay, a particular antioxidant activity trend, similar to that obtained using the DDPH assay, is found among the samples (Fig. 4b). The samples showing the significantly ($p < 0.05$) higher antioxidant activity are always those immersed in brine containing 20% M-RLE for 12, 8 and 4 d. The samples immersed in brine added with M-RLE concentration equal to 5% and 10% have a maximum value of antioxidant activity after 8 days of storage, thereafter decreasing at 12 d of storage up to values almost equaling those obtained during the first 4 d of storage (Fig. 4b).

The antioxidant activity found in the mozzarella samples is correlated to the concentration of total polyphenols absorbed by the cheese matrix (see paragraph 3.2.1) and attributable to the presence of the most represented compounds, iriflophenone 3-*C*-glucoside and mangiferin (see Table 1), despite the trace presence of other polyphenols, as previously reported for other vegetable matrix (Scavo et al., 2019), can greatly contribute to the antioxidant activity and explain the increase in antioxidant activity even when polyphenols concentration seem to decrease in mozzarella matrix (Fig. 2). A recent review sudy of Kumar et al. (2021) reported as the young leaves of three different mango cultivars, evidenced as the TEAC (Trolox equivalent antioxidant capacity) value was highest in compounds such as maclurin

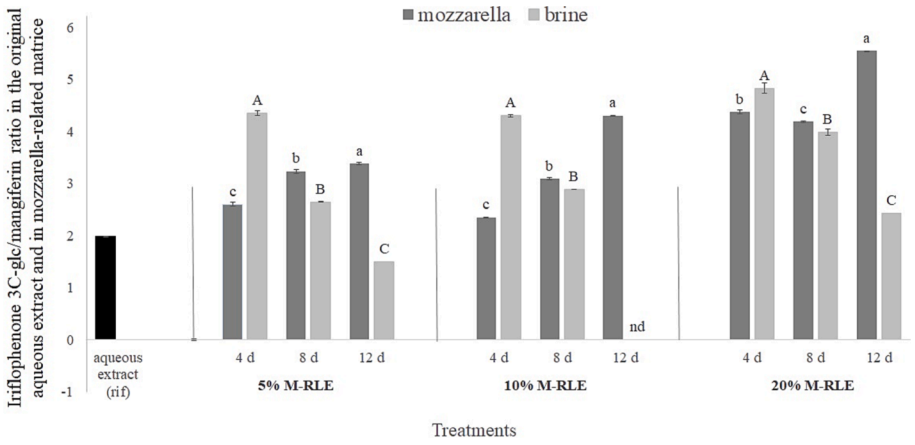

Fig. 3. Iriflophenone 3-*C*- glucoside / mangiferin ratio in the original aqueous extract (black bar), in mozzarella (grey bars) and corresponding brine (light grey bars). Samples were evaluated after 4, 8 and 12 d of refrigerated storage (4 ± 1 °C). Within each extract concentration (5%, 10% and 20%), column comparing mozzarella or brine samples, respectively followed by different lower letter or capital letter, are significantly diffentent according to Fisher's least significant difference test ($p < 0.05$). Vertical bars indicate the standard deviation of the mean.

