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Natural products for the prevention of infectious diseases and for the mitigation of the environmental impact of livestock

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Abstract

In recent years, world livestock production has increased constantly, mainly due to an increased demand for animal-derived products for human consumption. At the same time, however, some concerns have emerged, such as the growing resistance to antibiotics and the increase in greenhouse gas emissions associated with intensive livestock farming. One of the most explored approaches in literature, both as an alternative to antimicrobials and for the reduction of emissions, particularly methane, is the use of plant-derived compound. In animal nutrition, the use of active compounds of plant sub-products, especially in free form, is often limited by their reduced bioavailability in the gastrointestinal tract as well as by their negative effect on digestibility when used at incorrect doses. Considering this, the aim of this study was to identify the most promising active compound of plant origin and to evaluate its encapsulation in a nanoliposome system to reduce its toxicity and increase its activity.

A screening was performed to assess the minimum inhibitory concentration (MIC) of the following Nature Identical Compounds (NICs): nerolidol, geraniol, D-carvone, β-caryophyllene, α-pinene, p-cimene, eucalyptol, β-pinene, R-limonene, curcumin and quercetin. The results indicated that nerolidol, geraniol and curcumin had the highest antimicrobial activity. On the other hand, the evaluation of their cytotoxicity (on MDBK line cell) and hemolytic activity (on defibrinated sheep blood) revealed that they were the most toxic compounds. Taking this into account, as well as the intrinsic characteristics of the different compounds, the project proceeded with the encapsulation of nerolidol. In particular, the nanoliposome suspension used to encapsulate nerolidol had a concentration of 5000 μg/mL; but a loading ratio of 1% was employed, meaning that nerolidol represented 1% of the total nanoliposome concentration (5 g/L). The antimicrobial activity of Unloaded nanoliposomes (UN), Loaded nanoliposomes (LN), and free-form Nerolidol was assessed using the Time-kill method. Experimental time points corresponded to the average retention times in different gastrointestinal segments of monogastric animals: 2, 4, 6, and 24 h. Results confirmed that free nerolidol retained its MIC results, showing activity only against Gram-positive bacteria, particularly *Enterococcus faecium*, even at low

concentrations, at each point. Nanoliposome formulations, however, demonstrated activity against all tested bacteria. Both LN and UN showed time- and dose-dependent antimicrobial activity with similar activity at the highest tested concentrations against *S.* Typhimurium (at 24 hours) and against MRSA and *E. coli* (at 6 hours). Overall, these results suggest that the nanoliposome carrier possesses antimicrobial activity itself, probably due to the membrane fusion mechanism. Furthermore, the inclusion of Nerolidol does not increase the antimicrobial activity of the formulation, probably due to the limited amount of Nerolidol loaded. The cytotoxicity of UN and LN were assessed using the same methods used for screening, but in addition their effect on the IPEC-J2 cell line was evaluated. Both LN and UN exhibited dose-dependent cytotoxicity, with IPEC-J2 cells being more sensitive. In addition, LN demonstrated higher toxicity than the UN; due to the presence of Nerolidol. Consequently, considering all results, both LN and UN showed interesting antimicrobial activity, in most cases with similar activity.

The third part of the study focused on the use of LN and UN at a theoretically equivalent *in vivo* dose of 100 g per 100 kg, corresponding to 0.1% of the substrate, to evaluate their influence on *in vitro* nutrients digestibility. The digestibility of dry matter (DMD), crude protein (CPD), and neutral detergent fibre (NDFD) were assessed using digestibility methodology specific to bovine and swine. In particular, DMD and CPD were assessed at the end of each digestion step for both bovine and swine. A multienzymes system was used for both digestibility methodology; additionally, for the bovine, the rumen fluid was used as inoculum for the ruminal digestion. Results showed no differences in DMD and CPD for either model. Furthermore, The LN depressed the NDFD at 48 h of fermentation, indicating a possible reduction in the methane production. Conversely, for the determination of NDFD in swine model, an inoculum of cecal liquid was used for the last step of *in vitro* fermentation, but no differences were observed between LN and UN.

In conclusion, free-form Nerolidol emerged as the plant-derived compound with the most promising antimicrobial properties, though limited to Gram-positive bacteria, but with high cytotoxicity. Encapsulation within a nanoliposome system at 1% of a 5000 μg/mL concentration resulted in a formulation with broad-spectrum antimicrobial activity, very similar to UN, especially after 6 hours.

However, the presence of Nerolidol increased cytotoxicity in the LN formulation. Both formulations did not negatively impact the DMD or CPD in bovine or swine, although a reduction in NDFD was observed at 48 hours in the bovine by LN.

The LN formulation has interesting antimicrobial and anti-methanogenic effects, without affecting feeds digestibility. However, further *in vivo* studies are necessary to evaluate the practical applications of these products in livestock systems.

State of Art

Health and Environmental issues

In recent years, world livestock production has grown constantly (FAOSTAT, 2024), and the excessive focus on productivity in the livestock system has impacted not only on animal health, but also on human health and on the environment (Sakadevan and Nguyen, 2017; Zhang et al., 2024). To address these issues, a holistic approach known as "One Health" has gained prominence in recent years within livestock production. This approach recognizes the interconnected nature of human, animal, and environmental health and aims to ensure a more sustainable production system. The One Health framework advocates for practices that not only meet consumer demand but also do so responsibly, with a commitment to minimizing ecological impacts and safeguarding public health and the environment (Destoumieux-Garzón et al., 2018; Zhang et al., 2024).

Antimicrobials in livestock production

Antimicrobial resistance (AMR) is a global problem involving both veterinary and human medicine, and the environment. As a result of drug resistance, antibiotics and antimicrobial medicines become ineffective and infections become difficult or impossible to treat, increasing the risk of disease spread, with increased frequency of severe illness, disability and death (World Health Organization, 2022). The spread of AMR among farm animals is therefore also a problem for humans, as several of the pathogenic bacteria in animals are ubiquitous in particular, the use of antibiotics in intensive animal husbandry is one of the main factors contributing negatively to the development of AMR (Haulisah et al., 2021). In fact, in the past, they have been used in livestock farming mainly for therapeutic use but also to prevent (prophylaxis) or control (metaphylaxis) the spread of bacterial infectious diseases (Brown et al., 2017; Callaway et al., 2021). Regarding therapeutic treatment, livestock may be treated by individual treatment (e.g. dairy cattle, beef cattle, sheep, goats) or through mass medication (e.g. poultry and fish) (Mcewen and Fedorka-Cray, 2002). Metaphylaxis typically involve administering drugs at therapeutic levels for short periods of time. It is a form of mass medication after the diagnosis

of a clinical disease in one part of the group, in order to treat clinically affected animals and to control the transmission of the disease to animals in closer contact and in danger and that may already be sub-clinically infected.

