In vivo effect of Origanum vulgare essential oil on microbiological quality of raw milk from subclinical mastitis affected cows

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Mohamed Walid HAMLAOUI[1], Ahmed HADEF[2], Fawzi Rostane MEKLATI[3], Meriem ALLEG[4], Bouchra GHOUILA[4]

IN VIVO EFFECT OF *Origanum vulgare* ESSENTIAL OIL ON MICROBIOLOGICAL QUALITY OF RAW MILK FROM SUBCLINICAL MASTITIS AFFECTED COWS

SUMMARY

The study aims to test under in vivo condition the effect of *Origanum vulgare* essential oil (OEO) on the bacteriological quality of raw milk from cows affected by subclinical mastitis. The tested oil extracted from Algerian endemic oregano collected in Setif province (eastern Algeria) was characterised as carvacrol-chemotype using gas chromatography-mass spectrometry (GC-MS). The in vitro inhibitory effect of diluted OEO was tested on control reference strains. *Staphylococcus aureus*, ATCC 700699 methicillin-resistant seemed more susceptible to OEO than ATCC 25923 methicillin-susceptible. The in vivo trial was conducted on lactating and non-pregnant cows selected according to their California mastitis test scores. The microbiological analysis was applied for 100 units of raw milk samples collected before and three days after the twice a day topical application of OEO diluted with vegetable oil (5%). Results showed the only presence of total aerobic mesophilic bacteria (TMAB) and coagulase-positive staphylococci (CPS) in all samples with values close to the critical limit. After treatment, a significant decrease ($P < 0.001$) in the mean count of TMAB and a non-significant decrease in the number of CPS ($P > 0.05$) were noted. Results indicate that aromatherapy is a promising approach for improving udder health and raw milk microbiota.

Keywords: Carvacrol, Essential oil, Mastitis, Milk, Oregano, *Staphylococcus*

[1]Mohamed Walid Hamlaoui, Institute of Veterinary Sciences, Frères Mentouri University of Constantine, El Khroub, Constantine, ALGERIA

[2]Ahmed Hadef, (corresponding author: hadef_vet@yahoo.fr), Department of Veterinary Sciences, Faculty of Nature and Life Sciences, Chadli Bendjedid University of El Tarf, PB 73, 36000 El-Tarf, and Laboratory of Development and Control of Hospital Pharmaceutical Preparations, University of Annaba, ALGERIA

[3]Fawzi Rostane Meklati, Centre de Recherche Scientifique et Technique en Analyses Physico-Chimiques (CRAPC), BP 384, Bou-Ismail, 42004, Tipaza, ALGERIA

[4]Meriem Alleg, Bouchra Ghouila, Department of Agronomy, University of El Tarf, ALGERIA.

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INTRODUCTION

On a global scale, Algeria is a major importer of milk powder and derived products with a global bill estimated in 2018 at US$1.40 billion, thus occupying second place behind cereals. The efforts and subsidy schemes put in place by the State over the past few decades with the aim to increase the production of raw milk and above all to encourage the emergence of a real agro-food industry (Meklati *et al.*, 2020), have come up against the difficulty posed by this raw material which remains complex and altered.

Few studies carried out in Algeria on the microbiological quality of raw milk samples taken individually on the production site have been published. The low hygienic bacteriological quality that exceeds the critical threshold was described in a study carried out on farms in the Tiaret region. The authors found the presence in the analysed samples of TMAB greater than 105 colony forming unit per millilitre (CFU/ml) (81.2%), faecal coliforms (18.06%), *Staphylococcus aureus* (81.93%) and faecal streptococci (80.64%) (Ghazi and Niar, 2011). This bacteriological quality was influenced in a study carried out in the Metidja region by the size of the dairy farm and the practice of udder disinfection (Hakem *et al.*, 2012).

