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In vitro antimicrobial activity of ozonated sunflower oil in milk against *Escherichia coli*: comparative study in cow, goat and sheep

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ABSTRACT
Mastitis is one of the most impacting diseases in dairy farming. Conventional treatment of mastitis using antibiotics is costly and has led to the emergence of antimicrobial resistance against most of the commonly used antibacterial agents. Research has begun to focus on molecules with antimicrobial potentials structurally different from conventional antibiotics We compared the antibacterial activity *in vitro* of ozonized sunflower oil (OSO) with different peroxide concentrations (150, 300, and 600 PI) against *E. coli* in goat, cow and sheep milk. It was found that the antibacterial effect, after 72 h, was more important for the goat's milk with OSO 150 ($p < 0.001$) and OSO 300 ($p < 0.001$). However, the effect was greater for cow's milk, when OSO 600 was used ($p < 0.001$). In the case of sheep's milk, it was observed that the antimicrobial effect was only significant with the use of OSO 600, however, this decrease in the concentration of *E. coli* ($p < 0.001$) remained practically unchanged from 24 h to 72 h of incubation. In conclusion, ozonated sunflower oil offers many therapeutic possibilities that would reduce the use of antibiotics for the prevention or treatments of mastitis, and its antimicrobial effect is greater with cow's milk and less with sheep's milk.

1. Introduction

Mastitis is one of the most impacting diseases in dairy farming. Their main cause is infections by bacteria and other microorganisms (Islas-Rodrìguez et al. 2009; Lakew et al. 2019) in addition to injuries and traumas in the udders (Megersa et al. 2010; Getaneh and Gebremedhin 2017). It is an important endemic disease that can affect all lactating animals (Ariffin et al. 2019). The most frequently found etiological agents in cows, goats and sheep suffering from infectious mastitis are staphylococci (*S. aureus*), streptococci (*S. dysgalactiae, S. uberis*) and coliform (*Escherichia coli*) (Tomasinsig et al. 2010; Ariffin et al. 2019; Giagu et al. 2022). It is estimated that approximately 10% of clinical coliform mastitis cause the death of animals (Hogan and Larry Smith 2003). The specie isolated in 80% of coliform cases is *Escherichia coli (E. coli)* (Bradley and Green 2001; Alekish et al. 2018). This microorganism usually infects the mammary glands during the dry period, progressing to inflammation and symptoms during early lactation (Balemi et al. 2021).

Controlling these infections is a long-sought ambition actually (Jingar et al. 2017). Conventional treatment of mastitis using antibiotics is costly and has led to the emergence of antimicrobial resistance (AMR) against most of the commonly used antibacterial agents (Bergonier et al. 2003; Jingar et al. 2017). The antibacterial sensitivity test results in different studies involving dairy cows, goats and sheep indicate an alarmingly increasing incidence of AMR among *S. aureus* and *E. coli* isolates against most commonly used antibacterial agents in many parts of the world (Vishnupriya et al. 2014; Jahan et al. 2015; Ismail 2017; Alekish et al. 2018) Therefore, not only AMR is regarded as a serious threat to global public health and food security, but it also increases animal suffering and production losses (Shinozuka et al. 2009; Alekish et al. 2018; de Prado-Taranilla et al. 2020). Therefore, research has begun to focus on drying off treatment protocols, vaccination and on molecules

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with antimicrobial and antiseptic potentials that are structurally different from conventional antibiotics (Whist et al. 2006; Lim et al. 2007; Sunder et al. 2013; Alekish et al. 2018; de Prado-Taranilla et al. 2020; Pollock et al. 2021; Rainard et al. 2022; Taga et al. 2012; H. Wang et al. 2022). One such molecule is ozone, which is the third most potent oxidant agent after fluor and persulfate and known for its antiseptic and anti-inflammatory properties. This molecule leads to the production of reactive oxygen species and the formation of hydrogen peroxide and lipid peroxidation products that are responsible for bacterial lysis and cell death (Grandi et al. 2022). Over the last few years, several therapeutic protocols using ozone have been developed to treat infections in human and animals. Thus, in the veterinary sector, ozone has been used due to its anti-inflammatory and antiseptic properties (Remondino and Valdenassi 2018). For example, it has been used in the prevention of epidemics from parasites in the poultry industry (da Soares et al. 2018), in urogenital tract infections (Koseman et al. 2019), in the purification of warehouses for animal feed (Conte et al. 2020; Kannan et al. 2021) in the disinfection of clinical equipment (Córdoba-Lanús et al. 2022; Torres-Mata et al. 2022); and in the prevention and treatment of mastitis also (Ogata and Nagahata 2000; Duričić et al. 2015). In these protocols, ozone is used in the following three different forms: gaseous ozone, ozonated water, and ozonated oil (Önyay et al. 2015; Fitzpatrick et al. 2018; Koseman et al. 2019; Grandi et al. 2022; Ramirez-Peña et al. 2022).

Ozonated vegetable oils are obtained after the oxidation generated by ozone to fatty acids and other substances present in vegetable oils. During this ozonation reaction, lipoperoxides, hydroperoxides, peroxides, ozonides, aldehydes and ketones are produced (Díaz et al. 2005) which, due to their high oxidant activity, affects polyunsaturated acids in the biological membranes of bacteria, molds, fungi, and viruses, while also oxidizing nucleic acids (Ayala et al. 2014; Torres-Mata et al. 2022; X. Wang et al. 2022) causing the destruction of the pathogenic agent. In recent years, ozonized sunflower oil (OSO) is one of the most used pharmaceutical presentations of ozone, which according to experimental and clinical studies, provides a large number of therapeutic effects against certain pathological agents including *E. coli* both in human and in veterinary medicine (Ginel et al. 2021; Grandi et al. 2022; Sechi et al. 2001; Zerillo et al. 2022).

