

## LEMON LEAF ESSENTIAL OIL AS NATURAL FOOD PRESERVATIVE IN FRESH CHEESE

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### ABSTRACT

In recent years, there has been a noticeable surge in exploring alternative methods for the conservation of dairy products, favoring the use of essential oils over synthetic preservatives. The trend toward natural solutions reflects a broader demand for healthier and more sustainable food preservation choices. The aim of this study is to monitor changes in the physico-chemical, sensory, and microbiological characteristics of a milk-derived product eventually induced by the incorporation of lemon essential oil (LEO) of leaf at different concentrations (0.125%, 0.5%, and 1.25%).

LEO incorporation showed an effect ( $P < 0.05$ ) on pH, dry matter, titratable acidity, ash, aerobic mesophilic flora, and lactic acid bacteria of the control and aromatized cheeses. In addition, no pathogenic flora growth was observed in aromatized cheese. Thus, LEO is a promoting agent in the preservation and aromatization of fresh cheese and can be applied at 0.5%.

**Keywords:** Lemon essential oil, preservative and flavoring agent, antimicrobial activity, fresh cheese, physicochemical, microbiological and sensory properties

## INTRODUCTION

Cheese is widely recognized as a popular milk derivative, made by coagulating milk. It's an excellent source of various essential nutrients, especially protein, carbohydrates, and lipids, as well as vitamins and minerals (López-Expósito *et al.*, 2012). Fresh cheese is made from fresh curds that have neither been pressed nor ripened. This type of cheese has a short shelf life due to its high moisture content, which makes it susceptible to the development of fungi and bacteria that cause surface deterioration (Lara-Castellanos *et al.*, 2021). Fresh cheese is also susceptible to fat oxidation. According to Pike and O'Keef (2017) and Takyar *et al.* (2019) fat oxidation is a key factor in the deterioration of food quality. It alters nutritional quality, modifies organoleptic characteristics, and promotes the formation harmful molecules.

Preservatives such as calcium lactate, benzoic acid, sodium benzoate, potassium sorbate, and calcium ascorbate are used to prevent the growth of yeasts and molds during the cheese-making process (Sloan, 2017).

Food safety is a vital issue for the food sector and consumers, making it one of the major challenges facing the sector, whose priority is to develop food production methods aimed at eliminating or significantly reducing the use of synthetic preservatives in food. The need to develop new non-toxic conservators with important antioxidant and antimicrobial properties has increased due to food spoilage and the rise in food-borne illnesses (Khorshidian *et al.*, 2018; Falleh *et al.*, 2020). In recent years, there has been an increasing interest in the use of plant essential oils (EOs) as suitable substitutes for synthetic preservatives in dairy products (Fancello *et al.*, 2020; Reis *et al.*, 2022).

EOs are volatile hydrophobic liquids with a characteristic aroma (Mishra *et al.*, 2020) extracted from various parts of medicinal and aromatic plants, such as seeds, stems, buds, leaves, roots, and fruit (Dhifi *et al.*, 2016; Laranjo *et al.*, 2017). They are mostly composed of a mixture of secondary metabolites, including frequently, terpenoids, terpene hydrocarbons, simple alcohols, ketones, aldehydes, phenols, esters, and more (Hyldgaard *et al.*, 2012). Numerous studies have reported that the EOs exhibit insecticidal, antioxidant, antifungal, and antiviral properties (Burt, 2004; Calo *et al.*, 2015; Dhifi *et al.*, 2016).

EOs oils are frequently used as aromatic additives in the food sector. However, due to their antimicrobial features, they can also play a role in extending the shelf life of food products (Khorshidian *et al.*, 2018). In this respect, previous research has confirmed the antimicrobial and antioxidant features of lemon leaf essential oil (Hojjati and Barzegar 2017; Klimek-Szczykutowicz *et al.*, 2020).

EOs are often used as aromatic additives in the food sector. However, owing to their antimicrobial properties, they can also contribute to prolonging the shelf life of food products (Khorshidian *et al.*, 2018). In this regard, prior research has

established the antimicrobial and antioxidant qualities of lemon leaf essential oil (Hojjati and Barzegar, 2017; Klimek-Szczykutowicz *et al.*, 2020).

This study aims to examine the impact of LEO on the physicochemical, microbial and sensory features of fresh cheese.

## MATERIAL AND METHODS

### Essential oil and microbial strains

Lemon (*Citrus limon* L.) Leaf EO was obtained from a private company located in Chiffa, in the wilaya of Blida, Algeria. LEO was tested against two Gram (+) bacteria: *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, and two Gram (-) bacteria: *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, and two fungi strains: *Candida albicans* ATCC 10231, and *Aspergillus niger* ATCC 16404. These microorganisms were obtained from the SAIDAL group, Algeria.

### Free-radical scavenging activity – total phenolic content

Total phenolic content (TPC) evaluation was carried out in accordance with the method described by Dambolena, Zunino *et al.* (2010), using the Folin-Ciocalteu reagent supplied by Sigma-Aldrich Chemie based in Steinheim, Germany. The results obtained were expressed in milligrams of gallic acid equivalent (GAE) per gram of LEO, through calibration curve established for gallic acid. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) test, as described by Džamić, Nikolić *et al.* (2015), was also performed. All tests, including TPC and DPPH, were performed in three replicates to ensure reliable results.

### Antimicrobial assessment of essential oil

Two methods, agar-well diffusion and macrodilution broth, were used to evaluate the antibacterial and antifungal properties of LEO. The aim was to determine the minimal inhibition concentration (MIC), minimal bactericidal concentration (MBC), and minimal fungicidal concentration (MFC) associated with LEO (Dellacasa *et al.*, 2003; Moosavy *et al.*, 2017).

The LEO was tested against six pathogenic microbial strains, including fungi, Gram-positive and Gram-negative bacteria. Initially, an aseptic process was used to culture all bacterial strains in nutrient broth at 37°C for a period of 24 hours. Fungal strains were placed on Sabouraud dextrose agar and maintained at 25°C for 7 days. Bacterial and fungal suspensions underwent adjustments until a final density of 10<sup>8</sup> CFU/mL and 10<sup>6</sup> CFU/mL respectively, by diluting fresh cultures

in sterilized saline solution and comparing them with the McFarland scale (Hayes and Markovic, 2002).

Various concentrations of LEO (2.5, 5, 7.5, and 10 mg/mL) (w/v) were prepared by dissolving them in dimethylsulphoxide (DMSO) 1% and Tween 20. A 0.22 µm membrane filter was used to sterilize each dilution. All experiments were carried out in three replicates.

Amoxicillin (25 µg/disc) (HIMEDIA, Inde) was used to control the sensitivity of microorganisms and served as a positive control. Amphotericin B (20 µg) and the mix DMSO/Tween 20 were used as positive and negative control. Three repetitions were performed for each test. The growth and sterility controls are sampled in the same manner.

### Fresh cheese making

The whole raw cow's milk from a local dairy farm (Ben Achour, Blida, Algeria) was subjected to microbiological and physicochemical analyses before cheese preparation.

The raw milk (pH  $6.7 \pm 0.01$ , total dry matter  $9.25 \pm 0.39\%$ , density  $1.03 \pm 0.002$ , fat  $20 \pm 0.001$  g/L, and ashes 7.2 g/L) was standardized with 10 % milk powder (0 % fat). After undergoing pasteurization at 90°C for 10 minutes, the mixture was subsequently cooled down to 30°C.

