USE OF ESSENTIAL OILS FROM AROMATIC AND MEDICINAL PLANTS TO INCREASE THE SHELF LIFE OF MOUNTAIN DAIRY PRODUCTS IN ROMANIA

Liviu GACEU[1,2,3], Raisa SAMOILA[1], Romulus GRUIA[1,2,3] Oana Bianca OPREA[1]

[1] Transilvania University of Brasov,
[2] researcher at CE-MONT/INCE/AR,
[3] Academy of Romanian Scientists

Abstract

The paper addresses the subject of the antimicrobial potential of essential oils from aromatic and medicinal plants present in the spontaneous mountain flora in Romania. Research shows the inhibitory effects of *Thymus vulgaris* essential oil on yeasts of the genera *Kluyveromyces* and *Candida* developed in sheep cheese (telemea) products. The general objective of the work is to extend the shelf life of sheep-type products, thus increasing the degree of food safety. The research methodology included the following steps: incorporation of *thyme* essential oil in sheep's milk at concentrations of: 0.5%, 1%, 2%; preservation of the product for periods of 5, 10, respectively 15 days; performing comparative microbiological analyzes between the control sample and the samples with embedded essential oil; interpretation of results and formulation of conclusions. The results obtained demonstrated the action of inhibiting the development of yeasts of the genera *Kluyveromyces* and *Candida*, the number of colonies developed after 5 days decreasing from 30,000 (in the case of the control sample, without the addition of *thyme* essential oil) to 5,700 (in the case of the addition of essential oil in concentration of 0.5%), 100 (in case of addition of essential oil in concentration of 1%), respectively at 0, (in case of addition of essential oil in concentration of 2%). It was also highlighted the fact that the developed number of colonies was kept between limits up to an interval of 2 weeks, thus properly extending the shelf life of the studied product. The paper opens wide perspectives for the use of essential oils from Romania's spontaneous mountain flora, to improve the preservation of mountain dairy products, future research taking into account different ways of dosing the antimicrobial agent in packaging, brine or biofilm applied during the technological process.

Key words: *aromatic plants, essential oil, sheep cheese, mountain flora, dairy products.*

INTRODUCTION

In recent years, essential oils and plant extracts have attracted great interest because of their potential as a source of natural antioxidants and biologically active compounds. The antimicrobial and antioxidant activities of essential oils have been the basis of many applications, including the preservation of fresh and processed foods, medicines, alternative medicine and natural therapies. (Alsirrag M.A., 2019, Bozin B., et al., 2007; Hussain A.I., et al., 2008; Tepe B., et al., 2007; Wannissorn B., et al., 2005; Abdelhamid, S.M., et al., 2021; Licon C.C., et al., 2020, Reyes-Jurado et al., 2019). As Bozin B. et al. says, the antimicrobial activity of essential oils is the basis of many applications, including the one in this study: essential oil from *Thymus vulgaris* for inhibition of yeasts of the genera *Kluyveromyces* and *Candida* present in sheep's cheese (telemea).

Thymus vulgaris L. (Figure 1) is an aromatic plant of the *Labiatae* family. An essential oil is extracted from this plant that contains more than 60 ingredients, most of which have important antioxidant and antimicrobial properties. (Baranauskiene R., et al., 2003, Bukvicki,

D., et al., 2018,, Giller, K., et al., 2020, Singh Chouhan, K.B. et al., 2019). Thyme essential oil (*Thymus vulgaris*), contains 20–54% thymol (PDR for Herbal Medicine). Thymol, an antiseptic, is an active ingredient in various oral cleansers (Pierce A., 1999). Thyme has been considered to be antiseptic, antimicrobial, medicinal, astringent, carminative, disinfectant, tonic. Thyme is useful in cases of combined intestinal infections and infestations, such as intestinal parasites, gram-positive and gram-negative bacteria, it also can improve liver function and stimulate appetite.

