In vitro and *in vivo* safety and efficacy of ozonized olive oil for the treatment of naturally occurring acute clinical mastitis in dairy cows: a preliminary study

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SUMMARY

Antimicrobial resistance is a critical concern in livestock health, necessitating alternative therapies to reduce antibiotic use, especially for non-severe bovine mastitis. This study investigated the safety and efficacy of ozonized extra virgin olive oil as a potential alternative treatment for acute clinical mastitis in dairy cows. In vitro analyses assessed its safety regarding skin irritation, barrier properties, and antimicrobial activity. In vivo, 40 lactating Friesian cows with mastitis were divided into two groups: one treated with ozonized olive oil and the other with a cefapirin/prednisolone-based suspension. The oil was non-irritating, with protective effects but no direct antimicrobial activity. Clinically, 80% of cows treated with ozonized olive oil achieved symptom remission, while 100% of those receiving antibiotics improved. Somatic cell counts (SCC) decreased in both groups, with a faster reduction in the antibiotic group. Four cows treated with ozonized olive oil required subsequent antibiotic therapy. The findings suggest ozonized olive oil as a promising, non-antibiotic option for managing mild to moderate mastitis, potentially reducing unnecessary antibiotic use. However, antibiotics remain necessary for severe cases or those involving systemic infection. This approach could support more sustainable dairy farming practices while addressing public health concerns about antibiotic resistance. Further controlled studies are required to confirm these findings and clarify its role in mastitis management.

KEY WORDS

Antimicrobial resistance; bovine mastitis; ozonized olive oil; somatic cell count (SCC).

INTRODUCTION

Antimicrobial resistance represents a significant and escalating global concern for human, animal, and environmental health, stemming from the emergence, spread, and persistence of multidrug-resistant bacteria [1]. In farm animal husbandry, the excessive use of antibiotics and the presence of their residues in animal-derived food products pose risks to consumer health [2]. On many farms, almost all cases of mastitis are treated with antibiotics, but in cases of non-severe clinical mastitis (CM) it doesn't give great benefits. Infections caused by pathogens like *Escherichia coli* often resolve without treatment, reducing the overall benefit of antibiotics in these cases [3]. Thus, there is a critical need to develop and implement new strategies to reduce the overuse of antibiotics in livestock farming.

Ozone (O_3), an unstable molecule consisting of three oxygen atoms, was first reported for sterilization in 1826. Initially applied empirically in medicine, significant advancements over the last decade have led to the development of precise medical ozone generators [4]. O_3 acts by producing reactive oxygen species (ROS), which modulate key signaling pathways like Nuclear factor erythroid 2-related factor 2 (Nrf2) and Nuclear Factor- Kappa B (NF- B), regulating redox reactions and inflammatory responses [5]. Additionally, O_3 demonstrates notable antibacterial activity, making it effective in treating infectious skin diseases and promoting wound healing [4, 6]. O_3 has also been explored in dentistry, where its antimicrobial properties are utilized to reduce bacterial load and support oral tissue healing in cases of infection or inflammation [7].

Olive oil is one of the most effective carriers of ozone due to its ability to stabilize O_3 within the double bonds of unsaturated fatty acids. During ozonation, olive oil traps O_3 in the form

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of stable ozonides. Ozonized vegetable oils have been extensively studied for their antimicrobial properties and their capacity to support tissue healing [8-9].
Ozonation, through the integration of ozone molecules into the oil, generates compounds with high oxidative activity that inhibit the growth of pathogenic microorganisms and promote cellular regeneration [10-11].
In the veterinary field, research on ozone and ozonized oils has focused on their antimicrobial properties, ability to modulate oxidative stress, and role in supporting tissue repair and healing. These studies have explored their use in wound care, infection management, and as an adjunct in treating inflammatory conditions, highlighting their potential therapeutic benefits [12]. To the best of our knowledge, two studies have explored the effect of ozonized vegetable oil in the management of bovine mastitis, emphasizing the need for additional research to validate its efficacy [13].
The aim of this study was to evaluate the safety and efficacy of an ozonized extra virgin olive oil formulation (MastO3zoil® - Reiyel S.r.l.-Italy), both in vitro and in vivo, for the endocanalicular treatment of mastitis in dairy cows. The efficacy of this treatment was compared with that resulting from empirical therapy based on cefapirin/prednisolone. The final aim was to determine whether the ozonized extra virgin olive oil approach can indeed provide a valid alternative to the use of antibiotics.

MATERIALS AND METHODS

Ozonized olive oil

The ozonized olive oil utilized in this study MastO3zoil® (hereafter referred to as ozoil) was locally produced in the laboratories of Rochel S.r.l. (Uggiano la Chiesa, Lecce, Italy). The ozonization process was conducted by subjecting the extra virgin olive oil to a continuous flow of oxygen for a period exceeding eight hours. To ensure uniform ozonization and prevent the formation of volatile products, a high gas flow was employed without the addition of any additives or solvents. Throughout the ozonization process, the formation of peroxides was continuously monitored through titrations. All operations were conducted in a controlled environment using ventilation systems and fume hoods.

In vitro safety and efficacy tests

MastO3zoil® is already commercially available in Italy at local level as a topical product for external use; specific tests were conducted ad hoc for this study to evaluate its in vitro safety of potential endocanalicular application and efficacy. The ozoil underwent two primary in vitro tests: *the skin irritation* test to assess potential dermal irritation and *the barrier effect test* to evaluate its protective properties. Furthermore, *in vitro antimicrobial activity* was tested.

