Effect of Rosemary and Oregano Aqueous Extracts Supplementation on Microbial Growth during Refrigerated Storage of Milk

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Summary

The aim of the present study is to evaluate the effect of adding rosemary and oregano aqueous extracts to raw cow milk on the dynamics of microbial growth during milk refrigeration. The aqueous extracts from plants leaves were prepared and supplemented to milk samples at different concentrations: 0, 0.25, 0.5, 0.75 and 1 mg mL^{-1} for rosemary and 0, 1.25, 2.5, 3.75, 5 mg mL^{-1} for oregano. The evolution of microbial growth was monitored at regular intervals of times during ten days of refrigerated storage at 4 °C. The enumeration of the microbial flora was conducted by the culture methods. The supplementation of the two plants extracts to refrigerated milk generated a prolongation of the lag phase duration and limited microbial growth. For rosemary, the lag phase length (λ) in milks without supplementation (0 mg mL^{-1}) was prolonged from 1.2 ± 0.80 day (28.8 h) to 1.66 ± 0.92 day (39.84 h) in milks added with 0.75 and 1 mg mL^{-1} while the maximum cell load (X_{max}) diminished from 7.00 ± 0.17 log CFU mL^{-1} in non-supplemented milks to 6.56 ± 0.19 log CFU mL^{-1} in milks added with the same concentrations. For oregano, λ was delayed from 4.12 ± 0.11 day (98.88 h) in milks without supplementation to 5.04 ± 0.97 day (120.96 h) in milks added with 1.25 mg mL^{-1}. A decrease of X_{max} was remarked for the whole of the concentrations, registering the lowest value of 4.45 ± 1.34 log CFU mL^{-1} at 2.5 and 3.75 mg mL^{-1}. The use of rosemary and oregano aqueous extracts as natural additives during refrigeration could offer opportunities as biopreservatives in the milk industry, to reduce heat pretreatment and the addition of chemical additives.

Key words

biopreservatives, cold storage, microbial dynamics, plants extracts, psychrotrophic bacteria

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Introduction

Worldwide, the major concern for consumers, food industries and regulatory agencies is the microbial safety of foods (Negi, 2012; Kumuda et al., 2017; Ritota and Manzi, 2020). Commonly, food additives are known to be a source of health risks to consumers such as nausea, headaches and weakness. Also, they can cause dangerous health risks such as allergy, hypersensitivity, asthma, neurological damage, hyperactivity and cancer. These factors motivate consumers to pay particular attention to healthy food products (Tarnavölgyi, 2003; Daniele et al., 2017; Sharma et al., 2018). Although milk is considered as a heat-stable system, some chemical and physico-chemical changes can appear during milk heat treatment. These modifications include non-enzymatic browning (Maillard), denaturation of whey proteins, damage to the creaming properties and eventually a heat-induced coagulation and hydrolysis of the caseins (Lan et al., 2010; Fox et al., 2015). Therefore, there is an increasing demand for alternatives to chemical antimicrobial additives, this leading to the exploration of novel natural compounds used as biopreservatives to reduce partially or totally chemical additives. A variety of natural products deriving from animals, plants and microorganisms can be used in biopreservation to extend foods shelf life (El-Saadany et al., 2017; Liu et al., 2017).

Since distant past, plants have been used in food not only for flavorings, but also for their therapeutic and preservative effects. There is a close association between food pattern and disease prevention. The literature documented well the effects of food on cardiovascular and hypertension disease, obesity, diabetes and osteoporosis (Abdel-Massih et al., 2010; Martins et al., 2014; Abkhoo and Jahani, 2017). The genus *Origanum* is used as food flavoring and seasoning. Due to its bioactive components, oregano exhibits antiparasitic, antimicrobial, antispasmodic, stomachic, diuretic and immunomodulator activities (Bendifallah et al., 2015; Rahmani Gohar et al., 2016; Alagawany et al., 2020). Moreover, *Rosmarinus* genus is a traditional medicinal aromatic herb widely used as a food ingredient for flavoring and is also recognized for its therapeutics benefits and strong antioxidant, antimutagenic, antimicrobial, anti-inflammatory, antirheumatic and antialgesic properties (Shama et al., 2014; Gad and Sayd, 2015; Hać-Szymańczuk et al., 2017). Consequently, the application of herbs in the production of food could improve their nutritional and medicinal values (Oraon et al., 2017; Shahbazi and Shavisi, 2019).

