# The rule of biological and microbial safety in *Hissopus officinalis* extract for influencing mozzarella cheese functionality

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## Abstract

Mozzarella was identified as one of the most extensively consumed dairy products. The aim of this study was to investigate the effect of *Hyssopus officinalis* extract on microbial properties, antioxidant activity, and mozzarella storage. Ultrasound was applied to gain extract and tests, such as total phenolics, antioxidant attributes, and microbial investigation were accomplished. Physicochemical features, peroxide, sensory evaluation and microbial population were assessed on mozzarella including 0, 1.13 and 1.40 µg/mL *Hissopus officinalis* extract during 45 days. The highest phenolic content was observed in rosmarinic acid (60.33±2.31 mg/g) and *Listeria monocytogenes* was recognized as the most resistant microorganism in *Hissopus officinalis*. The physicochemical results were found to be standard and the lowest peroxide was detected in the sample with the highest extract amount (1.40 µg/mL) on the 45th day. The microbial evaluation showed that mentioned extract was effective to minimize survival of *Escherichia coli* and *Listeria monocytogenes* during storage. The reduction in contamination hazard of *Escherichia coli* almost 1 log CFU/mL was detected in treatment samples during storage. The *Hissopus officinalis* extract was spotted as the most appropriate agent to improve functional and sensory properties of mozzarella.

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Key words: *Hyssopus officinalis*; phenolic content; mozzarella; storage.

Conflict of interest: The author declares no conflict of interest.

Availability of data and materials: All data generated or analyzed during this study are included in this published article.

Received for publication: 9 November 2021.
Revision received: 6 May 2022.
Accepted for publication: 3 June 2022.

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*Journal of Biological Research 2022; 95:10272*
*doi:10.4081/jbr.2022.10272*





## Introduction

Mozzarella is a typical "pasta filata" cheese from southern Italy, which is produced in distinct shapes and brine.[1] It is distinguished by a remarkable economic growth due to stable progress in manufacture and consumption for various foods such as pizza.[2] The consumption of pizza cheese was extended considerably all over the world; subsequently, its industry was expanded, and investigation represented a significant potential in improving quality and safety.[1,2] Mozzarella is characterized by a rather short storage and numerous researches have been conducted so far.[2] One trend for extending mozzarella storage was to introduce antimicrobial and natural preservatives.[3] Recently, notable attention is paid to medicinal herbs due to their antibacterial resistance and absence of side effect.[4]

Shelf life of low moisture mozzarella is about 10 days at 4°C as perishable food, which is caused by raw milk, microorganism contamination, and spoil.[1]

*Hissopus officinalis* belongs to the Lamiaceae family; it is a flowering herb widely cultivated in European countries such as Russia, Spain, France and Italy.[5] It has been employed in folk medicine for sundry purposes such as antibacterial, anti-inflammatory, antispasmodic, antipyretic and antihyperlipidemic.[6]

From past until now, antioxidant and antimicrobial functions of several extracts such as thyme,[7] rosemary and thyme,[8] and tomato juice[9] have been investigated on quality attributes of mozzarella. The purpose of present research was to evaluate the influence of *H. officinalis* extract on mozzarella to improve its microbial and antioxidant attributes during storage.

## Materials and Methods

### Materials

*H. officinalis* was collected from foothills around Zashk (located near Mashhad) in spring 2020 and liquid rennet was achieved [Rennilase, 55 International Milk Clotting Unites (IMCU)/mL, DSM, France]. Microorganisms, including gram-positive bacteria *Staphylococcus aureus*: *S. aureus* (ATCC 25923), and *Listeria monocytogenes*: *L. monocytogenes* (ATCC 6633), gram-negative bacteria *Salmonella typhimurium*: *S. typhimurium* (ATCC 14028), and *Escherichia coli: E. coli* (ATCC 8739) and yeast *Candida albi-*

*cans* were chosen for antimicrobial screening and purchased from the Microorganism Collection Center of Iran Scientific and Industrial Research Organization.