Fig. 1. Thymus vulgaris L.
Source: https://radiosimplu.ro/20-de-beneficii-ale-uleiului-de-cimbru/)

It is used in the treatment of cartilage, bronchial and urinary tract infections, and also useful in the treatment of laryngitis and inflammation (Saleh H., et al., 2015). As shown the antimicrobial activity of *Thymus vulgaris* oil compared to 3 other Thymus species (*Thymus serpyllum, Thymus pulegioides, Thymus glabrescens*) it is obviously the most effective because it inhibited all strains of bacteria and yeast tested for both types of concentration (Varga E., Bardocz A., Belák A., Maráz A., Boros B., Felinger A., Böszörményi A., Horváth G., 2015, Zeinhom, M.A.M et al., 2021).

MATERIALS AND RESEARCH METHOD

Figure 2 schematically shows the research methodology used in the current study in which the main stages are: preparation of cheese (telemea), incorporation of *Thymus vulgaris* oil, vacuum packaging, storage for 5, 10 and 15 days at 10°C, sampling, preparation, making the necessary dilutions for analysis, transferring the required amount of each dilution to sterile petri dishes, pouring the culture, thermostating the previously solidified plates for 5 days at 25°C and reading and interpreting the results.

Regarding the preparation of cheese (telemea), the necessary steps are the standard ones from the dairy industry. Before the start of the study, the analysis of the microbiologically expired sheep cheese (telemea) was performed. In the first part of the study, the essential oil of *Thymus vulgaris* was incorporated into the sheep cheese with a exceeded shelf life, for 0.5%, 1%, 2%, concentrations related to the amount of milk needed to make the cheese.

Concentrations were chosen based on the study conducted in Indian Journal Dairy Science, in 2019 by Yasser H. et al. Thus, for 1 kg of telemea 4 liters of sheep's milk are needed, resulting that for 100 g of cheese, for a concentration of 1% oil, 4 ml of oil is needed. The chosen concentrations were 0.5%, 1% and 2% for 50 g of cheese (Table 1).

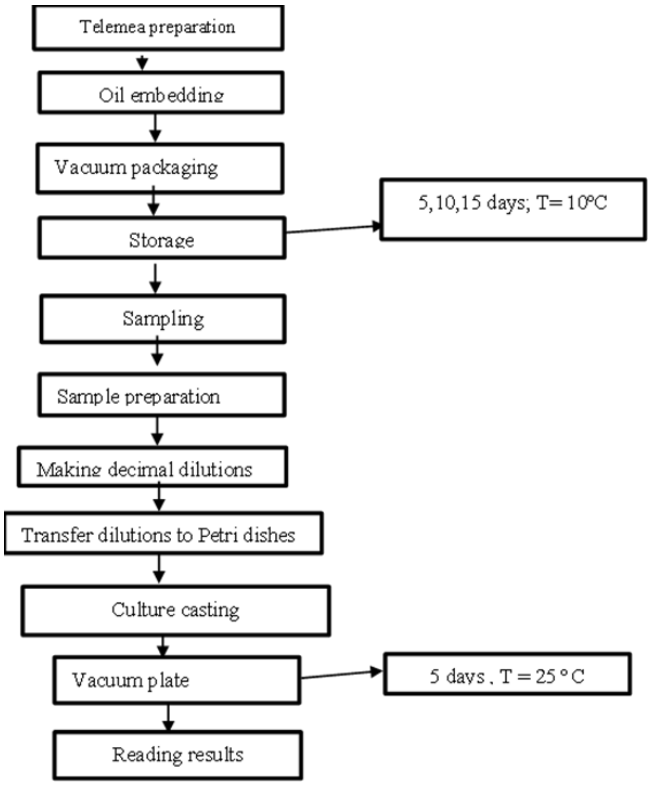

Fig. 2. Scheme of research methodology

Source: the authors, 2021

Table 1. The quantities of oil required for each concentration chosen for 50 g of telemea

Concentration	Oil quantities (ml)
0.5%	1
1%	2
2%	4
Total	7

Three samples were taken from each concentration, which will be analyzed at 5, 10, respectively 15 days after the oil is incorporated and the product is packaged. The total amount of oil used in the study was 21ml. As can be seen in Figure 3 the embedding of the oil began by crushing the cheese (telemea). The shredding was done with a blender type BEKO CHP5550W, for 60s, with a frequency of 50 rpm. The previously crushed cheese (telemea) was weighed for each concentration with the help of the electronic balance, after which the dosing and mixing with oil was performed. The required amount of oil was measured using sterile syringes of 2, and 10 ml. The embedding of the oil was done with the same blender used to grind the cheese (telemea), but this time for 180 s.