Although there are several studies in which ozone therapy has been used for the treatment of mastitis (Ogata and Nagahata 2000; Ohtsuka et al. 2006; Shinozuka et al. 2009; Duričić et al. 2015; Koseman et al. 2019) some of them do not contain microbiological analysis; do not mention the concentration of ozone or time of the ozone treatment (Afonso et al. 2022). Taking into account that lipid oxidation products may exert antimicrobial and anti-inflammatory properties (Ogata and Nagahata 2000; Zamora Rodríguez et al. 2007; Skalska et al. 2009; Koseman et al. 2019) the aim of this study was to compare the antibacterial activity of OSO against *E. coli* on the three most common milk of animal origin in our geographical and socio-economic environment (goat, cow and sheep), evaluating *in vitro*, different peroxide concentrations and treatment times.

2. Materials and methods

2.1. Experimental design

Briefly in each type of milk (goat, G; cow, C; and sheep, S), the procedure was identical and consisted of evaluating the antimicrobial capacity against *E. coli*, of each concentration of OSO (PI 150, PI 300, PI 600) at different incubation times (0, 8, 12, 24, 48 and 72 h) at 37°C. Thus, each milk sample (990 µl) was inoculated with 1 µl of the *E. coli* EBETAN-1 suspension, as described in the corresponding section on material and methods. In addition, 10 µl of OSO was added to each sample, of the corresponding concentration according to the study group.

There is not much scientific bibliography regarding this matter, and aspects such as microbial analysis, dosage, volumes, or excipients of ozonized products, nor the action times, are usually not well explained or standardized. Thus, and calculating a volume of milk in the udder after milking of 200 mL for sheep and goats and about 400 mL for cows (Davis and Reinemann 2001; Cording et al. 2013; Meyer et al. 2022), we simulate the application of an intramammary cannula inside the udder of a cow, goat and sheep with a proportionally similar volume (1–2%) to that is used routinely in commercial presentations of intramammary cannulas for the treatment of mastitis in dairy cattle (Johns et al. 2001; Gonzalo et al. 2009; Bradley et al. 2010), avoiding the effect of dilution of the bacterial load that could be caused with larger volumes of product. Next, we decided to assess the effectiveness against a unique microorganism such as *E. coli*, which has been referred as one of the most common pathogens in mastitis in dairy cattle (Alekish et al. 2018; Ariffin et al. 2019; H. Wang et al. 2022). Finally, we focus on the study of antibacterial efficacy in the first 72 h.

As a summary, in each type of milk, the different concentrations of OSO were evaluated at six different times and each times was evaluated with eight samples. In summary, the total number of samples in the study was 576 (192 samples/ type of milk).

Figure 1 summarizes the experimental design.

2.2. Preparation of milk

Goat and sheep milk were obtained from local farms. After milking they were kept at 4°C until use, while a commercial brand (Asturiana®) was used for the cow's milk group. At the beginning of each test, the milk was pasteurized in a water bath, guaranteeing 75°C, in the centre of the sample, for 15 min.

2.3. Preparation of ozonated sunflower oil (OSO)

Standardization of the preparation of OSO was carried out according to peroxide index (PI), which indicates the quantity of peroxide (active oxygen) available per kilogram of OSO (mmol/kg).

Ozonized sunflower oil (OSO) was commercially provided by Laboratorio Pérez del Toro (Gran Canaria, Spain) produced using a portable medical ozone generator (Ozonobaric P®, Sedecal, Madrid, Spain), and medical grade O_2, obtaining three different concentrations of O_3/sunflower oil: 150, 300
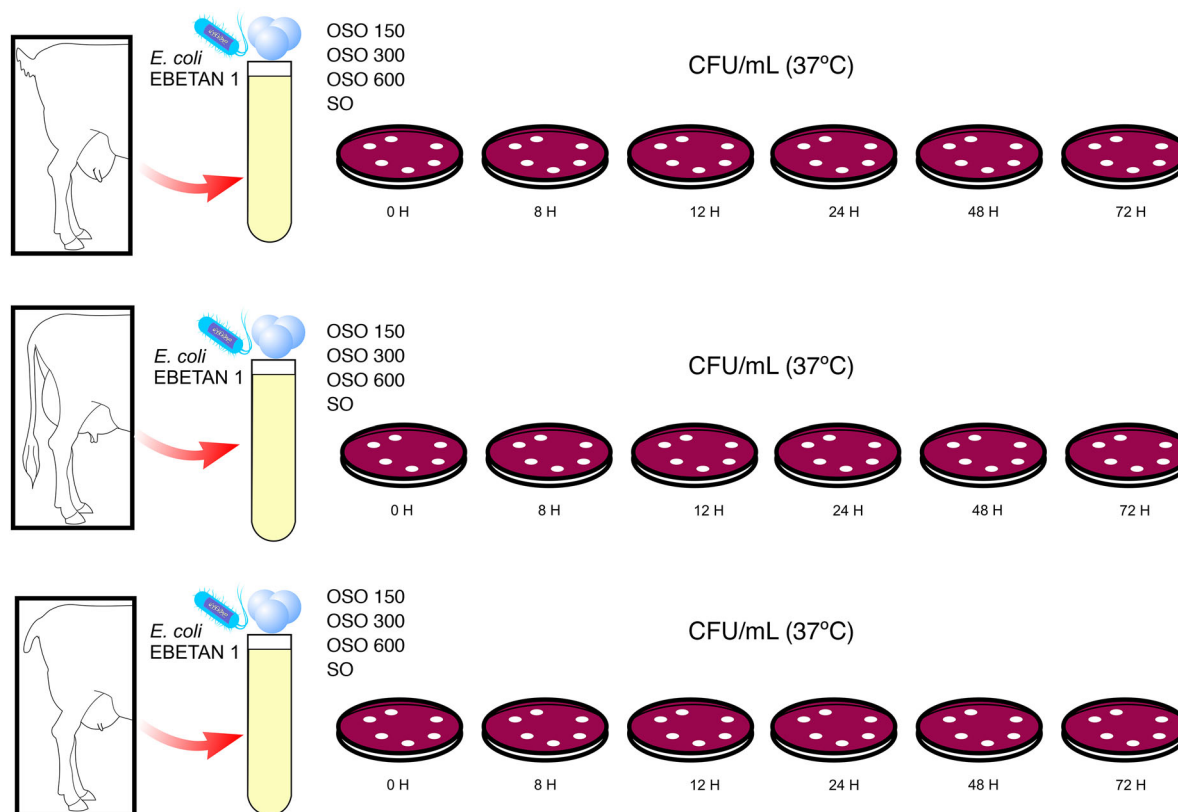
Figure 1. Illustration of the experimental design used in this study and timeline. Goat, cow and sheep milk (G, C and S groups respectively) inoculated with *E. coli* EBETAN-1 HUGCDN. In each subgroup (*n* = 12), resulting from the combination of types of milk (G, C and S) with different types of ozonated sunflower oil (OSO150, OSO 300 and OSO 600) or not- ozonated sunflower oil (SO) the colony-forming units of *E. coli* EBETAN-1 HUGCDN per mL of milk (CFU/mL), at 0, 8, 12, 24, 48 and 72H incubation at 37°C were calculated.

