Thymus capitatus essential oil ameliorates pasteurization efficiency

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Abstract This work aims to characterize *Thymus capitatus* essential oil and to investigate its preservative effects on raw milk quality alone or combined to pasteurization heat treatment. To study its preservative effect, 1 mg l⁻¹ of *T. capitatus* essential oil, characterized by GC-MS, was added to pasteurized or raw milk. The evaluation of milk quality was made by consulting samples total acidities, peroxide values and their total microbial counts, as compared to control milks. Assays were made immediately and after every 48 h of incubation at room temperature throughout 6 days. Results showed that the incorporation of *T. capitatus* EO to pasteurized milk was the most efficient treatment that inhibited milk deterioration. Combining pasteurization to EO incorporation into raw milk inhibited completely the contaminant bacterial growth to the second day of incubation. Moreover, until the fourth day, no statistical differences have been recorded on the total acidity of incorporated and pasteurized milk samples, while raw milk acidity exceeded 55 g of lactic acid equivalent l⁻¹. Considering milk fat oxidation, adding *T. capitatus* EO to pasteurized milk has significantly retarded milk peroxide production to day 4. In conclusion, *Thymus capitatus* essential oil, combined to pasteurization, presents an interesting potency to act as milk stabilizer.

Keywords *Thymus capitatus* essential oil · Raw milk · Pasteurization · Milk quality

Introduction

Milk and dairy products are important food of many diets around the world, as it can provide essential nutrients for human health (Kandpal et al. 2012). Meanwhile, milk safety, mainly its bacteriological quality, remains a concerning topic for dairy industries and public health communities (Oliver et al. 2009). As a matter of fact, bacterial spoilage is the most limiting factor in extending the shelf life of raw milk. Indeed, due to its unique biochemical composition and its high-water activity, milk presents an excellent medium for the growth and multiplication of many spoilage and pathogenic microorganisms (Smigic et al. 2012; Singh et al. 2011). Thus, raw milk has to be sanitized in order to render it adequate for human consumption and prevent pathogenic bacteria spoilage.

Milk pasteurization process consists on heating milk to 63 °C for 30 min to limit its fermentation. Indeed, pasteurization is designed to kill and/or inactivate raw milk contaminant bacteria (Garedew et al. 2012). However, pasteurized milk commercialization is limited due to quality defects caused by microbial contamination, growth and spoilage. Indeed, pasteurization inactivates organisms that grow rapidly in milk, but it does not destroy organisms that grow slowly or produce spores (Godefay and Molla 2000). The stability decrease of pasteurized milk during storage is mainly related to protein degradation caused by heat-resistant bacteria capable of surviving pasteurization (Cattaneo et al. 2014). Additionally, milk fat is very susceptible to oxidation, which is a mainly triggered by heat increase used during the pasteurization process of milk and

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dairy products (Aguiar et al. 2014). Such microbial deficiencies become usually obvious in the finished product through consumer complaints or shelf-life evaluations.

To prevent post pasteurization and/or residual contamination of pasteurized milk, other sanitation techniques could be used in combination with pasteurization, such as the use of antimicrobial bioactive molecules (Tserennadmid et al. 2011). Indeed, new approaches have been adopted in food preservation including the addition of natural compounds with inhibitory or bactericidal activities, like essential oils as safe/effective preservatives (Selim 2011; Donsi et al. 2011). Essential Oils (EOs) added, even in small quantity to foodstuffs, would reduce bacterial contamination (Selim 2011; Ben Jemaa et al. 2017) and extend therefore foods shelf lives. In this context, *T. capitatus* essential oil is used as flavor ingredients in a wide variety of food, beverage and confectionery products (Cosentino et al. 1999). Because its antioxidant and antimicrobial properties, it is also utilized for preservatives purposes as it is generally recognized as safe or GRAS (Lucera et al. 2012; Panizzi et al. 1993).