After the homogenization was performed, the cheese had a creamy texture, compared to the cheese (telemea) taken in the analysis, due to the used oil. As it can be seen, the color has changed from white to pale yellow.

Fig. 3. The result of mixing the essential oil with cheese (telemea)

Source: the authors – 2021

The next step of the research was to put the mixed cheese with oil into the molds of the vacuum machine using stainless steel and plastic spatulas as exemplified in Figure 4.

The packaging took place at a vacuum time of 4000 ms, gluing time-3700 ms, temperature of 134°C which helps to perform the correct vacuum and long-term resistance of the gluing between the two films used in the process – the one in which the cheese (telemea) is put and the upper one that wraps the package.

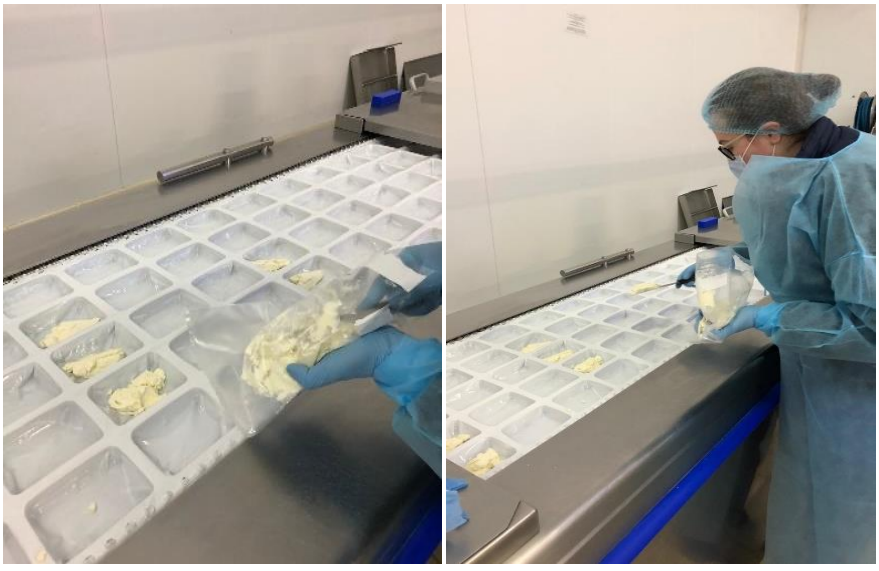

Fig. 4. Placing the cheese (telemea) in the shapes of the packaging machine

Source: the authors – 2021

Each sample was inscribed before being stored at 10°C for a total of 15 days. Along with the samples of cheese with essential oil, the control samples that did not contain thyme oil were also packed to observe the differences that appear in the inhibition of the yeasts of the *Kluyveromyces* and *Candida* genera.

The diagnostic procedure establishes the general guidelines for counting viable yeasts and molds in products intended for human consumption by the colony counting technique at 25°C. Yeasts and molds are microorganisms that at 25°C form specific colonies on a selective medium. The reference document is SR ISO 21527/1 or 2. The principle of the method is to use a selective medium and a quantity of the decimal dilution in the case of solid and semi-solid samples. The sample together with the culture is left to incubate under aerobic conditions at 25°C for 120 hours. The specific colonies are counted, after which the number of micro-organisms are calculated (CFU)/ml or (CFU)/g.

An appropriate amount of the culture is weighted into an Erlenmeyer flask, an amount of distilled water required for dilution is added, according to the instructions on the package. If necessary, the pH can be adjusted so that after sterilization the pH is 7.0 (+2) at 25°C. The solution is brought to boiling point while continuous stirring, then it is sterilized in an autoclave at 115°C for 10 minutes. It is kept in a water bath at a temperature of 45–50°C.

The samples are prepared and decimal dilutions are made with Ringers dilution solution, shaking completely so that the mixture is well homogenized. Approximately 10–15 ml of the culture is poured onto each sample-inoculated Petri dish.