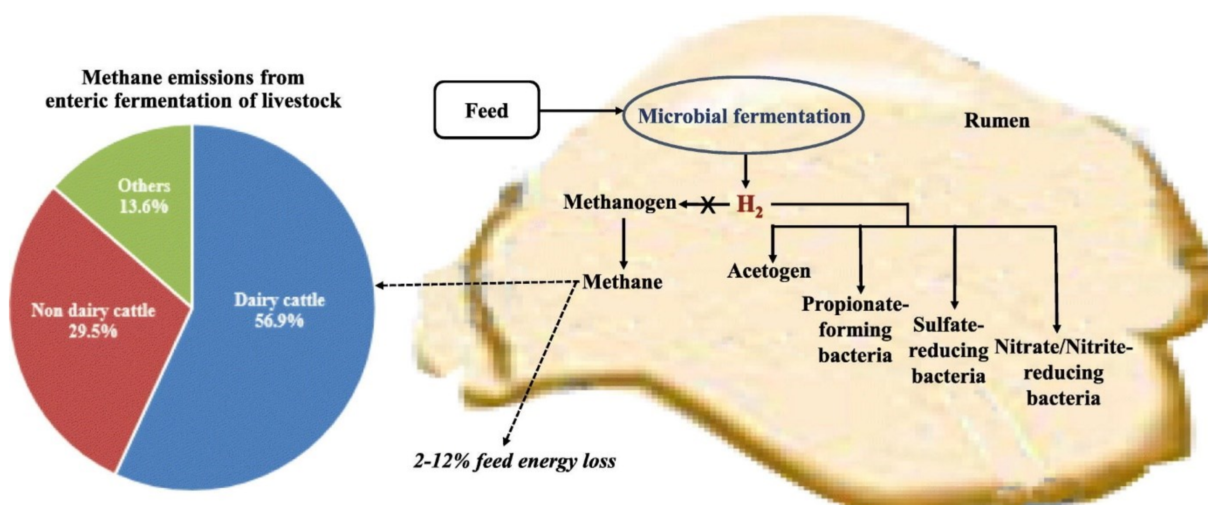
A widespread practice in the past, due to the absence of legislative restrictions, was to use antimicrobials at sub-therapeutic doses in livestock in order to improve their production performance (Mcewen and Fedorka-Cray, 2002; Callaway et al., 2021; Haulisah et al., 2021). Historically, the growth promoter effect of antibiotics was revealed in the 1940s; when an animal fed with dried mycelia of *Streptomyces aureofaciens* containing chlortetracycline residues enhanced their growth (Ramesh C. Gupta et al., 2019). This observation has led to the widespread use of antibiotics not only for therapeutic purposes, but also as additives to increase growth and improve the performance of livestock (Mcewen and Fedorka-Cray, 2002). By the 1950s and 1960s, a variety of antibiotic compounds, including tetracyclines, penicillin, and macrolides, were being used as antimicrobial-growth promoters in poultry, swine, and cattle production. Most of the antimicrobial-growth promoter registered for use in Australia, EU, and USA are antimicrobial agents used in human medicine (e.g. tetracyclines, penicillin, aminoglycosides (Brown et al., 2017). An antimicrobial can be considered an "Antibiotic growth promoter" (AGP) when it is administered at sub-therapeutic doses to farm animals to promote growth and improve feed efficiency; depending on the drug and the animal, the dosage is between 2.5 and 125 mg/kg (Mcewen and Fedorka-Cray, 2002). The economic and health-related benefits of AGP in animal husbandry have been well-documented over the past several decades; in particular these antibiotics, when administered in subtherapeutic doses, were found to improve feed conversion rates and reduce mortality, making livestock farming more economically viable, particularly in intensive poultry and swine production systems (Butaye et al., 2003; Dibner and Richards, 2005). Additionally, the routine use of AGP also reduces the need for therapeutic antibiotic treatments, as animals are less likely to develop infections. For example, chlortetracycline, penicillin and bacitracin were approved for growth promotion and feed efficiency in broilers, turkeys and egg layers (Mcewen and Fedorka-Cray, 2002).

Despite the advantages of using AGPs in livestock production, the routine use of antibiotics at subtherapeutic levels in animal feeds created selective pressure that promoted the proliferation of antibiotic-resistant bacteria contributing to the rising incidence of antibiotic-resistant infections in human medicine (e.g. *S. aureus*, *E.coli*, *Enterococcus faecium* and *Salmonella enterica* serovar Typhimurium). Taking these factors into account, many countries have introduced regulations to limit the use of AGPs in livestock production. Particularly, the European Union (EU) took a leading role in this effort, banning the use of antibiotics as growth promoters in animal feed starting in 2006 (Regulation 1831/2003/EC). Regulatory restrictions on AGPs have encouraged research into alternative strategies to maintain animal health and efficiency without using antibiotics. Alternatives to AGPs include probiotics, prebiotics, organic acids, enzymes, and phytogenic compounds, which support gut health and enhance growth in livestock (Ramesh C. Gupta et al., 2019).

Environmental Issue

In recent years, one of the most important environmental issues has been the rise in earth surface temperature, attributed to the increased rate of Greenhouse Gas (GHG) emissions into the atmosphere (Yusuf et al., 2014). Greenhouse gas emissions seem to derive from five sectors: energy systems, industry, construction, transport and AFOLU (Agriculture, Forestry, and Other Land Uses). Within the last sector, the emissions are split into two major components: methane (CH_4) and nitrous oxide (N_2O-Lamb et al., 2021). Methane is second to CO_2 for contributing to total GHG emission, and its main source is related to animal husbandry. The production of CH_4 in the livestock is physiological and is related to the methanogenic bacteria fermentative activity in the rumen and large intestine (Yusuf et al., 2014; Lamb et al., 2021).