Subclinical mastitis is one of the causes of the deterioration of the quality of raw milk collected from the farm often due to infections by potentially pathogenic germs, namely coagulase-positive staphylococci, enterobacteria (*E. coli*) and streptococci (FAO, 1989). However, the emergence of mastitis due to minor germs, especially coagulase-negative staphylococci, has been reported (Pyörälä and Taponen, 2009). In eastern Algeria, *Staphylococcus aureus* has been considered one of the major pathogens causing subclinical bovine mastitis (Zaatout *et al.*, 2020). It represents one of the germs often implying the use of antibiotic-based applications, but their efficacy remains low (10-30%) due to the potential of resistance of these bacteria (Gomes and Henriques, 2016). In addition to the risk to public health linked to the toxi-infectious potential of this germ, one of the consequences of this pathology is the marketing of milk with low industrial value due to the presence of antibiotic residues (Sachi *et al.*, 2019). As a result, the use of essential oils as a possible alternative therapeutic approach against mastitis to solve the problem of antibiotic resistance has been the subject of several in vitro experiments, but their properties have been very little tested in vivo on livestock (Baskaran *et al.*, 2009; Mushtaq *et al.*, 2018; Alagawany *et al.*, 2020; Gupta *et al.*, 2020). These studies have confirmed the efficacy of the antibacterial activity of the compounds of these oils on germs isolated from mastitis milk, particularly the phenolic compounds, including carvacrol. Its use as a feed supplement compound to improve animal health was reviewed by Sharifi-Rad *et al.* (2018). This phenol is generally present in significant percentages in Lamiaceae that grow spontaneously in North Africa (Algeria, Tunisia), especially *Origanum vulgare* (Sari *et al.*, 2006; Béjaoui *et al.*, 2013; Ali *et al.*, 2020; Nabti *et al.*, 2020).

The present study involves an in vivo trial preceded by an in vitro test on reference control strains, a first in Algeria at our level of knowledge, in order to test the effect of the essential oil of *Origanum vulgare* on the quality and bacteriological composition of the raw milk of cows exposed to the risk of subclinical mastitis. This approach aims to contribute to the development of new therapeutic option for bovine mastitis in accordance with the "One Health" concept.

MATERIAL AND METHODS

Essential Oils origin

The essential oils used in the study were obtained after steam extraction of the dried aerial parts (leaves and flowers) of *Origanum vulgare* subsp. *Glandulosum* (Desf.), an aromatic medicinal plant endemic to Algeria (Sari *et al.*, 2006). The plant material was collected during the flowering period in June 2019 in the mountains of Amoucha located in Sétif province in eastern Algeria (Fig. 1). The taxonomic identification of oregano specimen was made by Dr Samia LAKEHEL (Badji Mohktar University of Annaba, Algeria).

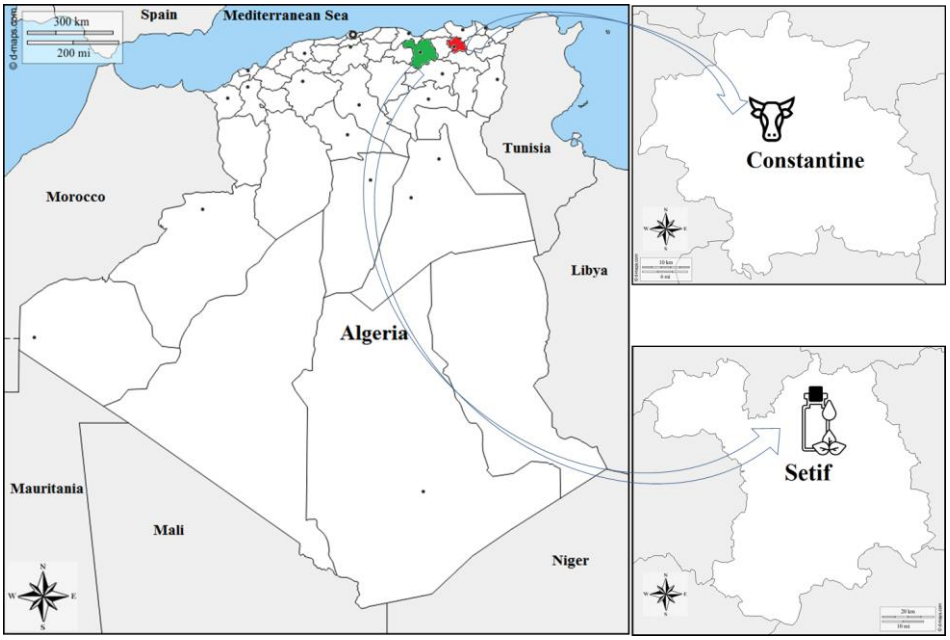

Figure 1. Location of the oregano collection region (Setif) and the study area of in vivo trial (Constantine)