Skin irritation test

The skin irritation test [14] involves the use of Reconstructed Human Epidermis tissues (RHE, MatTek in vitro Life Science Laboratories, Bratislava, Slovak Republic) and is based on exposing the tissues to the tested sample and then measuring the tissue viability through the conversion of the yellow tetrazolium dye MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] into a purple formazan salt by cellular reductase enzymes present in viable cells [15].
Briefly, before performing the skin irritation test, the ozonized olive oil underwent a preliminary test, in order to evaluate its ability to directly reduce MTT to formazan, which may interfere with cell viability measurements and require the use of adapted controls for corrections (killed tissue replicates that undergo the entire testing procedure to generate a non-specific MTT reduction, NSMTT).
For the skin irritation test, the tissues were pre-incubated in their specific growth medium for at least 2 hr at 37±1°C with 5% CO_2. Subsequently, each tissue was treated with 30 l of ozonized olive oil. The tissues were incubated for 35±1 min at 37°C, followed by 25±1 min at room temperature. Each experiment was conducted in triplicate, including positive control (sodium dodecyl sulfate, SDS, 5%) and negative control (phosphate buffered saline, DPBS). After incubation, the tissues were washed with DPBS to remove any residues of the treatments, placed in a 24-well plate containing 0.3 mL per well of MTT (1 mg/mL in tissues culture medium), and incubated for 3 hr. After the incubation, the resulting formazan salt was extracted from the tissues by placing them in a 24 well plate containing 2 mL of isopropanol per well. The plates were placed in a sealed plastic bag to prevent evaporation and set on an orbital shaker for 2 hr at room temperature. The optical density (OD) at 570 nm was measured on a spectrophotometer with isopropanol used as a blank. The percent viability of each treated tissue was calculated relative to negative control using the following equation:

$$\% \text{ viability} = \frac{OD_{TS}}{OD_{NC}} \cdot 100$$

Where ODTS is the optical density of the tissue treated with the ozonized olive oil and ODNC is the mean optical density of the negative control tissues. The percent viability of the positive control tissues was calculated in the same way.

Barrier effect test

The barrier action of the ozonized oil was assessed by measuring its ability to reduce the amount of cytokines in RHE tissues subjected to an irritant stimulus. In particular, the production of interleukin-1 (IL-1), a key cytokine released from keratinocytes during irritation, was evaluated [16]. The production of cytokines was induced using 1% SDS. One series of tissues was not stimulated (NC, negative control), one series of tissues was stimulated with SDS, a third series of tissues was treated with the tested product and then stimulated with SDS, and a fourth series was treated with the tested product, washed, and subsequently stimulated with SDS.
After a 60-min treatment period, the tissues were washed with DPBS, post-incubated in culture medium for 24 hr, and then the medium was collected for the cytokines analysis. The concentration of IL-1 released from the RHE tissues into the assay medium during the exposure period was measured by enzyme-linked immunosorbent assay (ELISA) (AssayGenie, Dublin, Ireland. Assay range: 31.25pg/mL - 1000pg-mL; Sensitivity: <10pg/mL; Specificity: recognizes both natural and recombinant human IL-1) according to the manufacturer's instructions and expressed as a percentage relative to the concentration of IL-1 in NC tissues.

In vitro antimicrobial activity

The antimicrobial activity of ozonized olive oil was tested against

clinical isolates from cow mastitis. Strains of *Pseudomonas spp, Staphylococcus aureus, Streptococcus spp.*, and *Escherichia coli* were tested, using the agar well diffusion test [17]. Briefly, plates of Mueller Hinton agar (Liofilchem, Teramo) were used and an inoculum of each isolate at McFarland's turbidity standard 2 was prepared. The inoculum was spread on the plate by sterile cotton swab and allowed to dry for 5-8 min. Wells (6 mm in diameter) were punched into the agar using a sterile stainless steel borer and filled with 50 L of the extract. A disk of gentamicin (GEN, 10 g, Oxoid, Hampshire, UK) was used as positive control. The plates were incubated at 37°C for 24 hr.

In vivo safety and efficacy study

Animals and study design

The study was conducted on a single farm located in the province of Taranto (Puglia, Italy). Lactating Friesian dairy cows in which the farm's veterinarian identified symptoms of acute clinical mastitis of probable bacterial etiology (alterations in milk and mammary gland) were included. For each cow enrolled in the study, the days in milk (DIM) were recorded, along with whether the animal was primiparous or multiparous. The cows were fed a diet composed of oat hay, a concentrate feed mix, water, and a blend of soft wheat silage and sorghum. The animals were not vaccinated against infectious mastitis and were milked twice daily. Milking represented the clinical evaluation moment used to identify cows with mastitis, and final inclusion criteria were based on a positive California Mastitis Test. To minimize variability in disease presentation and obtain a more uniform study population, only cows with a single affected mammary quarter were enrolled. Cows that had received antibiotic and/or anti-inflammatory treatment within the thirty days prior to enrollment, as well as those showing symptoms of mastitis in more than one mammary quarter, were considered ineligible for the study. The enrolled cows were evenly divided into two treatment groups: the Ozoil Group (OG) and the Control Group (CG). Each cow was numbered in chronological order of enrollment, and the enrolled animals were alternately assigned to the two groups. Cows with odd numbers were allocated to the OG, while those with even numbers were allocated to the CG. This randomized allocation was suitable for our barn study context, ensuring a random distribution of subjects across treatment groups.