Fig. 5. Sample preparation

Source: the authors – 2021

The solid samples taken are weighed 10 g of product and diluted with 90 g of diluent. It is homogenized in the Stomacher, after which 1 ml of the sample thus prepared can be inoculated (Fig. 5.).

After preparation of the samples, for subsequent dilutions, the procedure in the same as for 10^{-1} dilutions, so: transfer with sterile 1 ml pipette of dilution 10-1, into a test tube with 9 ml of Ringer's diluent, shake the dilution 10^{-2} (Figure 6.). Transfer 1 ml with the sterile pipette from the dilution 10^{-2} in the Petri dish inscribed with the sample code and dilution, and for dilution 10^{-3} the same process is applied as in the case of 10^{-1} dilution (Figure 7). The critical dilution steps – 2 consecutive decimal dilutions – are selected for

inoculation of Petri dishes, which could enter between 15 and 300 colonies per plate, as far as possible.

Fig. 6. Stirring the newly formed dilution
Source: the authors – 2021

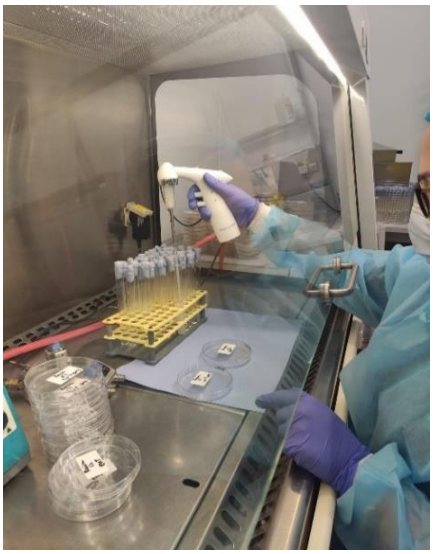

Fig. 7. Performing decimal dilutions
Source: the authors – 2021

Two sterile Petri dishes are taken, in each of which 1 ml of the initial sample or 1 ml of the desired decimal dilution is transferred using a sterile pipette (Fig. 8). For each sample, a new sterile pipette will be used from each dilution.

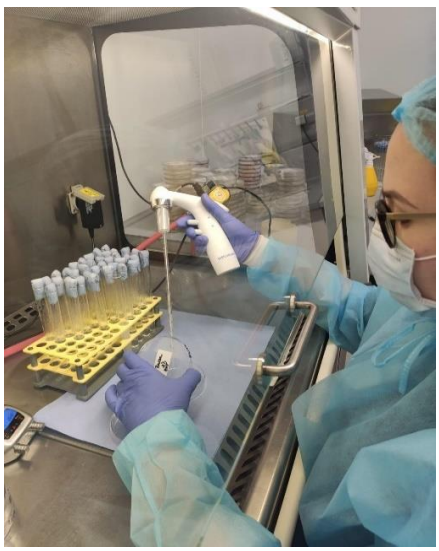

Fig. 8. Transfer the dilution to the Petri dish
Source: the authors – 2021

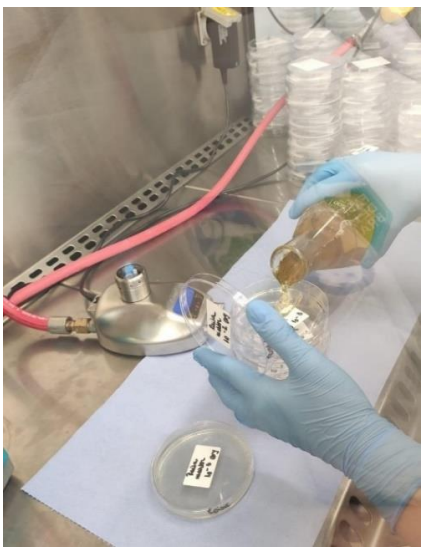

Fig. 9. Culture casting
Source: the authors – 2021

For inoculation, the culture is cooled to 45–50° C, and 10–15ml are poured into the Petri dishes (Fig. 9) inoculated with the sample. After inoculation of the culture in the Petri dishes, are covered with a lid, and shaked by turning it clockwise several times, counterclockwise several times in a horizontal direction so that the sample is well homogenized with the culture. The inoculated Petri dishes are left at room temperature until the culture medium has solidified (a few minutes). At this point, the inoculated Petri dishes are stacked, one on top of the other, with the lid down, so that any condensation drops do not affect the sample and are placed in the oven for thermostating, previously brought to the appropriate temperature (25°C), and are left in the oven for 5 days.