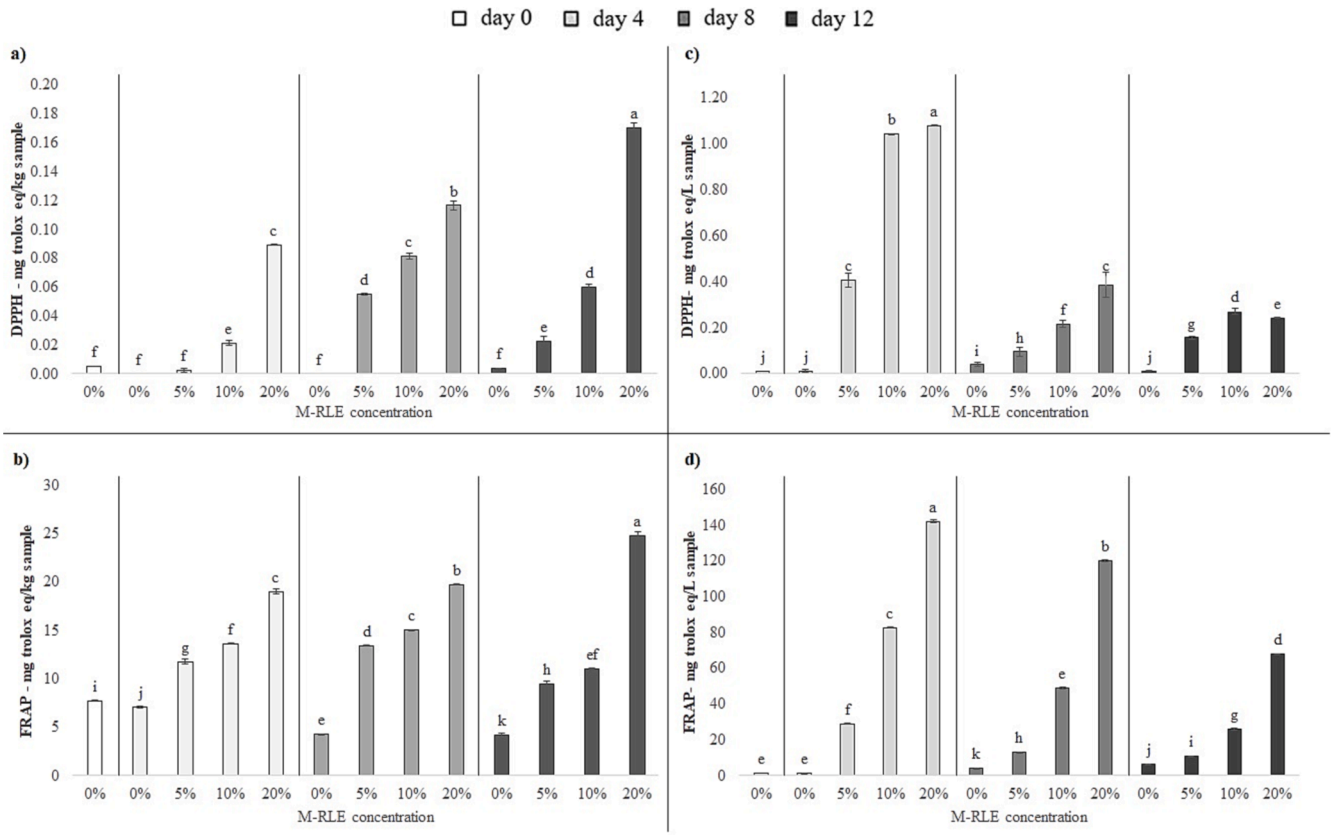

Fig. 4. Impact of different concentration of mango red leaf extract (M-RLE) on the antioxidant activity of mozzarella evaluated through DDPH assay (a) and FRAP assay (b) and on the antioxidant activity of brine samples evaluated through DPPH (c) and FRAP assay (d) up to 12 d of refiregerated storage (4 ± 1 °C). In each graph (a, b, c or d) column chart with different letter are significantly different according to Fisher's least significant difference test ($p < 0.05$). Vertical bars indicate the standard deviation of the mean.