Figure 1. Ruminant fermentations (Lan and Yang, 2019)



Enteric CH_4 Emission in Livestock production

Every year, approximately 80 million tonnes of CH_4 are produced by ruminant livestock production systems, accounting for about 28% of global anthropogenic CH_4 emissions. The majority of CH_4 emissions from dairy cows come from enteric fermentation as a by-product of anaerobic fermentation in the rumen (87% of the total) carried out by the ruminal microflora in order to digest feed for energy for the animal (Shibata and Terada, 2010; Zhao et al., 2020). The rumen can be considered a vast

habitat, housing a complex community of microorganisms including ciliated protozoa, anaerobic fungi, anaerobic bacteria, and archaea. Bacteria typically represent the most prevalent component of microbial biomass. Methanogenesis in the rumen follows two primary pathways, both carried out by archaea. Specifically, the hydrogenotrophic pathway converts H_2 and CO_2, produced by protozoa, bacteria, and fungi, into CH_4. The second type of substrate involves the methyl group, which can be present in methylamines and methanol; methylamines originate from glycine betaine and choline, while methanol derives from the hydrolysis of plant polysaccharides. The main genera of methanogenic archaea involved in these pathways include *Methanobrevibacter*, *Methanosphaera*, and *Methanimicrococcus* (hydrogenotrophic), as well as *Methanosarcinales* (methylotrophic). Several studies indicate that there is no strong correlation between the number of methanogenic archaea and CH_4 production, and when a correlation does exist, it tends to be weak. Methane production in the rumen is indirectly associated with protozoa, bacteria, and fungi. Specifically, within ruminal bacteria, various types exist that can utilize fibers, starch, proteins, and sugars. Overall, CH_4 emissions depend on the abundance of bacteria capable of producing H_2. Among fiber-degrading bacteria, cellulolytic species like *Ruminococcus* and certain *Eubacterium* spp. are known for their ability to produce H_2, whereas other cellulolytic bacteria, such as *Fibrobacter*, do not produce H_2 (Tapio et al., 2017). Various studies evaluated the ruminal microbiome and how shifts in different microbial populations within the rumen may influence CH_4 emission; however, other factors such as the animal's health status, productivity, feed intake, and, most importantly, the quality and chemical composition of the diet consumed by the animal, can influence its emission. The CH_4 production tends to decrease with an increase in the protein content of the diet, while it increases as fiber content rises (Shibata and Terada, 2010; Tapio et al., 2017). Diets with a high intake and low digestibility increase volatile solids, while high Indigestible Neutral Detergent Fibre (iNDF) reduces digestibility, affect CH_4 emissions (Ramin et al., 2021). Lastly, according to Zhao et al. (2020), several methods exist to measure enteric CH_4 emissions in ruminants, like respiration chambers, SF_6 tracer, and GreenFeed. However, mathematical models can predict emissions without costly experiments, using common dietary data like Dry Matter Intake (DMI), metabolizable energy, NDF, ADF, ether extract, lignin, and forage proportion. In beef

cattle, CH_4 emissions correlate more with dietary NDF than DMI, while in dairy cows, DMI shows a stronger correlation with CH_4 output. Overall, these models offer an effective alternative for emission prediction (Tapio et al., 2017; Ramin et al., 2021). To mitigate CH_4 emissions, various strategies have been developed, with nutritional approaches being the most studied. These include optimizing feed composition and intake, supplementing diets with lipids, and improving feed digestibility. Several compounds, tested both *in vitro* and *in vivo*, influence methanogenesis by modulating microbial populations or enhancing feed digestibility; among the most studied the natural plant-derived compounds seem to have a good potential in reducing methane production (Beauchemin et al., 2022).

Plants Feed Additives

To date, the use of additives for zootechnical use in Europe is regulated by regulated by Article 5 of Regulation (EC) No. 1831/2003 which outlines the criteria that feed additives must meet to ensure their safety and proper use. Feed additives are broadly defined as “substances, microorganisms, or preparations intentionally added to animal feed or water to achieve specific purposes”. Specifically, these substances must not pose risks to animal or human health, nor should they harm the environment. Furthermore, feed additives must not mislead users or negatively influence consumers by altering the characteristics of animal products or creating misconceptions about them. These purposes, as described in the regulation, include improving the nutritional or physical characteristics of feed, enhancing the quality or yield of animal products, influencing the coloration of ornamental animals, reducing the environmental footprint of animal production, or supporting overall animal health and productivity (Ramesh C. Gupta et al., 2019).

Feed additives should be allocated to one or more of the following categories:

- **Technological additives**: Substances added for technological purposes to improve the product’s properties, such as extending shelf life.
- **Sensory additives**: Substances that enhance or modify the organoleptic properties of feed, such as its color or flavor.
- **Nutritional additives**: These are added to meet the animals' nutritional needs and support growth.
- **Zoo-technical additives**: Additives used to improve animal performance and gut health.

Among the most interesting alternative additives there are organic acids, enzymes, prebiotics, probiotics and Plants Feed Additives (PFA) (Ramesh C. Gupta et al., 2019) which, due to their wide biological properties as antioxidant, anti-methanogenesis and antimicrobial are nowadays of great interest (Righi et al., 2021; Beauchemin et al., 2022; Spadini et al., 2024). Additionally, these compounds are effective, non-resistance forming, renewable and environmentally friendly. The term “PFA” in the literature refers to a series of compounds and active principles of plant origin, including