and 600 PI, (OSO 150, OSO 300 and OSO 600) equivalent to 150, 300 and 600 mmol/mL, respectively. Room temperature (22.0–23.7°C) and relative humidity (53–65%) were controlled by the air conditioning system of the Hospital de Gran Canaria Dr. Negrín. OSO was kept refrigerated at 4°C, and protected from light until used within 30 days of preparation. Non-ozonated sunflower oil (SO) was used as a control.

2.4. E. coli *inocula preparation*

The *E. coli* strain used in this study was isolated from intestinal microbiota of healthy Sprague Dawley rats from the animal facility of the Hospital Universitario de Gran Canaria, Dr. Negrín. The MALDI-TOF MS (Vitek®MS, Biomerieux) technique (Bizzini and Greub 2010) was used for the strain identification. The strain was designated as *E. coli* EBETAN-1 HUGCDN (Gutiérrez-Falcón et al. 2021). *E. coli* strain cultures were stored at −80°C with 20% glycerol (v/v) addition in Brain Heart Infusion broth (PanReac-AppliChem). Fresh cultures were made prior the assays, and the strain was aerobically incubated in Trypticase Soy broth (Becton Dickinson) medium for 18 h at 37°C with shaking (120 rpm). Before the challenge, the bacteria were centrifuged at 2500×g for 10 min and washed three times with sterile 0.9% saline solution. The bacterial concentration was measured with a spectrometer at 600 nm. Sterile 0.9% saline solution was used to adjust the suspension to the desired bacterial concentration (5×10^8 CFU/mL).

2.5. E. coli *counts in milk*

To determine the survival capability of *E. coli* EBETAN-1 HUGCDN in milk, the number of colony-forming units per mL of milk (CFU/mL) was measured. Milk samples were inoculated with the amount described in the experimental design section and the corresponding amount of OSO was added, according to the experimental group. To examine bacterial loads, serial tenfold dilutions were made in 0.9% sterile saline and plated on McConkey Agar (Beckton Dickinson). After incubation at 37°C for 0, 8, 12 24, 48 and 72 h, we calculated total viable counts of original samples (CFU/mL milk). Colonies were separated and isolated 2–3 times. We identified bacterial species using colony morphology and Gram stain. MALDI-TOF MS (Vitek®MS, Biomerieux) technique (Bizzini and Greub 2010)was used for advanced identification.

2.6. Statistical analysis

Analyses were done using Statistical Package R Core Team 2022 version 4.2 (R Foundation for Statistical Computing, Vienna, Austria). The Kolmogorov–Smirnov test has been used to check the normality of the data. Quantitative variables are expressed as medians and 25th and 75th percentiles. To calculate the differences between incubation times and types of milk with OSO, the linear model of repeated measures with interaction has been used. Data are expressed as median. All tests were two-tailed and statistical significance was considered at $p < 0.05$.

Table 1. Minimum (Min), maximum (Max), 25th, 50th and 75th percentiles (P25, P50 and P75, respectively) of the number of colony-forming units of *E. coli* EBETAN-1 HUGCDN per mL of milk (CFU/mL) during 0, 8, 12, 24, 48 and 72 h of incubation (0H, 8H, 12H, 24H, 48H and 72H respectively).