This study focuses on the stabilization of raw milk physicochemical and bacteriological qualities *via* testing *T. capitatus* EO incorporation, milk pasteurization and their combination. Milk samples quality was monitored during 6 days incubation at room temperature. Samples were tested immediately and every 48 h of incubation for their total viable count, their acidity and their peroxide value.

Materials and methods

Plant material and essential oil extraction

Aerial parts of *Thymus capitatus* were collected from naturally diversified mountain in the northeast of Tunisia which is at 27 m and latitude of 37°02'_N, 10°59'_E. Samples were shade dried at room temperature for 15 days. Essential oil was extracted from the dried material (200 g) for 3 h at 80 °C using cleverger hydrodistillation. The obtained essential oil was collected and stored in amber vials at − 20 °C.

GC-MS analysis

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) was used to analyzed *Thymus capitatus* EO chemical composition. The injection of helium was set in the split mode (1/10) at 1.2 ml min^{−1}. The temperatures of the injector and the detector were 250 and 280 °C, respectively. The temperature of ion source was 175 °C

and the ionization was made by electron impact at 70 eV. According to Snoussi et al. (2016), mass spectral data were attained in the scan mode (50–550 m/z). Component identification was based on comparing retention times with those of authentic standards, comparing their Linear Retention Indices relative to the series of n-hydrocarbons, and by computer matching against commercial libraries (NIST 98 and ADAMS 95) and a home-made library of mass spectra built up from pure substances and components of known essential oils and MS literature data. 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”.

Milk treatments

To evaluate the preservative effect of *Thymus capitatus*'s EO alone or combined to pasteurization heat treatment on raw milk, samples were once incorporated with 1 mg l^{−1} of *T. capitatus* EO (RMEO), once pasteurized (RMP), and both conservation methods were combined one more time (RMPEO). Results were compared to raw milk quality as negative control (RM). Initial raw milk was purchased from local market. For milk pasteurization, samples were heated for 30 min at 63 °C. For the sample dealing with combined effect (RMPEO), *T. capitatus* EO was incorporated after milk pasteurization at a concentration of 1 mg l^{−1}. Once prepared, all milk samples were incubated for 6 days at room temperature. Milk quality tests were done immediately and after every 48 h of incubation.

Milk quality analysis

Total viable count

To test the microbial survivability of raw milk bacteria in different treated samples, the pour plate method, was applied to enumerate viable populations (Qiumin et al. 2013). Experimentally, 1 ml of milk sample was serially diluted with saline water. The appropriate dilution was inoculated in Petri dish containing the Muller Hinton medium for total count. Then, Petri dishes were incubated for 24 h at 37 °C. The results were expressed as colony forming units (CFU ml^{−1}) with 10³ CFU ml^{−1} as a limit of detection.

Total acidity

Milk acidity was monitored using 0.1 N NaOH solution and phenolphthalein as indicator (Smigic et al. 2012). The required volume of NaOH to neutralize milk acids was used to calculate milk acids content 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^{-1} . Results were expressed as g of lactic acid equivalent l^{-1} .

Peroxide value (PV)

To assess milk fat oxidation, 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. PV was expressed as milli-equivalents of peroxide per kilogram of sample (meq Kg^{-1}).

Statistical analysis

For all chemical tested parameters, three replicates were used. Means were compared using one way-ANOVA analysis (the Duncan test). Significant differences were found at 5% of confidence level, using the Statistical package SAS 9.1 (2002, 525). Graphics were obtained using Microsoft Office Excel 2003 (Microsoft Corp. Washington, USA).

Results

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 (Fig. 1). The major fraction of *Thymus capitatus* EO was constituted by oxygenated monoterpenes (81.0%), distantly followed by monoterpene hydrocarbons (15.1%). Carvacrol was distinguished as the major compounds representing more than 75% of the total EO components.