Treatments

The cows in the OG received a treatment consisting of the infusion of 10 mL of 100% ozonized extra virgin olive oil (MastO3zoil® - Reiyel S.r.l.-Italy), via the endocanalicular route, into the mammary quarter affected by mastitis. This infusion was performed at the end of milking, twice daily, for 5 consecutive days. The cows in the CG received a suspension containing 300 mg of cefapirin and 20 mg of prednisolone, infused into the mammary quarter affected by mastitis (MastiPlan® - MSD Animal Health S.r.l.-Italy). The infusion was administered at the end of milking, twice daily, for 5 consecutive days.

Sampling and Laboratory Analyses

Samples of milk and blood were collected from the enrolled cows. Four mL of milk were obtained aseptically from the affected mammary quarter. Milk was collected prior to treatment initiation (T0), at the end of treatment (T1), and ten days after therapy suspension (T2). Blood samples were collected from the mammary vein using serum tubes before treatment initiation (T0) and at the end of treatment (T1). Milk samples obtained at T0 and T1 underwent somatic cell count (SCC) analysis and bacterial examination, while only SCC analysis was performed ten days post-therapy suspension (T2) using flow cytometry (Fossmatic^{tm} 7DC, Foss). The bacteriological examination of milk samples was conducted using Blood Agar and a selective medium for Enterobacteriaceae. The protocol followed was based on test method MP 01/067, a standardized method for microbiological analysis [18]. Blood samples were analyzed to determine the levels of acute phase proteins (APPs), specifically haptoglobin (Hp) and serum amyloid A (SAA), using the Olympus AU 5811 - Beckman Coulter instrument (Brea, California, USA).

Statistical Analysis

All statistical analyses were performed using JASP (version 0.18.3, JASP Team, 2024, Amsterdam, Netherlands; https://jasp-stats.org). Descriptive statistics and the Shapiro-Wilk test were used to assess whether the somatic cell count (SCC) values for each group followed a normal distribution. To compare the initial values and evaluate the homogeneity between the two treatment groups (OG vs CG) at T0, Levene's Test was conducted. Differences in SCC values over time between the Ozoil Group vs Control Group were analyzed using Student t-test. For the analysis of acute phase protein (APP) levels over time, the Wilcoxon Signed-Rank Test was applied due to the non-parametric nature of the data. This test was used to assess whether the treatments had a significant impact on APP concentrations within the study groups. Comparisons were considered statistically significant at $p < 0.05$.

RESULTS

In vitro tests results

Skin irritation test results

Results of the preliminary tests aimed to evaluate Ozoil's ability to reduce MTT to formazan confirmed the presence of possible interferences with cellular viability measurements. To account for this interaction, the skin irritation test was also performed on non-viable tissues. After NSMTT correction, the sample was classified as non-irritating with mean tissue viability of 100.4%.

Barrier action test results:

The levels of IL-1 in tissues treated with Ozoil and stimulated with SDS were significantly lower than those in tissues stimulated with SDS and left untreated. In tissues where the product was washed before applying the stimulus, they were observed significantly higher levels of IL-1 compared to unwashed tissues; however, these levels still remained significantly lower than those of tissues stimulated with SDS, indicating that the product maintains its barrier action even after washing (**Figure 1**).

Antimicrobial activity test results

No inhibition zones were observed, indicating no antimicrobial activity against the tested bacterial strains.

In vivo study results

Clinical-pathological results

Forty Holstein cows met the inclusion criteria. For each ani-

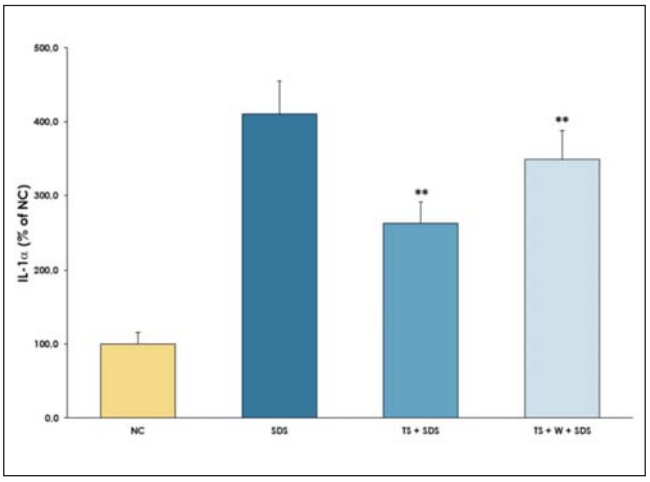

Figure 1 - Absorbance at 450 nm reflects IL-1 production. Data are expressed as % of negative control (NC), mean ± SD. Statistical analysis was performed by Student's t-test ($p<0.05$ considered significant). NC = untreated/unstimulated tissues; SDS = sodium dodecyl sulphate; TS+SDS = tissues pretreated with test sample before SDS; TS+W+SDS = tissues pretreated, washed, then stimulated with SDS. **$p<0.01$ vs SDS.