Characterisation of oregano essential oil used in the clinical trial

The chemical composition of OEO to be used in the in vivo test was identified by gas chromatography-mass spectrometry (GC-MS). The analyses were carried out on an HP 6890 gas chromatograph coupled to an HP 5973 mass

spectrometer (Agilent, CA, USA) using HP-5MS capillary column (30 m × 0.25 mm, 0.25 µm film thickness). GC-MS spectra were obtained using the following conditions: injected essential oil volume was 1 µl in splitless mode; injection temperature was 250 °C; helium was the carrier gas at a flow rate of 0.5 ml/min; oven temperature program (60 °C for 8 min, increased at a rate of 2 °C/min to 250 °C, then held at 250 °C for 10 min). The mass spectral database used for essential oil component identification was Wiley 7N and NIST 02 (National Institute of Standards and Technology, USA).

In vitro efficacy of several dilutions of oregano essential oils on control reference strains

Control reference strains were used to evaluate the effectiveness of the oregano essential oils in inhibiting bacteria growth. *Staphylococcus aureus* ATCC 700699 (Mu50) applied in drug discovery was used as the reference control for methicillin-resistant (MRSA) strains. *Staphylococcus aureus* ATCC 25923 applied in the examination of dairy products and susceptibility disc testing was involved to control methicillin-susceptible (MSSA) strains. They were grown after spreading on Chapman's agar and incubated at 37 °C for 24 hours. After growing, a few well-isolated colonies of bacteria were taken and diluted in sterile physiological saline, then homogenised until an opacity equivalent to 0.5 units of the Mc Farland scale was obtained. Afterwards, 1 ml of inoculum was deposited and inoculated over the entire surface with Mueller-Hinton Agar (MHA) previously poured into a Petri dish.

The susceptibility of the reference strains to OEO diluted in the vegetable oil (VO), used both for in vitro and in vivo test, at the habitual (1%), recommended (5%) and double recommended (10%) concentrations for topical use (Franchomme *et al.*, 2001) was evaluated by measuring the diameter of the inhibition zone (aromatogram). For this purpose, 6 mm paper discs impregnated with 10 µl of each of the three doses mentioned above (1% , 5% , 10%) were used according to the disc-diffusion agar method for the evaluation of the activity of topical antimicrobial agents as described by Glasser *et al.* (2010).

The experiment was repeated for each dose and the data were presented as the mean and range of inhibition zone diameters. A threshold value of 8 mm was used to classify the bacteria tested as sensitive to the topical antimicrobial agent (Holder and Boyce, 1994). Also, the vegetal oil diluent without OEO was used as a positive control. A disc impregnated with 10 µl pure vegetable oil (100%) was applied on the MHA to check the sensitivity of the strains tested to the essential oil vehicle. Furthermore, a diffusion control test was performed using dimethyl sulfoxide (DMSO) as essential oil vehicle at the same tested concentrations.

In vivo assay of OEO effect

Study area: The clinical trial was carried out in March 2020 on a pilot dairy farm located in the province of Constantine (Figure 1) in north-eastern Algeria (36° 16' 17.1 "N 6° 40' 11.9 "E). This farm was chosen based on its

prevalence of subclinical mastitis that we have previously assessed (Hamlaoui *et al.*, 2019).

Animals: The studied cows were selected from a herd of 85 dairy cows based on their California mastitis test (CMT) score. These females of Prim'Holstein breed, aged two to six years, were in lactation (post-partum period) and not pregnant. They had not previously received any antibiotic-based treatment.

Essential oil application: In the selected cows, the OEO oily solution recommended dose (Franchomme *et al.*, 2001) for topical use (5%) was applied twice a day, after morning and evening milking, by skin massage on the selected quarters for three successive days.

Sampling: A total of 20 raw milk samples were taken individually from 10 quarters, 5 affected (high somatic cells score) and 5 healthy (normal somatic cells score). They were taken twice, 10 samples before the application of the essential oil of oregano (day 0) and 10 samples 3 days later, each time keeping the same quarter of origin for each sample. The samples were taken aseptically, in compliance with the hygiene rules required for microbiological analysis, in sterile tubes labelled with a code linking it to the mammary quarter of origin. These samples were kept cold and sent to an analysis and quality control laboratory.