Milk quality assessment

The total viable count assessment

The Total Viable Count results shown in Fig. 2 demonstrated a significant variability ($p < 0.05$) between all tested milk samples since the beginning of the experimentation. Indeed, at day 0 RM had the highest bacterial load (TVC exceeded $300 \times 10^3 \text{ CFU ml}^{-1}$ at day = 0), while the TVC of RMPEO was zero (at day 0). Meanwhile, except RMPEO sample, milk incubation at room temperature has increased the TVC of different samples with different extend. After 48 h of incubation, RMP bacterial load increased significantly and exceeded $300 \times 10^3 \text{ CFU ml}^{-1}$, while for RMPEO samples bacterial contaminant remained inexistent.

The focus on the pasteurization effect shows that such heat treatment was able to prevail milk bacterial spoilage only at the beginning of experimentation. Indeed, at day = 0, milk pasteurization decreased considerably the initial bacterial population load ($93 \times 10^3 \text{ CFU ml}^{-1}$) as compared to raw milk ($> 300 \times 10^3 \text{ CFU ml}^{-1}$). However, after the second day of incubation, bacterial multiplication has been revived and RMP TVC increased considerably to exceed $300 \times 10^3 \text{ CFU ml}^{-1}$.

Concerning the effect of *T. capitatus* EO incorporation on raw milk quality, the obtained data (Fig. 2) reveal that this EO decreased significantly milk initial bacterial load. Indeed, at $t = 0$, bacterial population in RMEO was significantly reduced, as compared to RM (218 and $> 300 \times 10^3 \text{ CFU ml}^{-1}$, respectively). Yet, the TVC of RMEO increased significantly to surpass $300 \times 10^3 \text{ CFU ml}^{-1}$ by the second day.

Meanwhile, when combining the pasteurization to the incorporation of EO into raw milk, the obtained data showed that the interaction of these two treatments blocked actively milk bacterial growth and ameliorated raw milk quality. Indeed, TVC of RMPEO was significantly lower since day 0, as compared to the other milk samples (0 and $> 90 \times 10^3 \text{ CFU ml}^{-1}$, respectively). Moreover, the fermentation of RMPEO was also decelerated in time, in comparison with other treated milks. Indeed, except RMPEO where the TVC was limited to $37 \times 10^3 \text{ CFU ml}^{-1}$ (day 4), the microbial populations of almost all milk samples reached their maximum, over $250 \times 10^3 \text{ CFU ml}^{-1}$.

Milk total acidity analysis

Data shown in Fig. 3 demonstrated that lactic acid content in all milk samples increased considerably during experimentation, particularly in RM. Indeed, the most important increase was recorded in RM sample where milk acidity at

Fig. 1 Chemical composition of essential oil of *T. capitatus* expressed as % of constituents

Fig. 2 Total viable count (TVC) of raw milk samples, pasteurized or not and treated or not with *T. capitatus* EO. The TVC (expressed as $\times 10^3$ CFU ml⁻¹) is measured every 48 h. The values followed by the same letter are not significantly different at $p < 0.05$

Fig. 3 Acidity of raw milk samples, pasteurized or not and treated or not with *T. capitatus* EO. The acidity (expressed as g of lactic acid equivalent l⁻¹) is measured every 48 h. The values followed by the same letter are not significantly different at $p < 0.05$

day 6 was more than 3 times higher than it was at day = 0. Figure 3 results showed that the incorporation of *T. capitatus* EO into raw milk, or its pasteurization decelerated significantly milk acidity increase. As a matter of fact, no statistical differences ($p > 0.05$) were recorded between RMP acidities at day 0 and day 2, while RM acidity was considerably (5.52 and 2.22 g of lactic acid equivalent l⁻¹, respectively). However, the total acidity of RMP and RMEO samples increased significantly from day 4 and exceeded 5 g of lactic acid equivalent l⁻¹ at the end of the study.