primarily Essential Oils (EOs), plant extracts, and their active components (Ramesh C. Gupta et al., 2019). They are described as primary or secondary components of plants that contain bioactive constituents which exert a positive effect on the productivity and health of animals. Many EOs and plant extract have beneficial multifunctional properties derived from their specific bioactive compounds, however among the different plants belonging to the same family could be observed a difference in their composition of bioactive compounds due to harvest conditions, growing localization, extraction/distillation, storage conditions (Turek and Stintzing, 2013; Ramesh C. Gupta et al., 2019). They are generally approved in the EU for the use in animal feed as "sensory additives" or "flavors", however this might have a negative impact on feed intake and digestibility of animal, especially if added at high dose (Calsamiglia et al., 2007). The use of PFA in animal nutrition has been known for many years thanks to their beneficial effects not only in animal health but also performance production improving the quality of the animal products (Manuelian et al., 2021; Pitino et al., 2021). Furthermore, EOs and NICs have been widely studied as natural rumen modifiers to optimize fermentation processes and enhance nutrient utilization in ruminants. These bioactive additives have shown the ability to reduce undesirable by-products such as ammonia-N, methane, and acetate while promoting the production of beneficial fermentation products like propionate and butyrate (Calsamiglia et al., 2007). However, when using natural molecules, there are also disadvantages in terms of the dose effect on the animal, as high doses of these molecules have also been associated with negative effects on animal performance (Manuelian et al., 2021; Righi et al., 2021). The effects of EOs and NIC are highly dependent; at appropriate concentrations, they can selectively inhibit specific microbial populations in the rumen, optimizing fermentation pathways. However, excessive doses may lead to a substantial reduction in fermentation activity, negatively impacting overall rumen function and diet digestibility (Calsamiglia et al., 2007). The specific outcomes of these additives also depend on the type of fermented substrate and the chemical composition of the plant extracts used, highlighting the importance of customized applications. Their influence on fiber digestibility, particularly Neutral Detergent Fibre Digestibility (NDFD), and Dry Matter Digestibility (DMD) is complex and varies based on the interactions between substrate, dose, and microbial ecosystem (Nanon et al., 2014; Righi

et al., 2017). Additionally, as extensively reviewed by Beauchemin et al., (2022), numerous *in vitro* studies have reported decrease in CH_4 production when EOs and NICs where used, demonstrating promising activity. However, the authors remark that the *in vivo* studies are less conclusive since the use of these molecules is often associated with a reduction in feed digestibility, which could lead to an increase in both manure production and enteric emissions. Given the broad spectrum of action of EOs, identifying specific EOs capable of selectively inhibiting methanogenesis without negatively impacting other digestive processes, and in addition acting on pathogen's bacteria could represent a general objective to be pursued.

Among the many active principles identified, some of the most notable and widely studied include those derived from aromatic plants, which are known for their antimicrobial, anti-inflammatory, antioxidant, and other beneficial effects. Below are some of the most interesting and promising active compounds:

- Geraniol (GER), the trans-isomer of 3,7-dimethyl-2,6-octadien-1-ol, it is a naturally occurring acyclic isoprenoid monoterpene found in the EOs of a variety of aromatic plants; *Cinnamomum tenuipilum*, *Valeriana officinalis*, *Phyla scaberrima*, and *Perilla frutescens*. This versatile compound is widely utilized in the fragrance industry, as an ingredient in perfumes and cosmetics, as well as in personal care products, pharmaceuticals, and as a flavoring agent in food products (Lei et al., 2019; Lira et al., 2020; Chen and Viljoen, 2022). Due to its favorable safety profile, geraniol has been approved by the United States Food and Drug Administration (FDA) as a food additive.
- Curcumin (CUR): it is a natural polyphenol found in turmeric (*Curcuma longa*), is widely known for its culinary use as a flavoring and coloring agent. Beyond this, it has potential therapeutic applications. However, CUR has limitations, including poor water solubility, instability in solutions, low bioavailability, and rapid elimination from the body. To address these issues, organic solvents like ethanol, methanol, acetone, and dimethyl sulfoxide (DMSO) are often used to solubilize CUR. This phenolic compound is widely studied for its biological properties such as antibacterial, antiviral and antifungal (Trigo-gutierrez et al., 2021).

- Nerolidol is a sesquiterpene alcohol found in the EOs of a variety of plants, such as *Piper claussenianum*, *Momordica charantia*, *Ginkgo biloba*, *Baccharis dracunculifolia*, *Myrocarpus frondosus*, and several fragrant flowering plants like lavender, neroli, lemongrass, tea tree, and ginger. Due to its beneficial biological properties, including antimicrobial, anti-inflammatory, and antibiofilm effects, Nerolidol has been explored for use in multiple sectors, such as the pharmaceutical, food, and cosmetic industries (Chan et al. 2016).
- Carvone, is a ketone monoterpene found mostly in the EOs of plants from genus of *Mentha,* and its present in two different isomeric forms: L or D. It's widely used in the cosmetics and agriculture sector thanks to its antioxidant and anti-inflammatory properties (Pina et al., 2022).
- *β*-Caryophyllene is a major component of EOs from various spice and food plants, including black pepper (*Piper nigrum*), rosemary (*Rosmarinus officinalis*), cinnamon (*Cinnamomum* spp.), oregano (*Origanum vulgare*), basil (*Ocimum* spp.), thyme (*Thymus vulgaris*), sage (*Salvia officinalis*), mint (*Mentha piperita*), ginger (*Zingiber officinale*), chinotto (*Citrus myrtifolia*). Approved by the FDA and EFSA, exhibits biological activities, including anti-inflammatory, antimicrobial, and cytotoxic effects against various cancer cell lines (Francomano et al., 2019).
- 1,8-Cineole, also known as Eucalyptol, is the primary component of Eucalyptus oil. Two three species used for the commercial production of cineole are *Eucalyptus globulus* and *Eucalyptus polybractea*. It is known for its therapeutic properties, including anti-inflammatory and antimicrobial effects, and is approved by the FDA for use in food (Campos and Berteina-Raboin, 2022).
- Pinenes and other bicyclic terpenes are present in the EOs of coniferous trees, rosemary, lavender, and turpentine. These compounds occur as optical isomers or enantiomers, which are non-superimposable mirror images, differing solely in how they interact with polarized light. Pinenes have two main isomers: α-pinene and β-pinene. Each has enantiomers, including (−)-α-pinene (commonly found in European pines), (+)-α-pinene, (−)-β-pinene, and (+)-β-pinene (Ana Cristina Rivas da Silva et al., 2012).

- *p*-cymene is a naturally occurring monoterpene found in the EOs of various plants (Asteraceae; Burseraceae, Lamiaceae and Mirtaceae) including thyme, oregano, and cumin. P-cymene also possesses biological properties, such as antioxidant, anti-inflammatory, and antimicrobial activities (Balahbib et al., 2021).
- R- (+) -limonene is a cyclic monoterpene primarily found in the rind of citrus fruits, such as oranges, lemons, and limes. It is a colorless liquid with a characteristic citrus scent and is widely used in the food, fragrance, and cleaning industries thanks to a wide range of biological activities, including antioxidants, anti-inflammatory, and anticancer properties (Ravichandran et al., 2018).
- Quercetin is a flavonoid found in a variety of fruits, vegetables, and herbs, such as apples, onions, and citrus fruits. Known for its antioxidant and anti-inflammatory properties, quercetin has gained attention for its potential health benefits (Salehi et al., 2020).