	G-0H	G-8H	G-12H	G-24H	G-48H	G-72H	S-0H	S-8H	S-12H	S-24H	S-48H	S-72H	C-0H	C-8H	C-12H	C-24H	C-48H	C-72H
BD (Control)																		
Min	4.6	8.21	11.2	15.62	15.68	10.3	5.15	8.78	11.3	13.34	14.78	13.75	5.08	12.25	15.75	14.64	15.57	16.61
P25	5.15	8.66	11.31	15.88	15.73	14.3	5.27	10.41	11.48	13.95	15.13	15.2	5.23	12.39	15.83	14.96	16.1	20.41
P50	5.15	8.9	12.3	16.64	15.94	14.3	5.3	10.6	11.7	14.08	15.32	15.7	5.26	12.64	15.9	15.08	16.73	20.67
P75	5.16	9.02	12.6	16.73	16.06	14.38	5.78	10.65	11.98	14.13	15.45	15.94	5.38	12.67	15.97	15.17	17	21
Max	5.3	9.08	13.26	17.03	16.18	14.78	5.9	11.08	13.08	15.15	15.56	16.16	5.53	12.72	15.99	15.48	17.33	21.34
PI OSO 150																		
Min	4.78	8.25	10.3	13.3	10.78	8.6	4.73	9.58	10.14	11.41	11.58	13.2	4.64	9.3	10.3	11	15.08	15
P25	4.87	8.3	10.9	14.52	12.3	9.04	4.91	9.9	10.21	11.64	11.71	14.52	4.75	9.3	10.73	11.26	15.52	15.56
P50	4.9	8.4	11	14.87	12.3	9.08	5.05	10.45	10.22	11.7	14.89	14.9	4.83	9.3	10.85	11.39	15.75	15.76
P75	5.06	8.6	11.12	14.97	12.38	9.14	5.33	10.78	10.4	11.7	15.12	15.43	4.88	9.3	11.37	11.51	15.99	16.03
Max	5.26	9.15	11.26	15	12.78	10.9	5.78	11.15	13.15	11.76	15.6	15.81	4.91	11.3	14.6	11.62	16.27	17.3
PI OSO 300																		
Min	4.3	8.3	10.9	13.3	10.3	8.6	4.68	10.3	9.78	11.64	11.26	13.2	4.66	9.3	10.3	11.15	15.2	14.9
P25	4.73	8.3	10.98	13.56	10.73	8.78	5.01	10.66	10.26	11.69	14.35	13.86	4.72	9.6	10.73	11.56	15.59	15.61
P50	4.9	8.6	11.15	14.34	10.9	9	5.04	10.9	11.38	11.78	14.99	14.51	4.76	9.6	11	11.87	16.03	15.79
P75	5	8.81	11.26	15.02	11.16	9.1	5.12	11.22	12.43	11.86	15.12	15.24	4.83	9.6	11.45	12.61	16.26	16.14
Max	5.3	9.38	11.56	15.03	12.3	9.2	5.52	11.51	13.7	12.06	15.38	16.15	4.89	13.3	12.3	15	16.67	16.3
PI OSO 600																		
Min	4.9	6.6	10.78	12.6	10.3	9.2	4.91	10.3	10.07	11.15	11.34	11.2	4.48	7.83	10.6	11	15.66	0
P25	4.98	6.83	10.87	13.76	10.53	10.3	5	10.66	10.13	11.53	11.46	11.59	4.58	8.08	10.87	11.37	15.94	0
P50	5.08	7.35	10.9	14.2	10.6	10.6	5.28	10.9	10.25	11.62	11.56	11.74	4.66	8.26	10.9	11.53	16.14	2.3
P75	5.23	8.16	10.9	14.42	11.96	10.9	5.38	11.05	10.75	11.64	11.65	11.93	4.8	8.32	10.93	11.59	16.29	2.38
Max	5.34	8.3	11.68	14.86	13.38	11.34	5.78	11.2	11.92	11.78	11.79	12.05	5.02	8.35	11.51	11.75	16.63	2.78

Peroxide Index (PI), Goat's milk (G), Sheep's milk (S), Cow's milk (C).
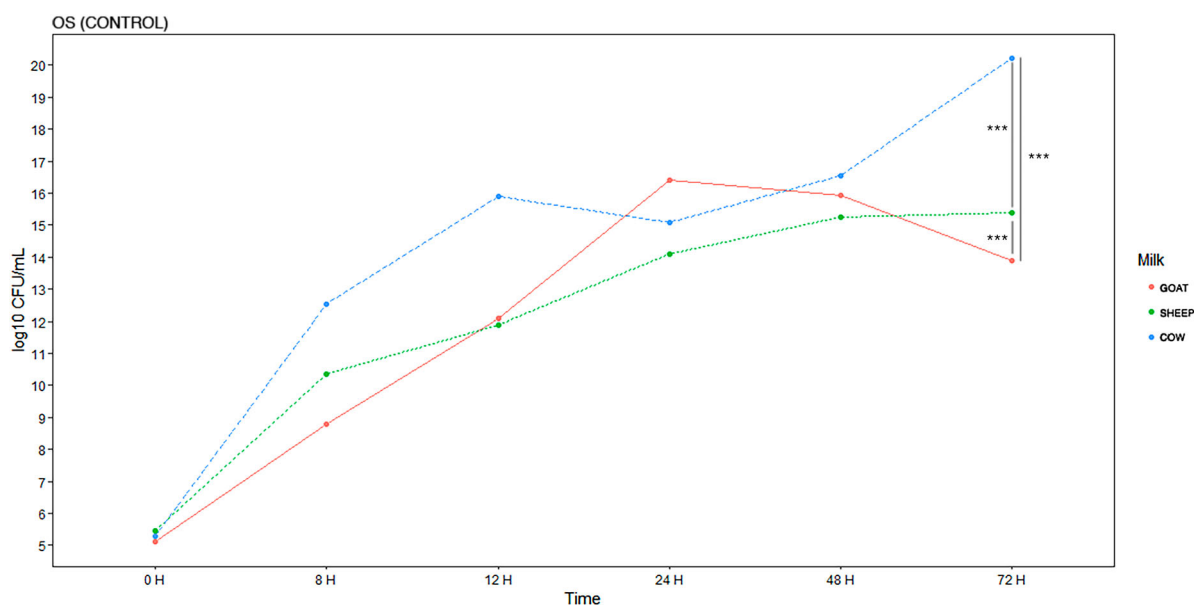Note: Date is expressed as log10 CFU/mL.

Figure 2. *E. coli* EBETAN-1 HUGCDN concentration (log10 CFU/mL) in cow's milk, goat's milk and sheep's milk containing non-ozonated sunflower oil (SO) during the first 72 h of incubation at 37°C. Data shown as median. Significant difference ***($p < 0.001$).