### Extraction and evaluation of the extract

*H. officinalis* leaves were dried under vacuum and powder were extracted with 100 mL ethanol (80 %) at 20 KHz in ultrasonic bath (at 45°C and 20 min). Afterwards, solvents were evaporated under vacuum.[10]

Separation and quantitative measurements of HPLC coupled to a diode array detector were performed for polyphenolic constituents. The separation was executed on a reverse phase column (5µm) Supelco, Discovery ® HS C18 with 25cm × 4.6mm at 28°C, and 280 and 320nm wave length.[11,12]

Phenolic components with Folin Ciocalteu solution and antioxidant with 1,1-diphenyl-2- picrylhydrazyl, known as DPPH, were determined by spectrophotometer (Thermo Scientific, Madison, WI, USA).[13]

### Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extract

Luria Bertani agar was utilized to culture experimental microorganisms and determines target values. Antimicrobial activity of *H. officinalis* extract was assessed by agar well diffusion procedure in Luria Bertani agar at 37°C for 24h to determine MIC and MBC against target bacteria.[3]

### Manufacture and analytical determination of mozzarella

Mozzarella samples were collected as previously reported;[14] mozzarella samples were prepared by pasteurized whole milk. The pH was regulated from 6.6 to 5.8 by citric acid in milk. Solution of double strength rennet was added up to milk and mixed, then the prepared curds were sliced. Before they were cooked, these slices were permitted to release in whey. Later, salt 2 % (W/W) and different concentration of extract were completely blended with curds. The tests were performed on 45 days and treatments are identified in Table 1. The mozzarella samples (about 3g) were put in aluminum plate and heated in oven (Blodgett Mark V-100) at 105°C for 3h; afterwards, the treatment moisture was measured according to standard AOAC procedure.[15] The pH of homogenized solutions was measured through a digital pH meter (Metrohm 827). Antioxidant capacity was noticed according to Mooliani and Nouri[13] method and stretchability test was performed according to Reid and Yan.[16]

Samples were stabilized in gluteraldehyde solution (2.8 %) by immersing instantly and maintained at 4 to 6°C about 6h and scanning electron microscope (JSMe6390 LV, Japan) was applied at speeding 5kV voltage. Panelists ranked all quality features by a hedonic scale of 1 to 5, 0 = unacceptable; 1 = poor; 2 = fair; 3 = good; 4 = very good, 5 = like much (Table 1).[13]

### Microbiological analysis

*L. monocytogenes* and *E. coli* bacteria were sub cultured twice in Triptic Soy Broth (0.6% w/v, yeast extract) at 30°C for 24h before utilization. To inoculate samples, cultures were instantly employed after incubation at 35°C for 2h. Cheese samples (10g) were homogenized with 90 mL sterile peptone water in a stomacher. The populations of *L. monocytogenes* and *E. coli* in cheese were measured through plating adequate dilutions on polymyxin Acriflavine Lithium chloride Ceftazidime Aesculin Mannitol agar (30°C at 48h) and Sorbitol Macconkey agar (37°C at 24h), respectively. Lactic acid bacteria were investigated by plating homogenized samples on M17 Agar at 30°C for 48h.[17]

### Statistical analysis

Initially, normality distribution was conducted for each data; then outcomes were assessed using factorial test in a completely randomized design with mean and standard deviations. The parameters were the concentration of *H. officinalis* extract and storage. Multiple range test of Duncan was applied to measure the difference among mean values and Minitab software 15 was performed for the statistical evaluation.

## Results

### *H. officinalis* extract assessment

The highest rosmarinic acid content (60.33±2.31mg/g extract) was detected followed by flavonoids with high biological potency (luteolin 0.72±0.05mg/g extract, apigenin 0.83±0.03mg/g extract and myristin 0.35±0.01mg/g extract), according to the results of phenolic in *H. officinalis* extract by HPLC coupled to a diode array detector. Total phenolic composition was found to be 110.24±5.35mg gallic acid equivalents (GAE)/100 g ethanol, which its capacity was 17.07±0.93 µg/mL. and indicated antioxidant defense.

### The extract effect on MIC and MBC assays

In Table 2 the MIC values of the extract on growth of *C. albicans* (0.142 µg/mL), *S. typhimurium* (0.253µg/mL), *E. coli* (0.298µg/mL), *S. aureus* (0.374µg/mL) and *L. monocytogenes*