The refrigerated storage of raw milk controls efficiently the proliferation of mesophilic spoilage populations, while it favors simultaneously the growth of psychrotrophic flora. The psychrotrophic bacterial populations developing in milk are represented mainly by Gram-negative genera including *Pseudomonas, Aeromonas, Achromobacter, Alcaligenes, Flavobacterium* and *Serratia* and a minor number of Gram-positive genera including *Bacillus, Clostridium, Corynebacterium, Lactobacillus, Streptococcus* and *Microbacterium* (Oliveira et al., 2015; Boubendir et al., 2016). Ahmed El-Sayed and Aly (2014) investigated the antibacterial and antitumor activities of *Rosmarinus* extracts. The inhibitory effect of aqueous extract against a number of pathogenic bacteria registered minimum inhibitory concentrations values for the species of *Pseudomonas aeruginosa* (0.15 mg mL^{-1}), *Staphylococcus aureus* (0.15 mg mL^{-1}), *Escherichia coli* (0.1 mg mL^{-1}), *Shigella dysenteriae* (0.1 mg mL^{-1}), *Klebsiella pneumoniae* (0.1 mg mL^{-1}), *Micrococcus roseus* (0.15 mg mL^{-1}) and *Bacillus subtilis* (0.15 mg mL^{-1}). Licina et al. (2013) evaluated the antimicrobial activity of *Origanum* extracts against bacteria and fungi. The aqueous extract demonstrated the best inhibitory effect against the bacterial species tested such as *P. aeruginosa* (5 mg mL^{-1}), *E. coli* (5 mg mL^{-1}), *Proteus mirabilis* (0.3 mg mL^{-1}), *K. pneumoniae* (2.5 mg mL^{-1}), *Salmonella typhimurium* (5 mg mL^{-1}), *S. aureus* (0.6 mg mL^{-1}), *Enterococcus faecalis* (5 mg mL^{-1}) and *Bacillus cereus* (0.3 mg mL^{-1}). Also, the antimicrobial activity of rosemary and oregano was tested at a concentration of 5 mg mL^{-1}. The two plants extracts showed an inhibitory activity against *P. fluorescens*, *E. coli*, *S. typhium*, *B. subtilis* and *S. aureus* (Kumuda et al., 2017). The data on the dynamics of microbial growth during the refrigerated storage of raw milk added by plants extracts is missing in the literature. The aim of the present study is to evaluate the effect of the supplementation of rosemary and oregano aqueous extracts to refrigerated milk on microbial growth dynamics.

Material and Methods

Plant Material

The leaves of *Rosmarinus* and *Origanum* used were collected in the region of Mila (36° 27´ N / 6° 16´ E) located in the North East of Algeria. The plant material was harvested during the period of February-April 2018. The leaves were then cleaned, dried in the open air and protected from the light for eight to 15 days at room temperature, finally ground into fine powder and stored for microbial analysis.

Milk Sampling

Raw milk samples were collected in a dairy farm located at the same region. A total of ten milk samples were taken from healthy cows breed "Française Frisonne Pie noir". The cows were fed by bran, barley, oats and hay. At the time of sampling, the teat ends were cleaned, the first jets were discarded and 25 mL of raw milk samples were gathered from the four udders (i.e., a total of 100 mL per cow) in a sterile bottle and shaded. The samples were transported to the laboratory at a temperature of 4 °C.

Preparation of Aqueous Extracts

To prepare the aqueous extract, 100 g of dry powder of each plant was dissolved in sterile distilled water (570 mL) and warmed at 40-50 °C. The maceration was conducted by a moderate stirring at 100 Hz during 48 h. The resulting extracts were filtered two times using standard filter paper. The filtrate obtained was spread on Petri dishes and left to dry for a week; the dried extract was recuperated, ground into a powder and stored in the refrigerator until use. The extraction yields were determined following the equation: Yield (%) = W_1 / W_2 * 100. W_1 is the weight of the extract after drying and W_2 is the weight of plant powder.

Preparation of the Aqueous Extract Stock Solution

Two aqueous extract stock solutions were prepared by adding 1 g of rosemary and 5 g of oregano extracts to 50 mL of sterile distilled water, the solutions were mixed and kept in the refrigerator until use. In order to obtain varied concentrations of plants extracts in milk, serial dilutions were calculated ($C_1 V_1 = C_2 V_2$) and different volumes from the stock solutions were supplemented to milk. The selected concentrations were: 1, 0.75, 0.5, 0.25 and 0 mg mL^{-1} for rosemary and 5, 3.75, 2.5, 1.25 and 0 mg mL^{-1} for oregano.