Pasteurized milk was inoculated with lyophilized mesophilic starter culture (DELVO DSL, DMS Food Specialties, Netherlands) (LL-50) (*Lactobacillus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*) according to the manufacturer's instructions using a concentration of 0.03% (w/v) and then incubated at a temperature of  $30 \pm 2^\circ\text{C}$ .

After 30 min, liquid rennet (MAXIREN 1800 GRANULATE, DMS Food Specialties, Netherlands) (100% pure calf Chymosin) (powdered rennet reconstituted at 6.2 g/100 mL) was added at 100 µL per 100 g of fresh curd. The mixture was placed into 100 mL plastic cups, and then incubated at  $30 \pm 2^\circ\text{C}$  until a firm curd was observed.

Then, the coagulum was cut into cubes with a diameter lower than 1 cm and the curd was stirred, transferred into pans repeatedly and pressed for 6 h to facilitate whey drainage.

Five lots of three repetitions each were prepared, and each batch was enhanced with a distinct concentration of LEO according to the following experiment design: FCC (-) - Fresh cheese without LEO (control -), FCC1 - Fresh cheese with 0.125% (w/w) LEO, FCC2 - Fresh cheese with 0.5% (w/w) LEO, FCC3 - Fresh cheese with 1.25% (w/w) LEO, FCC (+) - Fresh cheese with 0.125% (w/w) nisin (control +). In order to obtain a homogeneous mixture, the added essential oil was mixed using a sterilized glass stirring rod under a laminar flow hood for 5 min. These three concentrations were selected because previous studies have demonstrated their bacteriostatic and bactericidal activity.

All cheese samples were packaged and sealed immediately in a sterilized polyethylene plastic container (30 g), then stored for 28 days in a refrigerator at  $4^\circ\text{C} \pm 1^\circ\text{C}$ . Every day, a mercury thermometer was used to check the temperature inside the refrigerator. Good manufacturing practices were followed to avoid cheese contamination. Cheese samples were set in triplicate.

The physicochemical, microbiological analysis and sensory test were performed at 0 hours (directly after production) and after refrigerated storage of 7, 14, 21, and 28 days.

### Analyses of cheese samples

#### Cheese yield and physicochemical evaluation

The yield of the fresh cheese was determined as the percentage of weight obtained in relation to the total weight of milk used in preparation (Oluwayemisi *et al.*, 2017).

In accordance with AFNOR standards (1993), analyses were carried out for pH, dry matter, titratable acidity, ash and fat. Cheese samples were subjected to pH measurement by means of a pH meter (HANNA INSTRUMENT, HI 2211 pH/ORP meter) that had been calibrated using standardized buffer solutions of pH 4.0 and 7.0. The titratable acidity (TA) was carried out using titration with NaOH (1/9 N) and expressed as a percentage of lactic acid.

The following parameters were determined in cheese samples: dry matter content by drying in oven (MEMMERT) at  $105^\circ\text{C}$  (Eroglu *et al.*, 2016), fat content by Gerber van Gulik method and expressed as fat in dry matter values (FDM), by means of the following formula:  $\text{FDM} = (\text{fat}/\text{totalsolid}) \times 100$  (Diamantino *et al.*, 2014), total ash content according to Gomes *et al.* (2011) using muffler furnace (NABERTHERM) at  $550^\circ\text{C}$  and total sugar by Dubois *et al.* (1956).

Mineral, trace and heavy elements in digested cheese (after mineralization with nitric acid) were quantified in triplicate using Flame Atomic Absorption Spectrometer (FAAS) (Agilent Technologies 200 Series AA) according to the standard method of International Organization for Standardization (IOS 8070: 2007). Measurements were carried out using an air/acetylene flame, except for calcium, which was determined using an inert gas.

The standard solutions of the mineral salts (Spectrosol 1000 ppm in 0.5% nitric acid) were obtained from MERCK (Darmstadt, Germany) REAGECON (Poole, UK). The calibration solutions for each mineral were prepared from single element

solutions. The linearity test was carried out in concentration ranges from 0 to 100 ppm (Ca), 0 to 50 ppm (Na), 0 to 100 ppm (K), 0 to 20 ppm (Mg), 0 to 2 ppm (Cu), 0 to 30 ppm (Fe), 0 to 2.5 ppm (Zn) and 0 to 4 ppm (Pb). The concentration of each following element Ca, K, Na, Cu, Fe, Zn and pb were determined, respectively, at the following Wavelength: 239.9 nm, 404.4 nm, 589 nm, 324.7 nm, 248.3 nm, 213.9 nm and 217 nm. A white sample was carried out in the same way. Concentrations were expressed in  $\text{mg} \cdot \text{L}^{-1}$ .

The physicochemical parameters (pH, titratable acidity, dry matter and ash) were measured at intervals of 7, 14, 21 and 28 days of refrigerated conditions.

### Microbial analysis

For each sample, 10 grams of cheese were blended with 90 ml of a sterilized 2.0% dipotassium phosphate solution at  $\text{pH } 7.5 \pm 0.2$  and subjected to a series of dilutions. During storage at 0, 7, 14, 21 and 28 days, microbial enumerations were performed using plate counting with appropriate solid media. Selective microbial groups were enumerated according to Zantar *et al.* (2014) and Official Journals of People's Democratic Republic of Algeria (2017) in the following manner:

- Total aerobic mesophilic flora (TAMF) was enumerated using the plate count agar (PCA) incubated aerobically at  $30^\circ\text{C}$  for 72 hours;
- Enterobacteriaceae on violet-red bile lactose (VRBL) incubated anaerobically at  $37^\circ\text{C}$  for total coliforms and at  $44^\circ\text{C}$  for *E. coli* for 24-48 hours;
- *Staphylococcus* on Baird Parker (BP) and coagulase positive *Staphylococcus* (CPS) on BP supplemented with Rabbit Plasma Fibrinogen (RPF) supplement, incubated aerobically at  $37^\circ\text{C}$  for 24 hours;
- The *Salmonella* was cultivated on nutrient broth media and isolated on Hektoen agar; lactic acid bacteria (LAB) on de Man-Rogosa-Sharpe (MRS) agar, incubated under anaerobic conditions at  $30^\circ\text{C}$  for 48 hours;
- *Lactococcus* on M17 agar, incubated under aerobic conditions at  $37^\circ\text{C}$  for 48 hours.

Samples were also analyzed for the presence of yeasts and molds on oxytetracycline Glucose yeast extract agar (OGA).

Microbial analysis was carried out in three replicates using triplicate cheese samples and results were expressed in  $\log$  (CFU/g of cheese).

### Sensory analysis

The sensory characteristics of samples were carried out. The Hedonic Rating Test according to ISO22935-1 (2009); ISO22935-2 (2009); ISO 8586 (2012) and ISO 11136 (2014) was used to evaluate the acceptance of consumers and detect the possibility difference between cheeses at J+1 of preparation that were conserved in plastic containers and stored at a temperature of  $4 \pm 2^\circ\text{C}$ .

The descriptive panel consisted of fourteen judges (laboratory members) (7 females and 7 males, 27 - 36 years old, with an average age of 29 years and three months) who performs regular sensory evaluations on food products. Tasting panel was selected and prepared in preliminary sessions following the ISO8586 (2012) and ISO 5492 (2016) in order to reach agreement on the sensory characteristics and the use of the evaluation scale.