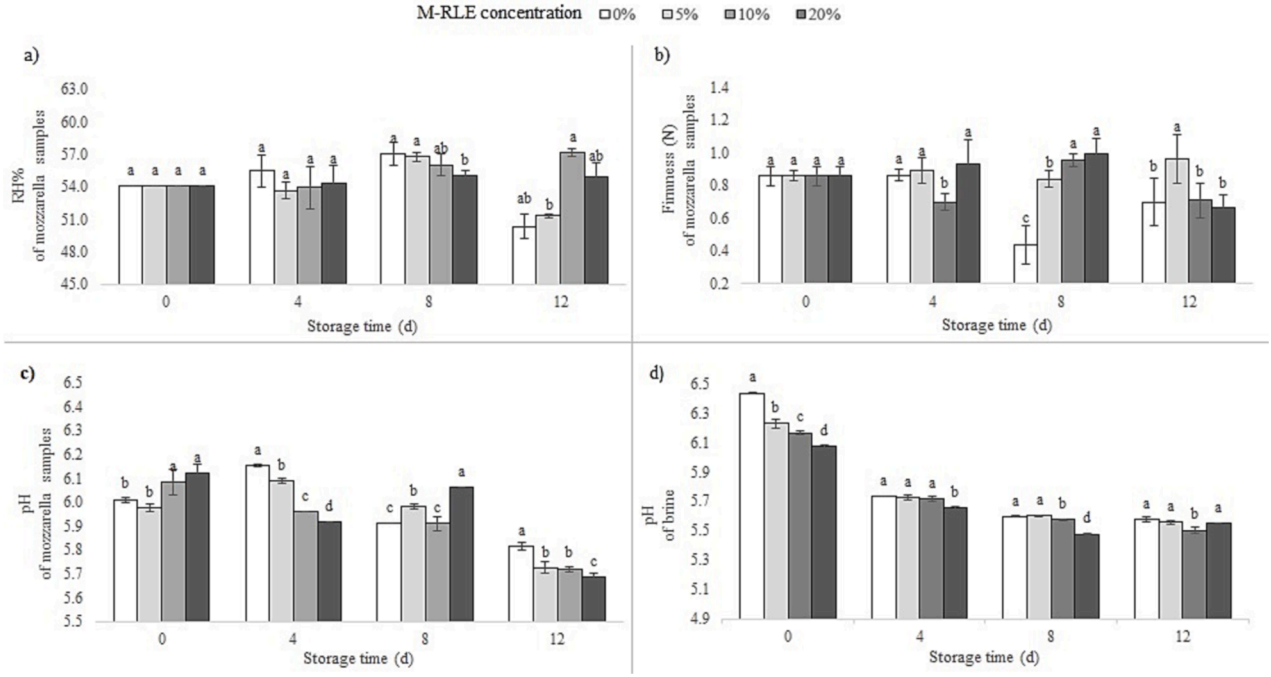

Fig. 5. Evaluation of **a)**, Relative Humidity (RH%), **b)** Firmness (N) and **c)** pH of mozzarella samples immersed in brine enriched with different amounts of M-RLE (0%, 5%, 10% and 20%), and **d)** pH of brine itself evaluated up to 12 d of refrigerated storage. Vertical bars indicate the standard deviation of the mean.

galloylglucoside, trigalloylglucose and iriflophenone galloyl hydroxybenzoyl glucoside. From the data obtained by the identification of antioxidants in young mango leaves by LC-ABTS and LC-MS, it is possible to hypothesize that the antioxidant activity increases in sample containing M-RLE, especially after 4 and 8 d of storage, for the direct connection of water retained by mozzarella (Kuo, Gunasekaran, Johnson, & Chen, 2001) and to the interaction of M-RLE polyphenols with milk protein that can bind the bioactive compounds in a reversible or irreversible form (Yildirim-Elikoglu & Erdem, 2018).

Conversely, the DPPH and FRAP analysis of brine containing different amount of M-RLE (5, 10, and 20%) evidenced a strong decrease in antioxidant activity during storage even when the brine contains the highest amount of M-RLE (Fig. 4 b and c). The decrease in brine antioxidant activity may be due to a minimal part of the absorption of polyphenols in the mozzarella matrix, but probably is also due to the instability of bioactive compounds to pH and overall brine conditions.

To the best of our knowledge, mango leaves were never used for improving the antioxidant activity of food; only Rambabu et al. (2019) demonstrated the successful application of mango leaves as a primary antioxidant ingredient for active food packaging films.

3.2.3. RH%, texture, pH and color trends of mozzarella samples during refrigerated storage

Fig. 5a shows the relative humidity (RH%) of each mozzarella sample stored in brine containing different amount of M-RLE.