**Table 1. Mozzarella cheese samples under study.**

| | |
|---|---|
| $CMC_0$ | Control mozzarella cheese on day 0 (production day) |
| $CMC_{15}$ | Control mozzarella cheese on day 15 |
| $CMC_{30}$ | Control mozzarella cheese on day 30 |
| $CMC_{45}$ | Control mozzarella cheese on day 45 |
| $EMCI_0$ | Experimental mozzarella cheese containing MIC on day 0 (production day) |
| $EMCI_{15}$ | Experimental mozzarella cheese containing MIC on day 15 |
| $EMCI_{30}$ | Experimental mozzarella cheese containing MIC on day 30 |
| $EMCI_{45}$ | Experimental mozzarella cheese containing MIC on day 45 |
| $EMCB_0$ | Experimental mozzarella cheese containing MBC on day 0 (production day) |
| $EMCB_{15}$ | Experimental mozzarella cheese containing MBC on day 15 |
| $EMCB_{30}$ | Experimental mozzarella cheese containing MBC on day 30 |
| $EMCB_{45}$ | Experimental mozzarella cheese containing MBC on day 45 |

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration.

**Table 2. Quantitative results of MIC and MBC of extract.**

| Target microorganisms | MIC (µg/mL) | MBC (µg/mL) |
|---|---|---|
| *Escherichia coli* | 0.298 | 0.475 |
| *Salmonella typhimurium* | 0.253 | 0.420 |
| *Staphylococcus aureus* | 0.374 | 0.605 |
| *Listeria monocytogenes* | 1.130 | 1.401 |
| *Candida albicans* | 0.142 | 0.207 |

MIC: Minimum Inhibitory Concentration; MBC: Minimum Bactericidal Concentration.

(1.130µg/mL) are shown. A similar trend was observed for MBCs on *C. albicans* (0.207µg/mL), *S. typhimurium* (0.420µg/mL), *E. coli* (0.475µg/mL), *S. aureus* (0.605µg/mL) and *L. monocytogenes* (1.401µg/mL) of extract.

As a result, samples were added in next steps to extend mozzarella storage, because *L. monocytogenes* had the highest MIC (1.13µg/mL) and MBC (1.40µg/mL).

## Analytical determination of mozzarella

pH decreased by over time however, it increased using extract addition. The standard range was 5.510 to 5.755 for samples during 45 days.

Higher moisture absorption 76 to 74% and lower level 66 to 61% were for samples with low and high fat in standard, respectively.[1] Since mozzarella moisture ranged from 60.18 to 61.00% (Table 3), samples were classified in two groups with high fat and low moisture content. The moisture did not exceed the standard maximum during 45 days and samples represented the highest level on 1st day and the lowest on 45th day. According to the results of Table 3, extract addition did not have a significant effect on physicochemical aspects.

The variance analysis indicated that extract and time had a significant effect ($p<0.05$) on free radicals inhibition. Also, extractability is promoted to prevent free radicals at higher levels (Table 3). The results of comparing mean peroxide illustrated $EMCB_{45}$ (experimental mozzarella cheese containing MBC) had the lowest peroxide 0.72 (meq/kg) and oxidation compared to others on similar day and storage effect was remarkable on peroxide level ($p<0.05$). This factor was developed over time and the lowest peroxide level was observed for 0.02 (meq/kg) $EMCB_0$ and the highest 1.52 (meq/kg) in $CMC_{45}$ (Control Mozzarella Cheese).

As portrayed in Table 3, a range from 197.5 to 201.5 mm was identified by tensile strength. *H. officinalis* extract had no significantly effect on stretching feature of samples. Figure 1 illustrated less surface porosity by adding extract compared to control during shelf life.

The impact of *H. officinalis* extract was observed on sensory attributes (Figure 2). No significant difference was found among samples in terms of color and texture ($p>0.05$), because of low extract (with no color). The main dissimilarities could be attributed to difference in flavor between treated and control samples. After 30 days, decrease in flavor was higher in treated samples than control. Sensory evaluation indicated that flavor is affected by *H. officinalis* extract in cheese. Overall acceptance showed favorable results and all samples were consumable in terms of sensory evaluators during 45 days.

## Results of microbial analysis

The highest contamination hazard 4.9 log Colony Forming Cells (CFU)/mL was observed in $CMC_{45}$. Contamination intensified with a slight slope from 1st to 45th day. In CMC, acid was produced by starter culture, which inhibited the growth of bacterial contaminants during fermentation (Figure 3a).

