**Quality preservation of deliberately contaminated milk using thyme free
and nanoemulsified essential oils**

Running title:

***Thymus capitatus* essential oil and nanoemulsion preserve milk quality**

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Abbreviations: CM: Contaminated Milk; CMNE: Contaminated Milk supplemented with 2 mg/ml NanoEmulsion; CMEO: Contaminated Milk supplemented with 2 mg/ml of Essential Oil; DPPH: 1,1-diphenyl-2-picrylhydrazyl ; L.R.I.: Linear Retention Index; NE: Nano-Emulsion; EO: Essential Oil; IC: Inhibitory Capacity; LA: Lactic Acid; PV: Peroxide Value; SDS: Sodium Dodecyl Sulfate; UHT: Ultra High Temperature; TVC: Total Viable Count.

Abstract.

The objective of this study is to evaluate the effect of either a solution of *Thymus capitatus* essential oil or its nanoemulsion on the quality of milk contaminated by bacteria. After 24h of *S. aureus* inoculation, bacterial growth reached 202×10^3 CFU/ml in the presence of the essential oil while it was limited to 132×10^3 CFU/ml when treated with nanoemulsion. The reduction of antioxidant capacity of milk treated with essential oil was higher when treated with nanoemulsion. Moreover, free essential oil was more efficient in protecting proteins from degradation than the nanoemulsion. For instance, after 24h of *E. hirae* contamination, 26% of the total proteins were consumed in the presence of nano-encapsulated essential oil, while only 14% of the initial content was consumed when free essential oil was added. Concerning milk acidity increase and the inhibition of peroxide production, no statistical differences have been recorded between the use of free essential oil or its nano-emulsion. In conclusion, bulk or nano-encapsulated *T. capitatus* essential oil preserve milk quality and can extend its shelf life.

Key words: Nanoemulsion, Essential Oil, Milk quality, Gram⁺ bacteria.

1. Introduction

Improving foods shelf-life can have an important economic impact in reducing losses attributed to spoilage and allowing products to reach distant and new markets. Food conservation can be assured with the use of synthetic conservatives which exhibit a strong preservative activity against food spoilage. However, due to their potential risks *in vivo*, the use of these artificial preservatives in foodstuffs is restricted or prohibited in some countries (Olmedo, Nepote, and Grosso, 2013). Therefore, safer conservators from natural sources have been developed (Olmedo et al., 2013). With this respect, studies on actual foods show that some botanicals have the potential to be effective bio-preservatives, especially spices rich on natural antimicrobials such as essential oils (EO) (Shobharani, Padmaja and Halami, 2015). Essential oils (EOs) added even in small quantity to foodstuffs, would reduce bacterial contamination (Selim, 2011). Most of these EOs are regarded as safe and accepted by consumers as natural food additives or preservatives (Burt, 2004). However, it has been confirmed that EOs incorporation to foods presents several drawbacks considering their hydrophobicity and easy oxidation (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny and Martín-Belloso, 2014). Nano-encapsulation of EO might be an attractive new approach. In fact, the nano-encapsulation of bioactive compounds represents a viable and efficient approach to increase the physical stability of the active substances, protect them from the interactions with the food ingredients, increase their bioactivity, and mask the undesirable taste and aroma of certain compounds (Selim, 2011).

Milk and dairy products are important food of many diets around the world. Milk can provide essential nutrients for human health, especially for the very young and the very old (Kandpal, Srivastava and Negi, 2012). Milk, as well as dairy products, due to their unique composition and properties, presents a great medium for the growth of many spoilage and pathogenic microorganisms (Smigic, Djekic, Tomasevic, Miocinovic and Gvozdenovic,

2012). Milk deterioration, mainly caused by microbial spoilage, can be evaluated according to sanitary, nutritional, technological and sensory parameters. All these criteria and their interactions depend on numerous factors which are linked to the main physicochemical components of milk (Morand-Fehr, Fedele, Decandia and Le Frileux, 2007). For example, proteolysis, caused especially via microbial enzymes, is the predominant factor that affects several of the technological properties of milk. In fact, the decreased stability of stored pasteurized milk and age gelation of UHT (Ultra High Temperature) milk are mainly dependent on enzymatic protein degradation (Cattaneo, Stuknyte, Pellegrino and DeNoni, 2014). Additionally, milk fat is very susceptible to oxidation, which is a major deteriorative reaction that occurs during the processing, distribution, and storage of milk and dairy products. This can cause changes that affect the nutritional quality, integrity, and safety of milk (Aguar, 2014).

Furthermore, microbial contamination of raw milk can occur from a variety of pathogen microorganisms, especially Gram-positive germs, which can pose public health concerns (Selim, 2011). Among the predominant bacteria involved in these pathogens, *S. aureus* is one of particular concern in milk safety. Indeed, *S. aureus* commonly exists in food processing environments, including dairy production plants (Xing, Zhang, Wu, Wang, Ge and Wu, 2015) and causes food poisoning by its enterotoxins which accounts for a large number of bacterial foods borne illnesses (Huang et al., 2015). Likewise, enterococci bacterial genus, such as *Enterococcus hirae*, figures among bacteria to fear in Milk Industry, since it is a leading cause of gastroenteritis resulting from the consumption of contaminated dairy products (Selim, 2011). By the same token, *Bacillus licheniformis*, as an example of Bacillus species, is among the predominant micro-organisms identified in milk. These bacteria can produce heat-resistant spores which presents a potential safety risk to dairy products (Behringer and Kessler, 1992).

This paper highlighted the possible protective effect of bulk and nanoencapsulated essential oil on milk contaminated with pathogenic bacteria. Samples quality was monitored based on fermentation indicators and oxidative stability at the beginning and after 24h of inoculation.

2. Material and methods

2.1. Plant material and Essential Oil extraction

Thymus capitatus aerial parts were collected, in March 2013, from one naturally diversified mountain in the northeast of Tunisia which is at 27 m and latitude of 37°02'_N, 10°59'_E. Plant identification was carried out by the botanist of the biotechnology center of Borj Cedria. All samples were shade dried for 15 days at room temperature. The dried material was cut into small pieces and subjected to hydrodistillation using a Clevenger type apparatus, for 3 hours. The obtained essential oil was collected and stored at -20 °C in amber vials before analysis.