The RH% increased after 4 days of storage in the Control sample, reaching the highest value after 8 d and then drastically decreasing after 12 d of storage. The samples immersed in brine containing 5% M-RLE evidenced the same trend of Control sample, while, increasing the concentration of M-RLE up to 10% and 20%, the mozzarella samples appeared to retain the absorbed water. In fact, mozzarella samples immersed in brine containing 10% of M-RLE registered, after 12 d of storage, the significantly ($p < 0.05$) highest RH% value of 57.2 ± 0.32 followed by samples immersed in 20% M-RLE that evidenced a value equal to 54.9 ± 1.31. The decreased RH% in the Control sample is a phenomenon that can be attributed to water diffusion through the cheese matrix, in order to reestablish an osmotic pressure balance (Jakobek, 2015). Luo et al. (2013) reported as the decreasing in water content can be correlated with a final production of cheese with a soft rind and evidenced as the content of brine calcium chloride could be directly correlated with the decreasing of mozzarella moisture during storage, modifying the cheese surface.

In our study mozzarella samples immersed in brine containing 10% and 20% of M-RLE evidenced the significantly ($p < 0.05$) highest retained water content, probably for diminution of sodium chloride present in brine, or for polyphenols' interaction with lipids, carbohydrates and proteins (Jakobek, 2015). Although further studies are needed, results of the present study are promising, because as already suggest by Faccia et al. (2019) a higher water retained by mozzarella cheese can increase the product yield and therefore bring economic benefit for the producers. Furthermore, the ability to hold water can greatly affect the texture of mozzarella samples. In the present work, the firmness, in particular after 4 and 8 d of storage, evidenced a significant ($p < 0.05$) difference between mozzarella sample added with M-RLE and the Control, which showed a significant ($p < 0.05$) decrease of firmness value. The use of M-RLE seems to help the preservation of the product surface, as observed by Zappia et al. (2020), who confirmed that natural additives on the mozzarella surface can influence the textural properties, in particular the hardness (Zappia et al., 2020).

The change of pH during storage was evaluated both in mozzarella cheese and in brine solution up to 12 d of storage (4 ± 1 °C) (Fig. 5c and d). Immediately after the addition of the extract (time 0), mozzarella cheese immersed in brine containing 10% and 20% of M-RLE showed a significantly ($p < 0.05$) slight increase of pH, if compared to the Control sample and to the sample containing 5% of M-RLE (Fig. 5c). Furthermore, while this last sample during the whole storage period maintained a pH trend very similar to the Control, the mozzarella samples immersed in brine containing higher quantities of M-RLE (10 and 20%), during storage and in particular after 4 days, evidenced a more significantly ($p < 0.05$) pronounced decrease in pH values (Fig. 5c). During storage, although with less marked differences compared to those found in the mozzarella samples, also the pH of the brine itself, containing the highest concentrations of M-RLE, has significantly ($p < 0.05$) lowered pH values compared to the Control sample (Fig. 5d). The slight decrease of pH in mozzarella and brine samples is probably given by the acidifying action of the M-RLE which has a pH of 5.13 ± 0.02 (as previously reported in the paragraph 3.1.2.) that can determine a change in ionic equilibria and can affect the type and extent of proteolysis in stored Mozzarella cheese (Feeney, Guinee, & Fox, 2002).

Table 2 shows the chromatic trend of the mozzarella samples immersed in brine containing different amount of M-RLE (5, 10 and 20% v/v).

The concentration of M-RLE extract that does not significantly influenced ($p > 0.05$) the color of mozzarella is 5%. However, this statement is valid only for time zero; during storage (4, 8 and 12 d), in fact, it is possible to note significant differences ($p < 0.05$) for all parameters L*, a*, b* and C*. Mozzarella samples containing the M-RLE extract at concentration equal to 10% and 20% evidenced a significant ($p < 0.05$) strong decrease in the L* parameter and a significant ($p < 0.05$) increase in the a*, b* and C* parameters.

With regard to the preserving liquid, all concentrations of M-RLE influenced the color of brine, showing significantly ($p < 0.05$) higher values of the parameters a*, b* and C*, while the parameter L* was less affected by M-RLE addition that in some cases showed not significant differences ($p > 0.05$) in comparison to the Control.