Encapsulation System: Nanoliposome

The PFA offers several benefits, but they also have some limitations. It is important to consider that the use of NICs in animal nutrition may be limited by several factors, such as their low solubility, their potential cytotoxicity, as well as by earlier adsorption (Majeed et al., 2015; Zhu et al., 2021; Nsairat et al., 2022). As reviewed by Majeed et al. (2015), terpenes and aldehydes are primarily absorbed in the stomach and proximal small intestine, making it difficult for them to reach the lower intestines. To address this limitation, techniques like encapsulation are being explored, allowing for a slow release of active ingredients (Rajendran et al., 2022). This method reduces early absorption and promotes prolonged action along the gastrointestinal tract, enhancing the local availability of bioactive compounds while protecting them from external factors like light, temperature, and pH changes. There are a wide variety of encapsulation systems available, each offering distinct advantages depending on the type of matrix used and the method of preparation. Furthermore, variations in preparation techniques—such as spray drying, coacervation, or solvent evaporation—can lead to differences in the size, stability, and release kinetics of the encapsulated compounds. Therefore, selecting the appropriate encapsulation system requires careful consideration of the specific characteristics of the bioactive

ingredients, as well as the desired outcome for their release and effectiveness. For instance, natural protein-based systems tend to be more suitable for applications where biocompatibility and biodegradability are crucial, while polysaccharide-based systems may be preferred when slower release or enhanced stability is required. Additionally, polysaccharide polymers, including substances like alginate, hyaluronic acid, and chitosan, are often used in encapsulation due to their biodegradability, non-toxicity, and the potential for controlled release (Rajendran et al., 2022). Liposomal systems, which consist of lipid bilayers encapsulating active ingredients, are further popular approaches (Taylor et al., 2005).

Liposome and liposomal systems

Liposomes are among the most widely used encapsulation technologies; they consist of a lipid bilayer with the hydrophilic heads of phospholipid molecules oriented towards the aqueous environment to form small spherical vehicles whose diameter range between 25 and 1000 nm (Taylor et al., 2005). Liposomes can be made from both natural and synthetic phospholipids, and their properties—including particle size, rigidity, fluidity, stability, and charge—are influenced by lipid composition. For example, liposomes made from unsaturated phosphatidylcholine (e.g., egg or soybean) are more permeable but less stable, while those made from saturated phospholipids (e.g., dipalmitoyl phosphatidylcholine) are rigid and less permeable. The hydrophilic head group can be negatively, positively, or zwitterionic, charged, affecting stability through electrostatic repulsion. The hydrophobic tail varies in acyl chain length, symmetry, and saturation. Liposomes can be formulated using various techniques, and the choice of preparation method, along with the type of phospholipids used, significantly impacts the final characteristics of the liposomes, including size, stability, and drug encapsulation efficiency. The fabrication procedures can be classified into several methods: Thin Film Hydration Method (Bangham Method), Reverse-Phase Evaporation Method, Solvent Injection Method, Detergent Removal Method, Dehydration-Rehydration Method, Heating Method, pH Jumping Method, Microfluidic Channel Method, Supercritical Fluidic Method. Each of these methods has its own advantages and is selected based on the desired liposome characteristics, such as size, encapsulation efficiency, and stability. The choice of phospholipids, as well as the preparation method, plays a crucial role in determining the

performance and application of the final liposomal formulation (Taylor et al., 2005; Nsairat et al., 2022). Liposomes offer the advantage of enhancing the solubility and bioavailability of hydrophobic compounds, while also protecting sensitive molecules from degradation.

Liposomes characterization

Liposome physiochemical characterization includes average size and size distribution or polydispersity index (PDI), surface charge (or Zeta potential), shape and morphology, lamellarity, encapsulation efficiency, phase behavior (or polymorphism) and *in vitro* release profile. Nanoliposomes are classified as liposomes which means diameter is comprised between 50-150 nanometers; in addition, considering the number of bilayers, the liposomes are classified into unilamellar vesicles (ULVs) or multilamellar vesicles (MLV) (Taylor et al., 2005). The PDI in nanoliposomes is a parameter measuring the distribution of particle sizes within a suspension. In other words, PDI indicates how uniform the liposome particles are in terms of size. A low PDI (close to 1) suggests a uniform distribution of particle sizes, whereas a high PDI indicates significant variation in particle sizes, with the presence of both larger and smaller particles compared to the average size. The PDI is calculated as the ratio of the standard deviation of particle diameter to the mean particle diameter. A PDI value close to 1 typically indicates good monodispersing, meaning the particles are similar in size, while a PDI greater than 1.5 suggests a wider distribution of sizes, which can impact the stability and effectiveness of the nanoliposomes, especially when used for controlled drug release(Danaei et al., 2018; Witika et al., 2022). The zeta potential is a parameter used to predict the stability of dispersions due to electrostatic interactions between particles in a suspension. These particles can exhibit surface charge either due to their intrinsic chemical composition or due to ionization or absorption of charged species. Charged particles are surrounded by several ionic layers in solution, which differ in composition from the bulk solution. When these particles move in solution, for example, due to Brownian motion, they move along with a double ionic layer. The zeta potential represents the main force of interaction (repulsion/attraction) between particles and is highly sensitive to the composition of the charged species in the dispersion. A high zeta potential value ($Z > 30$ mV) ensures that particles suspended in bulk liquids remain separated from each other, repelling each other and thus reducing the likelihood of

agglomeration or flocculation, thereby making the suspension stable over time (Honary and Zahir, 2013).

Aim of the project

The objective of the project is to evaluate the antimicrobial activities of NIC against important bacteria implicated in infectious diseases in livestock. Specifically, *in vitro* tests will initially be conducted to evaluate the Minimum Inhibitory Concentration of NICs, as well as cytotoxicity tests to understand their real and possible future use.