The results demonstrated that *H. officinalis* extract had a significant effect on *L. monocytogenes* survival during storage (Figure 3b). The higher growth of *L. monocytogenes* was detected over time, which may be considered as a potential risk to public

**Table 3. Mean interaction effects of distinct levels (extract and time) on pH, moisture peroxide level and stretching value for mozzarella samples (mean±standard error).**

| Storage time, days | pH | | |
|---|---|---|---|
| | CMC | EMCI | EMCB |
| 0 | $5.753^{a}$±0.013 | $5.750^{a}$±0.022 | $5.755^{a}$±0.024 |
| 15 | $5.665^{b}$±0.020 | $5.670^{b}$±0.010 | $5.660^{b}$±0.003 |
| 30 | $5.555^{c}$±0.011 | $5.600^{c}$±0.031 | $5.601^{c}$±0.015 |
| 45 | $5.515^{d}$±0.043 | $5.510^{d}$±0.017 | $5.505^{d}$±0.018 |
| | Moisture (%) | | |
| 0 | $60.97^{a}$±0.04 | $61.00^{a}$±0.01 | $60.98^{a}$±0.04 |
| 15 | $60.74^{b}$±0.02 | $60.75^{b}$±0.07 | $60.73^{b}$±0.05 |
| 30 | $60.51^{c}$±0.06 | $60.50^{c}$±0.03 | $60.52^{c}$±0.03 |
| 45 | $60.18^{d}$±0.03 | $60.19^{d}$±0.05 | $60.20^{d}$±0.02 |
| | Peroxide level (mEq/kg) | | |
| 0 | $0.23^{c}$±0.03 | $0.05^{b}$±0.00 | $0.02^{a}$±0.00 |
| 15 | $0.44^{d}$±0.06 | $0.8^{b}$±0.05 | $0.05^{b}$±0.02 |
| 30 | $0.45^{de}$±0.05 | $0.14^{bc}$±0.03 | $0.50^{de}$±0.04 |
| 45 | 1.52g±0.01 | $0.92^{f}$±0.04 | $0.72^{e}$±0.05 |
| | Stretching value (mm) | | |
| 0 | $200.8^{a}$±0.5 | $200.5^{a}$±1.3 | $200.3^{a}$±0.9 |
| 15 | $201.5^{a}$±0.7 | $200.6^{a}$±0.8 | $201.5^{a}$±1.1 |
| 30 | $198.1^{b}$±1.2 | $197.5^{b}$±0.9 | $198.0^{b}$±0.8 |
| 45 | $197.6^{b}$±0.9 | $197.5^{b}$±1.3 | $197.7^{b}$±0.6 |

Significant differences are indicated by $^{a}$ to $^{g}$ index letters ($p<0.05$).
CMC: Control mozzarella cheese, EMCI: Experimental mozzarella cheese containing MIC, EMCB: Experimental mozzarella cheese containing MBC.



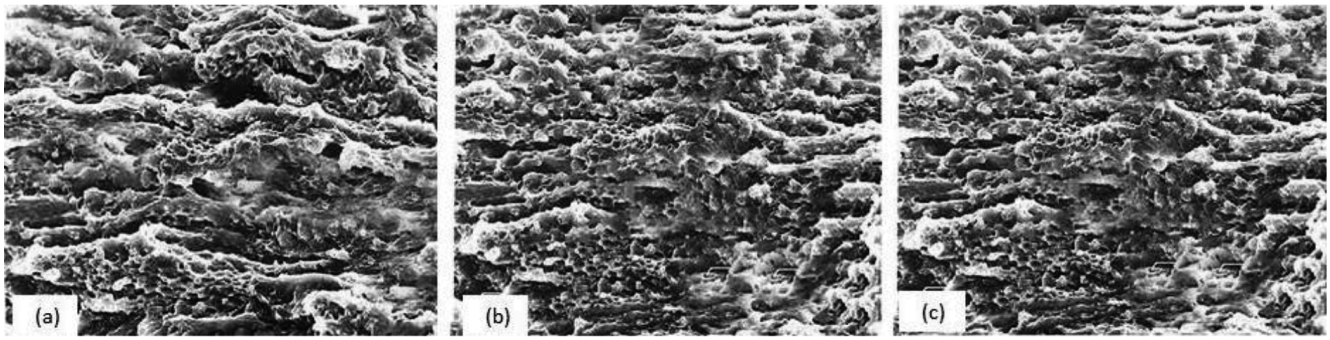


**Figure 1. Scanning electron micrograph of mozzarella cheese, 10 µm magnification: a) control mozzarella cheese; b) experimental mozzarella cheese containing MIC; c) Experimental mozzarella cheese containing MBC.**