Bacterial Culture

Raw milk samples were stored at 4 °C to promote the growth of the psychotropic flora in the microbiota and different concentrations of plants aqueous extract were added initially (day 0^{st}) to milk samples. The evolution of microbial growth was controlled at regular intervals of time: 0, 2, 4, 6, 8, 10 days. The samples were diluted (10^{-1} - 10^{-5}) and 1 mL of each dilution was spread onto the surface of Standard Plate Count (SPC) agar, a standard medium corresponding to the American Public Health Association formulation for milk, water, food, and dairy products (Oxoid CM0463; Oxoid Ltd., Basingstoke, UK). The plates were incubated at 37 °C for 48 h, those with the number of colonies 30 to 300 were considered for counting. The whole of the experiments was completed in three replications per milk sample at each time point and the results were expressed as arithmetic mean values.

Biomathematical Analysis

The predicted values of the microbial growth parameters, i.e., maximum growth rate (μ_{max}), lag phase length (λ) and maximum cell load attained (X_{max}), were obtained using Baranyi and Roberts Model (Baranyi and Roberts, 1995).

Results

The microbial growth curves of refrigerated milk supplemented with different concentrations of rosemary and oregano aqueous extracts are shown in (Fig. 1). The results of microbial growth parameters (X_{max}, μ_{max} and λ) obtained using Baranyi and Roberts Model, are represented in (Fig. 2) for *Rosmarinus* and (Fig. 3) for *Origanum*. The linear regression relationship between the maximum microbial growth and *Rosmarinus* and *Origanum* aqueous extracts concentrations is given in Fig. 4. The extraction yields of rosemary and oregano aqueous extracts gave 9 and 13 % respectively and the initial microbial contamination of milks registered the mean value of 3.54 log CFU mL^{-1} (3500 CFU mL^{-1}) for rosemary and 3.19 log CFU mL^{-1} (1550 CFU mL^{-1}) for oregano.

For rosemary plant, the results showed a delay in microbial growth characterized by a prolongation of the generation time (λ) with 11.04 h, from 1.2 ± 0.80 day (28.8 h) in milk without supplementation to 1.66 ± 0.92 day (39.84 h) in both milks enriched with 1 and 0.75 mg mL^{-1} of rosemary aqueous extract. However, the concentrations 0.5 and 0.25 mg mL^{-1} showed reduced generation times of 0.22 ± 1.26 and 0.92 ± 0.78 day respectively. In addition, a slight diminution in the maximum growth level (X_{max}) was remarked from 7.00 ± 0.17 log CFU mL^{-1} in non-supplemented milk to 6.56 ± 0.19 log CFU mL^{-1} in both milks enriched with 1 and 0.75 mg mL^{-1} of rosemary aqueous extract. However, the maximum microbial growth in milks supplemented with 0.5 and 0.25 mg mL^{-1} reached 7.04 ± 0.18 and 7.00 ± 0.12 log CFU mL^{-1} respectively. These rates are almost similar to the ones of non-supplemented milk. A decrease in the maximum growth rate (μ_{max}) was observed from 0.78 ± 0.23 Δlog CFU mL^{-1} day in non-added milk to 0.55 ± 0.17 Δlog CFU mL^{-1} day in milk supplemented with 0.5 mg mL^{-1} of rosemary aqueous extract, while a slight increase in maximum growth rates was remarked in the other concentrations ranging from 0.82 ± 0.26 to 0.84 ± 0.44 Δlog CFU mL^{-1} day.