The best candidates will be formulated with support systems to become food additives, that is, these molecules will be carried by a carrier that will prevent their degradation once they enter the gastro-enteric tract. Finally, *in vitro* digestibility and *fermentation* tests will be carried out on both mono-gastric and poly-gastric models.

First Study: Evaluation of Antimicrobial and Cytotoxicity activity of different NIC for veterinary applications

Introduction

In recent years, the increasing spread of antimicrobial-resistant microorganisms has posed a major challenge for both human and veterinary medicine. In veterinary medicine, one of the sectors with the

highest antibiotic consumption is livestock farming. For years, antibiotics in livestock farming were used not only for therapeutic purposes but also at sub-therapeutic doses to promote animal growth, due to the beneficial properties of certain active ingredients on microbiome (Ramesh C. Gupta et al., 2019). However, since January 1, 2006, following the implementation of EU Regulation No. 1831/2003, the use of antimicrobials at sub-therapeutic doses as growth promoters has been banned. For this reason, researchers have started an active search for alternatives to antimicrobials to prevent infectious diseases in animal husbandry. In this context, plants by-product, especially NIC has emerged as a promising area of research for the development of innovative agents. These compounds, chemically identical to naturally occurring molecules, exhibit potential antimicrobial activity combined with a safety profile that could support their use in livestock farming as new alternative feed additives (Spaggiari et al., 2023; Spadini et al., 2024).

For this reason, the aim of this phase of the project was to screen different NICs, nerolidol; geraniol; D-carvone, β-caryophyllene; α-pinene; P-cymene; eucalyptol; β-pinene; R-limonene; curcumin; quercetin, against a selection of livestock pathogens; *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus* (MRSA), *E. coli* ATCC 25922, *Salmonella* Typhimurium, and a bacteria belong to commensal flora, *Lactobacillus acidophilus.* In addition, this study included an evaluation of their safety by analyzing: Cytotoxicity on an MDBK (Madin-Darby Bovine Kidney) cell line, and hemolytic activity on defibrinated sheep blood.

Materials and Method

NIC

All compounds were purchased from SIGMA ALDRICH: nerolidol (H59605-25G, batch number STBK5509); geraniol (163333-25G, batch number SHBL9235); D-Carvone (W224928-100G-K, batch number SHBN0831); β-caryophyllene (W225207-1KG-K, batch number SHBL8590); α-pinene (147524-250ML, batch number MKCQ4002); P-cymene (batch number 1003445330); eucalyptol (C80601, batch number BCCG5791); β-pinene (89335, batch number 123961714); R-limonene (183164, batch number MKCQ8289); curcumin (C1386-10G, batch number 0000160804); quercetin

(337951, batch number WXBD8240V). All compounds were stored under the conditions specified by the manufacturer.

Bacterial Strains

The antimicrobial activity of NICs was tested against *Enterococcus faecium* ATCC 19434, methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300, *E. coli* ATCC 25922, *Salmonella* Typhimurium ATCC 14028 and *Lactobacillus acidophilus* ATCC 4356.

Preparation for bacterial suspension

The bacterial suspension was set up following the Clinical and Laboratory Standards Institute (2019). Briefly, for each reference strain, three to five morphologically similar colonies were selected from plates of suitable solid medium and inoculated into tubes containing suitable liquid culture medium. For all bacteria Mueller Hinton Broth (DIFCO, Sparks, USA; -MHB) was used as medium, while for *Lactobacillus acidophilus* MRS broth (DIFCO, Sparks, USA;) was used. The tubes were incubated at 37°C for 24 h; *Enterococcus faecium* and *Lactobacillus acidophilus* were incubated in microaerophilic condition (5 % CO_2). Next, the bacterial suspension was centrifuged at 2000 rpm for 20 min. The obtained pellet was resuspended and turbidity adjusted with a Biophotometer plus (Eppendorf, Hamburg, Germany) (k¼600nm) until a bacterial suspension containing approximately 10^8 CFU/ml (OD_{600}, range 0.08-0.13) was obtained. Finally, the suspension was then diluted 1:100 in a suitable culture medium. Within 30 minutes of preparation, 50 µL of this suspension (10^6 CFU/mL) was inoculated into each well of a 96-well microtiter plate to obtain a final concentration of approximately $5*10^5$ CFU/mL.

MIC assay of NICs

MIC assay was evaluated following the literature with minor modifications (Spadini et al., 2024). Nerolidol, geraniol, D-carvone, β-caryophyllene, α-pinene, R-limonene, eucalyptol, β-pinene, curcumin, and quercetin were diluted into a stock solution in DMSO at 400 mg/mL: while P-cymene at 20 mg/mL. Two-fold dilutions in DMSO of each compound from stock solution were performed in a separate 96-well microtiter plate and then, one microliter of each was added to the wells of the plates

(Greiner, Milan, Italy). Then, 50 µL of previously standardized bacterial suspension was added to each well. For nerolidol, geraniol, D-carvone, β-caryophyllene, α-pinene, R-limonene, eucalyptol, β-pinene, curcumin, and quercetin the final dilution range was between 4000 to 7.8 µg/mL, while for P-cymene was among 2000 to 3.9 µg/mL. Growth controls (absence of compounds) and sterility controls (absence of compounds and bacteria) were set up for each test. The microtiter plate was incubated at 37°C for 24 h in aerobiosis condition for MRSA, *Salmonella* Typhimurium and *E. coli*, while *Enterococcus faecium* and *Lactobacillus acidophilus* were incubated in microaerophilic condition (5% CO_2). Three independent experiments for each bacterial, with three replicates for each experiment were performed.

After incubation, MIC was read by an unaided eye.

Cytotoxicity Assay

Cytotoxic activity of NIC was evaluated on 10^5 cells/mL Madin-Darby bovine kidney (MDBK) ATCC CRL-6071 by the MTT colorimetric assay, following the protocol reported in literature (Donofrio et al., 2008). 1 μL of diluted NIC (4000 to 31.25 μg/mL or 2000 to 15.63 μg/mL) on DMSO was added to each well. The plates were incubated for 24 hours at 37 °C in the presence of 5% of CO_2. After incubation, 10 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide) was added to each well of the assay and incubated at 37°C for 6 h. At the end of incubation, 100 μL of the solubilization solution (10% SDS in 0.01 M HCl) was added to each well and incubated overnight. The yellow MTT tetrazolium salt is reduced in metabolically active cells to the insoluble form of purple-colored formazan crystals, which is solubilized by the addition of a detergent. After incubation, the plates were read with a spectrophotometer (Biophotometer plus, Eppendorf, Hamburg, Germany 620 nm). Positive and negative controls were made for each plate. Three replicates were made for each assay for two independent experiments. Finally, Statistical differences with negative control were tested by Student's t test.