Raw milk analysis

CMT test: To screen the individual quarter milk for somatic cells, the CMT test was done before milking at the beginning, before the application of the essential oil, and at the end of the trial. CMT scores ranging from 0 (negative) to 4 (strong positive) were recorded according to the interpretation of test results described by Lévesque (2004).

Microbiological analyses: Repeated microbiological analyses (n= 5 units) were performed for each sample in a quality control laboratory according to the methods described in the Official Journal of the Algerian Republic for counting of: Total mesophilic aerobic bacteria (TMAB) on the Agar medium, with incubation at 30 °C for 72 hours. Coagulase-positive staphylococci (CPS) by plating 1 ml of raw milk on Baird Parker agar with incubation at 37° ±1 °C for 24-48 hours. Thermotolerant coliforms using lactose agar with incubation at 30 °C ± 1 °C for 24 hours ± 2 h. *Salmonella* on Hektoen agar, with storage in an oven at 37 °C for 18 to 24 hours. *Listeria monocytogenes* via Oxford agar. Incubation was carried out at 37 °C for 24 to 48 hours.

Statistical Analysis

The statistical analysis was performed using IBM SPSS Statistics 26 software. The comparison of the cytological and microbiological values with the accepted standard criteria was made through a Student 't' test. The Z test using the Bonferroni correction was performed for comparison between prevalence of groups categories formed according to levels of TMAB and CPS counts and time of treatment (before and after).

The linear model was applied to highlight the fixed effect of the time factor reflecting the oregano essential oil effects on the cytological (CMT scores and

ASC) and microbiological parameters (bacteria count and proportion to the total mesophilic flora). These parameters were considered as dependent variables. Also, the pairwise comparison t-test was used to compare the results of the affected and healthy quarters before and after treatment. A significance threshold of 0.05 was retained for all the tests.

RESULTS AND DISCUSSION

Oregano essential oil characterisation

The result of the GC-MS analysis of *Origanum* essential oil showed that the prevalent compound is carvacrol (39.27%) followed by thymol with 25.83% (Table 1 and Figure 2). This composition is similar to those reported in subsequent studies carried out in our study area (Sétif) on the same species, *O. vulgare* L. subsp. *Glandulosum*, which showed the presence of 04 major compounds thymol, carvacrol, p-cymene and γ-terpinene with the majority of samples belonging to both chemotypes, carvacrol and/or thymol (Sari *et al.*, 2006; Khalfi *et al.*, 2008). The predominance of carvacrol recorded was described in a recent study on samples of the same species collected in the Bougaa region of Sétif with a percentage of 26.29% (Ali *et al.*, 2020). Considering the endemic character of this plant in North Africa, a study in the Mediterranean Phyto-region, North-East Tunisia, showed that carvacrol with a value ranging from 61.08 to 83.37% was also the main compound of this *Origanum* species (Béjaoui *et al.*, 2013). On the other hand, in the same region, from data published in 2020 (Nabti *et al.*, 2020), carvacrol was present in a small percentage (2.8%) with thymol as chemotype (56.3%) in the chemical composition of the oil extracted from the same species. However, these authors (Nabti *et al.*, 2020) found a dominance of carvacrol (59.6%) in a region bordering ours (Bordj-Bou Arreridj) for the oil of the species studied.

In vitro effect of oregano essential oils on control reference strains

The results of the aromatogram (Table 2) show that the highest diameters of inhibition were obtained with the solubilisation of the oil in dimethyl sulphoxide (DMSO) for the two strains ATCC MU 50 and ATCC 25923, for a concentration of 10% with a diameter of 31.72 mm and 17.42 mm respectively. The efficacy of this oil against *Staphylococcus aureus* ATCC 25923 was also found by Bouhaddouda *et al.* (2016) who obtained a high inhibition diameter of 51.83 ± 2.56 mm using 5 µl of undiluted essential oil of *O. vulgare* L. *ssp. glandulosum* (Desf.) Ietswaart collected from the province of Guelma (northeastern Algeria) with para-cymene (25.615%), thymol (23.129%), carvacrol (20.321%) as the main components using the agar disc method. It appears that the effectiveness of the essential oil is dependent on the diffusion ability of the vehicle (DMSO or vegetable oil). For both vehicles, MRSA were more susceptible to OEO than MSSA strain with a largest diameter obtained at 10% concentration. The 5% concentration seemed to be the limiting dilution among those tested allowing an inhibitory effect.