Interestingly, the interaction between EO incorporation into raw milk and its pasteurization decelerated considerably milk acidity increase. As a matter of fact, after 4 days of incubation at room temperature, only a negligible increase of RMPEO total acidity was detected (1.9 and 2.0 g of lactic acid equivalent l⁻¹ at day 0 and day 4 respectively). Moreover, at the end of the study, RMPEO total acidity remains always the least (3.6 g of lactic acid equivalent l⁻¹), as compared to other treated milk which acidities exceeded 5 g of lactic acid equivalent l⁻¹.

Peroxide value variation

The data shown in Fig. 4 demonstrated that the peroxide values of milk samples increased significantly ($p < 0.05$) during this experimentation. Indeed, at day = 2, PV increase exceeded 6 meq O₂ kg fat⁻¹ (RM). After that, day 4 results demonstrated a significant decrease of milk PV (except RMPEO sample) from 12.53 meq O₂ kg fat⁻¹ (RMEO) to reach zero at the fourth day.

Considering milk pasteurization, PV results suggested that the process generated raw milk oxidative stability degradation. Indeed, the formation of peroxides in RMP

Fig. 4 Peroxide Value of raw milk samples, pasteurized or not and treated or not with *T. capitatus* EO. The PV (expressed as meq O₂ kg fat⁻¹) is measured every 48 h. The values followed by the same letter are not significantly different at $p < 0.05$

samples was more intense at day 2, as compared to RM (9.97 and 6.39 meq O₂ kg fat⁻¹, respectively).

Additionally, results demonstrated that EO addition in pasteurized milk blocked the production of peroxides when compared to other milk samples (Fig. 4). In fact, PV of RMPEO increased only after the fourth day of incubation and was limited to 7.41 meq O₂ kg fat⁻¹ at the end of the experimentation.

Discussion

Raw milk quality remains a considerable public concern due to its high nutraceutical quality and its vulnerability to alteration. The present study investigated the combined effect of *Thymus capitatus* EO incorporation and the pasteurization of raw milk. The experimentation consisted on the measurement of milk's total viable counts, its total acidity and, its peroxide value through 6 days. The gathered data demonstrated that combining raw milk pasteurization to its incorporation with *T. capitatus* EO ameliorated actively raw milk quality and extended its shelf life.

The obtained results demonstrated that raw milk bacterial load was rapidly amplified during the first fourth days of incubation. Due to its unique biochemical composition and its high water activity, raw milk presents a great medium for the growth of many pathogenic microorganisms (Singh et al. 2011). Indeed, when no preservation method is used, contaminant bacteria degrade available nutrients in raw milk to extend their populations, inducing milk alteration and its quality deterioration (Smigic et al. 2012). However, once bacterial expansion had exhausted

milk nutrients (by day 6), milk becomes inappropriate for their growth. In such critical conditions, bacteria would be dead due to autophagy process (Glick et al. 2010), leading to a decrease of the TVC in contaminated milk. On the other hand, when raw milk was pasteurized, its initial bacterial load was restricted, and microbial multiplication was decelerated. Actually, milk pasteurization has been practiced as an effective method for contamination reduces and preventing infections caused by contaminated milks (Garedew et al. 2012). However, from the second day of incubation at room temperature bacterial multiplication was resumed and RMP bacterial load increased considerably. This bacterial recovery could be due to the residual bacteria that resist milk pasteurization. Indeed, as low heat treatment, pasteurization can only prevent organisms that grow rapidly in milk (thermo-sensitive bacteria), but it does not destroy organisms that grow slowly or produce spores (Godefay and Molla 2000). Such thermo-resistant microorganisms contribute to the alteration of post pasteurized milk and limit its shelf life (Cattaneo et al. 2014). Considering the effect of *T. capitatus* EO incorporation into raw milk, results showed that this EO was able to reduce the initial bacterial load in raw milk. However, remaining bacteria restarted their growth and reached their maximum by the second day of incubation. These results could be explained by the fact that the initial microbial charge of raw milk was rather high that the used concentration of EO has not totally blocked it. Indeed, Celikel and Kavas (2008) declared that the antibacterial activity of EO is strongly related to the concentration of target microorganisms in the medium. Meanwhile, combining the effect of pasteurization to the incorporation of *T. capitatus* EO into raw milk showed the best preservation. Indeed, for RMPEO not only initial bacterial load was considerably decreased, but also milk fermentation was significantly decelerated in time. As a matter of fact, milk initial high load was, firstly, decreased by the pasteurization process, than *T. capitatus* EO regain its efficiency to fight the residual bacteria which resisted to milk pasteurization. This antibacterial efficiency is probably due to the carvacrol, as it was distinguished, in previous work (Ben Jemaa et al. 2017), as the major compound in *T. capitatus* essential oil. This molecule was recognized as very effective against bacterial spoilage (Teixeira et al. 2013). Indeed, carvacrol acts as protonophore (carrier of protons across the lipid bilayers) causing the dissipation of bacterial proton motive force (Ultee et al. 2002). In particular, carvacrol molecule is inserted into the bacterial membrane, disrupts its structure and exchanges its hydroxyl group for another ion (Ultee et al. 2002).