IL-1 levels in tissues treated with Ozoil and stimulated with SDS (TS+SDS) were significantly lower than those in tissues stimulated only with SDS (SDS). In tissues where the product was washed before applying the stimulus (TS+W+SDS), they were observed significantly higher levels of IL-1 compared to unwashed tissues but significantly lower than those of tissues stimulated with SDS.

mal in the two treatment groups, acute mastitis was diagnosed at T0 and classified according to the International Dairy Federation (IDF) definitions into mild, moderate, and severe forms, based on the presence of milk alterations, mammary gland changes, and systemic symptoms [19]. In the OG, four cows (4/20; 20%) were considered non-responsive to the treatment, as they continued to exhibit symptoms of clinical mastitis (such as clots and flakes in milk, heat and redness of the udder, and dullness). In these four animals, after five days of unresponsiveness to ozonized oil treatment and in accordance with ethical guidelines on animal welfare, antibiotic and anti-inflammatory therapy (MastiPlan®) was administered. In the remaining 80% (16/20) of cows in the OG, a progressive improvement in both clinical and milk scores was observed during treatment, leading to complete symptom remission. In the CG, all 20 cows (100%) exhibited progressive improvement in clinical parameters and milk alterations, achieving complete remission by the end of the treatment period. No adverse effects on feed intake, general behavior, or milk yield were observed during the study period.

All data related to the clinical-pathological parameters recorded for the cows enrolled in the study, both before and after treatment, are detailed in **Tables 1, 2, 3 and 4**.

Regarding APPs, in the OG, the median concentration of Hp at T0 was 1.26 mg/dl, with values ranging from 0.1 to 4.4 mg/dl; at T1, the median concentration of Hp was 2.44 mg/dl, with values ranging from 0.1 to 9.07 mg/dl. The analysis showed no significant difference between T0 and T1 ($p = 0.135$; Wilcoxon Signed-Rank Test). In the same treatment group, the median concentration of SAA at T0 was 1 μg/mL, ranging from 0.23 to 4.36 μg/mL. At T1, the median SAA concentration was 0.79 μg/mL, ranging from 0.18 to 2.1 μg/mL. The analysis indicated no significant difference between T0 and T1 ($p = 0.205$; Wilcoxon Signed-Rank Test). In CG cows, the median concentration of Hp at T0 was 1.07 mg/dl, with observed values

Table 1 - Ozoil group - Clinical and laboratory parameters of cows with clinical mastitis at enrollment (T0). Legend: C: Clots; F: Flakes; H: Heat; R: Redness; S: Swelling; P: Pain; D: Dullness; A: Anorexia; SCC: Somatic Cell Count; HP: Haptoglobin; SAA: Serum Amyloid A.

Cows	Days in milk	Milk alteration	Udder alteration	Systemic signs	Clinical grading	Microbiology	SCC(cells/mL*1000)	Hp t0 (mg/dL)	SAA t0 (μg/mL)
c1	108	C			Mild		4306	0.2	0.23
c3	85	C/F			Mild		5533	4.4	4.36
c5	175	F	H/P		Moderate	*E. coli*	5532	0.2	0.62
c7	56	C	H/R		Moderate	*E. coli*	8592	3.46	0.71
c9	4	C	H/P	D	Severe		9135	2.54	0.27
c11	200	C			Mild		7871	0.2	0.89
c13	63	C	P/R/S		Moderate	*S.uberis*	9057	0.3	0.44
c15	33	F			Mild		7775	1.06	0.89
c17	159	F	H		Moderate		9903	0.17	0.62
c19	25	C			Mild		8765	0.15	0.89
c21	106	C			Mild		6970	0.4	3.2
c23	10	C	H/P	A	Severe	*E. coli*	9102	3.8	0.89
c25	166	C	R		Moderate		9485	0.5	0.58
c27	65	C/F			Mild		5812	2.95	0.42
c29	12	F			Mild		6233	3.1	0.77
c31	198	F			Mild		9783	0.3	0.65
c33	72	C	H/P		Moderate		5487	0.1	0.52
c35	50	C			Mild		8236	0.95	0.9
c37	6	F	H/P	A	Severe		9953	0.2	0.45
c39	35	C			Mild		4458	0.4	1.75

Table 2 - Control group - Clinical and laboratory parameters of cows with clinical mastitis at enrollment (T0). Legend: C: Clots; F: Flakes; H: Heat; R: Redness; S: Swelling; P: Pain; D: Dullness; A: Anorexia; SCC: Somatic Cell Count; HP: Haptoglobin; SAA: Serum Amyloid A.

Cows	Days in milk	Milk alteration	Udder alteration	Systemic signs	Clinical grading	Microbiology	SCC(cells/ mL*1000)	Hp t0 (mg/dL)	SAA t0 (µg/mL)
c2	32	C			Mild		6211	0.63	1.12
c4	65	F	P		Moderate		4286	2.15	1.63
c6	206	F			Mild		5495	4.6	1.32
c8	115	C			Mild		5395	1.89	0.57
c10	85	C/F	H/P		Moderate	*Strept. spp.*	8753	1.15	0.35
c12	18	C			Mild		5704	1.2	1.23
c14	32	C/F	R/S		Moderate		7946	0.9	0.7
c16	66	F			Mild		4270	0.87	1.52
c18	198	C	H/P	D	Severe		8985	0.32	0.84
c20	410	F			Mild		7985	0.18	0.62
c22	16	C	P/S	D/A	Severe	*E. coli*	9911	0	0.98
c24	22	C	H/R	D/A	Severe	*S. uberis*	9977	2.8	1.42
c26	74	C			Mild		9925	3.1	0.62
c28	223	C			Mild		6274	0.1	0.44
c30	410	F	H/S		Moderate		9188	0.1	0.27
c32	6	C			Mild	Mixed flora	5378	0.9	0.71
c34	1	F	P		Moderate		8335	0.2	0.8
c36	246	C	H/P	D	Severe		9432	0	1.25
c38	129	C/F	H/R		Moderate		7592	0.22	0.71
c40	74	F			Mild		4069	0.12	0.89

Table 3 - Comparison of pre and post-treatment Somatic Cell Count (SCC), Haptoglobin (Hp), and Serum Amyloid A (SAA) levels in the Ozoil Group and Control Group.