The panelists were asked to rate the samples and the effects of adding EOs to fresh cheese using an acceptance test. They were also asked on the overall acceptability of samples using a descending order scale from 'not' to 'most' appreciable, using a scale from 1 to 5, where 1 corresponds to the mention "no appreciable" and 5 to "most appreciable" (Elsamanin *et al.*, 2014).

The parameters related to texture and appearance (elasticity, resistance, firmness or friability, and creaminess), color (white, lowly white or yellow), odor (refreshing, and rancid), and flavors (acid, astringent, and rancid) were assessed.

### Statistical analysis

The data collected from the experimental designs FCC (-), FCC1, FCC2, FCC3, and FCC (+) ( $n = 3$  for each sample) and analytical procedures (all done in triplicate) have been statistically analyzed. Experimental results were presented using MS-Excel 2010, and expressed as mean  $\pm$  standard deviation (SD). ANOVA (one-way) analysis was used to determine statistical significance at  $p < 0.05$ . Then, Tukey's post-hoc test was performed by applying SPSS 21.0 software.

## RESULTS AND DISCUSSION

### TPC and FRSA

The studied EO showed a high FRSA, demonstrating a high percentage of DPPH free radical inhibition, with half-maximal inhibitory concentration (IC50) measured at  $0.321 \mu\text{g}/\text{mL}$ , although it was more efficient than ascorbic acid ( $\text{IC}_{50} = 0.622 \mu\text{g}/\text{mL}$ ).

The average TPC of EO the extracted from lemon leaves was  $22.76 \pm 0.35$  mg GAE/g of LEO. However, Hojjati and Barzegar (2017) recorded TPC of 14.73 mg GAE/g dry plant matter, and its IC50 value in the DPPH test was 980 µg/mL.

While **Maaroufi et al. (2012)** indicated TPC values of  $(3.90 \pm 0.42) - (0.21 \pm 0.2)$  mg EGA/g dried extract. These results were expressed on dry extract basis or dry plant material, making it difficult to make comparison with our results. These variations result from various factors such as extraction conditions, the different varieties of used lemon and geographical area of origin.

Phenolic compounds are frequently associated with antioxidant activity because of their ability to act in free radical reactions, as electron donors. However, according to **Hojjati and Barzegar (2017)** peel had lower antioxidant activity than *Citrus* leaf E.O. It was suggested that the E.O may enclose components with significant proton-releasing properties.

The composition of EO, in particular secondary metabolites and conjugated double shows strong antioxidant activity (**Dhifi et al., 2016**). Previous studies have reported that, the presence of monoterpenes hydrocarbons in the tested E.O would be responsible on the significant antioxidant activity, more specially  $\beta$ -pinene, D-limonene,  $\gamma$ -terpinene and  $\rho$ -cymene which are the major components, alcohols, particularly Linalool,  $\alpha$ -Terpineol, Geraniol and ester, particularly Geranyl acetate, Neryl acetate, Linalyl acetate,  $\alpha$ -Terpinyl acetate (**Lota et al., 2002; Alfonzo et al., 2017; Hojjati and Barzegar, 2017**). These compounds have redox properties, which aid in the decomposition of peroxides and neutralize free radicals (**Jugreet et al., 2020**). So, the LEO is able to decrease the lipid peroxidation and therefore improve the quality and stability of food products (**Ben Hsoune et al., 2017**).

**Antimicrobial activities**

The antimicrobial effect of LEO on fungi, Gram-positive and Gram-negative bacteria was evaluated. Agar well diffusion method was used to assess antimicrobial effects on bacteria and fungi. The results are reported in table 1.

All strains tested were sensitive to the essential oil and their inhibition zone diameter (IZD) was dependent on the concentration of LEO. According to previously published studies, IZD were rated in this way: Extremely sensitive (diameter  $\geq 20.0$  mm), sensitive ( $14.0 < \text{diameter} < 20.0$  mm), moderately sensitive ( $8.0 < \text{diameter} < 14.0$  mm) and not sensitive (diameter  $\leq 8.0$  mm) (**Djabou et al., 2013; Li et al., 2019**). IZD results indicated that LEO was more effective against Gram (+) than Gram (-) (Table 1).

Other authors have also reported comparable results demonstrating that the EO of *Citrus* leaf showed significant efficacy whether, against Gram-positive or Gram-negative bacteria (**Dongmo et al., 2008; Saeb et al., 2016; Hojjati and Barzegar, 2017**) due to the presence of an outer membrane surrounding their cell wall, in fact,

the lipopolysaccharide coating can limit the diffusion of hydrophobic compounds due to its outer membrane properties (**Loizzo et al., 2009**). In few cases, Gram positive-bacteria may be more receptive to EOs than gram-negative bacteria (**Wilkinson and Cavanagh, 2005; Dhifi et al., 2016**), because the hydrophobic components of EO can penetrate cell membranes more effectively than to the lipophilic ends of lipoteichoic acid present in these membranes (**Rodriguez-Garcia et al., 2016**).

The results show that the LEO exhibits against all tested bacteria a moderate antibacterial activity at the low concentration (2.5 mg/mL) except for *S. aureus*.

The LEO exhibited highly effective antibacterial activity against *Staphylococcus aureus* between 5 and 10 mg/mL and against *B. subtilis* at concentration of 7.5-10 mg/mL. Whereas LEO efficacy against *Escherichia coli* was only observed at 10 mg/mL.

Besides, LEO shows average antibacterial activity against *B. subtilis* at the concentration 5 mg/mL, *E. coli* at the concentration 7.5 mg/mL, and *P. aeruginosa* at the concentration 7.5 - 10 mg/mL.

Our results are in line with findings previously reported on the antimicrobial activities of LEO (**Guerra et al., 2013; Al-Jabri and Hossain, 2014; Hsoune et al., 2017**). The ability of EOs to permeate the cytoplasmic membrane of cells and mitochondria and permeabilize their various layers of fatty acids, polysaccharides, and phospholipids may be the cause of their antimicrobial effectiveness (**Burt, 2004; Falleh et al., 2020**).

The antimicrobial efficacy of EOs may result from their ability to permeate the cytoplasmic membrane of cells and mitochondria, and to permeabilize the various layers of fatty acids, phospholipids, and polysaccharides that make it up. When the bacterial cell wall and cytoplasmic membrane structures are disrupted, several consequences occur. These include a drop in membrane potential, a significant release of ions and other cellular components, a decrease in ATP pools, proton pump collapse, weakening of the cell membrane and, ultimately, the loss of macromolecules (**Gutiérrez-del-Río et al., 2018**) and it's the main cause of inevitably cell lysis. Moreover, according to **Burt (2004)**, EOs can coagulate the cytoplasm and inhibit several of enzyme systems. These include those responsible for energy regulation and synthesis of structural components.

The result of antifungal activity showed that the foodborne pathogens *A. niger* and *C. albicans* were extremely sensitive to the LEO at the concentration 7.5-10 mg/mL.

The tested bacteria were not inhibited by the negative control.