3. Results

The number of colony-forming units of *E. coli* EBETAN-1 HUGCDN per mL of milk, according to type of animal species, type of OSO and incubation time are shown in Table 1.

3.1. Antimicrobial effect depending on the type of OSO

The concentrations of colony-forming units over time, depending on the type of OSO used, are shown in Figures 2–5. The first result that we can highlight is that the concentration of *E. coli*

EBETAN-1 HUGCDN continues to increase in cow's milk, reaches the plateau of the curve in sheep's milk, and decreases in goat's milk after 72 h of incubation at 37°C.

Comparing with the normal evolution of the bacterial load in the milk samples treated with SO, it was found that the antibacterial effect, on the studied strain, after 72 h of incubation, is more important for the goat milk with OSO 150 (14.3 vs 9.08 log10 CFU/mL, $p < 0.001$) and OSO 300 (14.3 vs 9 log10 CFU/mL, $p < 0.001$). However, the effect was greater for cow's milk, when OSO 600 was used (20.67 vs 2.3 log10 CFU/mL, $p < 0.001$).
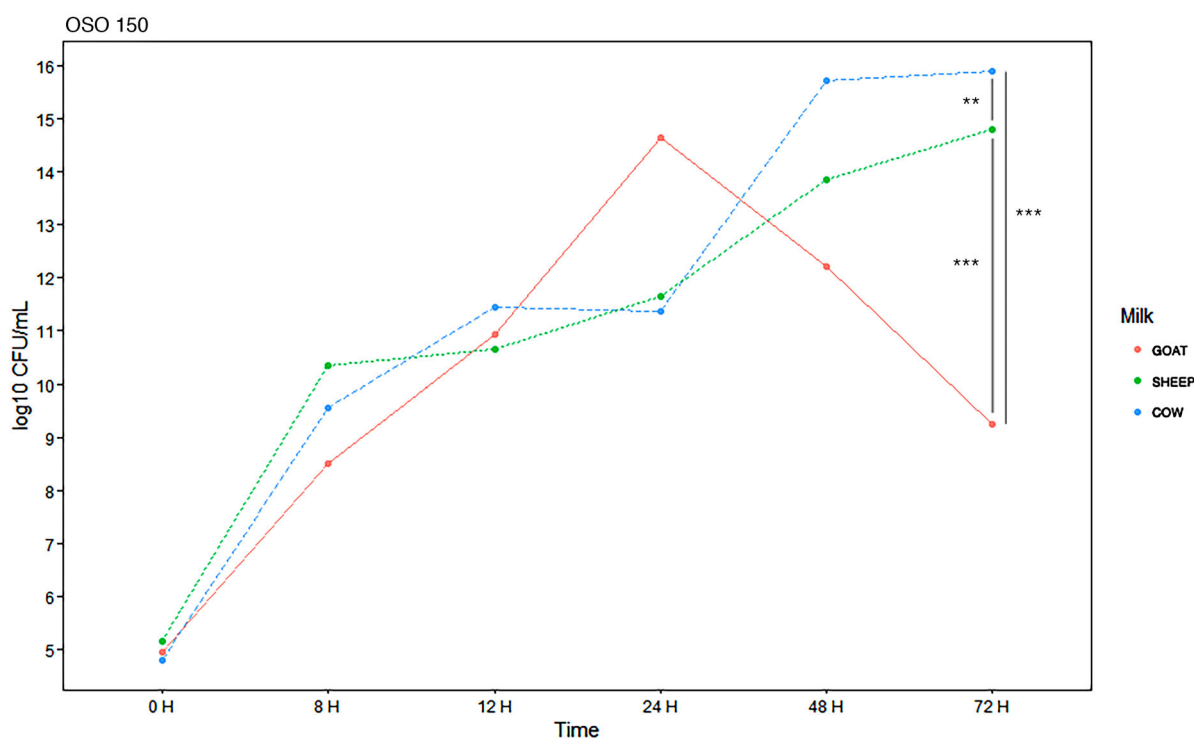

Figure 3. *E. coli* EBETAN-1 HUGCDN concentration (log10 CFU/mL) in cow's milk, goat's milk and sheep's milk containing ozonated sunflower oil 150 PI (OSO 150) during the first 72 h of incubation at 37°C. Data shown as median. Significant difference **($0.001 > p < 0.01$) and ***($p < 0.001$).
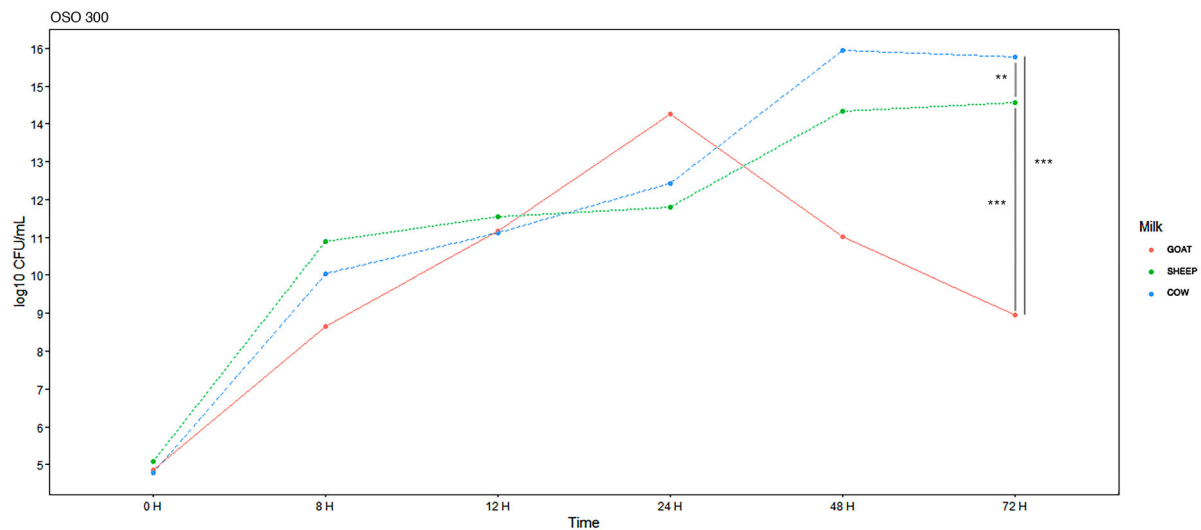
Figure 4. *E. coli* EBETAN-1 HUGCDN concentration (log10 CFU/mL) in cow's milk, goat's milk and sheep's milk containing ozonated sunflower oil 300 PI (OSO 300) during the first 72 h of incubation at 37°C. Data shown as median. Significant difference **(0.001 > p < 0.01) and ***(p < 0.001).