Ozoil group								Control group							
	SCC (cells/mL*1000)			Hp (mg/dL)		SAA (µg/mL)			SCC (cells/mL*1000)			Hp (mg/dL)		SAA (µg/mL)	
Cows	T0	T1	T2	T0	T1	T0	T1	Cows	T0	T1	T2	T0	T1	T0	T1
c1	4306	2461	210	0.2	0.10	0.23	0.44	c2	6211	396	345	0.63	0.42	1.12	0.98
c3	5533	4341	479	4.4	0.6	4.36	1.42	c4	4286	59	42	2.15	1.6	1.63	0.32
c5	5532	1486	82	0.2	0.89	0.62	0.36	c6	5495	509	95	4.6	2.95	1.32	0.93
c7	8592	2846	305	3.46	0.15	0.71	0.98	c8	5395	146	22	1.89	2.35	0.57	0.79
c9	9135	8630	-	2.54	8.68	0.27	0.18	c10	8753	250	47	1.15	4.65	0.35	0.74
c11	7871	2368	65	0.2	0.32	0.89	1.69	c12	5704	363	34	1.2	0.12	1.23	0.81
c13	9057	7792	-	0.3	6.3	0.44	0.44	c14	7946	1552	166	0.9	3.6	0.7	0.91
c15	7775	1282	43	1.06	0.2	0.89	0.27	c16	4270	324	116	0.87	0.46	1.52	0.4
c17	9903	8570	370	0.17	0.25	0.62	0.49	c18	8985	3538	15	0.32	0.2	0.84	2.05
c19	8765	3587	17	0.15	7.8	0.89	0.53	c20	7985	3754	102	0.18	1.08	0.62	0.21
c21	6970	2983	317	0.4	0.8	3.2	1.85	c22	9911	3408	14	0	0.22	0.98	0.89
c23	9102	8729	-	3.8	5.4	0.89	0.42	c24	9977	3617	67	2.8	0.1	1.42	0.27
c25	9485	7779	504	0.5	0.89	0.58	1.01	c26	9925	381	328	3.1	3.5	0.62	0.8
c27	5812	1557	87	2.95	0.3	0.42	2.1	c28	6274	57	40	0.1	7.6	0.44	0.71
c29	6233	1342	45	3.1	9.07	0.77	0.52	c30	9188	490	89	0.1	5.5	0.27	0.62
c31	9783	9053	388	0.3	0.4	0.65	0.34	c32	5378	141	21	0.9	0.23	0.71	0.89
c33	5487	3784	17	0.1	7.76	0.52	0.35	c34	8335	94	45	0.2	4.8	0.8	0.89
c35	8236	2490	68	0.95	0.35	0.9	0.85	c36	9432	350	33	0	0.10	1.25	0.8
c37	9953	8202	-	0.2	2.1	0.45	0.53	c38	7592	1509	158	0.22	0	0.71	1.16
c39	4458	2569	220	0.4	4.2	1.75	1.05	c40	4069	313	52	0.12	0.11	0.89	0.18

Table 4 - Clinical grading of cows enrolled in the study before and after treatment.

	Ozoil group		Control group	
	T0	T1	T0	T1
Mild	11/20	0/20	10/20	0/20
Moderate	6/20	1/20	6/20	0/20
Severe	3/20	3/20	4/20	0/20

ranging from 0 to 4.6 mg/dl; at T1, the median concentration of Hp was 1.98 mg/dl, with observed values ranging from 0 to 7.6 mg/dl. The analysis showed no significant difference between T0 and T1 (p = 0.455; Wilcoxon Signed-Rank Test). In the same treatment group, the median concentration of SAA at T0 was 0.88 μg/mL, ranging from 0.27 to 1.5 μg/mL. At T1, the median SAA concentration was 0.76 μg/mL, ranging from 0.18 to 2.05 μg/mL. The analysis indicated no significant difference between T0 and T1 (p = 0.478 Wilcoxon Signed-Rank Test). In the same treatment group, the median concentration of SAA at T0 was 0.88 μg/mL, ranging from 0.27 to 1.5 μg/mL. At T1, the median SAA concentration was 0.76 μg/mL, ranging from 0.18 to 2.05 μg/mL. The analysis indicated no significant difference between T0 and T1 (p = 0.478 Wilcoxon Signed-Rank Test).

Milk Laboratory test results

Milk SCC results

Descriptive statistics revealed that the mean SCC for the OG at T0 was 7599.4 (SD = 1891.02), while the CG exhibited a slightly lower mean of 7255.55 (SD = 2052.08). The Shapiro-Wilk test indicated that the distribution of SCC for the OG at T0 had a W-statistic of 0.906 (p = 0.055), and the CG at T0 had a W-statistic of 0.912 (p = 0.070). Both groups demonstrated a data distribution that did not significantly differ from a normal distribution (p > 0.05) (**Table 5**). The independent samples Levene's test comparing SCC levels between the OG and the CG yielded non-significant results (F (38) = 0.314, p = 0.579) (**Table 6; Figure 2**).

For the CG, values from all enrolled animals were analyzed (20/20), while for the OG, only animals that responded to the treatment were analyzed (16/20), excluding the four subjects that still showed mastitis symptoms after the 5-day treatment with ozonized oil. Significant differences in SCC were observed over time between the Ozoil Group vs Control Group, at T1 and T2 particularly (**Figure 3**).