2.2. GC-MS analysis

The EO was analyzed using a gas chromatograph (HP 5890-SERIE II) coupled to a mass spectrometer (HP-MSD 5972 A) equipped with an HP INNOWAX polar column (30 m × 0.25 mm, film thickness, 0.25 µm). Helium (1.2 ml/min) injection was set in the split mode (1/ 10). Injector and detector temperatures were 250 and 280°C, respectively. Ionization was by electron impact at 70 eV and the ion source temperature was 175°C. Mass spectral data were acquired in the scan mode in the m/z range of 50 to 550 (Snoussi, Dehmani, Noumi, Flamini, & Papetti, 2016). The components were identified by comparing their relative retention times and mass spectra with the data from the Baser library of essential oil constituents, Wiley, Mass Finder and Adams GC/MS libraries. Peak areas were quantified as a percentage of the total ion count. Peaks contributing to the total area by more than 0.01%

were identified. The retention indices (L.R.I.) were calculated according to Van Den Dool and Kratz (1963) formula:

$$\text{L.R.I.} = 100n + 100 (t_x - t_n) / (t_{n+1} - t_n)$$

Where t_n and t_{n+1} are retention times of the reference n-alkane hydrocarbons eluting immediately before and after the target chemical compound “X”; t_x is the retention time of compound “X”.

2.3. Essential oil ‘s nano encapsulation

To enhance its activity, *Thymus capitatus*’s essential oil was encapsulated into Oil in Water nanoemulsion. The nanoencapsulation of the EO was performed using a high pressure micro-fluidizer (NanoVater NV200, Yoshida Kikai CO. Ltd., Japan). Actually, 90% of the NE was composed by 1% aqueous SDS while the remaining 10% consisted of 70% *T. capitatus* EO and 30% soybean oil. The average droplets diameter of the obtained nanoemulsion was analyzed using a Laser Diffraction Particle Size Analyzer (LS13320, Beckman Coulter, Inc., Brea, CA), and was around 110 nm and the preparation was stable for 12 days.

2.4. Milk contamination

To evaluate the preservative effect of *Thymus capitatus*’s EO and its nanoemulsion on contaminated Milk, 3 Gram-positive bacteria were selected: *Staphylococcus aureus subsp* (ATCC 6538) , *Bacillus licheniformis* (ATCC 8480), and *Enterococcus hirae Farrow and Collins* (ATCC 10541). The chosen food matrix was semi-skimmed UHT milk (greater frequency of consumption) purchased from a local supermarket. Each bacterial strain was added to milk, to obtain a final bacterial population of about 10^5 CFU/ml. Contaminated milk was afterwards divided into three samples:

(1) Contaminated Milk, labeled “CM”;

(2) Contaminated Milk supplemented with 2 mg/ml EO, labeled “CMEO”; and

(3) Contaminated Milk supplemented with 2 mg/ml NanoEmulsion, labeled “CMNE”.

Then, all milk samples were incubated 24 hours at 37°C. Milk quality tests were done at t=0h and after 24 hours of incubation.

2.5. Milk quality analysis

Microbial survivability: To test the microbial survivability of the different tested bacteria in milk, supplemented or not with of *Thymus capitatus*'s EO or its nanoemulsion, the pour plate method, which had a limit of detection of 10^3 CFU/ml, was used to enumerate viable bacteria (Qiumin, Davidson and Zhong, 2013). 1 ml of milk sample was serially diluted several times, with saline water. 1 mL from appropriate dilution was put in petri dish and put on Muller Hinton medium for total count. Petri dishes were incubated for 24 h at 37°C. The result was expressed as colony forming units (CFU/ ml).

Total Proteins: Diluted milk samples (1/20) were treated, referring to the Bradford Method (Bradford, 1976), to evaluate the effect of milk's bacterial fermentation on total protein concentration.

Total acidity: The acidity of milk was analysed by titratable method using 0,1N NaOH solution and phenolphthalein as indicator (Smigic et al., 2012). The volume of NaOH required to neutralize milk acids was recorded and used to calculate the content of titratable acids referring to the following equation:

$$\text{Total acidity (g of lactic acid equivalent/l)} = (V_{\text{NaOH}} * 0,009) / (V_{\text{Sample}} * 0,01)$$

Where V_{NaOH} is the volume of NaOH used to neutralize the lactic acid (ml); 0.009 conversion factor whereby 1 ml NaOH (0.01 N) neutralizes 0.009 g of lactic acid; V_{Sample} is the volume of milk sample for titration (ml); and 0,01 conversion factor from % of acidity to g of lactic acid equivalent/l.

Peroxide Value (PV): Total milk lipids were extracted using a chloroform/methanol/water (2:2:1.8; v/v/v) mixture at a 20:1 solvent to sample volume ratio (Cruz-Hernandez et al.,

2004). PV was measured, using the International Dairy Foundation method described by AOAC (2000), directly on the fat extract from Milk Samples.

DPPH free radical-scavenging assay: The antioxidant activity of EO and NE was studied by evaluating their free-radical-scavenging effect on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical based on the method proposed by Boroski, Giroux, Sabik, Petit and Visentainer (2012) with slight modifications. 250µl of methanol diluted milk samples, were added, on a 96 well plate, to 50µl of a DPPH solution (0.2 mM in methanol). The mixture was left standing in the dark for 30 min. The absorbance was then measured at 517 nm employing a Varioskan Flash spectral scanning multimode reader (Thermo Scientific). The ability to scavenge DPPH was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/A_0] * 100$$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample.

2.6. Statistical analysis

For all tested parameters, three replicates were used. Means were compared using the Duncan test at the $p < 0.05$ level, when significant differences were found. Using the Statistical package SAS 9.1 (2002, 525), Pearson's correlation test was conducted to determine the linear correlation among the variables. A value of $p > 0.5$ was considered significant. Graphics were obtained using Microsoft Office Excel 2003 (Microsoft Corp. Washington, USA).

3. Results

3.1. Essential oil compound identification

GC/MS chemical analyses of the *T. capitatus* essential oil allowed the identification of 29 components, representing 99.4% of the total GC/MS peak areas (Table1). Among these 29 identified compounds, carvacrol was distinguished as the major compounds representing more than 75% of the total essential oil components. Oxygenated monoterpenes constituted

the major fraction of this oil distantly followed by monoterpene hydrocarbons, 81.0% and 15.1% of the total essential oil, respectively.