**Table 1** Antibacterial and antifungal activities of LEO. Mean values (n = 3)  $\pm$  SD.

Bacterial or fungal strain	IZD (mm)*				Ampicillin***	Amphotericin B****
	DMSO + Tween 20	<i>C. limon</i> EO **				
		2.5 (mg/mL)	5 (mg/mL)	7.5 (mg/mL)	10 (mg/mL)	
<i>Staphylococcus aureus</i>	-	20 $\pm$ 1.14 <sup>a</sup>	25 $\pm$ 2.1 <sup>b</sup>	29 $\pm$ 0.1 <sup>c</sup>	30.3 $\pm$ 0.6 <sup>d</sup>	27 $\pm$ 0.3
<i>Bacillus subtilis</i>	-	12.33 $\pm$ 2.12 <sup>a</sup>	18.6 $\pm$ 1.73 <sup>b</sup>	23.3 $\pm$ 1.5 <sup>c</sup>	28.3 $\pm$ 1.2 <sup>d</sup>	20 $\pm$ 0.15
<i>Escherichia coli</i>	-	8.25 $\pm$ 1.19 <sup>a</sup>	11.25 $\pm$ 0.85 <sup>b</sup>	16.3 $\pm$ 4.2 <sup>c</sup>	21.05 $\pm$ 0.1 <sup>d</sup>	16 $\pm$ 0.2
<i>Pseudomonas aeruginosa</i>	-	8.1 $\pm$ 1.4 <sup>a</sup>	11.06 $\pm$ 0.94 <sup>b</sup>	16.72 $\pm$ 1.9 <sup>c</sup>	18.3 $\pm$ 2.1 <sup>d</sup>	21 $\pm$ 0.34
<i>Candida albicans</i>	-	9.8 $\pm$ 1.3 <sup>a</sup>	13.19 $\pm$ 2.3 <sup>b</sup>	20.6 $\pm$ 0.8 <sup>c</sup>	21.2 $\pm$ 0.4 <sup>d</sup>	ND
<i>Aspergillus niger</i>	-	10.06 $\pm$ 1.74 <sup>a</sup>	13.86 $\pm$ 1.07 <sup>b</sup>	21.8 $\pm$ 0.1 <sup>c</sup>	23 $\pm$ 0.6 <sup>d</sup>	ND

**Legend:** ND - not determined/Detected. \*IZD (mm) including well diameter of 6 mm. \*\**Citrus limon* essential oil (50  $\mu$ l/well). \*\*\* The used concentration of ampicillin was 25  $\mu$ g \*\*\*\* The used concentration of Amphotericin B was 20  $\mu$ g. a, b, c, d, e, f, g Different superscripts indicate significant differences (ANOVA y test LSD,  $\alpha = 0.05$ ) among EOs concentration (mg/mL).

Table 2 summarized the results of the antimicrobial activity test assessed using the dilution method. The LEO showed MICs  $< 0.125$   $\mu$ g/mL against the all tested bacteria. The presence of chemical components in the essential oil is responsible for its significant antimicrobial activity (**Hsoune et al., 2017**).

The ability of EO components to act as antimicrobial agents is based on the interaction between their hydrocarbon skeleton, which is lipophilic, and the functional groups present, which are hydrophilic (**Kalembe and Kunicka, 2003; Wei and Shibamoto, 2010**).

**Table 2** Minimal Inhibitory Concentrations (MIC), Minimum bactericidal concentration (MCB) and Minimal fungicidal Concentrations (MFC) values (as % v/v) for LEO.

	<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Candida albicans</i>	<i>Aspergillus niger</i>
<b>LEO</b>						
MIC ( $\mu$ g/mL)	$< 0.125$	$< 0.125$	$< 0.125$	0.125	$< 0.125$	$< 0.125$
MBC ( $\mu$ g/mL)	2.00	$> 2.5$	$> 2.5$	$> 2.5$	ND	ND
MFC ( $\mu$ g/mL)	ND	ND	ND	ND	2.00	0.125
<b>Control [a]</b>						
MIC ( $\mu$ g/mL)	0.04	0.12	0.1	0.5	ND	ND
MBC ( $\mu$ g/mL)	0.10	0.25	0.3	0.85	ND	ND
<b>Control [b]</b>						
MIC ( $\mu$ g/mL)	ND	ND	ND	ND	0.01	0.08
MFC ( $\mu$ g/mL)	ND	ND	ND	ND	0.15	0.2

**Legend:** LEO - Lemon essential oil, Control [a] - Ampicillin; Control [b] - Amphotericin B. Values are given as mean  $\pm$  SD of triplicate experiments.

**Yield and physicochemical composition of Cheese**

Table 3 shows data on the yield and average physicochemical composition of fresh cheese. The results present the means, standard deviations, and ANOVA of pH, titratable acidity, moisture, fat, ash, macro-elements like Ca, Na, and K, and traces of heavy elements (Cu, Fe, Zn, and Pb).

Cheese yield is one of the most economically important aspects of cheese manufacturing (Mehaia, 1993). It is given as the percentage ratio between the quantity of cheese produced and the quantity of milk processed (% CY) (Fritzen-Freire et al., 2010; Stocco et al., 2018).

Previous research has shown that the yield of fresh soft cheese was 14–19 kg/100 Kg (Topçu and Saldamli, 2006; Sant’Ana et al., 2013). In the present study, the average yield of fresh cheese obtained was 44.51 % (table 3). This result was better than what the other authors had found. This discrepancy between the results could be due to milk’s constituent elements, in particular its fat and protein content (Mehaia, 1993; Guo et al., 2004), more specifically, the casein micelles present in cheese play an essential role in shaping its structure and the technological properties of milk (Law and Tamine, 2010), but also in the recovering the various milk components in the curd (REC traits), which affects the overall efficiency of cheese making (Stocco et al., 2018). However, Guo et al. (2004) point out that there is a significant correlation between the yield of cheese, salt and starter addition, and milk concentration. Additionally, minerals such as calcium, phosphorus, magnesium, and zinc, which bind to casein, had an impact on the yield of fresh cheese.

Fresh cheese is produced by coagulating pasteurized milk with enzymes and adding lactic acid bacteria. On the first day of preservation, samples of all treated cheeses showed pH values between 4.59 and 4.68. Cheese treated with control (-) showed a relatively low pH (4.59), which differed significantly (P<0.05) from the other cheeses subjected to various treatments (Table 3). The increase in acidity is observed following a change in pH, measured in grams of lactic acid / 100 grams of cheese. Acidity values ranged from 0.39 to 0.54 for the different treatments. Some cheese samples show significant variations in terms of titratable acidity (P<0.05). Diamantino et al. (2014) observed comparable results for Minas fresh cheese, which is elaborated from full-cream cow’s milk. Lactic acid bacteria produce an amount of lactic acid during lactose fermentation, and this production shows a significant correlation with the progressive increase in acidity in all treatments (Zarroug et al., 2020).

All cheese samples had moisture contents ranging from 71.32% to 75.92%, indicating a high moisture product. Moisture levels were above 54%, according to Codex Alimentarius (283-1978) (Alimentarius, 1978).

Cheese quality is influenced by a number of factors, such as the composition of the raw milk, according to Cichoski et al. (2002), who also noted the importance of the processing methods used.

The amount of fat measured in this study was similar to those established by Abdalla and Mohamed (2009) and Sant’Ana et al. (2013), who obtained values for white soft cheese equal to 25.13 g/100 g and 17.44 g/100 g, respectively.

The ash content value of all cheese samples ranged from 4.09% to 5.63% (P<0.05). These results concur with those obtained by Belewu et al. (2012) which showed values of 5.00 ± 1.79%. According to Šnirc et al. (2020), there are differences in the nutrition and metabolism of ewes and cows, which account for the higher mineral content of ewe’s milk than that of the latter.

The calcium content values of all the cheese samples reached about 91.26 and 97.95 mg/100 g. However, the sodium content value reached between 30.17 and 31.82 mg/100 g. In cheeses with lower moisture content compared to the other treatments, we recorded a high calcium content and a low sodium content (P<0.05).