The focus on milk total acidity exhibited a significant increase in the lactic acid content in all milk samples particularly in RM. As a matter of fact, contaminant

bacteria consume, for their growth, raw milk carbohydrates leading to the production of lactic and formic acids as well as a small amount of CO₂ that induce a significant increase in milk acidity (Ghalem and Zouaoui 2013). Thus, the more milk is highly populated, the more milk acidity increases rapidly, since contaminated milk acidity is highly correlated to its microbial loads (Ben Jemaa et al. 2017). Therefore, milk preservation designed to reduce raw milk bacterial load, will theoretically induce a decrease on the lactic acid liberation on the medium (Hahn 1996). Consequently, the efficiency of the used method for milk preservation will be the major factor to control the liberation of acidic molecules. These statements were confirmed by the obtained results, since in RMPEO, the most efficient method in controlling bacterial spoilage, the production of lactic acids were the most limited and decelerated, as compared to other milk samples.

Considering the fermentation effect on milk fats quality, the obtained results suggested that bacterial spoilage induce milk fats oxidation, confirmed by the considerable increase of PV in all milk samples. Actually, milk caseins, with antioxidant activity, are used as substrates by the microbial contaminant (Cattaneo et al. 2014), inducing the antioxidant activity loss of contaminated milk (Smet et al. 2008). Thus, milk fats would be exposed to oxidation since caseins protection would be decreased (Ben Jemaa et al. 2017). Conversely, although pasteurization has limited partially milk bacterial growth, the peroxide production in RMP was accelerated. These results could be attributed to the fact that milk pasteurization use intensive heat treatment to destroy contaminant bacteria. Such high temperature will trigger milk fat oxidation, leading to the formation of peroxides on the medium (Aguar et al. 2014) and the alteration of milk fat quality (Aguar et al. 2014). On the other way, milk treated with both pasteurization and EO incorporation showed the best milk fat quality preservation. In fact, in RMPEO the production of peroxides decelerated and its PV was the lowest after 6 days of incubation. In RMPEO, milk fat was protected from oxidation via the dual action of pasteurization process and *T. capitatus* EO. As a matter of fact, carvacrol, the major compound of *T. capitatus* EO (Ben Jemaa et al. 2017), inhibited milk caseins degradation by controlling bacterial spoilage, leading to the maintain of its antioxidant activity (Ben Jemaa et al. 2017). As well, carvacrol limited peroxide formation using its own antioxidant efficiency. More specifically, carvacrol works against oxidation by donating its hydrogen atom to the lipid free radicals and stop therefore the oxidation chain reaction proceed (Waraho et al. 2011).

Conclusion

Overall, the results suggested that the incorporation of *Thymus capitatus* essential oil was effective against bacterial growth in pasteurized milk and increased consequently its shelf live. Thus, the addition of *Thymus capitatus* essential oil ameliorated pasteurization efficiency in preserving raw milk quality.

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