3.2.4. Microbiological analysis

Table 3 displays the microbial counts determined over 12 d of refrigerated storage (4 ± 1 °C) on mozzarella samples immersed in brine with different concentrations of M-RLE and on the brine itself.

With reference to the samples of mozzarella, we can observe as the initial TMB in all samples was 5.4 ± 0.1 log CFU/g, and it rapidly increased after 4 d of storage, in samples containing 20% of M-RLE and in Control sample, while the samples immersed in 5% and 10% M-RLE evidenced the significantly ($p < 0.05$) lower values. After 8 and 12 days of storage not significant differences ($p > 0.05$) were observed between sample containing 20% of M-RLE and Control sample. Samples containing 5% and 10% M-RLE showed a delayed growth of TMB only after 4 d of storage, whereas the same samples evidenced significantly ($p > 0.05$) higher values, in comparison to the Control, after 8 and 12 days of storage.

The starting *Staphylococcus* spp. count was for all sample 4.4 ± 0.3 log CFU/g. After 4 days of storage, samples containing 10% and 20% of M-RLE showed significantly lower *Staphylococcus* spp. values compared to the Control (Table 3). Also, the sample containing 5% M-RLE evidenced a decrease in *Staphylococcus* spp. in comparison to the Control that showed the highest value. After 8 d of storage, only samples containing 10% and 20% of M-RLE still maintained significantly lower *Staphylococcus* spp. counts in comparison to the Control. The *Pseudomonas* spp. count, starting from 4.3 ± 0.0 log CFU/g, after 4 d of storage seemed to be affected only by the presence of 20 % M-RLE, evidencing the significantly ($p < 0.05$) lower value in comparison to the Control. Moreover, sample containing 20% of M-RLE registered a lower *Pseudomonas* spp. count, in comparison to the Control, also after 8 and 12 d of storage. *Enterobacteriaceae* count was not affected by M-RLE addition on the whole, while LAB population seemed to be positively affected by M-RLE addition, both at 10 and 20%, after 4 and 8 d of storage ($p < 0.05$), while after 12 d no significant ($p > 0.05$) difference was evidenced if compared to the Control.

The microbiological analyses carried out on brine evidenced a different trend. Total mesophilic bacteria were not significantly affected by extract addition, at any of the considered concentrations throughout

Table 2
Color trends of mozzarella cheese and brine containing different amount of mango leaf extract over 12 days of storage at 4 ± 1 °C.