Table 1. Chemical composition of the tested oregano essential oil

Peak Number	Compounds	Time retention (min)	Peak area percentage (%)
1	β-Thujene	9.72	0.31
2	α-Pinene	10.10	0.29
3	Camphene	10.96	0.04
4	β-Pinene	12.69	0.06
5	Octen-3-ol	13.33	0.26
6	3-Heptanone	13.49	0.17
7	β-Myrcene	13.77	0.72
8	α-Phellandrene	14.57	0.11
9	3-Carene	14.92	0.04
10	α-Terpinene	15.45	1.27
11	p-Cymene	16.29	12.27
12	D-Limonene	16.39	0.68
13	γ-Terpinene	18.74	13.58
14	2-Hepten-4-ol	19.23	0.08
15	(+)-4-Carene	20.49	0.05
16	nd	21.48	0.03
17	Linalool	21.73	0.51
18	Terpinen-4-ol	27.11	0.25
19	α-Terpineol	28.25	0.15
20	Estragole	28.65	0.31
21	Thymol methyl ether	31.11	0.13
22	Isothymol methyl ether	31.74	0.24
23	Thymol	36.80	25.83
24	Carvacrol	37.72	39.27
25	Caryophyllene	43.20	1.11
26	α-Caryophyllene	45.31	0.03
27	β-Bisabolene	48.84	1.01
28	β-Sesquiphellandrene	49.68	0.22
29	α-Bisabolene (Z)	50.81	0.41
30	Caryophyllene oxide	53.11	0.28
Not-identified compounds			2.7
Total identified compounds			97.30

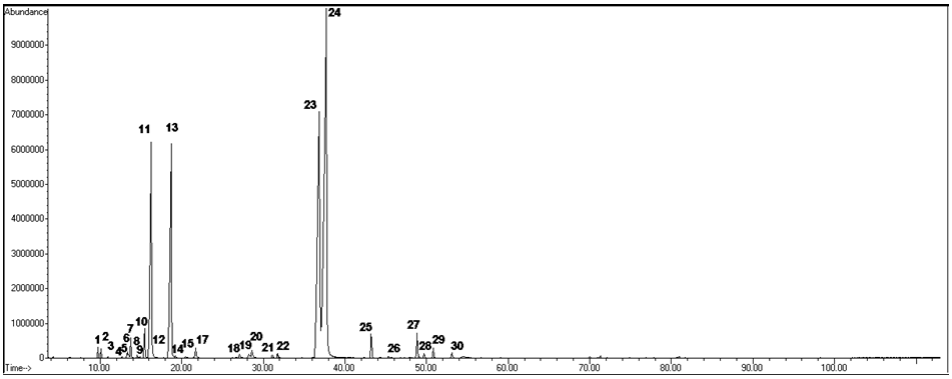

Figure 2. GC-MS Chromatograms and peak number of oregano essential oil compounds.

No control disc impregnated with vegetable oil alone inhibited the growth of the two reference strains. Regarding the 8 mm susceptibility threshold used to test the antimicrobial effect of the solutions for topical use (Holder and Boyce, 1994), The inhibiting effect was only obtained with the OEO diluted at 10% in vegetable oil. The susceptibility of ATCC 700699 strain to the essential oil of *Origanum vulgare* diluted to 10% was also found in a recent study by Cabrera-Contreras *et al.* (2020). These authors noted the absence of growth of this MRSA strain when it was inoculated on culture medium in mixture with essential oil undiluted or diluted in coconut oil at 10% , 1% , 0.5% and 0.25% (Cabrera-Contreras *et al.*, 2020). However, this adopted antibacterial activity test, which is different from our agar diffusion method, did not allow the MIC and inhibition diameter to be calculated.