3.2. Milk quality analysis

3.2.1. Fermentation indicators evaluation

Microbial survivability. Regardless the used bacterium and milk treatment, there were major differences between CM when compared to CMEO and CMNE at the beginning of the experiment (Figure 1). In fact, at $t=0$ h, the Total Viable Count (TVC) reached and exceeded 3×10^3 CFU/ml (the three CM), while for supplemented milk samples, TVC was inferior to 15×10^3 CFU/ml (CMNE-*E. hirae*). After 24h of incubation, there were no significant ($p>0.05$) changes on surviving bacteria counting on CM regardless the used bacteria. On the other side, the incubation for 24h at 37°C of CMEO and CMNE induced a notable increase of total microbial count. In fact, the microbial population in supplemented sample had increased in 24 hours from around 0 to 250×10^3 CFU/ml (CMEO-*S. aureus* and CMEO-*E. hirae*, respectively).

Considering the effect of *T. capitatus* EO or its NE, Figure 1 exhibited that their addition has inhibited considerably the development of all bacterial population at $t=0$ and $t=24$ h. As compared to CM, EO and NE were able to reduce greatly the bacterial total count and they were able to contain the contamination with an inhibition percentage ranged about 95% of bacterial population (CMEO-*E. hirae* and CMEO-*S. aureus*, respectively). Moreover, in the CMEO the total bacterium population did not exceed 250×10^3 CFU/ml and for CMNE, a better activity was recorded with a total bacterial count inferior to 160×10^3 CFU/ml (*E. hirae* at $t=24$ h).

Regardless incubation time and milk treatment, the tested bacteria showed different sensitivities towards EO and NE (Figure 1). In fact, even if at $t=0$ h the three bacteria exhibited the highest population counts in CM (over 300×10^3 CFU/ml), they were significantly affected by the milk supplementation especially for the most sensitive strain *B. licheniformis*.

In fact, for the latest bacteria, at t=0h, the total bacterial counts decreased from $>300 \times 10^3$ CFU/ml to 1.7×10^3 and 1×10^3 CFU/ml for CMEO and CMNE, respectively. Besides, after 24h of incubation, the total population count of the three tested bacteria increased significantly but with different extend depending on strain sensitivity. With this respect, *B. licheniformis* showed the lowest bacterial expansion (4.7×10^3 CFU/ml) after 24h of incubation in treated milk, whereas, *E. hirae* presented the highest resistance to the EO and NE. Actually, after 24h of incubation, *E. hirae* bacterial population count reached 250×10^3 CFU/ml (CMEO).

Total protein quantification. Considering the incubation period and for every bacterial strain, general trend in protein content results showed no statistical differences at t=0h in CM, CMEO and CMNE (Table 2). The fermentation of milk caused a significant degradation ($p<0.05$) of the total proteins in all samples when compared to the same sample at t=0h (Table 2). For instance, while at t=0h total protein contents was comprised between 22.2 and 31.6 mg/ml (MNE-*B. licheniformis* and CM-*S. aureus*, respectively), at t=24h this content did not exceed 23 mg/ml (CM-*E. hirae*). The data from table 2 draw attention that the additions of EO or NE significantly protect proteins from degradation when comparing to the CM at t=24h, regardless the used bacteria. Indeed, after 24h of contamination, more than 50% of the total proteins were consumed in the later samples (CM) while this degradation was inferior to 37% in CM-EO and CM-NE. The most effective protection was registered for CMEO-*E. hirae* where the total protein content was reduced by only 14%. Moreover, at t=24h, CMEO-*S. aureus* and CMNE-*B. licheniformis* showed significantly the same efficiency, around 24%, to conserve the total proteins content.

Considering bacterial strains, the three tested bacteria were able to decrease considerably the total protein contents in absence of EO or NE (Table 2). The most important decrease was caused by *Enterococcus hirae* (62% in CM) followed equally by *Staphylococcus aureus* and *Bacillus licheniformis* (51% in CM). Besides, after 24 hours incubation, *E. hirae* and *S.*

247 *aureus* seem to be more sensitive to essential oil action (14 and 23%, respectively) than to the
248 nanoemulsion effect (26 and 38 % of t=0, respectively); whereas *B. licheniformis* was more
249 affected by the nanomelusion addition.

250 **Total acidity measurement.** As shown in Figure 2, the average acidity at t=0h was estimated
251 to 1.8 g of lactic acid/l of milk. After t=24h, the lactic acid contents in all milk samples
252 increased considerably and was comprised between 2.9 and 4.8 g lactic acid/l of milk
253 (CMEO-*S. aureus* and CM-*E. hirae*, respectively). Indeed, the most important increase was
254 equal to 3-fold (CM-*E. hirae*) and the lowest one was 1.6-fold for CMEO-*S. aureus*.

255 Independently of time intervals and bacterial strains, *Thymus capitatus* EO and its
256 nanoemulsion were able to inhibit equally and actively milk acidity increase (Figure 2). While
257 in CM acidity varied from 3.8 to 4.8 g lactic acid/l (*B. licheniformis* and *E. hirae*), in CMEO
258 and CMNE the maximum of lactic acid content was equal to 3.9 g lactic acid/l (*E. hirae*).

259 Considering bacterial strain, the three tested pathogenic bacteria caused a severe
260 acidification of milk after 24h, especially in the absence of EO or NE. In CM, *E. hirae* and *S.*
261 *aureus* were statistically more harmful to milk acidity than *B. licheniformis*. The first two
262 bacteria enhanced the lactic acid content to reach 4.8 g lactic acid/l while in the case of *B.*
263 *licheniformis* the acidification were equal to 3.8 g lactic acid/l (Figure. 2). When comparing
264 the tested strains at t=24h, results demonstrated that *S. aureus* was the most affected by the
265 addition of EO and NE followed by *B. licheniformis* than finally by *E. hirae* (3, 3.3 and 3.8 g
266 lactic acid/l, respectively).