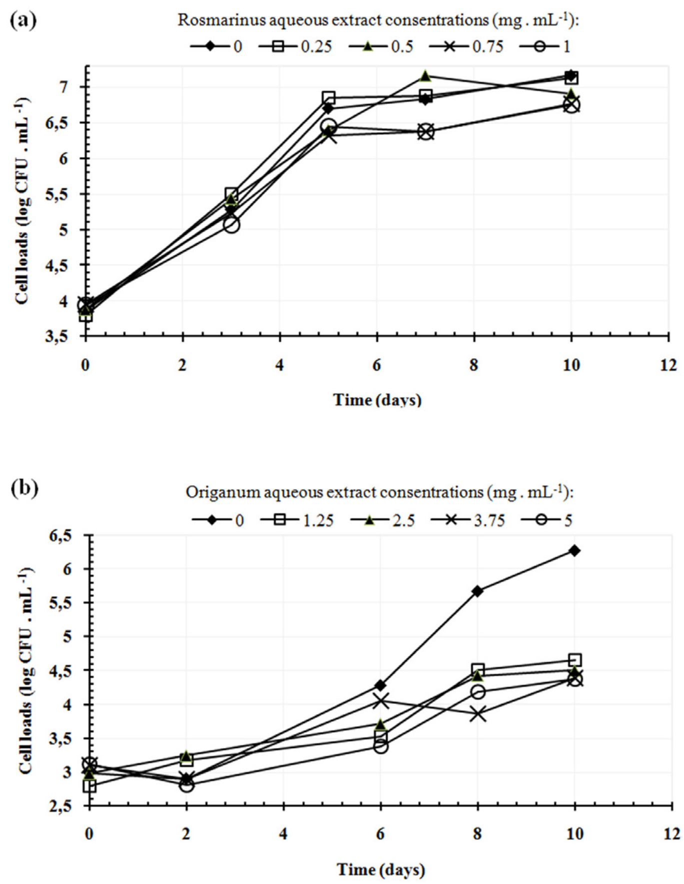


Figure 1. Growth curves of total bacterial count in raw milk supplemented with different concentrations of *Rosmarinus* (a) and *Origanum* (b) aqueous extracts and refrigerated during 10 days at 4 °C

For oregano plant, the results showed a delay in microbial growth characterized by a prolongation of the generation time (λ) with 22.08 h, from 4.12 ± 0.11 day (98.88 h) in milk without supplementation to 5.04 ± 0.97 day (120.96 h) in milk supplemented with 1.25 mg mL^{-1} of oregano aqueous extract while the concentration 5 mg mL^{-1} gave a generation time of 4.02 ± 1.70 (96.48 h), which is almost similar to the one of non-added milk. However, the concentrations 2.5 and 3.75 registered a reduced generation time of 1.93 ± 6.52 day. An important diminution in the maximum growth level (X_{max}) was remarked for the whole of oregano concentrations, registering the lowest value of 4.45 ± 1.34 log CFU mL^{-1} with the concentrations 2.5 and 3.75 mg mL^{-1} of aqueous extract, compared to the milk without supplementation which registered an X_{max} of 6.29 ± 0.03 log CFU mL^{-1}. On the other hand, a decrease in the maximum growth rate (μ_{max}) was observed in the totality of concentrations, registering the lowest value of 0.18 ± 0.2 Δlog CFU mL^{-1} day with the concentrations 2.5 and 3.75 mg mL^{-1} of aqueous extract, compared to the milk without addition which registered a higher μ_{max} of 0.71 ± 0.02 Δlog CFU mL^{-1} day.