Gomes et al. (2011) suggested that removal of sodium promotes a decrease in calcium solubilization from the paracasein casein matrix, with a direct effect on the colloidal calcium level, resulting in improved calcium retention within the cheese structure. The low sodium content of this cheese is due to the fact that no sodium chloride is added during production.

Potassium content value reached about 112.11 and 114.18 mg/100 g for all cheese samples (P<0.05). The potassium content varies according to the animal origin of the milk, with cow’s milk cheeses showing the highest concentrations of this mineral, compared with other raw milk cheeses.

The magnesium values of all cheese samples varied from 10.29 to 10.38 mg/100 g (P<0.05). Along with the prolonged coagulation times (20 h), the pronounced acidification with a pH of 4.6 causes significant demineralization of casein micelles. This is why cheeses have relatively low concentrations of calcium and magnesium.

All measurements of iron content in the cheese samples ranged from 0.280 to 0.490 mg/100 g (P<0.05). Copper concentrations ranged from 0.0575 to 0.1154 mg per 100 g for all treatments (P<0.05). These results are in line with previous studies on fresh cheese, where copper concentration was observed to increase from 2.83 ± 0.97 mg/kg to 3.25 ± 1.06 mg/kg in raw milk and fresh cheese, respectively (Elbarbary and Hamouda, 2013). This increase in copper content from raw milk to fresh cheese is attributed to its ability to bind to casein (Coni et al., 1996). Similarly, Elbarbary and Hamouda (2013) pointed out that the curdling process and subsequent salting of cheese are the main factors affecting the concentration of certain heavy metals, as opposed to the heating method.

According to Coni et al. (1996), Cu and Fe are released from the alloys of the materials and tools used to milk dairy products.

The zinc values of all the cheese samples reached about 0.253 and 0.287 mg/100 g (P<0.05). Substances in contact with galvanized copper or plastic pipes may contain more zinc than usual. However, the lead values of samples varied from 0.12 to 0.26 µg/100 g (P<0.05).

**Table 3** Yield and physico-chemical composition of fresh cheese at the beginning of the process (day 0), expressed in g per 100 g of cheese.

Chemical composition	FCC (-)	FCC (+)	FCC1	FCC2	FCC3
Yield (%) (g cheese/100 g milk)	44.51				
pH	4.64 ± 0.05 <sup>b</sup>	4.59 ± 0.04 <sup>a</sup>	4.64 ± 0.02 <sup>b</sup>	4.66 ± 0.01 <sup>bc</sup>	4.68 ± 0.01 <sup>c</sup>
Titratable acidity (g lactic acid/100 g of cheese)	0.46 ± 0.02 <sup>c</sup>	0.54 ± 0.08 <sup>d</sup>	0.45 ± 0.01 <sup>c</sup>	0.42 ± 0.03 <sup>b</sup>	0.39 ± 0.05 <sup>a</sup>
Dry matter (g/100 g of cheese)	25.75 <sup>b</sup>	24.08 <sup>a</sup>	28.49 <sup>c</sup>	28.12 <sup>c</sup>	28.68 <sup>c</sup>
Moisture (g/100 g of cheese)	74.25 ± 1.17 <sup>b</sup>	75.92 ± 0.9 <sup>b</sup>	71.51 ± 1.21 <sup>a</sup>	71.88 ± 1.18 <sup>a</sup>	71.32 ± 2.33 <sup>a</sup>
Fat (g/100 g of cheese)	20	20	20	20	20
FDM* (g/100 g)	77.76 <sup>b</sup>	83.06 <sup>b</sup>	70.21 <sup>a</sup>	71.12 <sup>a</sup>	69.73 <sup>a</sup>
Ash* (g/100 g)	4.82 ± 0.02 <sup>d</sup>	5.63 ± 0.05 <sup>e</sup>	4.09 ± 0.1 <sup>a</sup>	4.5 ± 0.02 <sup>b</sup>	4.56 ± 0.04 <sup>c</sup>
<b>Mineral composition (mg/100 g)</b>					
Ca	93.42 <sup>b</sup>	91.26 <sup>a</sup>	97.81 <sup>d</sup>	97.05 <sup>c</sup>	97.95 <sup>d</sup>
Na	30.59 <sup>d</sup>	31.82 <sup>e</sup>	30.17 <sup>a</sup>	30.23 <sup>b</sup>	30.33 <sup>c</sup>
K	112.11 <sup>a</sup>	114.18 <sup>c</sup>	112.25 <sup>a</sup>	112.90 <sup>b</sup>	113.00 <sup>b</sup>
Mg	10.38 <sup>c</sup>	10.38 <sup>c</sup>	10.29 <sup>a</sup>	10.31 <sup>ab</sup>	10.33 <sup>b</sup>
Cu	0.0839 <sup>c</sup>	0.1154 <sup>d</sup>	0.0575 <sup>a</sup>	0.0841 <sup>c</sup>	0.0695 <sup>b</sup>
Fe	0.425 <sup>b</sup>	0.490 <sup>c</sup>	0.283 <sup>a</sup>	0.289 <sup>a</sup>	0.280 <sup>a</sup>
Zn	0.275 <sup>c</sup>	0.287 <sup>d</sup>	0.261 <sup>b</sup>	0.263 <sup>b</sup>	0.253 <sup>a</sup>
Pb (µg/100 g)	0.24 <sup>cd</sup>	0.26 <sup>d</sup>	0.18 <sup>b</sup>	0.22 <sup>c</sup>	0.12 <sup>a</sup>

Legend: FCC (-), FCC1, FCC2, FCC3 - Fresh cheese with 0%, 0.125%, 0.5%, and 1.25% (w/w) of LEO, respectively, FCC (+) - Fresh cheese with 0.125% (w/w) nisin (control +).

\*: Fat and ash in dry matter.

a,b,c,d,e Within a line, different superscript lowercase letters denote significant differences (P < 0.05) amongst the different studied cheeses (three repetitions).

Values are means of triplicate samples (±SD), (n=3).

**Evolution of the physico-chemical parameters of fresh cheese**

Variations in pH, titratable acidity, dry matter and ash content for the different treatments over a storage period of 28 days at 4 ± 2°C, are illustrated in figures 1-a, 1-b, 1-c and 1-d. Differences were observed in all parameters analyzed among the different treatments (P<0.05) in the entire storage system.

All pH values measured for treated cheeses were between 4.60 and 4.68 (Figure 1-a), and there was a significant difference between these values (P<0.05). These values were similar as reported by several studies for white cheese made for cow’s milk (Topçu and Saldamli, 2006; Soltani et al., 2015).

The pH reduction accompanied an increase of titratable acidity for all treated cheese (Figure 1-a, 1-b) during the entire evaluated storage period. Similar increases in titratable acidity and pH reduction were observed by Sangaletti et al. (2009), Soltani et al. (2015), and Dimitreli et al. (2017).

There was an interaction between the pH reduction, the addition of the LEO, and the time of storage (P<0.05). The samples under control treatment (FCC (-) and FCC (+)) have a low pH value (4.45, 4.49, respectively) (Figure 1-a) (ΔpH = 0.19 and 0.10, respectively) (Data not reported) after 28 days of storage, there was a significant difference (P<0.05) between the value of these cheeses and those treated differently.