Hemolysis assay

Hemolytic activity on all NIC was evaluated on defibrinated sheep blood (Thermofisher Diagnostics, lot 39997900) following the method reported in literature (Sala et al., 2018). In a sterile 96-well U-bottom microtiter plate, 50 μL of defibrinated sheep blood was placed and incubated with 49 μL of sterile saline and 1 μL of NIC for 24 h at room temperature. The positive control (100% hemoglobin release) and negative control (0% hemoglobin release) were set up with sterile water and sterile saline, respectively. After incubation, the plate was centrifuged at 1400 rpm for 15 min, and after transferring the supernatant to sterile plate, hemolysis was measured spectrophotometrically at 450 nm (Biophotometer plus, Eppendorf, Hamburg, Germany).

The percentage of hemolysis was calculated by the following formula:

[1- 〖(A〗 _comp-A_NC)/ (A_(PC)- A_NC)] x 100

Where:

- A_{comp} represents the optical density of the sample (NIC) at 450 nm
- APC the optical density of the positive control
- ANC the optical density of negative control.
- For each assay, three replicates were performed for three independent experiments.

Results

MIC of NIC

Table 1 presents data on the antimicrobial activity of NICs against bacteria of veterinary interest. No antimicrobial activity was detected for most NICs, with MIC values above maximum tested concentration (4000 or 2000 µg/ml). However, Nerolidol exhibited inhibitory effects against *E. faecium* ATCC 19434 (76.39 ± 27.56 µg/mL) and moderate activity against *L. acidophilus* ATCC 4356 (250 ± 0 µg/mL) and MRSA (4000 ± 0 µg/ml). Geraniol demonstrated antimicrobial activity, with MIC values of 777.8 ± 263 µg/mL against *E. coli* ATCC 25922, 1666.7 ± 500 µg/mL against *S.* Typhimurium ATCC 14028, and lower values 666.7 ± 250 µg/mL against *E. faecium* and MRSA. Curcumin displayed moderate antimicrobial activity against MRSA ATCC 43300, with a MIC of 208.33 ± 63 µg/mL.

Table 1 Minimal Inhibitory Concentration (µg/mL) of Nature Identical Compounds (NIC) against reference bacteria strains of zootechnical interest (mean± standard deviations)

NIC	*E. coli* ATCC 25922	*S.* Typhimurium ATCC 14028	*E. faecium* ATCC 19434	MRSA ATCC 43300	*L. acidophilus* ATCC 4356
Nerolidol	>4000 ± 0	>4000 ± 0	76.39 ± 27.56	4000 ± 0	250 ± 0
Geraniol	777.8 ± 263	1666.7 ± 500	666.7 ± 250	666.7 ± 250	1000 ± 0
D-Carvone	>4000 ± 0	>4000 ± 0	>4000 ± 0	>4000 ± 0	>4000 ± 0
β-caryiophillene	>4000 ± 0	>4000 ± 0	>4000 ± 0	>4000 ± 0	>4000 ± 0
α-pinene	>4000 ± 0	>4000 ± 0	>4000 ± 0	>4000 ± 0	>4000 ± 0
P-cymene	>2000 ± 0	>2000 ± 0	>2000 ± 0	>2000 ± 0	>2000 ± 0
Eucaliptol	>4000 ± 0	>4000 ± 0	>4000 ± 0	>4000 ± 0	>4000 ± 0
β-pinene	>4000 ± 0	>4000 ± 0	>4000 ± 0	>4000 ± 0	>4000 ± 0
R-limonene	>4000 ± 0	>4000 ± 0	>4000 ± 0	>4000 ± 0	>4000 ± 0
Curcumina	>4000 ± 0	>4000 ± 0	>4000 ± 0	208.33 ± 63	>4000 ± 0
Quercitin	>4000 ± 0	>4000 ± 0	>4000 ± 0	>4000 ± 0	>4000 ± 0

Cytotoxicity assay of NIC

Table 2 presents the cytotoxicity results of the NICs tested, assessed using the MTT assay on the MDBK cell line. All tested NICs at all tested concentrations showed significative difference than control negative. High cytotoxicity was observed for Nerolidol and Geraniol, with cell survival rates below 45% at all concentrations tested. In contrast, α-pinene, eucalyptol, and R-limonene showed cell survival rates ≥ 80% at concentrations ≤ 250, 1000, and 250 µg/mL, respectively.

Table 2 Survival (%) of MDBK cell lines after 24h contact with different Nature Identical Compounds (NIC).

NIC	% Survival							
	4000 (µg/mL)	2000 (µg/mL)	1000 (µg/mL)	500 (µg/mL)	250 (µg/mL)	125 (µg/mL)	62.5 (µg/mL)	31.25 (µg/mL)

Nerolidol	14.3	14.1	14.4	18.1	14.2	14.2	14.2	33
Geraniol	14	13.8	12.8	12.7	12.8	24.8	30.7	42.6
D-Carvone	48.4	32.2	17.1	23.8	30.6	40.9	66.9	67.6
β-caryiophillene	11.8	11.4	11.5	13.3	16.5	44.8	66.3	83.4
A-pinene	25.1	40.9	61.9	73.4	81.3	84.7	86.5	90.9
Eucaliptol	83.8	73.4	81.6	83.9	84.6	84.9	85.5	91.3
β-pinene	11.5	10.7	12.4	24.7	52.4	67.6	75.9	70.2
R-limonene	15.6	26.9	44.4	72.3	89.4	92.2	89.5	94
Curcumina	47.1	47.5	43.7	36.1	28.1	22.3	14.1	12.9
Quercitina	46.1	39.3	32.9	18.5	99.8	64.6	43.3	48.6

NIC	% Survival							
	2000 (µg/mL)	1000 (µg/mL)	500 (µg/mL)	250 (µg/mL)	125 (µg/mL)	62.5 (µg/mL)	31.25 (µg/mL)	15.63 (µg/mL)
P-cymene	10.2	12.2	10.3	10	10	10.5	15.6	21.6

Hemolysis assay of NIC

Table 3 presents the hemolysis results on defibrinated sheep blood for the various NICs tested in this study. β-Caryophyllene and R-limonene were the only NICs that showed no hemolytic activity at any concentration tested. In contrast, α-pinene, eucalyptol, and β-pinene demonstrated hemolysis levels below 10% at all concentrations tested. For Nerolidol and Geraniol, hemolytic activity was observed until 250 and 500 µg/mL, respectively. Finally, Curcumin and Quercetin exhibited high percentages of hemolysis at all concentrations tested.