3.2. Antimicrobial effect depending on the animal origin of the milk

The concentrations of colony-forming units over time, depending on the type of milk used, are shown in Figures 6–8. When we analysed the values in cow's milk, it was observed that the greatest difference appeared at 72 h, when using OSO 600, the bacterial population significantly decreased to concentration below 3 log10 CFU/mL (p < 0.05), while the groups with OSO 150 and OSO 300, maintained similar values (15.76 and 15.75 log10 CFU/mL respectively).

In the case of goat milk, decreases in bacterial concentration began to be noted after 24 h, regardless of the type of OSO used. We observed the greatest effect at 72 h with the use of OSO 150 or OSO 300 in a very similar way.

Finally, in the case of sheep's milk, it was observed that the antimicrobial effect was only significant with the use of OSO 600, however, this decrease in the concentration of *E. coli* EBETAN-1 HUGCDN (0.001 > p < 0.01) remained practically unchanged from 24 h to 72 h of milk incubation (11.62–11.74 log10 CFU/mL).
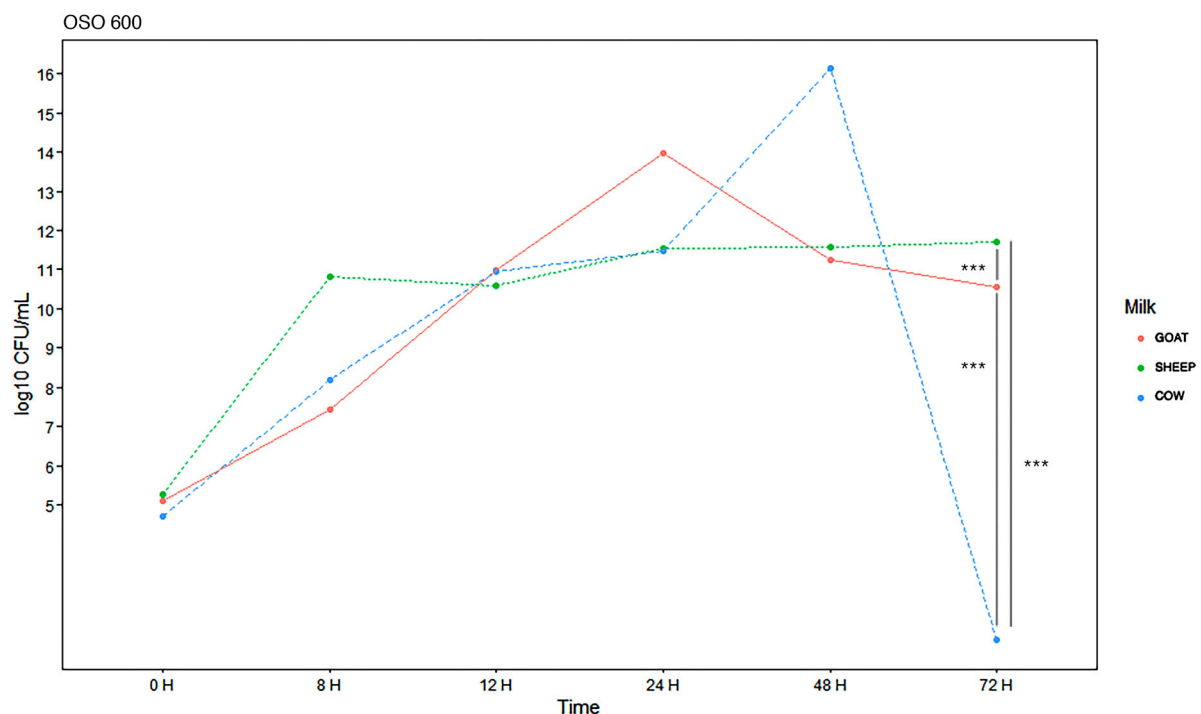