There was a significant difference ( $P < 0.05$ ) in pH after 21 days of refrigerated storage in the following treatments: FCC (-), FCC (+), and FCC1. Whereas, in the treatments FCC2 and FCC3, the pH value decreased slightly during storage, and a significant difference ( $P < 0.05$ ) was noted after 28 days of storage. Similarly, the titratable acidity highly increased ( $P < 0.05$ ) in all treatments during preservation, and the acidity value was similar in the treatments FCC (-) and FCC1 ( $P > 0.05$ ) but different from the treatments FCC2 and FCC3 ( $P < 0.05$ ).

The increase in pH led to a rise in acidity, measured as a percentage of lactic acid. The result showed that the acidity levels differ according to the various treatments, from 1.60 g lactic acid/100 g to 1.72 g lactic acid/100 g in the beginning on the first day of conservation, showing a significant difference ( $P < 0.05$ ).

The drop in pH can be explained by the transformation of the lactose remaining in the cheeses by the starter culture, resulting in acid production. The results of the study are in line with the results of **Topçu and Saldamli (2006)** and **Soltani et al. (2015)**, who observed high decreases in pH during the conservation of white cheeses made from cow's milk using a homofermentative mesophilic dairy culture similar to the one used in the study.

Maintaining acidity control during the storage process is essential in preserving the quality of fresh cheese. Acidification plays an important role in determining shelf-life (**Fernandes et al., 2017**).

Moisture percentages ranged from 71.32% to 75.92% for all treatments on the first day. During storage, the amount of moisture contained has decreased (figure 1-c) and the amount of dry matter content has increased considerably ( $P < 0.05$ ) during the refrigerated storage for the treatments FCC1, FCC2, and FCC3 from that of cheeses under control (FCC (-), FCC (+)). The constant loss of moisture from the curd (syneresis) during the storage period may be the cause of the rise in total solid contents. This loss of moisture was in correspondence with the acidification of the coagulum and a high disparity was observed between treatments and the control group (Figure 1-c).

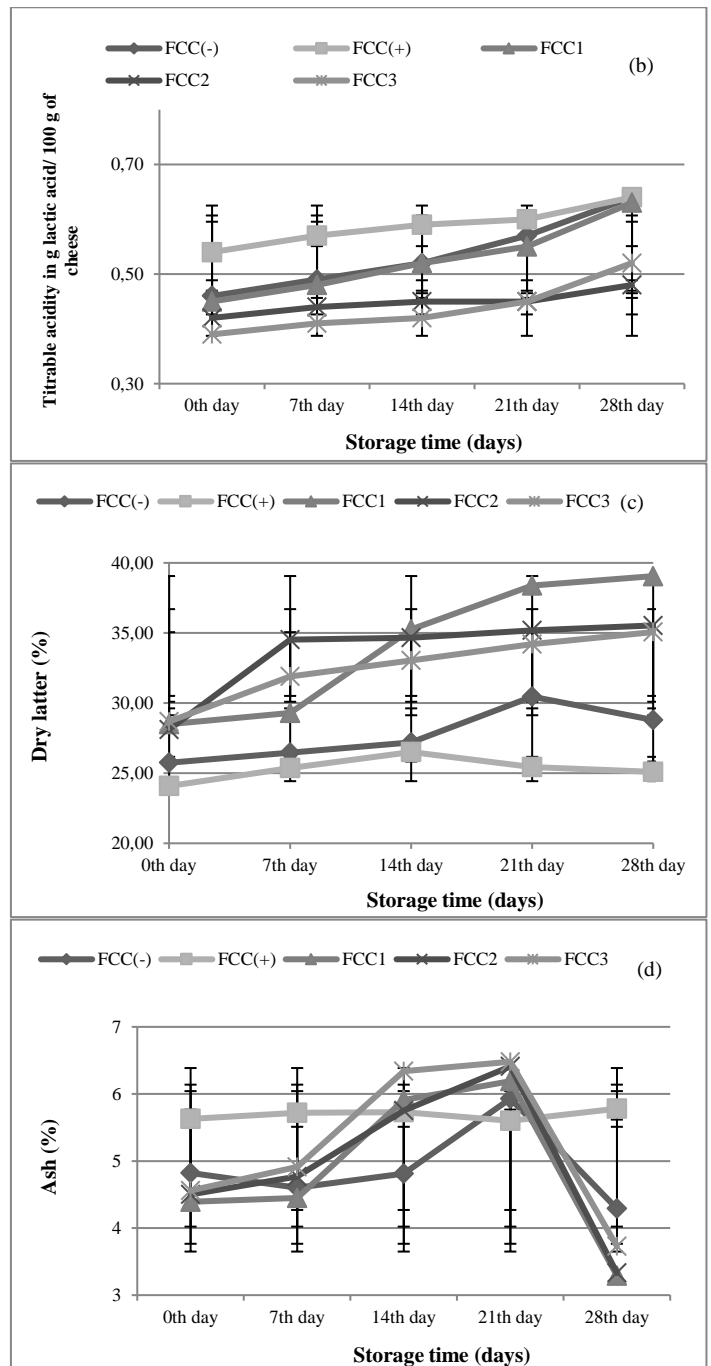
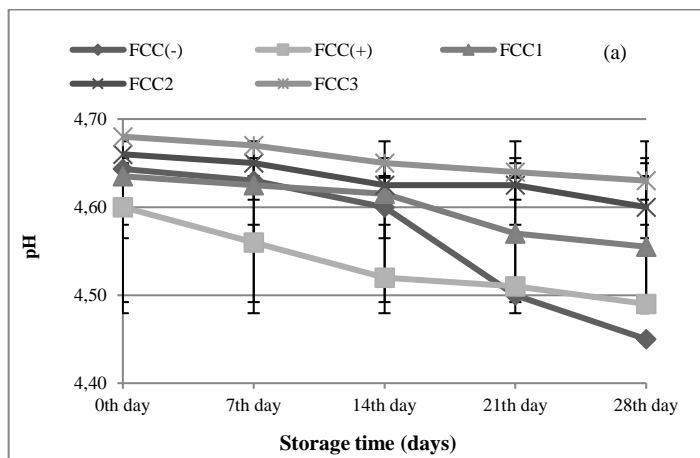
The treatment FCC1 resulted in a notably greater rise in syneresis compared to FCC2 and FCC3 following a 28-day storage period. This increase may stem from the lower proportion of LEO (0.125%) in FCC1, in contrast to the amounts present in FCC2 (0.5%) and FCC3 (1.25%).

LEO influenced the variation in pH and acidity of cheese by reducing the conversion of lactose to lactic acid, these results in lower levels of acidity in the cheese than those produced by the control process. Also, the addition of LEO was effective in decrease syneresis of cheese by preventing reducing pH and the whey expulsion. This decrease on syneresis could be as a result of the increase in the negative charge on casein which related the increase of the repulsive forces between casein micelles and that preventing aggregation.

The pH values and dry matter results are similar to those reported by **Sant'Ana et al. (2013)**.

Ash content increased highly ( $P < 0.05$ ) in all treatment cheeses during a 21-days refrigerated conservation period except for cheese under control (+) FCC (+) ( $P > 0.05$ ) (figure 1-d). There was an interaction between the increase in ash content and pH reduction because the increase in dry matter could be attributable to the loss of moisture from the coagulum. Therefore, as the dry matter content of cheese increases, so does its ash content.

However, an important ( $P < 0.05$ ) ash loss on the last 7 days of storage was noted in all treatments except for cheese under control (+) FCC (+), possibly as a consequence of dissolving minerals loss (e.g., sodium, potassium, and chloride) during the syneresis and which represent a significant proportion of residues from milk combustion.