health. Microbial load was detected 1.2 log CFU/mL and 1.7 log CFU/mL for $EMCI_{45}$ (Experimental mozzarella cheese containing MIC) and $EMCB_{45}$, respectively, which was significantly different from 6.3 log CFU/mL for $CMC_{45}$. *L. monocytogenes* population varied from 3.29 log CFU/mL on 1st day to 1.39 log CFU/mL on 15th day in EMCI, and from 3.12 log CFU/mL on 1st day to 1.21 log CFU/mL on 15th day for EMCB; therefore, a significant reduction was ascertained in the microbial population.

## Discussion

Same components with distinct values indicated similar results.[16] Higher phenolics were extracted using ultrasound diagnosis at less time compared to solvent method and total phenolic 117.43 (mg GAE/100g ethanol) was detected.[10] The previous results reported phenolic levels of 16.37µg/mL,[18] 156.6µg/mL,[12] 6.45µg/mL[11] and 24µg/mL[19] for *H. officinalis* depending on diverse parameters such as structure, concentration, temperature, light, substrate, physical state of system, etc.

The previous results proved that extract of Lamiaceae family had an antibacterial effect against a group of microorganisms, among which *E. coli* was more susceptible to extracts compared with *S. aureus*. *H. officinalis* was identified as more effective extracts,[20,21] which is in line with our research. The isopinocamphone was reported as major phenolic component of *H. officinalis* extract, which had desired level of antibacterial activity.[19]

Ripening time, acid level and starter consumption affected mozzarella acidity, which were consistent with the present study and pH lowered by time.[1] In contrast, some results were different in pH during storage, which promoted by over time and remained constant for 28 days.[22] High moisture was noticed in low fat mozzarella during storage, but no alternation was forced in non-fat mozzarella, which is due to complex interaction between fat substitute, casein and fat during production and storage up to 28 days.[22]

The components of *H. officinalis* were reduced in cheese during storage due to catabolism and protein matrix, which can sustain oxidation and decompose in acidic status.[23] Phenolic contents,[11] rosemary and thyme[8] and tomato juice[9] had improved antioxidant defense or reduced peroxide number, in agreement with the results of the present study.[8,9,11] The *H. officinalis* extract with antioxidant features caused a reduction in fat oxidation of cooked pork.[24]

This could be because of lower calcium in curd, which has been stretched at a low pH level. Milk components, calcium, pH and acidity influenced on mozzarella stretching.[4,17]

The aim is to produce mozzarella cheese and its flavor is minimized by oven heating and removing odor.[8] Concentrations of *H. officinalis* extract had not a negative effect on sensory activities of cheese. The obtained chitosan and phenolic contents from olive mill showed no significant difference on control and treated samples in cheese by sensory evaluation.[23]

The presence of *E. coli* was recognized as a crucial factor indicating fecal contamination and poor hygienic conditions in mozzarella (surface spoilage and proteolysis), which caused consumer rejection.[3] However, *E. coli* could compete with starter culture (*Lactococcus lactiscremoris*) and its survival was investigated in

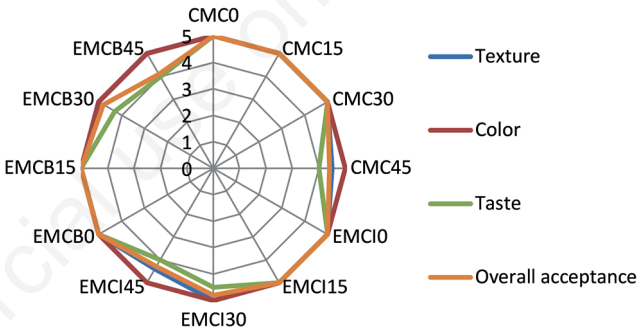


**Figure 2. Mean interaction effects of distinct levels (extract and time) on sensory properties for mozzarella samples. CMC: Control mozzarella cheese, EMCI: Experimental mozzarella cheese containing MIC and EMCB: Experimental mozzarella cheese containing MBC.**