267 **3.2.2. Oxidative stability evaluation**

268 **Peroxide Value.** Regardless the used bacteria, adding *T. capitatus* EO or its nanoemulsion to
269 milk induced a significant decrease in the peroxide value (PV) at t=0h, as compared to the
270 CM. Indeed, CM peroxide values were comprised between 0.7 and 3.3 meq O₂/kg fat (CM- *B.*
271 *licheniformis* and CM-*E. hirae*, respectively). While for CMEO or CMNE the PV, at t=0h,

272 did not exceed 2.3 meq O₂/kg fat (CMEO-*S. aureus*). Besides, after 24h of incubation at
273 37°C, excepting CMEO milk samples, the incubation of milk samples at 37°C for 24h has
274 induced a significant increase of the PV that reach 5.5 meq O₂/kg fat (CM-*S. aureus*). For this
275 sample, the PV increase at t= 24h, was about 1.7 folds comparatively with t=0h (Figure 3).

276 Considering the supplementation effect, results demonstrated that after 24 h incubation,
277 EO and NE addition blocked the production of peroxides in all samples when compared to the
278 CM (Figure 3). In fact, PV of both CMEO and CMNE were limited and inferior to 2.7 meq
279 O₂/kg fat, when the lowest PV of CM was equal to 3.7 meq O₂/kg fat and even attained 5.6
280 meq O₂/kg fat (CM- *B. licheniformis* and CM-*S. aureus*, respectively).

281 The focus on bacterial behavior showed that the three tested pathogens exhibited the same
282 behavior with different extend (Figure 3). As a matter of fact, at t=0h, *E. hirae* was very
283 sensitive to the NE since its PV decreased from 3.3 to 0 meq O₂/kg fat. The same tendency
284 was observed for CMNE-*B. licheniformis* with about 80% peroxide content reduction of the
285 CM- *B. licheniformis*. The same trend was found after 24h of incubation with *B. licheniformis*
286 as the most sensitive bacteria to *T. capitatus* EO, since CMEO-*B. licheniformis* had the
287 minimum peroxides content (1.3 meq O₂/kg fat).

288 **DPPH free radical-scavenging assay.** The DPPH radical stabilization activity measurements,
289 suggested that at t=0, no matter the used bacteria, adding *T. capitatus* EO or its NE had
290 induced a significant increase on the antioxidant capacity of milk samples as showed in
291 Figure 4 (p<0.05). In fact, CM presented the lower inhibitory capacity (IC) which did not
292 exceed 22.75% for CM- *E. hirae*, comparing to supplemented samples where this IC had
293 generally surpassed 30%. After 24h hours at 37°C, the antioxidant capacity of all samples has
294 been reduced. For example, for CM- *B. licheniformis*, the IC had decreased from 19.57% at
295 t=0, to 11.26 % at t=24h. However, this reduction was less significant in CMEO and CMNE

samples, than in CM. Indeed, the activity reduction in CMNE was lower than 21% and even statistically insignificant in CMNE-*B. licheniformis* and CMNE-*S. aureus*.

According to Figure 4, there were no statistical differences between EO and NE addition. For example, the inhibition percentage was statistically the same for CMNE-*B. licheniformis* (at t=0 and t=24h) and CMEO-*B. licheniformis* at t=24h. In the same way, the antioxidant activity was also the same for CMNE-*S. aureus* (at t=0 and t=24h) and CMEO-*S. aureus* (t=0 h).

Considering bacterial variability, at t=0 h, the three CM as well as the six treated samples, and for all tested bacteria, showed separately comparable antiradical efficiencies (Figure 4). The measurements of IC suggested that after 24h of incubation, the three tested bacteria caused considerable decrease on the antioxidant activity. The most harmful bacteria was *S. aureus* which had the highest impact in CM (without any supplement), since its DPPH results were lowermost both at t=0h and at t=24h (16.27% and 6.26%, respectively).

4. Discussion

4.1. Fermentation indicators evaluation

Bacterial survivability. The obtained results demonstrated that *T. capitatus* EO and NE were efficient in inhibiting the fermentation process in contaminated milk samples. This inhibition capacity is probably related to the known antimicrobial effect of carvacrol as it was identified as the major compound in *T. capitatus* essential oil. This molecule was recognized as very effective against gram-positive bacteria growth (Teixeira et al. 2013), and it was therefore used as safe food additive in frozen dairy foods and beverages (Arrieta, Peltzer, Garrigós and Jiménez, 2011). Actually, milk, as well as dairy products, presents a great medium for the growth of many spoilage and pathogenic microorganisms. Thus, in the absence of EO or NE, Gram-positive bacteria have the benefit of degrading available nutrients in this rich medium, and can influence deeply milk quality and cause its deterioration (Smigic et al., 2012). Adding

Thymus capitatus essential oil or its emulsion to contaminated milk, showed a good capacity to control bacterial growth, and consequently, to protect milk from deterioration and extend its shelf life. As well, Nostro and Papalia (2012) have declared that the important antibacterial efficiency of *Thymus capitatus* EO against *S. aureus*, *E. hirae* and *B. licheniformis* is probably fund on two levels. The primary site of antimicrobial action of carvacrol is at the membrane, where this monoterpenoid phenol interacts with the fatty acid chains of the lipid bilayer causing its destabilization and collapse of the membrane. Inside the bacterial cytoplasm, the secondary site, the efficiency of this compound leads to the disintegration of the proton motive force and depletion of the ATP pool.

Referring to this study and considering bacterial behavior, *Bacillus licheniformis* was the most responsive bacteria to milk treatments. For almost all tested parameters, *B. licheniformis* showed more sensibility to the antibacterial effect of *T. capitatus* EO or its NE than the two other tested bacteria. Actually, this finding can be considered as promising, since *Bacillus licheniformis* not only has been isolated from the udder of a clinically healthy cow recovered from mastitis (Salkinoja-Salonen et al., 1999), but also can produce resistant mesophilic spores (Behringer, and Kessler, 1992). These spores produce heat-stable toxins which can introduce a potential safety risk to dairy products because both endospores and toxins can survive current dairy processes like pasteurization and whey evaporation (Nieminen et al., 2007). Besides, *B. licheniformis* is a doubted bacterium in Milk Industry. In fact, toxinogenic *B. licheniformis* biofilm formation can increase contamination risk via strengthening *Bacillus* to environmental conditions in dairy products (Uraz and Gündüz, 2013).