Table 5 - Descriptive Statistics and Shapiro-Wilk Test Results for Somatic Cell Count in Ozoil and Control groups at T0.

	Ozoil group	Control group
Valid	20	20
Missing	0	0
Mean	7599.400	7255.550
Std. Deviation	1891.019	2052.080
Shapiro-Wilk	0.906	0.912
P-value of Shapiro-Wilk	0.055	0.070
Minimum	4306.000	4069.000
Maximum	9953.000	9977.000

Table 6 - Somatic Cell Counts (SCC) in Cows Affected by Mastitis: Assessing Group Homogeneity at T0 using Levene's Test.

	F	df1	df2	p
SCC	0.314	1	38	0.579

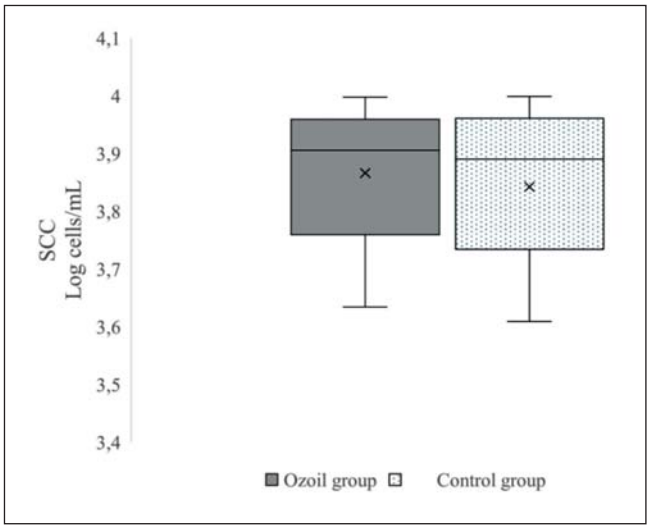

Figure 2 - Assessing Group Homogeneity at T0 using Levene's Test. Comparison of mean SCC levels between the Ozoil Group (OG) and the Control Group (CG) at T0. The independent samples test shows no difference between the variances of the two groups indicating that the samples come from populations with the same variance.

Milk microbiological Examination

The bacteriological examination of milk from the twenty OG cows revealed four positive samples at T0 (Table 1). Among these, three samples tested positive for *E. coli* (C5, C7, C23) and one for *Streptococcus uberis* (C13). At T1 two of these cows still exhibited clinical signs of mastitis and tested positive on the bacteriological examination (C13 for *S. uberis*; C23 for *E. coli*). The other two cows, initially positive for *E. coli*, tested negative at T1, with complete resolution of symptoms. In the CG, four cows tested positive in the milk microbiological examination at T0. Specifically, C24 for *S. uberis*, C22 for *E. coli*, C10 for *Streptococcus spp*., while C32, tested positive for mixed flora (Table 2). However, at T1 all four cows tested negative, showing a complete remission of symptoms.

DISCUSSION

In vitro and in vivo safety and efficacy of treatment with ozonized extra virgin olive oil (Ozoil) were evaluated in this study to expand the knowledge regarding alternative therapies for bovine mastitis. In vitro tests showed that Ozoil is not irritating to the skin and has a barrier action that remains significant even after washing. To evaluate the safety of ozonized olive oil in vitro, the Reconstructed Human Epidermis (RHE) model was selected, as it represents a validated and widely used system for simulating epithelial reactivity [14]. Recent studies have demonstrated that reconstructed epidermis models are adaptable to various types of exposures and environments, extending beyond the skin, thus providing a useful assessment of epithelial responses in diverse contexts [20]. Therefore, the