Table 2. Inhibition effect of oregano essential oil on control reference strains at several dilutions

Reference strains	OEO vehicle	Diameter (mm) of inhibition (mean ± SD) for OEO dilutions		
		1%	5%	10%
Staphylococcus aureus (ATCC® 700699™)	VO	3.65 ± 5.16	7.03 ± 0.06	8.84 ± 0.21*
	DMSO	9.22	25.91	31.72
Staphylococcus aureus (ATCC® 25923™)	VO	3.13 ± 4.43	7.36 ± 0.02	8.37 ± 0.83*
	DMSO	7.31	8.58	17.42

*: Presence of inhibition effect of essential oil on the reference strain DMSO: dimethyl sulfoxide; VO: vegetable oil

The use of the dilution method in agar medium allowed Nostro *et al.* (2004) to confirm the susceptibility of the MRSA and MSSA strains, including

ATCC 25923, to the essential oil of *Origanum vulgare* with thymol (24%) and carvacrol (14%). Their MIC was 0.125 after dilution in dimethyl sulfoxide (DMSO).

Results of in vitro test were in agreement with those of the authors mentioned above and confirm the antimicrobial efficacy of oregano oil against control strains. For further in vitro evaluation of the efficacy of topical solution or ointment containing essential oil, we suggest for the choice of the threshold value of the inhibition diameter to take into consideration the nature of the diluent, the diameter of the disc or the well and the dose to be impregnated on the disc or to be deposited in the well.

Microbiological quality in raw milk samples

The present trial revealed the occurrence of subclinical mastitis indicated by the CMT test without the presence at critical levels of major pathogens often investigated in quality control. These results are probably an expression of damage due to the minor germs that were not the subject of this control. These are mainly represented by coagulase-negative staphylococci (CNS) (e.g. *Staphylococcus chromogenes*) which cause an increase in SCCs but without effect on milk composition and production (Pyörälä and Taponen, 2009; Tomazi *et al.*, 2015). This finding was also cited by Bradley *et al.* (2007) in Great Britain and Wales who found the absence of major pathogens in 39% of samples representing subclinical mastitis and by Sampimon *et al.* (2009) in the Netherlands with an incidence of 20.8% in milk samples with a high somatic cell count greater than 250,000 cells/ml. In the present study, the somatic cell count was subjectively monitored by the CMT test which did not accurately reflect the status of cell immunity which could have been better controlled if an automated counter had been used, although in Algeria the cell count is not yet taken into account in the regulations as a criterion for the quality control of commercialised raw milk.

The microbiological analysis showed the presence of TMAB and coagulase-positive staphylococci in all the samples without their numbers reaching or exceeding the limits required by the Algerian state (Algerian Ministry of commerce, 2017). However, an absence of coliform, *Listeria monocytogenese*, and *Salmonella* in all samples, either at the beginning or the end of the trial was noted. Compared to Algerian standards (Algerian Ministry of commerce, 2017), the hygienic quality of all milk samples analysed was satisfactory regarding the limit criterion of major pathogenic germs counts independently of the sampling time, before or after the application of the essential oil.

Based on the overall results of the microbiological analysis (Table 3), a significant decrease ($P < 0.05$) in the number of TMAB after three days of essential oil application from values between 10^4 CFU/ml and 10^5 CFU/ml (90% of the samples) to values between 10^2 CFU/ml and 10^3 CFU/ml (70% of the samples) was observed. In agreement with the present finding, the supplementation in the concentrate mixture for healthy Holstein dairy cows with individual dose (2.5 and 5 g/head/day) of another oregano species (*O. majorana*),

mixed with clove and juniper in equal proportions as an essential oils blend improved the microbial profile of milk during the post-treatment period by a 20.7 and 17.9% reduction in total milk bacterial count (TMAB), respectively, compared to the control group (Al-Suwaiegh *et al.*, 2020).

For coagulase-positive staphylococci, there is a significant absence in 40% of the unit component samples ($P < 0.05$) after the application of the essential oil with the maintenance of a population counting from 1 CFU/ml to 50 CFU/ml in 60% of the samples before or after treatment ($P > 0.05$). Concerning the other bacteria (Coliforms, *Listeria* and *Salmonella*), a total absence of these bacteria was observed in all milk samples before and after the application of the essential oil. Therefore, the effect of this oil on them will not be retained for the rest of the discussion although other authors (Özkalp *et al.*, 2010) have observed in vitro an antibacterial activity of the oregano essential oil with different MICs against *E. coli* (250 µg/ml), *Listeria monocytogenes* (250 µg/ml) and *Salmonella enteritidis* (128 µg/ml).