Storage time (d)	Parameters	Mozzarella cheese				Brine			
		Control	5%	10%	20%	Control	5%	10%	20%
0	L*	93.4 ± 0.5a	93.1 ± 0.4a	91.8 ± 0.9b	90.7 ± 0.3c	55.5 ± 3.1a	57.1 ± 1.3a	46.8 ± 1.2b	39.1 ± 1.0c
	a*	−2.57 ± 0.1c	−2.44 ± 0.1bc	−2.26 ± 0.3ab	−2.23 ± 0.1a	−1.48 ± 0.0b	−2.21 ± 0.2b	−2.05 ± 1.3b	−0.64 ± 1.2a
	b*	10.7 ± 0.6b	10.5 ± 0.3b	10.5 ± 0.7b	13.1 ± 0.4a	2.87 ± 0.1ab	1.55 ± 1.4c	2.42 ± 0.7bc	3.43 ± 0.4a
	C*	11.04 ± 0.6b	10.77 ± 0.4b	10.74 ± 0.7b	13.26 ± 0.4a	3.23 ± 0.2a	2.83 ± 1.1a	3.19 ± 0.7a	3.64 ± 0.4a
4	L*	91.1 ± 2.3a	89.1 ± 1.8b	90.5 ± 0.4ab	88.9 ± 0.8b	70.0 ± 2.1b	72.0 ± 2.9b	74.7 ± 0.3a	71.9 ± 1.6b
	a*	−2.72 ± 0.1c	−2.35 ± 0.1c	−1.86 ± 0.1a	−1.75 ± 0.2a	−2.15 ± 0.0c	−1.74 ± 0.1b	−1.44 ± 0.0a	−1.51 ± 0.1a
	b*	9.7 ± 0.2d	11.0 ± 0.7c	12.1 ± 0.3b	13.9 ± 0.5a	0.38 ± 0.1c	1.23 ± 0.6b	6.01 ± 0.3a	6.14 ± 0.5a
	C*	10.09 ± 0.3d	11.26 ± 0.7c	12.22 ± 0.3b	13.99 ± 0.5a	2.18 ± 0.1b	2.19 ± 0.2b	6.18 ± 0.4a	6.33 ± 0.5a
8	L*	91.9 ± 1.0a	89.2 ± 1.2b	88.9 ± 1.1b	88.7 ± 1.0b	83.1 ± 1.2a	83.3 ± 1.4a	82.9 ± 0.9a	81.0 ± 1.0b
	a*	−2.88 ± 0.2d	−2.54 ± 0.1c	−2.18 ± 0.1b	−1.80 ± 0.3a	−2.30 ± 0.1c	−1.68 ± 0.5b	−1.06 ± 0.1a	−0.78 ± 0.1a
	b*	10.7 ± 0.3c	11.1 ± 0.9bc	11.6 ± 0.3b	12.5 ± 0.40a	2.72 ± 0.1c	4.99 ± 0.1b	6.43 ± 0.4a	6.22 ± 1.2a
	C*	11.06 ± 0.3c	11.39 ± 0.9bc	11.84 ± 0.3b	12.59 ± 0.4a	3.56 ± 0.2b	5.28 ± 0.2a	6.51 ± 0.5a	6.27 ± 1.3a
12	L*	90.9 ± 1.43a	90.0 ± 1.56ab	91.2 ± 0.64a	88.3 ± 1.93b	91.0 ± 1.9a	76.6 ± 3.0c	87.7 ± 1.7b	87.5 ± 1.9b
	a*	−3.5 ± 0.15d	−3.2 ± 0.22c	−2.4 ± 0.16b	−2.1 ± 0.12a	−2.18 ± 0.1c	−2.25 ± 0.0c	−0.53 ± 0.2b	−0.20 ± 0.1a
	b*	12.7 ± 0.24c	13.9 ± 0.47b	14.8 ± 0.65a	15.0 ± 0.83a	5.76 ± 0.3d	7.95 ± 0.1c	8.97 ± 0.6b	9.8 ± 0.6a
	C*	13.12 ± 0.3c	14.22 ± 0.9b	14.99 ± 0.3a	15.11 ± 0.4a	6.16 ± 0.4c	8.26 ± 0.1b	8.99 ± 0.7ab	9.82 ± 0.7a

Data presented as mean ± standard deviation of the mean. Parameter (L*: lightness; a*: redness; b*: yellowness; C*: chroma) in each row, within the same sample (mozzarella cheese or brine), followed by different letter are significantly different according to Fisher's least significant difference test ($p < 0.05$).

Table 3
Microbial counts evaluated during refrigerated (4 ± 1 °C) storage.