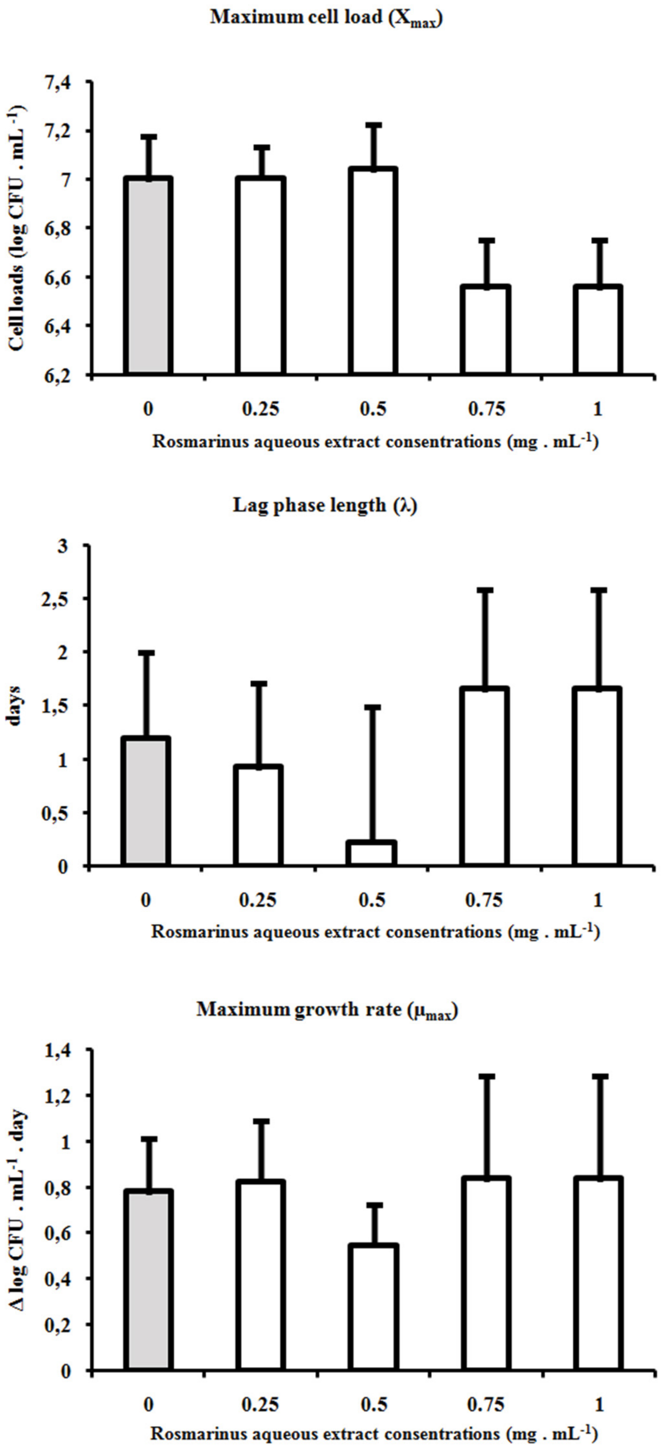


Figure 2. Microbial growth parameters obtained by Baranyi and Roberts Model of mean count data of total bacteria in raw milk supplemented with different concentrations of *Rosmarinus* aqueous extract and refrigerated during 10 days at 4 °C. The mean values are expressed with the standard deviation.

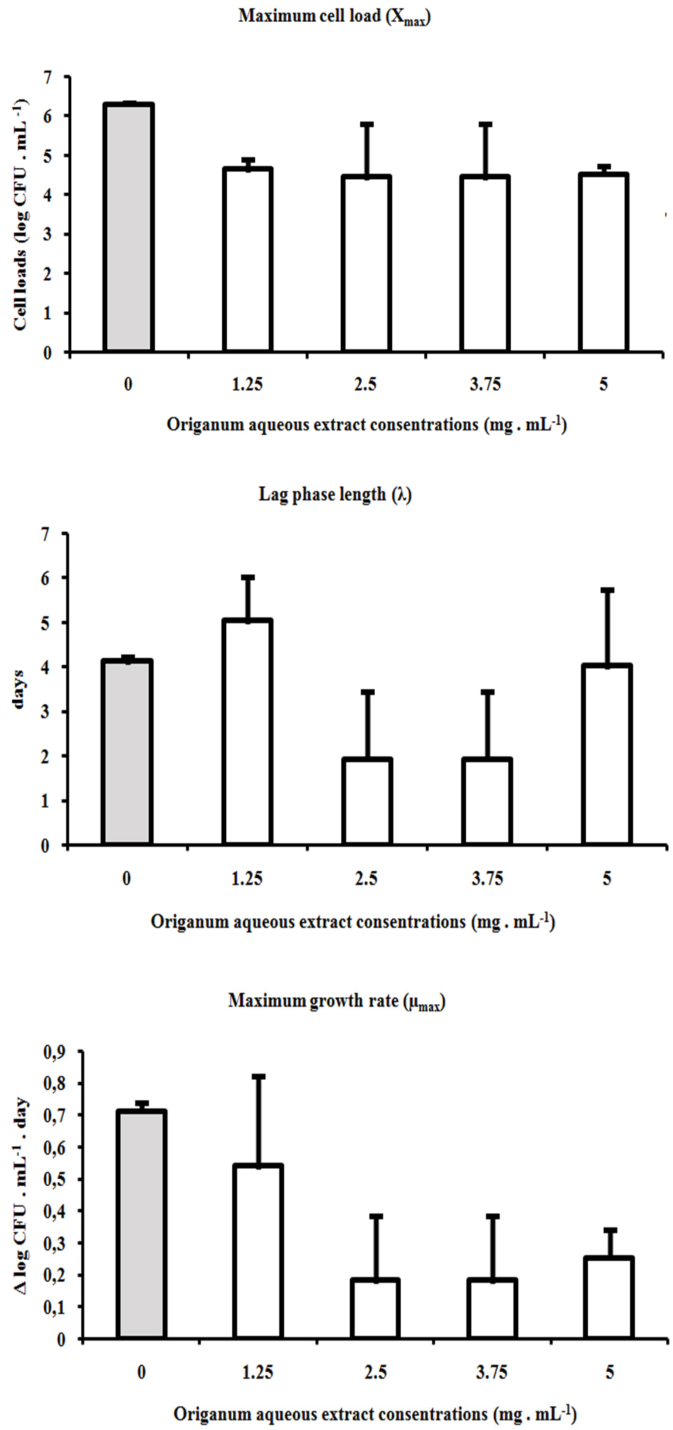
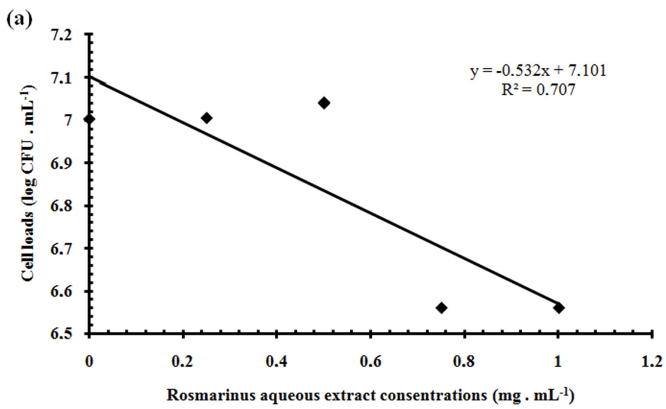