Expressing the results

Colonies developed on the plate are counted, after which they are multiplied by the dilution factor. Ex. 54 numbered colonies x 10-3 = 54 x 1000 = 54,000 CFU/g.

RESULTS

After carrying out the analyzes according to the reference document SR ISO 21527/1 or 2, after 5 days from the introduction of the plates in the oven, at a constant temperature of 25°C, these were removed to read the results using the colony counting device.

The first step was the analysis performed 5 days after the incorporation of the essential oil of *Thymus vulgaris* for inhibiting yeasts of the genera *Kluyveromyces* and *Candida* present in sheep cheese (telemea) after the expiry date.

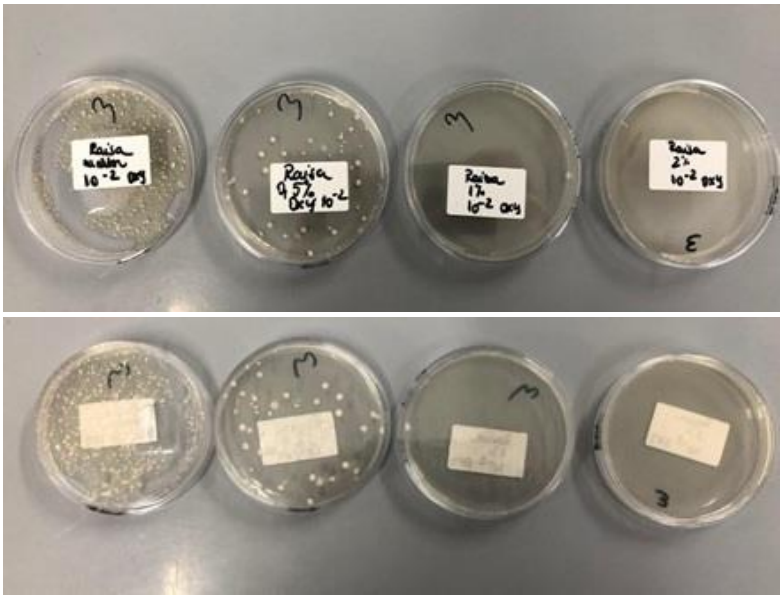

Fig. 10. Plates representing analyzes performed 5 days after incorporation of *Thymus vulgaris* (up) essential oil, after 5 days of incubation at 25°C, 10^{-2} dilution (down)

Source: the authors – 2021

As can be seen in Figure 10 the fungicidal effect is obvious. Compared to the control plate that had an oil concentration of 0% with over 30,000 CFU/g, the plate with the highest concentration, of 2%, had antifungal activity on these microbial species.

The next plate with high sensitivity to the action of the oil was represented by the one with a concentration of 1%. The cheese (telemea) with 0.5% oil is the one for which you can clearly see the difference between the one with and without the addition of vegetable oil.