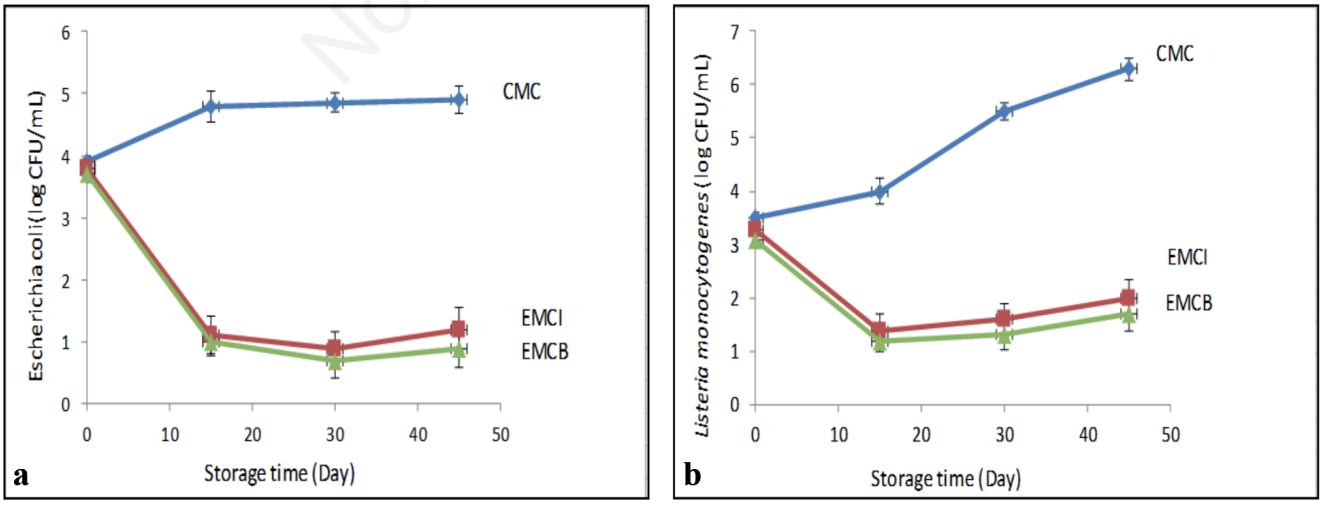


**Figure 3. Correlation of *Escherichia coli* (a) and *Listeria monocytogenes* counts (b) in Mozzarella samples with storage time. CMC: Control mozzarella cheese, EMCI: Experimental mozzarella cheese containing MIC and EMCB: Experimental mozzarella cheese containing MBC.**

milk by a lactic starter culture.[18] The growth rate of *Salmonella enterica* was seen to decrease in vitro by adding extract of *Capparis spinosa*.[3] For Egyptian Kareish cheese, growth reduction and total coliforms were recorded by adding distinct extracts of plants.[24] *Cinnamomum zeylanicum* essential oil with a suitable anti-*Brucella* activity was employed as an effective natural preservative for manufacture of fresh Baladi cheese.[21] This result was consistent with previous research that showed polyphenolic compounds were applied to diminish undesirable microorganisms such as *Pseudomonas fluorescens* and *Enterobacteriaceae* in *fior di latte* cheese.[23] The impact of herbal phenols on *Enterobacteriaceae* strains were also detected in fresh cheeses.[2]

Citric acid was applied in cheese manufacture, so a reduction in *L. monocytogenes* and short chain organic acids were observed due to acidic pH. Rosemary and thyme extracts were more efficient against *L. monocytogenes* in low-fat than in full-fat cheese and an inverse relationship was reported between lipid and antimicrobial activity of the extract.[8] Lactic acid bacteria were not investigated because their growth was not affected by adding *H. officinalis* extract and their microbial group was similar in all samples. The referred results confirmed previous findings, which reported that the inhibitory effect on lactic acid bacteria in fermented and enriched milk with phenolic contents was extremely limited.[23] Some researches showed that the antagonistic ability of natural microflora, especially of lactic acid bacteria, against *L. monocytogenes* indicated a bioprotective potential due to nutritional competition and active component production. Nevertheless, the antagonistic activity was not determined under substrate and temperature conditions of the present study, which is in line with previous results.[14,24,25]

## Conclusions

The results of the present research illustrated that antioxidants, phenolics and antimicrobial components were observed *in H. officinalis* extract. This extract was manufactured by two concentrations and employed for mozzarella to assess their influences on physicochemical, microbial, biological and sensory features up to 45 days. Extract addition was detected as the most appropriate agent to produce functional mozzarella.

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