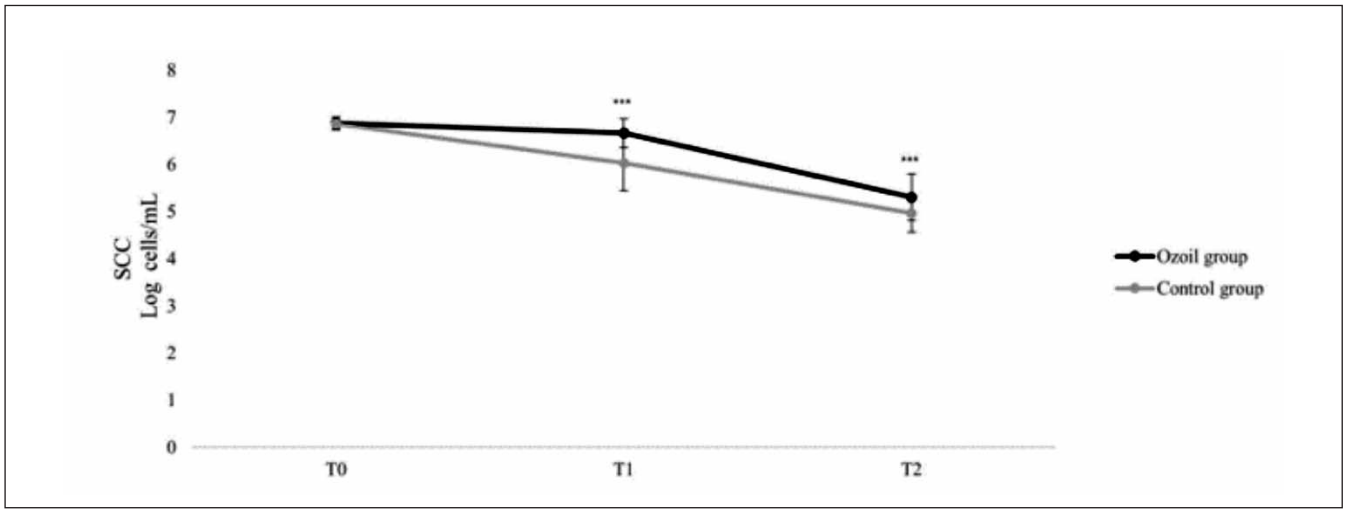

Figure 3 - Comparison of SCC reduction over time following administration of Ozonized extra-virgin olive oil and cefapiridin with prednisolone. The values are expressed as means ± standard deviation. Statistical data processing was performed by Student's t-test. Significant differences in somatic cell counts were observed over time between the Ozoil Group vs Control Group, at T1 and T2 particularly (***p <0.001).

RHE model is appropriate for obtaining an initial indication of product safety, in accordance with international guidelines for in vitro testing [14].

Based on the antimicrobial activity test results, no visible inhibition zones were observed in this study, indicating that the oil used here shows a lack of antimicrobial activity against the tested bacterial strains. This is not surprising, as the antimicrobial efficacy of ozonized vegetable oils is closely related to the concentration of peroxides generated during the ozonation process. In fact, recent studies on ozonated olive oils reported that peroxide values exceeding 1,200 meq O_3/kg are associated with significant antimicrobial activity against both Gram-positive and Gram-negative bacteria, whereas lower peroxide levels result in minimal or absent effects (Domínguez-Lacueva et al., 2025) [27]. Conversely, oils with lower peroxide levels demonstrate minimal or absent antimicrobial effects. MastO3zoil® exhibited a peroxide concentration of only 163 meq O_3/kg at the conclusion of the ozonation process, likely accounting for the lack of observed inhibitory activity. Despite the lack of antibacterial activity observed in vitro, clinical improvement was achieved in 80% of the cows treated with Ozoil. This discrepancy may reflect the barrier effect of Ozoil, which adheres to epithelial surfaces, reduces irritation, and supports tissue healing. Additional mechanisms, including modulation of the local inflammatory response and tissue-protective effects described in the literature [4-5], may also contribute to the observed therapeutic benefit.

In vivo results showed lack of adverse events and a positive clinical response to treatment associated with improvement in SCC in 80% (16/ 20) of the subjects treated with Ozoil. In particular, no adverse effects on feed intake, general behavior, or milk yield were observed during the study, supporting the preliminary safety of the preparation. However, quantitative measurements of these parameters were not performed, and future studies should include objective monitoring to better assess potential systemic effects.

Somatic cell count (SCC) results, in this study, indicate that standard treatment resulted in a more rapid and significant reduction in SCC compared to Ozoil. However, both treatments demonstrated the ability to induce a positive impact over time, showing significant reductions in SCC. These findings suggest that while cefapirin and prednisolone exhibited a more pronounced initial effect, Ozoil also contributed to a notable decrease in SCC, highlighting its potential as an alternative therapeutic option. The bacteriological examination of milk samples at T0 revealed positivity in just 4 cows in both groups. The low number of bacteriologically positive samples at time T0, with only 4 cows testing positive in both groups, aligns with findings in the literature. Previous studies reported nearly 50% of samples from cattle with mastitis exhibiting no bacterial growth. In addition, culture yield is influenced by sampling timing and intermittent shedding of pathogens, and conventional culture methods may fail to detect some environmental or fastidious organisms [22, 3]. This trend may be attributed to an efficacious inflammatory response that reduces the number of bacteria below the detection limits of laboratory tests. Furthermore, it is important to consider that mastitis may also be caused by environmental pathogens, which are not always detected by standard available tests [3]. Five days after treatment, conventional therapy was able to solve the infection in all the 4 positive animals in CG, while in the OG two cows remained positives.

Acute phase proteins (APPs), haptoglobin (Hp) and serum amyloid A (SAA) values at T0, in both the OG and the CG, were within the reference ranges established in the literature. After five days of treatment (T1), the values remained within acceptable limits in both groups, suggesting that the treatment was effective in controlling inflammation. The biomarkers selected for this study (SCC, Hp, SAA) are well-established indicators of inflammation in bovine mastitis and are widely applied in both experimental and field studies[19]. However, given the heterogeneity of clinical cases included (ranging from mild to severe forms and involving different pathogens), these markers alone may not fully capture the complexity of the inflammatory response. Future studies should therefore integrate broader panels of biomarkers and stratify animals according to severity and etiology, to achieve a more comprehensive evaluation of the therapeutic efficacy of ozonized oil. Four out of 20 cows did not respond positively to treatment with ozonized olive oil. Three of these cows (C9, C23, and C37) developed clinically severe mastitis in the immediate postpartum period, a time known for high immune stress and immuno-

suppression, which compromises the cows' ability to respond to intramammary inflammation (IMI) and predisposes them to more severe conditions [23, 3]. In particular, in cow C9 and cow C37, systemic manifestations such as dullness and anorexia were associated with mastitis. In cow C23, mastitis was complicated by *E. coli* infection. These particularly critical contexts were not adequately managed by the ozonized oil, suggesting the need for a more specific therapeutic intervention to achieve effective resolution. Lastly, in cow C13, which developed mastitis 64 days after calving, a *Streptococcus uberis* infection was detected at T0, which persisted even after five days of treatment with ozonized oil. This specific pathogen is known to be particularly resistant to non-antibiotic supportive therapies, often requiring antibiotic treatment for complete resolution [24]. Another limitation of this study is the absence of a comparison with non-ozonized olive oil. Since extra virgin olive oil itself possesses emollient and soothing properties, it cannot be excluded that part of the observed clinical improvement was related to its intrinsic effects rather than to ozonation. Future studies should therefore include an additional group treated with plain olive oil to clarify whether the therapeutic benefit observed is specifically attributable to ozonation.