	Days	Mozzarella cheese (log CFU/g)				Brine (log CFU/mL)			
		Control	5%	10%	20%	Control	5%	10%	20%
Total mesophilic bacteria (TMB)	4	7.1 ± 0.1b	6.5 ± 0.0d	6.8 ± 0.2c	7.4 ± 0.1a	7.2 ± 0.2c	7.5 ± 0.0a	7.4 ± 0.0ab	7.3 ± 0.0bc
	8	6.5 ± 0.5b	7.3 ± 0.2a	7.2 ± 0.1a	7.1 ± 0.2ab	7.2 ± 0.3c	8.0 ± 0.2a	7.7 ± 0.1b	8.1 ± 0.1a
	12	7.0 ± 0.3bc	7.5 ± 0.6ab	8.0 ± 0.0a	6.9 ± 0.1c	7.6 ± 0.6ab	7.1 ± 0.1b	7.8 ± 0.3a	7.8 ± 0.3a
Staphylococcus spp.	4	6.9 ± 0.2a	6.6 ± 0.0b	5.7 ± 0.1d	5.9 ± 0.0c	7.3 ± 0.0a	7.4 ± 0.1a	7.0 ± 0.0b	6.9 ± 0.0c
	8	6.2 ± 0.1a	6.0 ± 0.1a	5.2 ± 0.3b	5.3 ± 0.3b	6.5 ± 0.1a	6.6 ± 0.2a	6.6 ± 0.4a	6.3 ± 0.4a
	12	4.0 ± 0.4c	4.2 ± 0.1bc	4.7 ± 0.4a	4.6 ± 0.0ab	5.1 ± 0.0a	5.1 ± 0.2a	5.0 ± 0.1a	4.6 ± 0.0b
Pseudomonas spp.	4	6.7 ± 0.0b	7.0 ± 0.1a	6.8 ± 0.2b	6.3 ± 0.1c	7.0 ± 0.1c	7.2 ± 0.0a	7.1 ± 0.0b	7.0 ± 0.0c
	8	7.2 ± 0.1a	7.2 ± 0.2a	6.4 ± 0.2c	6.8 ± 0.0b	7.7 ± 0.2a	7.4 ± 0.0ab	7.4 ± 0.1a	6.6 ± 0.2b
	12	6.6 ± 0.4a	6.7 ± 0.1a	6.8 ± 0.2a	6.2 ± 0.2b	7.4 ± 0.3a	7.6 ± 0.1a	7.4 ± 0.2ab	6.8 ± 0.1b
Enterobacteriaceae	4	6.6 ± 0.0a	6.6 ± 0.0a	6.5 ± 0.0b	6.5 ± 0.0c	6.8 ± 0.0b	7.1 ± 0.0a	7.0 ± 0.0ab	6.4 ± 0.3c
	8	6.1 ± 0.2a	6.0 ± 0.0a	6.0 ± 0.1a	6.2 ± 0.0a	6.7 ± 0.0b	7.0 ± 0.2a	7.0 ± 0.0a	7.0 ± 0.1ab
	12	5.6 ± 0.1a	5.7 ± 0.1a	5.4 ± 0.1b	5.5 ± 0.1ab	5.4 ± 0.2bc	5.2 ± 0.2c	6.0 ± 0.1a	5.6 ± 0.1ab
Lactobacillus spp.	4	6.5 ± 0.0c	6.7 ± 0.1b	6.6 ± 0.0b	7.1 ± 0.0a	6.2 ± 0.0c	6.7 ± 0.1b	6.8 ± 0.0a	6.8 ± 0.0ab
	8	6.2 ± 0.0b	6.3 ± 0.3b	6.9 ± 0.0a	6.9 ± 0.1a	6.4 ± 0.1d	7.5 ± 0.1a	7.2 ± 0.0b	7.0 ± 0.0c
	12	6.5 ± 0.1b	6.5 ± 0.2a	6.4 ± 0.1a	6.4 ± 0.1a	7.9 ± 0.4a	7.5 ± 0.1b	7.6 ± 0.1ab	7.4 ± 0.0b

Data presented as mean ± standard deviation of the mean. In each row, within the same sample (mozzarella cheese or brine), value followed by different letter are significantly different according to Fisher's least significant difference test ($p < 0.05$).