Considering the effect of encapsulating *T. capitatus* EO, it has been discussed repeatedly that encapsulating essential oils ameliorate their biological capacities (Shah, Davidson and Zhong, 2012b; Bonnaud, Weiss and McClements, 2010). With this respect, TVC results showed a better efficacy of the nanoemulsion than the non encapsulated essential oil in

inhibiting the growth of the three tested bacteria. In fact, in the CMNE a better activity was recorded with a total bacterial count inferior to 160×10^3 CFU/ml while in CMEO the total bacterium population reached 250×10^3 CFU/ml. Moreover, the antiradical activity results confirmed that nanoencapsulated EO was more effective in preserving milk quality than the non encapsulated one. These assertions could be explained by the poor water-solubility of bulk essential oil, makes it difficult to incorporate into milk. Besides, bulk essential oil has a tendency to bind with hydrophobic milk proteins which reduces its availability in fighting Gram⁺ bacteria (Shah et al., 2012; Bonnaud et al., 2010). Furthermore, interactions between bacteria and food components such as proteins and lipids affect the localization of oil droplets in the matrix and thus their antimicrobial efficacy (Pérez-Conesa, Cao, Chen, McLandsborough and Weiss, 2011). In contrast, nanodispersed *T. capitatus* EO is evenly distributed in the liquid facilitating the contact between bioactive compounds and their target *i.e.* Gram-positive bacteria (Shah et al., 2012).

Total Protein quantification. Protein content decrease more than 50% in CM while only 37% was lost in CMEO and CMNE after 24h of contamination. This decline of protein content in CM was most probably due to their degradation by the alterative bacteria. In this context, Vignola (2002) reported that different proteolytic enzymes secreted by microorganisms cause alterations of milk constituents especially proteins, causing the milk deterioration during preservation. Data shown in Table 3 are in harmony with this justification, since protein content and TVC were strongly correlated ($R=-0.816$) in CM. While for treated milk, these two parameters were no longer connected to each other ($R=-0.342$). When the antibacterial capacity of *T. capitatus* EO or its NE omitted, available nutrients in CM have stimulated the growth of contaminant culture. More proteolytic enzymes were subsequently released leading to more extensive milk protein degradation during incubation. However, in treated milk samples, proteins were significantly preserved since bacterial growth was blocked by the use

of EO or NE. These results are in agreement with those cited by Ghalem and Zouaoui (2013) who studied the effect of the incorporation of Lavander and Chamaemelum essential oils in steamed yogurt. These authors reported that the incorporation of those essential oils had a marked effect on inhibiting bacterial growth. By the same token, Abdalla, Christen and Davidson, (1993) described a strong relationship between the presence of thermostable bacteria proteases and the reduction of dairy product shelf life. According to these authors, the decrease in protein content during storage might be related to protein degradation leading to formation of soluble compounds. With this respect, Table 3 analysis exhibited a strong negative correlation between the total proteins content and the titrable acidity in both contaminated and treated milk ($R = -0.916$ and -0.799 , respectively). Proteolytic enzymes secreted by contaminant bacteria degrade milk proteins into free amino acids. This liberation of free amino acids in the medium, contributes actively to the increase of acidity during milk fermentation (Widyastuti and Febrisiantosa, 2014).

Total Acidity. The obtained data exhibited a considerable increase in the lactic acid contents in all milk samples particularly in CM. Strong correlations (Table 3) were found between titrable acidity and TVC in both contaminated and treated milk ($R = 0.828$ and 0.681 , respectively). The increase of the total acidity in Gram-positive contaminated milk might be attributed to the utilization of carbohydrates by those pathogenic bacteria. In addition to the amino acid liberation in the medium, the consumption of lactose leads to the production of lactic and formic acids as well as a small amount of CO_2 that induce a significant increase of milk acidity in CM (Ghalem and Zouaoui, 2013). However, the addition of antimicrobial components in CM reduced significantly the TVC and therefore the bacterial metabolism and the liberation of acidic molecules in milk. Thus, titrable acidity was always lower in treated milk than in the contaminated one. In the same context, Olmedo et al. (2013) also have shared that incorporating EO in cheese decelerate the bacterial production of lactic acid.

4.2. Oxidative stability evaluation

Peroxide Value. CM exhibited higher PV after 24h of incubation as compared to treated milk.

In that case, the decrease of caseins antioxidant protection exposes milk fats to oxidation confirmed by the peroxide value increase during incubation at 37°C, while in the presence of EO and NE, caseins was preserved as well its lipid oxidation protection (Smet, Raesb, De Blocka, Hermana, Dewettinckc and Coudijzera, 2008). For treated milk, in addition to casein antioxidant activity, *T. capitatus* EO also had contributed to the protection of milk fat from oxidation. Actually, antioxidants molecules from *T. capitatus* EO work against oxidation by donating their hydrogen atom to lipid free radicals to stop the chain reaction from proceeding further, while the antioxidant residuals in milk remain stable (Waraho, McClements and Decker, 2011). These statements were confirmed by Table 3 data exhibiting a significant relationship between protein content and peroxide value in CM but this correlation was no longer significant in treated milk ($R = -0.636$ and -0.248 , respectively). In agreement with these results, Rafalowski, Zegarska, Kunciewicz and Borejszo (2014) showed a linear correlation obtained between milk oxidative stability and its contents of bioactive compounds with confirmed antioxidative properties. Also, Lorenzo, Pateiro, Fontán and Carballo (2014) have reported that a positive correlation between lipid oxidation products and protein has been detected during the storage of foal liver pâté. Thus, results dealing with milk oxidative stability visibly confirmed the antioxidant preservative effect of using *T. capitatus* EO and NE.

Antiradical Activity. The study of milk oxidative stability using the measurement of its antiradical activity showed an important decrease in CM efficiency as compared to treated one. This significant reduction in CM could be related to the proteins degradation, especially caseins, in that sample. Actually, milk caseins possess an antioxidant activity due to the oxidation of its amino-acid residues (Zulueta, Maurizi, Frigola, Esteve, Coli and Burini,

2009). Thus, proteins degradation caused by pathogenic bacteria could be the reason behind the antioxidant activity reduces. Those statements were confirmed by data shown in Table 3 where a negative correlations between TVC and the DPPH radical stabilization activity measurements were found in both contaminated and treated milk ($R = -0.563$ and -0.552 , respectively). With this respect, in treated milk, where the protein content was nearly conserved, the antiradical activity was relatively preserved too. Besides, for treated milk, the antiradical activity was reinforced by the use of the EO or NE as the antioxidant activity of *Thymus capitatus*'s essential oil is widely known (Akrou, El Jani, Amouri and Neffati, 2010).