**Figure 1** Mean values of pH (a), titratable acidity in lactic acid (b), dry matter (c), and ash (d) of fresh cheese over 28 days storage period at 4 °C. Error bars represent the standard deviation of the measurements.  
**Legend:** FCC (-), FCC1, FCC2, FCC3 - Fresh cheese with 0%, 0.125%, 0.5%, and 1.25% (w/w) of LEO, FCC (+): Fresh cheese with 0.125% (w/w) nisin (control +).

**Evolution of microbiological parameters and lactic acid bacteria (LAB) of cheese samples during storage at 4 °C**

Cheese samples were subjected to a plate counting method to assess the population of microorganisms in the total aerobic mesophilic flora (TAMF), coliforms, *Staphylococcus*, *Salmonella*, *Lactobacillus*, and *Lactococcus*. Figures 2 and 3 illustrate the results presented.

Quantifying the number of mesophilic microorganisms enables the assessment of the hygienic quality of food, thereby providing an estimate of its shelf life (**Fernandes et al., 2017**). The initial concentration of TAMF was 4.83 log CFU/g (Figure 2-a). Our results agree with those obtained by **Fernandes et al. (2017)**, who found a bacterial count of 4.88 log CFU/g in Minas frescal cheese at the beginning of the storage period.

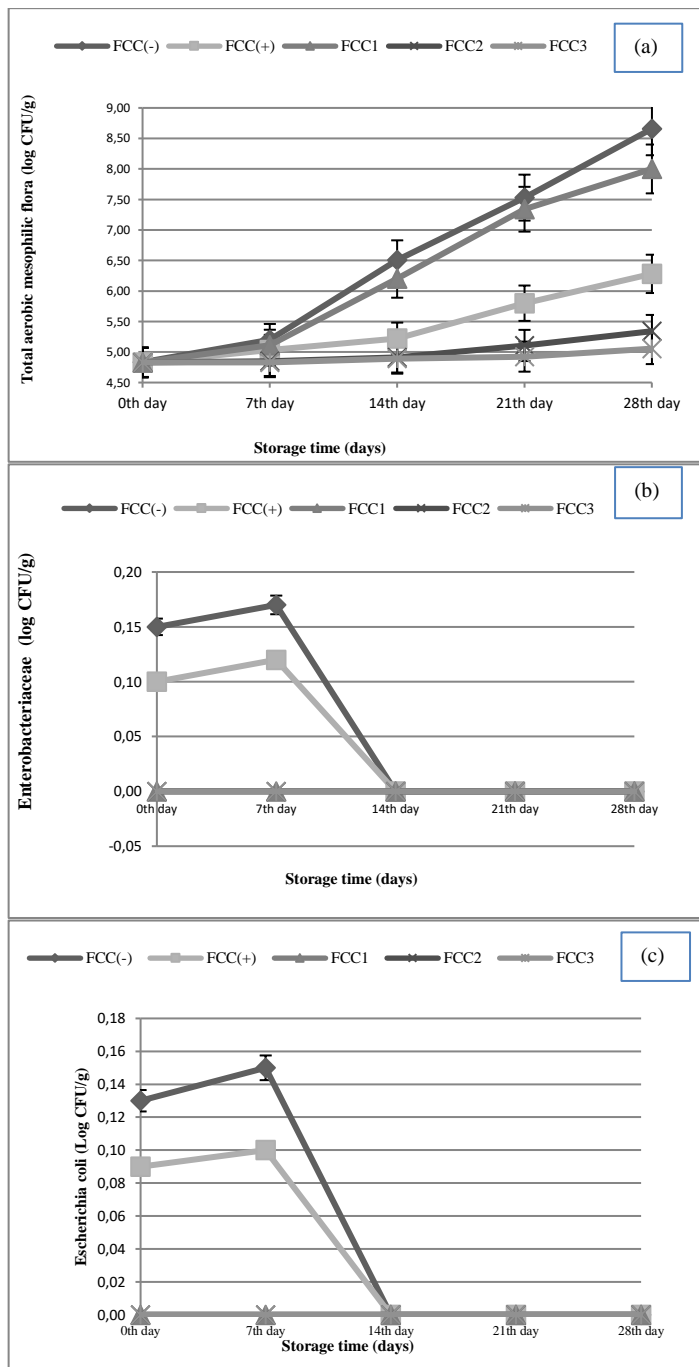
From the end of 14 days to 28 days of storage, LEO at concentration of 0.5% and 1.25% showed a high antimicrobial impact ( $P < 0.05$ ) on TAMF. The FCC2 and FCC3 cheese samples showed a reduction of 0.51 log cycles and 0.23 log cycles, respectively, compared to the control (without LEO) FCC (-). Moreover, they showed a reduction of 0.94 log cycles and 1.23 log cycles, respectively, compared to the FCC (+) control with the addition of nisin. **Melo et al. (2009)** considered the

maximum tolerable limit for aerobic mesophilic bacteria counts in Minas frescal cheese was found to be 8.00 log CFU/g.

Figure 2 shows variations in total coliform in control and flavored cheeses throughout their storage at 4°C. No coliforms, *Staphylococcus*, *Salmonella*, fungi, and molds were detected in flavored cheeses from day 0. While the controls cheeses FCC (-), FCC (+) presented, respectively, a total coliforms concentration of 0.15 log CFU/g and 0.10 log CFU/g. A correlation can be established between total coliform, *E. coli*, *Staphylococcus*, *Salmonella*, fungi, and mold development with the addition of LEO in fresh cheese.

Concentrations of total coliforms and *Escherichia coli* were acceptable according to the national standards (Official Journals of the People's Democratic Republic of Algeria 2017). Coliforms found in dairy products are a sign that heat treatment methods are inappropriate, equipment has not been properly disinfected, and handling has been neglected. The existence of coliforms in the dairy industry is worrying, as they can alter the taste, texture, and aroma of dairy products in undesirable ways (Fernandes et al., 2017).

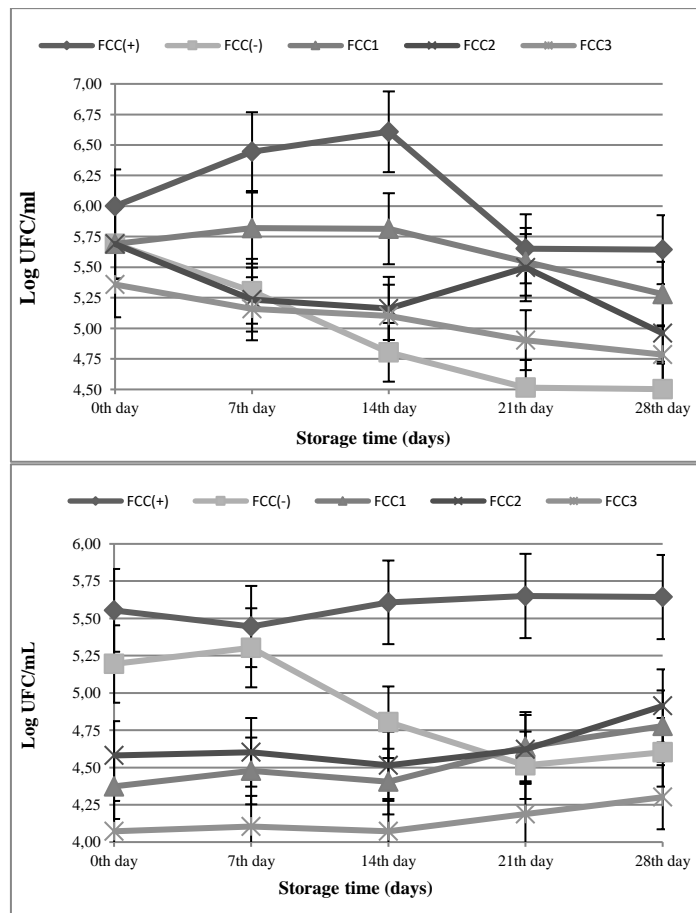
The concentration of total coliforms in controls FCC (-), FCC (+) showed a moderate decrease from 0.15 to 0.17 log CFU/g and 0.10 to 0.12 log CFU/g, respectively, at 14 days of conversation, with a total disappearance at the end of 14 days. This finding can be attributed to the fairly low pH of soft cheese and the lower sugar content.