Figure 3. Microbial growth parameters obtained by Baranyi and Roberts Model of mean count data of total bacteria in raw milk supplemented with different concentrations of *Origanum* aqueous extract and refrigerated during 10 days at 4 °C. The mean values are expressed with the standard deviation.

The statistical evaluation of the relation between X_{max} variations and plants aqueous extracts concentrations resulted in the equations of the regression line $y = -0.532x + 7.101$ for rosemary and $y = -0.298x + 5.624$ for oregano. The correlation coefficients (R^2) registered the values of 0.71 and 0.55 for rosemary and oregano respectively.



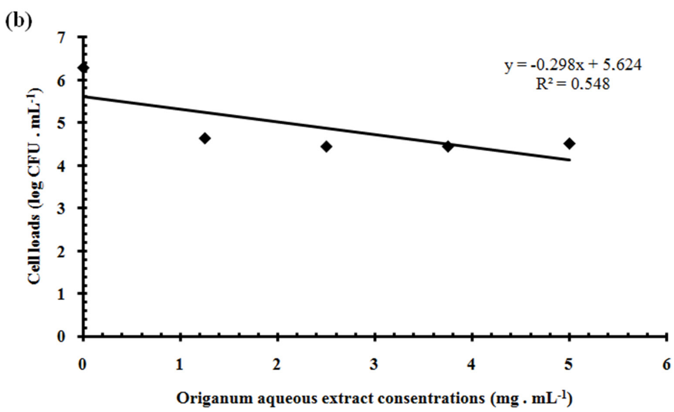


Figure 4. The linear regression relationship between the maximum microbial growth and *Rosmarinus* (a) and *Origanum* (b) aqueous extract concentrations supplemented to refrigerated raw milk at 4 °C

Discussion

The initial microbial contamination registered is under the hygiene limit for psychrotrophic bacteria in milk 4.69 (50 000 CFU mL^{-1}), which reflects the good level of hygiene of the herd and the respect hygienic principles of milk acquisition and storage (Cempírková, 2007; Cempírková and Mikulová, 2009). The delay and the limitation of microbial growth remarked, suggest the inhibiting effect on microbial growth of the natural bioactive compounds present in the plants extracts supplemented to milk. It is important to notify that the data on the dynamics of microbial growth in refrigerated milk to which plants extracts have been added is missing in the literature.