Conclusion

Considering the results obtained from this study, incorporating *Thymus capitatus* essential oil or its nanoemulsion have positively fought bacterial growth in different contaminated milk and ameliorated its physicochemical and microbiological quality as well. Thus, it can be said that the addition of *Thymus capitatus* essential oil or its nanoemulsion improves the oxidative and fermentative stability of semi skimmed UHT milk. Taken in consideration all the tested parameters, the general trend highlighted that essential oil nanoencapsulation enhanced its efficiency in preserving milk quality. These results provide an exciting potential for the future, especially about the shift away from artificial preservatives and the move toward more natural alternatives, and milk industries should consider the addition of encapsulated essential oils as a natural antioxidant/antimicrobial agent useful for the preservation of quality parameters in this kind of product. Nevertheless and considering the strong aroma and flavor of essential oils like Thyme, the next step in this research is to determine the impact of the inclusion of these antimicrobials in milk on consumer acceptability.

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References

- Abdalla, O.M., Christen, G.L., & Davidson, P.M. (1993). Chemical composition of and *Listeria monocytogenes* survival in white pickled cheese. *Journal of Food Protection*, 56, 841-846.
- Aguiara, S.C., Cotticab, S.M., Boeingb, J.S., Samensaria, R.B., Santosa, G.T., Visentainerb, J.V., & Zeoulaa, L.M., (2014). Effect of feeding phenolic compounds from propolis extracts to dairy cows on milk production, milk fatty acid composition, and the antioxidant capacity of milk. *Animal Feed Science and Technology*, 193, 148–154.
- Akrout, A., El Jani, H., Amouri, S., & Neffati, M., (2010). Screening of antiradical and antibacterial activities of essential oils of *Artemisia campestris* L., *Artemisia herbaalba* Asso, and *Thymus capitatus* Hoff. Et Link. growing wild in the southern of Tunisia. *Recent Research in Science and Technology*, 2, 29–39.
- AOAC, (2000). Official methods of analysis of the association of official analytical chemists. In W. Horwitz (Ed.) (17th ed.). Washington: AOAC.
- Arrieta, M.P., Peltzer, M.A., Garrigós, M., & Jiménez, A., (2013). Structure and mechanical properties of sodium and calcium caseinate edible active films with carvacrol. *Journal of Food Engineering*, 114, 486–494.
- Behringer, R., & Kessler, H. G., (1992). Influence of individual Milk Constituents on the Heat Resistance of *Bacillus licheniformis* and *Bacillus stearothermophilus* Spores. *International Dairy Journal*, 2, 243-255.

470 Belda-Galbis, C.M., Martínez, A., & Rodrigo, D., (2011). Effect of carvacrol on *Listeria*
471 innocula growth at different incubation temperatures in reference media. In: 1st CIGR
472 International Workshop on Food Safety: *Advances and Trends*; 14th-15th April; Dijon
473 (France).

474 Bonnaud, M., Weiss, J., & McClements, D.J., (2010). Interaction of a food-grade cationic
475 surfactant (lauric arginate) with food-grade biopolymers (pectin, carrageenan, xanthan,
476 alginate, dextran, and chitosan). *Journal of Agricultural and Food Chemistry*, 58, 9770–
477 9777.

478 Boroski, M., Giroux, H. J., Sabik, H., Petit, H.V., & Visentainer, J.V., (2012). Use of oregano
479 extract and oregano essential oil as antioxidants in functional dairy beverage formulations.
480 *Food Science and Technology*, 47, 167-174.

481 Bounatirou, S., Smiti, S., Miguel, M.G., Rejeb, M.N., Neffati, M., Costa, M.M., Faleiro, L.,
482 Figueiredo, A.C., Barroso, J.G., Pedro, L.G., (2007a). Chemical composition, antioxidant
483 and antibacterial activities of the essential oils isolated from Tunisian *Thymus capitatus*
484 Hoff. Et Link. *Food Chemistry*, 105: 146-155.

485 Bounatirou, S.G., Zouar,i S., Figueiredo, A.C.S., Barroso, J.G., Pedro, LG., Neffati, M.,
486 Rejeb, M.N., & Smiti, S. (2007b). Chemical homogeneity of *Thymus capitatus* Hoff. et
487 Link. essential oils from Tunisia. *Revue des Régions Arides*, 2 : 679-686.

488 Bounatirou, S., Smiti, S., Miguel, M.G., Rejeb, M.N., Neffati, M., Costa, M.M., Faleiro, L.,
489 Figueiredo, A.C., Barroso, J.G., Pedro, L.G., (2008). *Tymus capitatus* grown in Tunisia:
490 Antioxidant ability of the essential oils on linoleic acid evaluated by different methods.
491 *Acta Horticulturae (ISHS)*, 765: 315-324.

492 Burt, S., (2004). Essential oils: their antibacterial properties and potential applications in
493 foods – a review. *International Journal of Food Microbiology*, 94, 223–253.

494 Cattaneo, S., Stuknyte, M., Pellegrino, L., & DeNoni, I., (2014). Targeted peptides for the
 495 quantitative evaluation of casein plasminolysis in drinking milk. *Food Chemistry*, 155,
 496 179–185.

497 Cruz-Hernandez, C., Kramer, J. K. G., Kennelly, J. J., Glimm, D. R., Sorensen, B. M., Okine,
 498 E. K., Goonewardene, L. A., & Weselake, R. J., (2004). Evaluating the Conjugated
 499 Linoleic Acid and Trans 18:1 Isomers in Milk Fat of Dairy Cows Fed Increasing Amounts
 500 of Sunflower Oil and a Constant Level of Fish Oil. *American Dairy Science Association*,
 501 90, 3786–3801.

502 Ghalem, B.R., & Zouaoui, B., (2013). Evaluation of the quality of steamed yogurt treated by
 503 Lavandula and Chamaemelum species essential oils. *Journal of Medicinal Plants*
 504 *Research*, 7(42), 3121-3126.