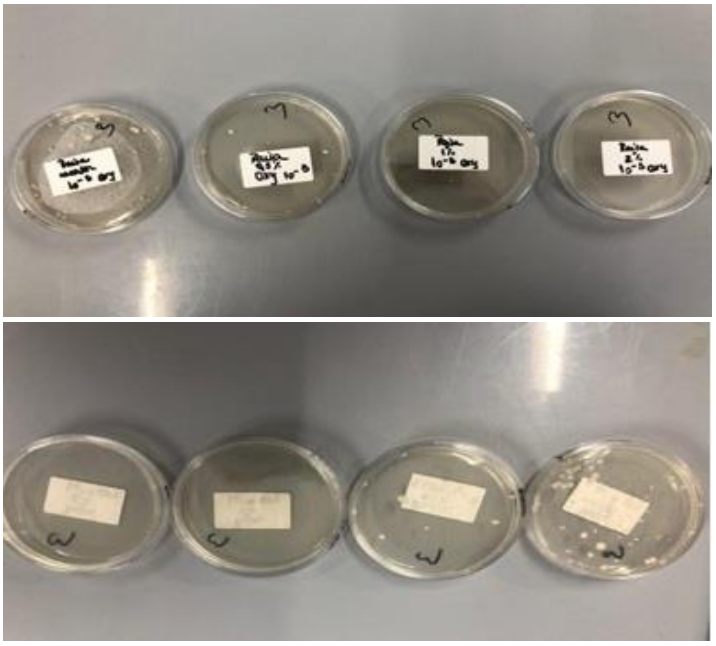

Fig. 11. The plates representing the analyzes performed 5 days after the incorporation of the essential oil from *Thymus vulgaris* (up), after 5 days of incubation at 25°C, 10^{-3} dilution (down)

Source: the authors – 2021

Figure 11 can confirm what was demonstrated in the 10-2 dilution. Although in all concentrations, except the control, the antifungal activity was gradually different, the number of CFU decreased with increasing concentration of the extract, the results being shown in Table 2. Similarly, results were obtained for steps 2 and 3 described above.

Table 2. Expression of test results for all samples, at 5, 10, 15 days after mixing with the essential oil and before the start of the study (CFU/g)

Oil addition	5 days		10 days		15 days		sample before adding oil	
	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}	10^{-2}	10^{-3}
0%	>30,000	59,000	>30,000	152,000	>30,000	152,000	>30,000	57,000
0.5%	5,700	14,000	1,600	4,000	2,400	4,000		
1%	100	1000	0	0	0	0		
2%	0	0	0	0	0	0		

With the end of the last stage, it was possible to centralize the results of the experiment performed in accordance with SR ISO 21527/1 or 2 (Table 2), in which the inhibitory effect of the *Thymus vulgaris* oil can be seen upon yeast of genera *Kluyberomyces* and *Candida* present in sheep cheese (telemea) with an expired expiry date.

As can be seen in Figure 12, the number of CFU / g for the control sample was continuously increasing up to 10 days, the values from 10 to 15 days being equal, in case of 10^{-3} dilution and the small difference between the sample before the oil was incorporated and 5 days after its addition can be explained by the fact that after sampling, they remained at a constant temperature of 4°C, and microorganisms did not develop at the same rate as after inoculation, at 10°C. For dilution 10^{-2} the values are constant because they have always been >30,000.

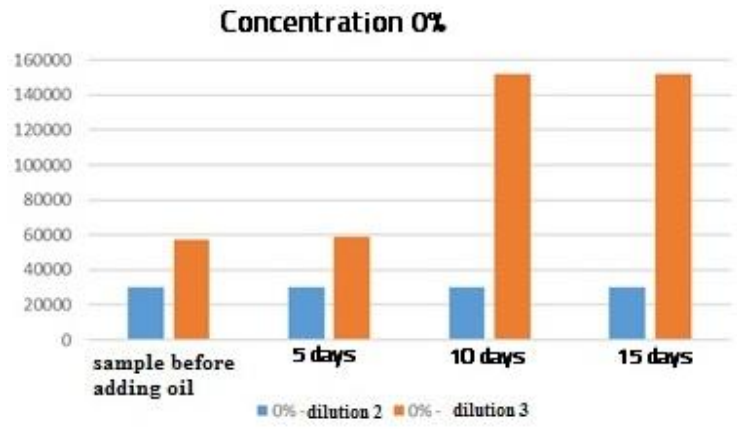

Fig. 12. Graphic interpretation of test results for control samples – 0% concentration, at 5, 10, 15 days and before the start of the study (CFU/g)

Source: the authors – 2021

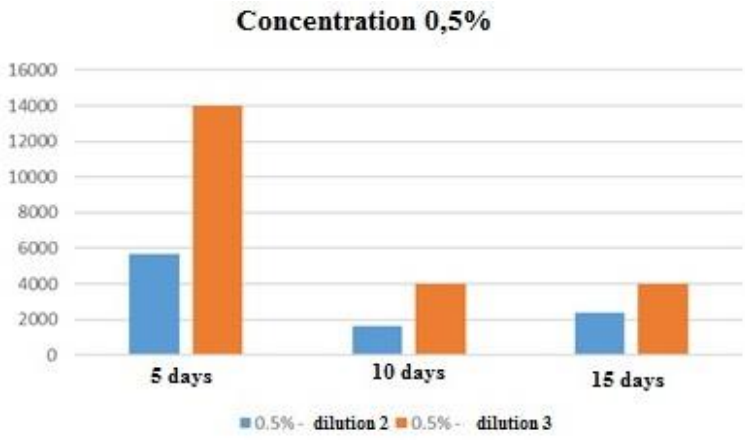

Fig. 13. Graphic interpretation of test results for 0.5% concentration samples, at 5, 10, 15 days (CFU/g)