Table 3. Prevalence of bacteria counts levels in raw milk samples before and after oregano essential oil applications

Bacteria	Counts levels (CFU/ml)	Prevalence (%) per unit component sample	
		Before	After
Total Mesophilic Aerobic Bacteria (TMAB) at 30 °C Limit*: 3×10^5 CFU/ml	$[10^2, 10^3[$	0^a (0/50)	70^b (35/50)
	$[10^3, 10^4[$	10^a (5/50)	30^b (15/50)
	$[10^4, 10^5[$	90^a (45/50)	0^b (0/50)
	$> 10^5$	0 (0/50)	0 (0/50)
Coagulase-positive staphylococci (CPS) Limit*: 100 CFU/ml	Absence	20^a (10/50)	40^b (20/50)
	[1, 50]	62^a (31/50)	80^a (30/50)
	]50, 80 [	10^a (5/50)	0^b (0/50)
	[80, 100 [	8^a (4/50)	0^b (0/50)
	> 100	0 (0/50)	0 (0/50)
Coliformes thermotolerant Limit*: 5×10^2 CFU/ml	Absence	100 (50/50)	100 (50/50)
Listeria monocytogenes Limit*:100 CFU/ml	Absence	100 (50/50)	100 (50/50)
Salmonella Limit*: Absence in 25 ml	Absence	100 (50/50)	100 (50/50)

Different superscript letters denotes a subset of TIME categories whose prevalence does differ significantly from each other at the 0.05 level using the z-test.

*: Algerian Ministry of Commerce (2017).

In vivo effect of oregano essential oil on counts of Total Mesophilic Aerobic Bacteria

Total aerobic mesophilic flora enumeration before and after the oily solution application shows that there is a most significant decrease ($P < 0.001$) in the number of bacteria in all samples. This finding is valid for both affected and healthy mammary quarters that showed counts below the limit value of 3×10^5 CFU/ml (Table 4). Comparison of the average count of the TMAB shows that there is a significant decrease ($P < 0.01$) from 13920.00 CFU/ml to 649.94 CFU/ml for healthy group and from 17040.00 to 1780.00 CFU/ml for affected quarters ($P < 0.05$) at the end of the trial (Table 4). These changes confirm the significant effect ($P < 0.01$) of EO on the overall mean TMAB number of all samples. This important antibacterial activity has been tested in vitro by the diffusion disc method on six standard strains of Gram-positive and Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Enterococcus hirae*). These strains showed a degree of sensitivity quite similar to the essential oils of O. vulgare with MIC values of 31.25 µg/ml to 125.00 µg/ml (Sari *et al.*, 2006). Recently, the antimicrobial activity of *Origanum vulgare* was reviewed by Alagawany *et al.* (2020). It was attributed to the particular richness of its oil in phenolic compounds. Among several compounds, carvacrol, citral, thymol and trans-cinnamaldehyde were effective at low MIC against the majority of mastitis-causing bacteria (Gupta *et al.*, 2020). Carvacrol is the major compound of the oil used in our trial. It was known by the US Food and Drug Administration (FDA) as a safe flavouring substance when used in or on food to preserve it (European Commission, 1999). This compound and thymol were the two phenolic compounds often present as major compounds in oregano oil particularly that collected in our study area as described above. Thus, the bactericidal activity of carvacrol has been observed in vitro on several bacteria (*Escherichia coli, Pseudomonas fluorescens, Staphylococcus aureus, Lactobacillus plantarum,* and *Bacillus subtilis*) with a MIC varying according to the bacteria, from 5 mg per Petri dish for *Escherichia coli* and *Staphylococcus aureus* to 20 mg for *Lactobacillus plantarum*. This activity has been linked to the hydrophobicity and chemical structure of this compound, whose presence of a free hydroxyl group and a delocalised system allows the exchange of protons (Ben Arfa *et al.*, 2006).

In vivo effect of oregano essential oil on coagulase-positive staphylococci counts

The counting of coagulase-positive staphylococci showed values below the permitted limits which decreased after the application of the essential oil, regardless of the status of the quarters, healthy or affected. A statistically significant decrease was only found for one unit in the sample ($p = 0.02$). A non-significant effect ($P > 0.05$) of OEO at the end of the trial on the mean number of staphylococci in the samples from healthy and affected quarters was recorded (Table 4) despite an overall decrease from 34.80 CFU/ml to 18.80 CFU/ml and 34.00 CFU/ml to 20.80 CFU/ml for healthy and affected quarters respectively.