Figure 5. *E. coli* EBETAN-1 HUGCDN concentration (log10 CFU/mL) in cow's milk, goat's milk and sheep's milk containing ozonated sunflower oil 600 PI (OSO 600) during the first 72 h of incubation at 37°C. Data shown as median. Significant difference ***(p < 0.001).
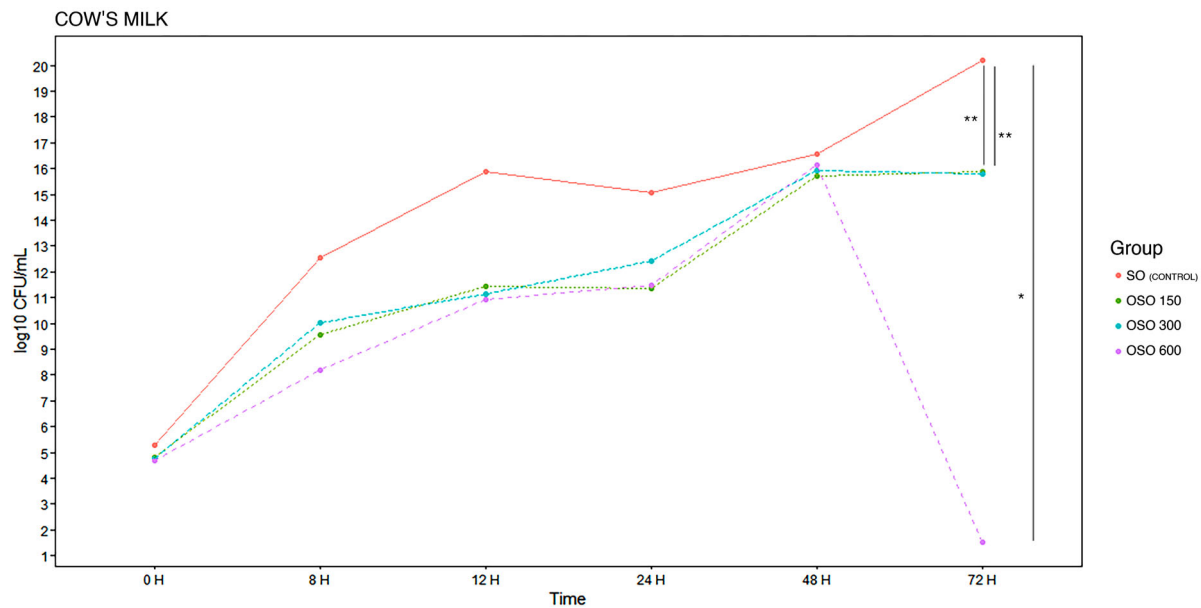
COW'S MILK

Figure 6. *E. coli* EBETAN-1 HUGCDN reduction (log10 CFU/mL) of cow's milk containing non-ozonated sunflower oil (SO), and ozonated sunflower oil with three different concentrations of peroxides (OSO 150, OSO 300 and OSO 600) during the first 72 h of incubation at 37°C. Data shown as median. Significant difference * ($p < 0.05$), and **($0.001 > p < 0.01$).
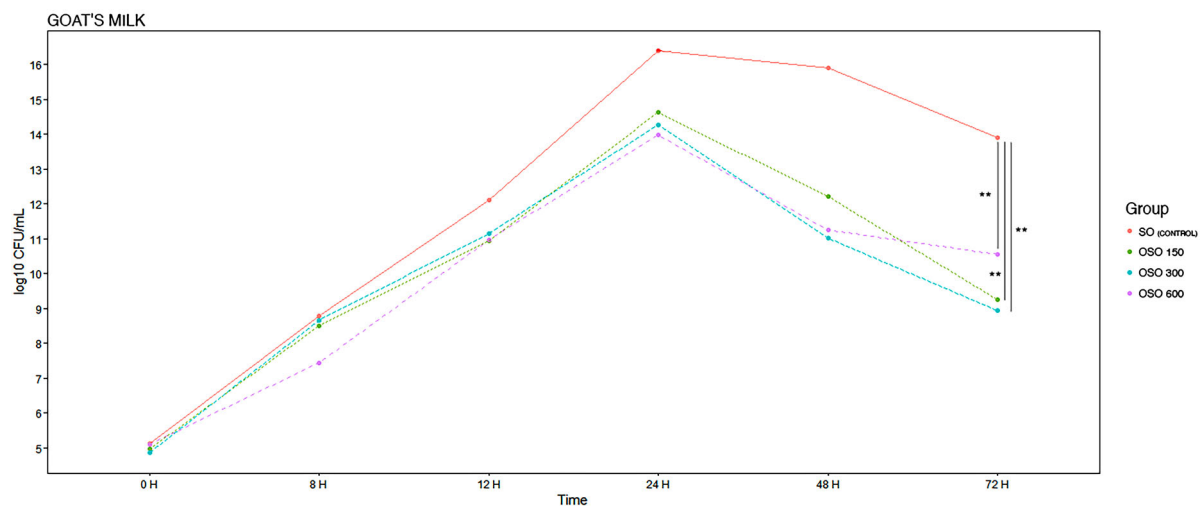
GOAT'S MILK

Figure 7. *E. coli* EBETAN-1 HUGCDN reduction (log10 CFU/mL) of goat's milk containing non-ozonated sunflower oil (SO), and ozonated sunflower oil with three different concentrations of peroxides (OSO 150, OSO 300 and OSO 600) during the first 72 h of incubation at 37°C. Data shown as median. Significant difference **($0.001 > p < 0.01$).

4. Discussion

In general, when the antimicrobial capacity of the different concentrations of OSO on the *E. coli* strain used was evaluated, we could to observe a clear germicidal effect dependent on the concentration of OSO and on the time of action. Several authors have demonstrated the efficacy of ozone's oxidative potential on bacteria or fungi, such as *Staphylococcus aureus* (Epelle et al. 2022), *Klebsiella pneumoniae* (Piletić et al. 2022), *E. coli* and *Pseudomonas aeruginosa* (Baghal Asghari et al. 2021), *Aspergillus fumigatus*, and *Candida albicans* (Epelle et al. 2022). In addition, other authors have indicated that Gram-negative bacteria, such as *E. coli*, are generally more sensitive to ozone than Gram-positive bacteria due to the lower amount of peptidoglycan in their cell walls and its protective

role against peroxidation of the cell membrane caused by reactive oxygen species (Rangel et al. 2022).