storage. *Staphylococcus* spp. growth in brine was negatively affected by the highest M-RLE concentration (20%) only after 4 and 12 d of storage ($p < 0.05$), while the same effect was evidenced against *Pseudomonas* spp. after 8 and 12 d of storage. *Enterobactariaceae* growth was significantly reduced only after 4 d by 20% M-RLE addition. LAB population in brine, similarly to that of mozzarella matrix, seemed to be increased by M-RLE addition, except at the end of the storage period (12 d) when a slight reduction, compared to the Control, was observed in the M-RLE added samples. Different effect of extract addition on microbiological parameters investigated on mozzarella and brine samples are probably attributable to the different distribution of bioactive compounds of M-RLE between brine and cheese matrix, as discussed in paragraph 3.2.1. The major phytochemicals responsible for the antimicrobial activity in mango leaves include phenolics, alkaloids, saponins, glycosides, terpenes, and tannins (Ebere Okwu & Ezenagu, 2008; Ediriweera et al., 2017). The concentration of the aforementioned compounds was previously measured as follows: flavonoid content was the highest at 11.25 mg/100 g; there was 3.23 mg/100 g of saponins; phenolic content was 0.08 mg/100 g; and tannins in leaves was at 0.46 mg/100 g (Kumar et al., 2021). Polyphenols and phenolic acids present in mango leaf extract include protocatechuic acid, gallic acid, hyperin, catechin, quercetin, kainic acid, ethyl digallate, ellagic acid, and shikimic acid, which can inhibit the growth of pathogens (Kumar et al., 2021). The mechanism of exertion of antimicrobial activity by bioactive compounds of mango leaves involves depleting intracellular ATP levels, depolarization of plasma membrane, cytoplasm leakage, damaging genetic material, and declining the concentration of microbial protein (Kumar et al., 2021). Antibacterial activity of leaf extract was mainly found against Gram positive bacteria, compared to Gram negative ones (Islam, Mannan, Kabir, Islam, & Olival, 1970), whose relative resistance is due to the presence of the lipopolysaccharide layer in their additional outer membrane additionally, mangiferin, a xanthone *C*-glycosyl compound extracted from mango leaf extract, has also shown to possess strong iron chelating activity, which favors antimicrobial activity (Kumar et al., 2021). However, other studies confirmed the ability of either *Lactobacillus casei* or effective microorganisms (EM), such as probiotic bacteria and/or other anaerobic organisms, to ferment hydro alcoholic mango leaf extract for conferring higher antioxidant activity (Park, Ku, & Yoo, 2015). The possibility that bioactive compounds of tropical fruits, including mango, have the potential to stimulate the growth of some LAB with probiotic properties such as lactobacilli (*Lacticaseibacillus casei*, *Lactiplantibacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp.*bulgaricus*) and Bifidobacteria (*B. animalis* subsp. *lactis*, *B. longum*) has been extensively reviewed by Borgonovi, Borghi Virgolin, Soares Janzantti, Casarotti, & Barretto Penna (2022).

4. Conclusions

Green-water extraction of mango red leaves allows to obtain an extract (M-RLE) rich in polyphenols where benzophenones and xanthone mangiferin are the dominant polyphenols. The use of the above-mentioned extract as an ingredient in mozzarella brine improves the antioxidant activity of mozzarella cheese stored up to 12 d under refrigerated conditions (4 ± 1 °C). Compositional analysis (HPLC/DAD and HPLC/ESI-MS) evidences as the retention of phenolic compounds, exerted by the cheese matrix, depends on the extract concentration and the storage time. It is interesting to note that iriflophenone 3-*C*-glucoside and mangiferin are preferentially retained by the cheese matrix. Therefore, the increase in antioxidant activity is attributable to the presence of the most represented compounds, iriflophenone 3-*C*-glucoside and mangiferin, despite a synergistic effect of the polyphenols present even in small quantities cannot be excluded. The use of the extract determined a slight change in color parameters but it does not negatively influence the *Lactobacillus* spp. load of mozzarella, even when used at the highest concentration; the extract addition rather maintained significantly lower *Staphylococcus* spp. counts with respect to the Control. Further studies must be carried out, in order to assess the final acceptability of mozzarella immersed in brine containing different amount of M-RLE through sensory tests.

CRediT authorship contribution statement

Lucia Parafati: Writing – original draft, Formal analysis. **Laura Siracusa:** Writing – review & editing, Writing – original draft, Formal analysis, Methodology. **Fabiola Pesce:** Writing – review & editing, Methodology. **Cristina Restuccia:** Writing – review & editing, Writing – original draft, Formal analysis, Methodology, Conceptualization. **Biagio Fallico:** Supervision, Conceptualization. **Rosa Palmeri:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136474.

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