505 Huang, Y., Zhang, H., Chen, X., Wang, X., Duan, N., Wu, S., Xu, B., & Wang, Z., (2015). A
 506 multicolor time-resolved fluorescence aptasensor for the simulta-neous detection of
 507 multiplex *Staphylococcus aureus* enterotoxins in the milk. *Biosensors and Bioelectronics*,
 508 74, 170–176.

509 Kandpal, S. D., Srivastava, A. K., & Negi, K. S., (2012). Estimation of quality of raw milk by
 510 milk adultrtion testing kit. *Indian Journal of Community Health*, 24(3), 188-192.

511 Lorenzo, J.M., Pateiro, M., Fontán, M.C.G., & Carballo, J., (2014). Effect of fat content on
 512 physical, microbial, lipid and protein changes during chill storage of foal liver pâté. *Food*
 513 *Chemistry*, 155: 57–63.

514 Morand-Fehr, P., Fedele, V., Decandia, M., & Le Frileux, Y., (2007). Influence of farming
 515 and feeding systems on composition and quality of goat and sheep milk. *Small Ruminant*
 516 *Research*, 68, 20–34.

517 Nieminen, T., Rintaluoma, N., Andersson, M., Taimisto, A.M., Ali-Vehmas, T., Seppala, A.,
518 Priha, O., & Salkinoja-Salonen, M., (2007). Toxinogenic *Bacillus pumilus* and *Bacillus*
519 *licheniformis* from mastitic milk. *Veterinary Microbiology*, 124, 329–339.

520 Nostro, A., & Papalia, T., (2012). Antimicrobial activity of carvacrol: current progress and
521 future perspectives. *Recent Patents on Anti-infective Drug Discover*, 7, 28-35.

522 Olmedo, R.H., Nepote, V., & Grosso, N.R., (2013). Preservation of sensory and chemical
523 properties in flavoured cheese prepared with cream cheese base using oregano and
524 rosemary essential oils. *Food Science and Technology*, 53,409-417.

525 Pérez-Conesa, D., Cao, J., Chen, L., McLandsborough, L., & Weiss, J., (2011). Inactivation
526 of *Listeria monocytogenes* and *Escherichia coli* O157:H7 biofilms by micelle-encapsulated
527 eugenol and carvacrol. *Journal of Food Protection*, 74, 55–62.

528 Qiumin, Ma., Davidson, P.M., & Zhong, Q., (2013). Antimicrobial properties of lauric
529 arginate alone or in combination with essential oils in tryptic soy broth and 2% reduced fat
530 milk. *International Journal of Food Microbiology*, 166, 77–84.

531 Rafalowski, R., Zegarska, Z., Kuncewicz, A., & Borejszo, Z., (2014). Oxidative stability of
532 milk fat in respect to its chemical composition. *International Dairy Journal*, 36, 82-87.

533 Salkinoja-Salonen, M.S., Vuorio, R., Andersson, M.A., Kampfer, P., Andersson, M.C.,
534 Honkanen-Buzalski, T., & Scoging, A.C., (1999). Toxigenic strains of *Bacillus*
535 *licheniformis* related to food poisoning. *Applied and Environmental Microbiology*, 65,
536 4637–4645

537 Salvia-Trujillo, L., Rojas-Graü, A., Soliva-Fortuny, R., & Martín-Belloso, O., (2014).
538 Physicochemical characterization and antimicrobial activity of foodgrade emulsions and
539 nanoemulsions incorporating essential oils. *Food Hydrocolloids*, 1-10.

540 Selim, S., (2011). Antimicrobial activity of essential oils against vancomycin-resistant
 541 *Enterococci* (vre) and *Escherichia coli* o157:h7 in feta soft cheese and minced beef meat.
 542 *Brazilian Journal of Microbiology*, 42, 187-196.

543 Shah, B., Davidson, P.M., & Zhong, Q., (2012). Nanocapsular Dispersion of Thymol for
 544 Enhanced Dispersibility and Increased Antimicrobial Effectiveness against *Escherichia*
 545 *coli* O157:H7 and *Listeria monocytogenes* in Model Food Systems. *Applied and*
 546 *Environmental Microbiology*, 78, 8448-8453.

547 Shobharani, P., Padmaja, R.J., & Halami, P.M., (2015). Diversity in the antibacterial potential
 548 of probiotic cultures *Bacillus Q5 licheniformis* MCC2514 and *B. licheniformis* MCC2512.
 549 *Research in Microbiology*, 1-9.

550 Smeta, K., Raesb, K., De Blocka, J., Hermana, L., Dewettinckc, K., & Coudijzera, K., (2008).
 551 A change in antioxidative capacity as a measure of onset to oxidation in pasteurized milk.
 552 *International Dairy Journal*, 18, 520–530.

553 Smigic, N., Djekic, I., Tomasevic, I., Miocinovic, J., & Gvozdenovic, R., (2012). Implication
 554 of food safety measures on microbiological quality of raw and pasteurized milk. *Food*
 555 *Control*, 25, 728-731.

556 Snoussi, M., Dehmani, A., Noumi, E., Flamini, G, & Papetti, A. (2016). Chemical
 557 composition and antibiofilm activity of *Petroselinum crispum* and *Ocimum basilicum*
 558 essential oils against *Vibrio* spp. strains. *Microbial Pathogenesis*, 90, 13–21.

559 Teixeira, B., Marquesa, A., Ramosa, C., Nengc, N.R., Nogueirac, J.M.F., Saraivab, J.A., &
 560 Nunes, M.L., (2013). Chemical composition and antibacterial and antioxidant properties of
 561 commercial essential oils. *Industrial Crops and Products*, 43, 587– 595.

562 Uraz, G., & Gündüz, S. T., (2013). Investigation of the presence of biofilm in *Bacillus*
 563 *subtilis*, *Bacillus licheniformis* and *Bacillus cereus* which are isolated from raw milks.
 564 *Current Opinion in Biotechnology*, 24, S101–S102.