Mastitis represents a significant problem in dairy farming, adversely affecting milk production and animal welfare. The therapeutic management of mastitis with antibiotics must be carefully evaluated, as they are often used unjustifiably, especially in the absence of precise diagnoses. In all cases of mastitis the adoption of culture-based protocols would be desirable to promote targeted therapy but, this practice takes time and could pose the risk of worsening the clinical condition. Furthermore, many cases of mastitis, especially those of mild or moderate severity, can solve spontaneously without the need for antibiotic treatments [25]. A negative control group (untreated cows) would have been useful to distinguish between spontaneous recovery and treatment effect. However, such an approach was considered unethical, as it would have implied leaving animals affected by clinical mastitis without therapy. It is therefore possible that a proportion of mild cases recovered spontaneously, as frequently reported in the literature. This aspect should be considered when interpreting the results. Future controlled trials, stratifying cows by severity and pathogen, will be essential to conclusively separate spontaneous recovery from the therapeutic contribution of ozonized oil. Studies indicate that a significant percentage of infections, such as those caused by *Escherichia coli* and some species of environmental streptococci, tend to heal spontaneously, making the use of antibiotics often superfluous and potentially harmful if not supported by an accurate diagnosis [26-27, 3]. Ozonized extra virgin olive oil appears to be a safe and effective alternative for mild to moderate mastitis, reducing reliance on antibiotics. However, in severe cases or systemic infections, antibiotics remain essential. Larger controlled trials are needed to confirm these findings.

CONCLUSIONS

Based on the preliminary results of this study, ozonized extra virgin olive oil appears to be a safe and effective alternative for mild to moderate mastitis, reducing reliance on antibiotics. Over the initial five days of therapy, clinical resolution was observed clinical resolution and a progressive improvement in somatic cell count as well as in abnormalities of milk and udder condition, culminating in complete healing, which was confirmed by examinations conducted 10 days after the end of treatment in most of the animals. This therapeutic approach can achieve full recovery without the need for additional treatments. Alternatively, in cases where improvement is insufficient or delayed, the time gained with initial treatment using ozonized olive oil can be used to perform specific microbiological tests that guide targeted therapies. However, in severe cases or systemic infections, antibiotics remain essential. Larger controlled trials are needed to confirm these findings.

Study limitations

These findings offer valuable preliminary evidence on the potential of ozonized olive oil as an alternative therapy for bovine mastitis, but several limitations must be considered. The study involved a relatively small sample, as cows were progressively enrolled as clinical cases emerged on a single farm. This pragmatic, clinically oriented design inevitably reduced statistical power and limited the generalizability of the results. At the same time, restricting the enrollment period helped to minimize environmental and management-related variability (such as seasonal temperature and feeding regimen) [3], thereby containing potential confounders and ensuring greater consistency in the data collected. A further limitation is the absence of a placebo control group. This choice was dictated by ethical concerns: given that the study dealt with cows naturally affected by mastitis, it would not have been acceptable to withhold therapy and expose animals, and indirectly the farmer, to a treatment with no therapeutic purpose. Nevertheless, the use of ozonized olive oil as an active treatment is supported by a substantial body of literature, documenting its antimicrobial, anti-inflammatory, and wound-healing properties in both human and veterinary medicine [15, 11, 30]. Thus, while the present results should be interpreted as indicative rather than conclusive, the study provides a meaningful feasibility assessment and highlights the need for larger, well-powered trials to confirm and expand upon these observations.

Ethical approval

The animal study protocol was approved by the Ethics Committee for Veterinary Clinical and Zootechnical Studies of the Department of Precision and Regenerative Medicine and Jonian Area at the University of Bari (Certificate of Approval No. 2001-2 III/13). Moreover, before enrolling the cows in the study, the experimental protocol was explained to the owner of the livestock farm, who subsequently signed an informed consent form.

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Author contributions

Conceptualization, Fabrizio Iarussi and Paola Paradies; Data curation, Flora Ferri, Federica Ferulli and Mariano Francesco Caratozzolo; Formal analysis, Flora Ferri, Federica Ferulli and

Mariano Francesco Caratozzolo; Investigation, Fabrizio Iarussi, Nicola Paradiso, Valentina De Monte, Martina Calvino and Alice Carbonari; Methodology, Fabrizio Iarussi; Project administration, Fabrizio Iarussi and Paola Paradies; Software, Fabrizio Iarussi, Flora Ferri, Federica Ferulli and Mariano Francesco Caratozzolo; Supervision, Paola Paradies; Validation, Flora Ferri, Federica Ferulli and Mariano Francesco Caratozzolo; Visualization, Fabrizio Iarussi; Writing - original draft, Fabrizio Iarussi; Writing - review & editing, Fabrizio Iarussi and Paola Paradies.

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Conflict of interest

The authors declare no personal commercial or financial interests in the findings of the research. The study was conducted independently, and the organization had no influence on its design, execution, or interpretation.

Data availability statement

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

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