Source: the authors – 2021

For sheepmeat with the addition of 0.5% essential oil, the results of the microbiological analyzes on antifungal activity, expressed graphically in Figure 13, show a sharp decrease from the fifth to the tenth day for both performed dilutions. Between the 10-day and the 15-day examination, it can be said that for the second dilution, although the number of CFU/g increased slightly, the fungicidal effect of the oil is present. In case of 10^{-3} dilution, *Thymus vulgaris* essential oil had a special inhibitory effect between the first and second analysis with a decrease of about 10,000 CFU/g, and between the second and third research closely the values remained constant, the development of microorganisms being stopped.

The addition of 1% essential oil to the sheep cheese caused a sudden fungicidal effect, with minimal values for both dilutions at the first analysis (Fig. 14). The next two examinations showed a total inhibition of yeasts from the genera *Kluyveromyces* and *Candida,* which shows that in the case of this oil concentration, the destruction of microorganisms was achieved over time.

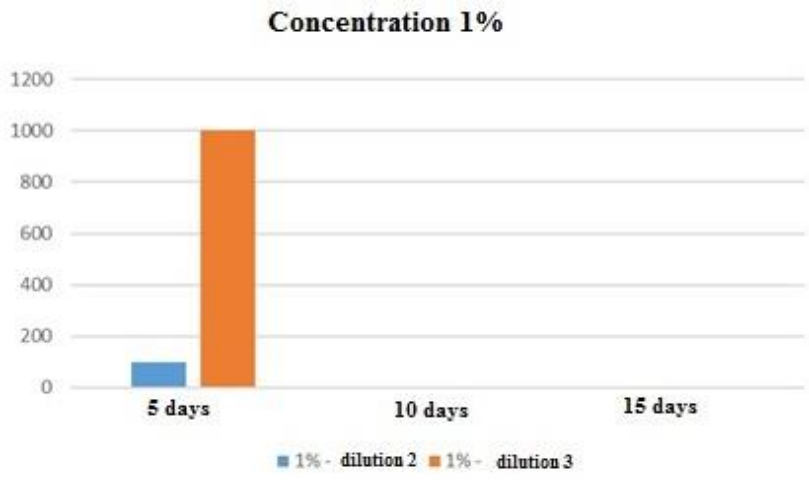

Fig. 14. Graphic interpretation of test results for 1% concentration samples, at 5, 10, 15 days (CFU/g)

Source: the authors – 2021

At the highest concentration of essential oil added to the cheese (2%) with exceeded shelf life the results were remarkable from the first stage of the research, the micro-organisms being sensitive to the action of the *Thymus vulgaris* oil. As shown in Figure 14, total inhibition of the two yeast strains was unchanged throughout the study. According to Table 2 it results that for the concentration of 2% the effect of preventing the development of yeasts takes place from the expertise performed after 5 days, which did not happen in any of the other samples. Similar results existed at 10 and 15 days for 1 and 2% oil concentrations (Figure 15).

Compared to the control sample, the antifungal effect of vegetable oil was evident for all concentrations applied to the product, the decrease in the number of CFU / g being inversely proportional to the applications performed regarding the concentrations in oil.