- Van Den Dool, H., & Kratz, P.D., (1963). A generalization of the retention index system including linear temperature programmed gas—liquid partition chromatography. *Journal of Chromatography*, 11, 463-471.
- Vignola, CL., (2002). *Science et technologie du lait : transformation du lait*. Ed. École polytechnique de Montréal. Québec. 600 p.
- Waraho, T., McClements, D.J., & Decker, E. A., (2011). Mechanisms of lipid oxidation in food dispersions. *Trends in Food Science & Technology*, 22, 3-13.
- Widyastuti, Y., & Febrisiantosa, R.A., (2014). The Role of Lactic Acid Bacteria in Milk Fermentation. *Food and Nutrition Sciences*, 5, 435-442.
- Xing, X., Zhang, Y., Wu, Q., Wang, X., Ge, W., & Wu, C., (2015). Prevalence and characterization of *Staphylococcus aureus* isolated from goat milk powder processing plants. *Food Control*, 59, 644-650.
- Zulueta, A., Maurizi, A., Frigola, M.J., Esteve, R., Coli, G., & Burini, (2009). Antioxidant capacity of cow milk, whey and deproteinized milk. *International Dairy Journal*, 19, 380–385.

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Tables

591 **Table1** Chemical composition of *T. capitatus* essential oil

<i>Constituents</i>	<i>L.R.I.</i>	<i>% of constituents</i>
tricyclene	928	tr
α -thujene	933	0.6
α -pinene	941	0.4
camphene	955	0.2
sabinene	977	tr
β -pinene	982	0.3
myrcene	993	1.1
3-octanol	995	0.1
α -phellandrene	1006	0.2
δ -3-carene	1013	tr
α -terpinene	1020	1.3
<i>p</i> -cymene	1027	3.9
limonene	1032	0.3
1,8-cineole	1034	tr
γ -terpinene	1063	6.7
<i>cis</i> -sabinene hydrate	1070	0.1
terpinolene	1090	0.1
linalool	1101	1.8
<i>cis-p</i> -menth-2-en-1-ol	1123	tr
borneol	1168	1.2
4-terpineol	1178	1.3
α -terpineol	1190	0.2
thymol	1292	0.3
carvacrol	1301	76.1
β -caryophyllene	1419	2.7
α -humulene	1455	tr
bicyclogermacrene	1496	0.2
spathulenol	1577	tr
caryophyllene oxide	1582	0.3
Monoterpene hydrocarbons		15.1
Oxygenated monoterpenes		81.0
Sesquiterpene hydrocarbons		2.9
Oxygenated sesquiterpenes		0.3
Diterpenes		0.0
Other derivatives		0.1
Total identified		99.4

592 **L.R.I.:** Linear Retention Index , tr: trace

Table 2 Total proteins content in milk samples contaminated either with *E. hirae*, *S. aureus* or *B. licheniformis*, and treated or not with *T. capitatus* EO or its NE. The protein content (expressed as mg/ml) is measured at t = 0 and t = 24 hours. The values followed by the same letter are not significantly different.

Treatments	Total Proteins (mg/ml)	
	t=0	t=24h
CM- <i>E. hirae</i>	26.15 ^{b,c}	10.05 ^g
CMEO- <i>E. hirae</i>	26.72 ^b	22.90 ^{c,d}
CMNE- <i>E. hirae</i>	28.30 ^{a,b}	20.91 ^{d,e}
CM- <i>S. aureus</i>	30.10 ^{a,b}	14.84 ^{f,g}
CMEO- <i>S. aureus</i>	29.42 ^{a,b}	22.52 ^{c,d}
CMNE- <i>S. aureus</i>	31.60 ^a	19.73 ^d
CM- <i>B. licheniformis</i>	22.94 ^c	11.18 ^g
CMEO- <i>B. licheniformis</i>	23.03 ^c	16.06 ^{e,f}
CMNE- <i>B. licheniformis</i>	22.17 ^{c,d}	16.88 ^{e,f}

*CM: contaminated Milk, CMEO: Contaminated Milk supplemented with EO and CMNE: Contaminated Milk supplemented with NE.

610 **Table 3.** R values of Pearson correlation's test

Pearson Test Correlation (R)	Contaminated Milk	Treated Milk
Total Acidity/DPPH	-0,532	-0,591
Total Acidity/Protein content	-0,916	-0,799
Total Acidity/Peroxyde Value	0,757	0,309
Total Acidity/TVC	0,828	0,681
DPPH/Protein content	0,382	0,523
DPPH/Peroxyde Value	-0,551	0,111
DPPH/TVC	-0,563	-0,552
Protein content/Peroxyde Value	-0,636	-0,248
Protein content/TVC	-0,816	-0,342
Peroxyde Value/TVC	0,597	0,464

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Figures and figure captions

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627 **Figure 1.** Total Viable Count (TVC) of milk samples contaminated either with *E. hirae*, *S.*
 628 *aureus* and *B. licheniformis*, and treated or not with *T. capitatus* EO or its NE. The TVC
 629 (expressed as *10³ CFU/ml) is measured at t = 0 and t = 24 hours. The values followed by the
 630 same letter are not significantly different.

631 CM: contaminated Milk, CMEO: Contaminated Milk supplemented with EO and CMNE: Contaminated Milk
 632 supplemented with NE.

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Figure 2. Acidity of milk samples contaminated either with *E. hirae*, *S. aureus* or *B. licheniformis*, and treated or not with *T. capitatus* EO or its NE. The acidity (expressed as g of lactic acid/ml) is measured at t = 0 and t = 24 hours. The values followed by the same letter are not significantly different.

CM: contaminated Milk, CMEO: Contaminated Milk supplemented with EO and CMNE: Contaminated Milk supplemented with NE.

Figure 3. Peroxide Value of milk samples contaminated either with *E. hirae*, *S. aureus* and *B. licheniformis*, and treated or not with *T. capitatus* EO or its NE. The PV (expressed as meq O₂/kg fat) is measured at t = 0 and t = 24 hours. The values followed by the same letter are not significantly different.

CM: contaminated Milk, CMEO: Contaminated Milk supplemented with EO and CMNE: Contaminated Milk supplemented with NE.

Figure 4. % of Inhibition Capacity of milk samples contaminated either with *E. hirae*, *S. aureus* and *B. licheniformis*, or treated or not with *T. capitatus* EO or its NE. The IC (expressed in %) is measured at t = 0 and t = 24 hours. The values followed by the same letter are not significantly different.

CM: contaminated Milk, CMEO: Contaminated Milk supplemented with EO and CMNE: Contaminated Milk supplemented with NE.