**Figure 2** Effect of the incorporation of LEO at 0%, 0.125%, 0.50%, and 1.25% on the evolution total aerobic mesophilic flora (a), *Enterobacteriaceae* (b), *Escherichia coli* (c) in fresh cheese, during the conservation at 4 °C.

Several studies have reported LEO antimicrobial properties, acting effectively against a variety of micro-organisms (Moosavy et al., 2017; Al-Jabri and Hossain, 2014).

Figure 3 illustrates the variations in lactic acid bacteria during the conservation period at 4°C, in both unflavored and flavored fresh cheese. At the end of the conservation period, disparities in the number of lactic acid bacteria were observed between all cheese samples. Similar observations were made by Zantar et al. (2014).



**Figure 3** Effect of the incorporation of LEO at 0, 0.125%, 0.50%, and 1.25% on the evolution of *Lactococcus lactis* (a), and *Lactobacillus lactis* (b) in fresh cheese, during conservation at 4 °C.

**Sensory properties**

The cheese sample was subjected to a consumer acceptance test, which was carried out using a hedonic evaluation. Figure 4 shows the intensity ratings of the attributes in a fresh product (stored for 0 day).

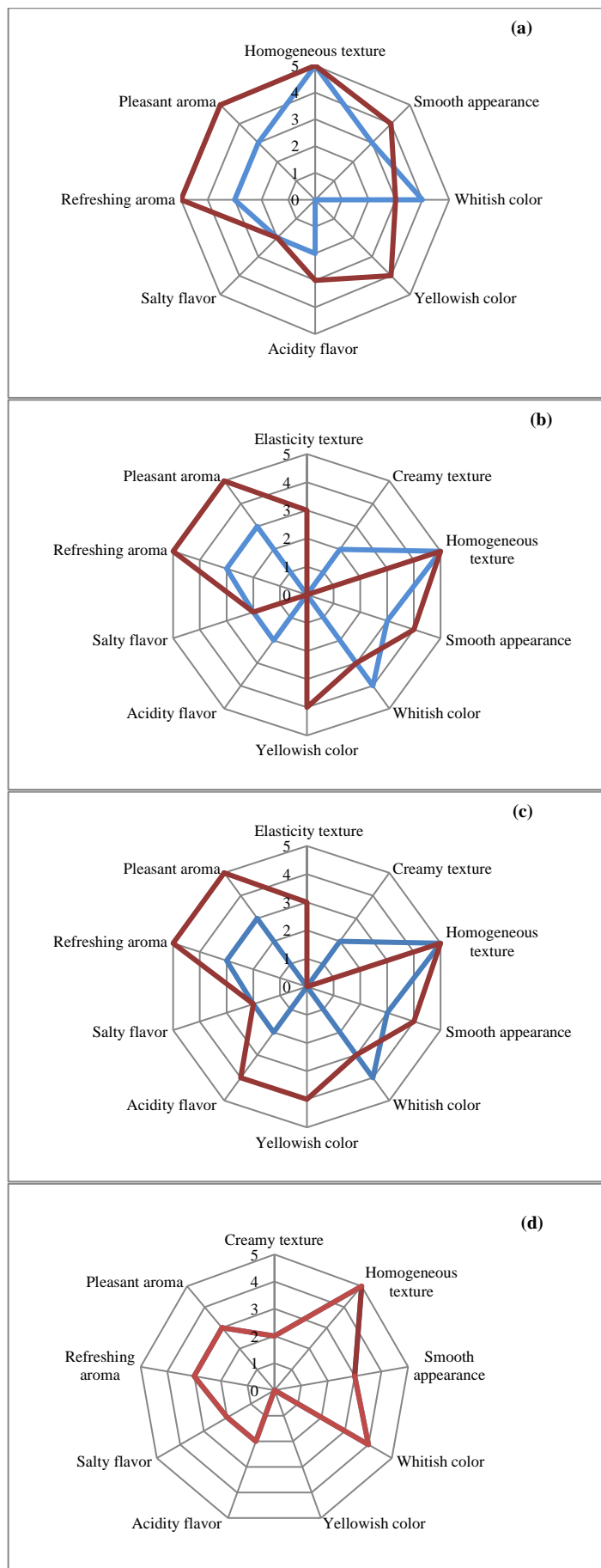
The cheeses assessed showed no marked differences (P>0.05) in terms of elasticity, homogeneous texture, and salty flavor. However, the acidic flavor has intensified (P<0.05) on fresh cheese addition of 1.25% (w/w) of LEO. Higher aroma score was obtained for cheese addition of LEO. Moreover, the lower one was observed in cheese sample control. The incorporation of LEO into the cheese induces a decrease (P<0.05) in whitish color, creamy texture and an increase in the yellow color of aromatized fresh cheese samples compared with controls.

Sensory evaluation results indicated that the addition of a higher concentration of LEO had an impact on consumer acceptability of the product.

The cheese sample was subjected to a consumer acceptance test, which was carried out using a hedonic evaluation. Figure 4 shows the intensity ratings of the attributes in a fresh product (stored for 0 day).

The cheeses assessed showed no marked differences (P>0.05) in terms of elasticity, homogeneous texture, and salty flavor. However, the acidic flavor has intensified (P<0.05) on fresh cheese with an addition of 1.25% (w/w) of LEO. A higher aroma score was obtained for the cheese addition of LEO. Moreover, the lower one was observed in the cheese sample control. The incorporation of LEO into the cheese induced a decrease (P<0.05) in whitish color and creamy texture and an increase in the yellow color of aromatized fresh cheese samples compared with controls.

Sensory evaluation results indicated that the addition of a higher concentration of LEO had an impact on consumer acceptance of the product.



**Figure 4** Graph illustrating sensory evaluation based on quantitative analysis of cheese samples, in comparison to the control (FCC (-)), FCC1 (a), FCC2 (b), FCC3 (c): Fresh cheese with 0%, 0.125%, 0.5%, and 1.25% (w/w) of LEO, FCC (+) (d): Fresh cheese with 0.125% (w/w) nisin (control +).

**CONCLUSION**

The findings of this study proved that adding LEO at concentrations of 0.5% to fresh cheese was more effective on its physico-chemical, microbial, and sensory properties. Nevertheless, the high tested concentrations of LEO (1.25%) may modify the sensory characteristics of fresh cheese, notably the flavor. LEO may be suggested as a natural ingredient to extend the shelf life of fresh cheese because of its high antibacterial and antiradical abilities *in vitro* and in cheese. However, numerous investigations should be carried out on their potential toxic effects with the aim of improving their potential application.

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