To the best of our knowledge, no studies in the literature investigate the time required for action of ozone or ozone-containing preparations to achieve an optimal antimicrobial effect in milk. There are studies in cows, in which it is shown that the intramammary application of foam at two different moments of the lactation period, offers better results on the concentration of *E. coli*, and somatic cell counting than if it is applied only once (Koseman et al. 2019). These results partially agree with ours, since the best antimicrobial effects were obtained with the longer OSO action times. However, we must be careful in our conclusions since the ozone-containing foam preparation used by these authors contained a mixture of ozone, olive oil,
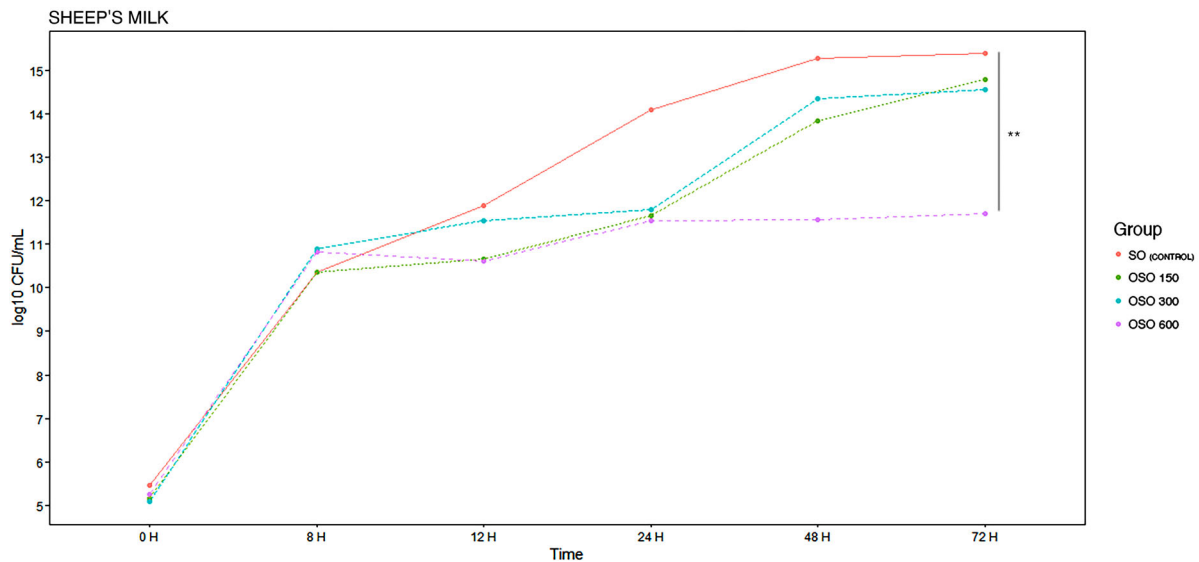
Figure 8. *E. coli* EBETAN-1 HUGCDN reduction (log10 CFU/mL) of sheep's milk containing non-ozonated sunflower oil (SO), and ozonated sunflower oil with three different concentrations of peroxides (OSO 150, OSO 300 and OSO 600) during the first 72 h of incubation at 37°C. Data shown as median. Significant difference **(0.001 > p < 0.01).

glycerin, propylene glycol and various caring oils. Ozone has a half-life of approximately 20 min in the gaseous phase, which has restricted some applications before low-concentration exposures for prolonged periods, with limited effectiveness (Rangel et al. 2022; Torres-Mata et al. 2022). For this reason, we believe that the application of ozone in the form of ozonated oil represents a therapeutic advantage and would explain that after 72 h of being added to milk, the antimicrobial effect was maintained.

In a study which the capacity of ozone against bacteria such as *S. aureus*, *E. coli* and *Bacillus stearothermophilus* was evaluated in different types of substrate (sterile buffer solution, whipping cream, locust bean gum solution, starch solution soluble and sodium caseinate solution) the authors concluded that the cream and sodium caseinate solution promoted a greater protective effect of the bacteria, hindering the action of ozone (Güzel-Seydim et al. 2004). Furthermore, the amount of fat can also interfere with the solubility of this gas in liquid media. In a study carried out with many different samples of milk exposed to ozone in different presentations, the best results in reducing the microorganism count, were obtained from samples that contained low levels of fat (Afonso et al. 2022). In another study whose object of analysis was orange juice, it was verified that organic matter exerts a negative effect on the oxidative capacity of ozone on the inoculated bacteria, in this case, *E. coli* (Patil et al. 2009). Our results could be explained by this fact, since the highest concentrations of bacteria at 72 h were in sheep's milk, followed by goat's milk and finally cow's milk, which corresponds to the order of higher to lower organic matter (Moatsou and Park 2017). Therefore, the amount of organic matter found in sheep's milk could affect the bactericidal capacity of OSO, giving significant results only in the group in which the highest concentration was used (OSO 600).

On the other hand, we must not forget that ozone is a powerful oxidizing agent with a potential toxic effect on animal cells. In this way, there are several studies that warn about this characteristic (Córdoba-Lanús et al. 2022; Rangel et al. 2022), even finding *in vivo* studies on infectious models that show that rectal pre-treatment with ozonized oxygen aggravates clinic status in septic rats treated with amoxicillin/clavulanate (Martín-Barrasa et al. 2015).

To conclude, we must indicate that the greatest strength of this study is that it is a work with a powerful statistical evaluation of three types of milk with different standardized ozone concentrations, using an excipient widely used in the formulation of commercial presentations of intramammary drugs, and its effectiveness is evaluated in defined and controlled times.

Although the use of ozonated sunflower oil offers many therapeutic possibilities that would reduce the use of antibiotics for the prevention or treatments of mastitis, new studies are necessary to evaluate the effect of this gas on other common pathogens in mastitis. In addition, *in vitro* tests on bactericidal effects or glandular toxicity must be complemented with *in vivo* studies that provide certainty about the use of suboptimal, therapeutic or excessive doses of this powerful antiseptic, and its impact on milk production, toxicity, residues in milk or meat and/or organoleptic alterations in foods of animal origin.

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