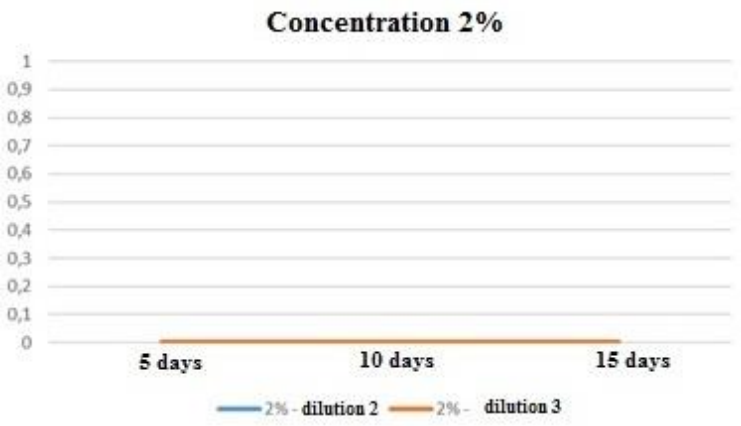

Fig. 15. Graphic interpretation of test results for 2% concentration samples, at 5, 10, 15 days (CFU/g)

Source: the authors – 2021

CONCLUSION

The essential oil of *Thymus vulgaris* has antifungal activity of wide spectrum proven in the literature, but also through its own research carried out so far. The research methodology used in the current study in which the main stages are: preparation of cheese, incorporation of *Thymus vulgaris* oil, vacuum packaging, storage for 5, 10 and 15 days at 10°C, sampling, preparation, dilution necessary for analysis, transfer the required amount of each dilution in sterile Petri dishes, pouring the culture medium, thermostating the previously solidified plates for 5 days at 25°C and reading and interpreting the results was performed according to the reference document SR ISO 21527/1 or 2.

In the first part of the study, the incorporation of the essential oil of *Thymus vulgaris* in sheep cheese with a shelf life exceeded, for 0.5%, 1%, 2% concentrations related to the amount of milk required for the manufacture of 50 g of cheese. The results of the present research on the inhibition of yeasts of the genera *Kluyveromyces* and *Candida* were as follows:

- The control sample presented the constant value for the 10-2 dilution, maximum value that can be determined for this and a staged increase up to 10 days (10-3), between the last part and the previous one, being equal;
- In the expertise of sheep cheese with the addition of 0.5% essential oil there was a sharp decrease from the fifth to the tenth day for both prepared dilutions. For dilution 10-3 the extract had a special inhibitory effect between the first and the second analysis with a decrease of about 10000 CFU/g;
- The effect of 1% essential oil in sheep cheese proved to be rapid, with minimal results after five days of incorporation of the extract and total inhibition of yeasts in the following stages, which shows that in the case of this concentration of oil the destruction of microorganisms was achieved in time;
- For yeasts *Kluyveromyces spp*. and *Candida spp.,* 2% concentration represented the total sensitivity of these two strains in each stage of the performed study, without anny CFU/g, a special effect that appeared only in this case;

- Comparing the last two dilutions, the difference appear in the first stage, in which for the 1% oil concentration there were minimum values while the last mentioned concentration resulted in 0 CFU/g, but the similarity appears for the second and third stage in which the antifungal effect was total, which shows that it is possible to go for a lower concentration for a period of time in which the oil could "work" in the product and inhibit the microorganism or to use a double amount of extract with an immediate effect;
- Even if the cheese with the lowest amount of added oil (0.5%) still has in the last stage for 10-2 (2400 CFU/g) and 10-3 (4000 CFU/g), the fungicidal effect is obvious, and the possibility that after 20 or 25 days of storage to reach close to 0 being very high.

The tested essential oil can be used for incorporation in various foods, in order to prevent the development of microorganisms, helping to increase shelf life and increase food safety, the effect of *Thymus vulgaris* oil on yeasts such as *Kluyveromyces spp.* and *Candida spp*. developed in the sheep cheese being obvious depending on the applied concentration.

Following the results, it is concluded that the shelf life can be extended by 15 days. The method can be recommended to enterprises with small processing flows, small entrepreneurs, even farmers for the creation of new products such as cream cheese with different concentrations of essential oil depending on the desired shelf life.

Future research directions will be continued on the idea of integrating in the sheep cheese (telemea), with the same method, several essential oils such as: basil, mint, rosemary, oregano, tea tree, but also the integration of the oil in the brine in which the telemeau is kept before packaging or final packaging in brine, respectively the integration of the essential oil in the packaging represented by the film in which the piece of cheese is packed.

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