The efficiency of the EO of *Origanum vulgare* with carvacrol (92%) has been observed in Brazil (Pozzo *et al.*, 2011) on coagulase-positive strains of *Staphylococcus spp* isolated from bovine mastitis regardless of their resistance profile to antibiotics (tetracycline, penicillin, erythromycin, ceftiofur, ampicillin, cephalothin and oxacillin) used in the treatment of mastitis with MICs lower than those obtained with the use of the majority pure compound carvacrol (99.5%). Its antibacterial activity was demonstrated in vitro in the United States on *Staphylococcus aureus* isolated from clinical bovine mastitis with a MIC of 0.5% and a minimum bactericidal concentration (MBC) of 1.2% (Baskaran *et al.*, 2009). At a concentration of 98% , carvacrol had strong antimicrobial activity on *Staphylococcus aureus* including MRSA strains isolated from tank cow's milk with a MIC of 0.058 - 0.234 mg/ml evaluated by microdilution method and inhibition zones ranging from 19 to 45 mm (Keyvan and Tutun, 2019). This susceptibility of MRSA strains to carvacrol explains that registered by the in vitro control test in our study.

Table 4. Effect of oregano essential oil on mean counts of total mesophilic bacteria, staphylococci and their ratio

Variables	Quarter Status	Time	Mean	SD	Time fixed effects	
					F	Sig.
TMAB MEAN (CFU/ml)	Healthy	Before	13 920.00*	4 520.18	95.14	0.00
		After	694.00*	320.44		
	Affected	Before	17 040.00*	4 879.34		
		After	1 780.00*	1 463.56		
CPS MEAN (CFU/ml)	Healthy	Before	34.80*	23.69	2.48	0.13
		After	18.80*	19.01		
	Affected	Before	34.00*	29.02		
		After	20.80*	19.42		
RATIO CPS/TMAB	Healthy	Before	0.003	0.002	3.23	0.09
		After	0.057	0.093		
	Affected	Before	0.002	0.001		
		After	0.022	0.031		

**:* Significant difference at $P < 0.01$ between the mean of variable and the lower microbiological limit (m) value using t test.
m= the threshold limit number (CFU/ml or CFU/g), below which the product is considered of satisfactory quality.
TMAB: total mesophilic aerobic bacteria; CPS: coagulase-positive staphylococci

Effect of oregano essential oil on the ratio of CPS count per TMAB

No significant change in the proportion of staphylococci to TMAB was noted (Table 4). However, an increase in ratios interpreted as an increase in the proportion of staphylococci relative to TMAB in both healthy and affected groups was recorded at the end of experimentation. The lack of significance of the effect of this oil on the mean overall ratio ($P > 0.05$) suggests that there was no significant change in the balance of the milk flora between the beginning and end of the trial (Table 4). This finding seems to be explained by the presence of multiple factors determining the SCP number and their proportion in the milk flora. Surveys on 27 dairy farms in France (Michel *et al.,* 2001) have shown that the level of microbial populations and the proportion between the flora of technological interest and spoilage flora are influenced by the hygienic practices of milking equipment and udder hygiene before and after milking. These measures had little influence on CPS which are very often present in non-negligible numbers either in milk samples with a low level of aerobic mesophilic flora (1.2×10^3 CFU/ml) where more than 50% of the milk contains at least 100 CFU/ml, or in milk with a higher level of mesophilic flora (1.5×10^4 CFU/ml) which have 37% of the samples at less than 100 CFU/ml. It has been suggested that the proportion of staphylococci is influenced by several factors such as the health status of the herd and, in particular, the presence or absence of cows with recurrent mastitis (Michel *et al.,* 2001).

CONCLUSIONS

The present in vivo study, reinforced by an in vitro control test, demonstrated an antibacterial effect of the topical oily solution containing essential oil of *Origanum vulgare* with carvacrol as a prevalent compound on the indicators of the microbiological hygienic quality of milk (TMAB) and mastitis-causing bacteria (CPS). It has also shown the ability of this oil to preserve the proportional balance of the bacterial population in milk. We suggest for further studies to take into consideration the emergence of minor germs and the recurrence potential of mammary infections due to antibiotic-resistant germs. Such research could help to assess the benefit-risk ratio of aromatherapy in the dairy sector and contribute to One Health.

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