Rosemary extracts have been the most widely used herbs for prolonging the shelf life of butter (Oraon et al., 2017). Najgebauer et al. (2009) evaluated the storage stability of butter added with dried rosemary; they concluded that the addition of rosemary herb was effective in delaying lipolysis. Also, Ghalem and Zouaoui (2013) supplemented yoghurt with *R. officinalis* essential oil kept up to 21 days and good scores for taste, flavor and texture of the herbal yoghurt were obtained. Marinho et al. (2015) provided evidence that coating of cheese with dehydrated rosemary leaves improved the physical and physicochemical properties of cheese made from raw or pasteurized milk as compared to cheese made without coating. On the other hand, it was demonstrated that the aqueous extract of *R. officinalis* was effective in inhibiting and delaying the growth of a number pathogenic bacteria, including *K. pneumoniae*, *P. aeruginosa*, *S. dysenteriae*, *S. typhimurium*, *Enterobacter sakazakii* and *E. coli* (Gram-negative) and *B. subtilis*, *S. aureus*, *M. roseus* and *Listeria monocytogenes* (Gram-positive) (Lee et al., 2009; Abkhoo and Jahani, 2017). According to Abdel-Hamied et al. (2009) a significant inhibition of psychrotrophic microorganisms in meat preserved at 4 and -18 °C was obtained using a mixture of rosemary and salvia extracts. Hać-Szymańczuk et al. (2017) determined the effect of rosemary on lipids oxidation and the microbial quality of chicken meat kept at -18 °C for four months.

Moreover, oregano extract is considered to be an effective inhibitory compound against *L. monocytogenes*, *S. aureus*, *B. subtilis*, *B. cereus*, *S. typhimurium* and *E. coli* (Licina et al., 2013; Shahbazi and Shavisi, 2019). Furthermore, Bankova and Popova (2017) confirmed that water extracts of the dried oregano herbs exhibited antimicrobial activity against *E. faecalis*, *K. pneumoniae*, *P. aeruginosa* and *Candida*. Martins et al. (2014) reported the efficiency of oregano water extracts against Gram-positive (Staphylococci) and Gram negative (Enterobacteria and *P. aeruginosa*) microorganisms. Oregano extract could be used as a natural compound to improve the shelf life of yogurt at refrigerated temperatures for 30 days (Shahbazi and Shavisi, 2019). In addition, the antibacterial effectiveness of oregano herbs extracts added to cheese against foodborne pathogens was demonstrated (El-Sayed and Youssef, 2019; Ritota and Manzi, 2020).

The results of the linear regression relationship between the two variables of maximum microbial growth and the concentrations of plants extracts supplemented to milk indicate a moderate negative correlation. This pattern of linear regression relationship could be explained by the intervention of more than two variables responsible for the plant antimicrobial activity, especially those of phytochemical diversity and the several mechanisms involved in antimicrobial activity of plants bioactive compounds. Indeed, phytochemicals represent a valuable source of bioactive compounds with potent antimicrobial activities and have exerted potential antibacterial activities against sensitive and resistant pathogens via different mechanisms of action. There are multiple targets for the antibacterial agents that comprise (I) bacterial protein biosynthesis; (II) bacterial cell-wall biosynthesis; (III) bacterial cell membrane destruction; (IV) bacterial DNA replication and repair, (V) inhibition of a metabolic pathway and efflux pumps (EPs) (Khameneh et al., 2019). The antimicrobial potential of oregano has been attributed mainly to carvacrol, thymol and rosmarinic acid (Bešta-Gajević et al., 2018),while the antibacterial activity of rosemary was associated with the presence of several phenolic diterpenes, such as carnosic acid, carnosol, rosmanol, rosmariquinone and rosmaridiphenol (Azizkhani and Tooryan, 2014; Silva et al., 2014).

Conclusion

We have demonstrated here that the supplementation of rosemary and oregano aqueous extracts to refrigerated milk generates a prolongation of the lag phase duration and a limitation of microbial growth. According to the results, the recommended concentrations of plants aqueous extracts providing a prolongation of the lag phase duration and a limitation of microbial growth are 0.75 mg mL^{-1} for rosemary and 1.25 mg mL^{-1} for oregano. Although this study is preliminary, the data obtained encourage the use of rosemary and oregano aqueous extracts to extend milk shelf life under refrigeration conditions without heat treatment or chemical addition. These two plants could be proposed as potential candidates for the biopreservation of milk. In perspective, a sensory analysis study will be performed to evaluate the acceptability of the products by the consumer.

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