Table 3 Results of hemolysis tests on defibrinated mutton blood of different Nature Identical Compounds (NIC).

NIC	% Haemolysis							
	4000 (μg/mL)	2000 (μg/mL)	1000 (μg/mL)	500 (μg/mL)	250 (μg/mL)	125 (μg/mL)	62.5 (μg/mL)	31.25 (μg/mL)
Nerolidol	52	44	30	29	4	0	0	0
Geraniol	98	96	100	3	0	0	0	0
D-Carvone	67	5	0	0	0	0	0	0
β-caryiophillene	0	0	0	0	0	0	0	0
α-pinene	2	4	0	0	0	0	0	0
Eucaliptol	3	0	0	0	0	2	5	7
β-pinene	6	7	7	4	4	4	1	3
R-limonene	0	0	0	0	0	0	0	0
Curcumina	65	84	98	84	77	62	80	86
Quercitina	19	39	26	44	39	36	58	23

NIC	% Haemolysis							
	2000 (μg/mL)	1000 (μg/mL)	500 (μg/mL)	250 (μg/mL)	125 (μg/mL)	62.5 (μg/mL)	31.25 (μg/mL)	15.63 (μg/mL)
P-cymene	0	0	37	1	15	48	3	24

Discussion

In the initial part of this study, we conducted preliminary screening aimed at evaluating both the antimicrobial activity against major pathogens of farm animals and the cytotoxicity of a selected group of NICs, known in the literature for their biological properties, as previously mentioned. The tested compounds included nerolidol, geraniol, D-carvone, β-caryophyllene, α-pinene, P-cymene, eucalyptol, β-pinene, R-limonene, curcumin, and quercetin. Regarding antimicrobial activity, the screening results revealed that most of the NICs tested showed no activity at the concentrations used. The only NICs that showed antimicrobial activity at the tested concentrations were nerolidol, geraniol and curcumin. geraniol, is an acyclic isoprenoid monoterpene, extracted from the essential oils of various aromatic plants, including *Cinnamomum tenuipilum*, *Valeriana officinalis*, *Phyla scaberrima*, and *Perilla frutescens var. hirtella* (Lei et al., 2019). To assess the antimicrobial activity of geraniol, the most widely used method in the literature is that of microdilution in broth for MIC determination. As reported in review by Lira et al. (2020) several studies evaluated the antimicrobial activity of geraniol by MIC and reported that against most pathogens, including *E, coli*, *Staphylococcus aureus*, *Salmonella* Typhimurium, MIC values were ≤ 600 µg/mL. However, for other pathogens, such as *Klebsiella pneumoniae* and certain strains of *S. aureus*, MIC values range from 600 to 1500 µg/mL. In our study, the results were generally comparable to those reported by Lira et al. (2020) although higher MIC values were observed for specific pathogens. Furthermore, our results are in line with, as reviewed of Chen and Viljoen (2022), where different studies evaluated the antimicrobial activity of geraniol against a variety of pathogens, including *S. aureus*, *Salmonella spp*. and *E. coli*. The ability of geraniol to effectively target both Gram-positive and Gram-negative pathogens underscores its broad-spectrum antimicrobial potential. This dual-spectrum efficacy highlights geraniol as a promising natural antimicrobial agent, with potential applications not only in animal health but also in sectors such as food safety and pharmaceuticals.

Nerolidol, a sesquiterpene commonly found in various plant extracts, has been widely studied for its antimicrobial and antibiofilm properties, particularly against Gram-positive bacteria (Chan et al., 2016; de Moura et al., 2021). As reviewed by Chan et al. (2016) numerous studies have highlighted the

antimicrobial activity of Nerolidol against both methicillin-sensitive and methicillin-resistant strains of *Staphylococci*, as well as *Streptococcus mutans*. Additionally, research has explored the effects of both racemic formulations and formulations containing only the -cis or -trans isomers of nerolidol (Inoue et al., 2004; Braca et al., 2008; Gonçalves et al., 2011). In our study, a racemic formulation of Nerolidol was tested to evaluate its antimicrobial activity. Nerolidol was found to be completely ineffective against both *E. coli* and *S.* Typhimurium, indicating an absence of activity against these Gram-negative bacteria. However, its activity against Gram-positive bacteria was much more notable, especially on *E. faecium* (MIC=76.39 ± 27.56 µg/mL). A strong antimicrobial effect was also observed against *L. acidophilus* (MIC=250 ± 0 µg/mL), whereas its activity against *MRSA* was relatively low (MIC=4000 ± 0 µg/mL). Studies on the mechanism of action of nerolidol, in relation to its antimicrobial activity, suggest that it primarily works by disrupting the bacterial membrane and affecting ion homeostasis. Specifically, Nerolidol is believed to interfere with potassium ion (K^+) homeostasis, which leads to alterations in membrane potential and cellular integrity (Chan et al., 2016). These findings underscore the potential of nerolidol as a targeted antimicrobial agent, particularly for infections caused by Gram-positive bacteria, where its activity appears to be most pronounced. Curcumin, along with curcuminoids, is a phenolic compound and one of the main active principles of *Curcuma longa L.* (family Zingiberaceae). The curcumin content in *Curcuma longa* varies significantly depending on the geographical area of cultivation (Trigo-gutierrez et al., 2021). Literature reports have highlighted the antimicrobial activity of aqueous and methanolic extracts of turmeric, with effectiveness against Gram-positive bacteria, although some activity against certain Gram-negative bacteria has also been documented (Zorofchian Moghadamtousi et al., 2014). In our study, we tested the active principal curcumin, dissolved in DMSO. Other studies in literature have used other types of solvents other than DMSO to dissolve curcumin, such as Tween 80, ethanol or edible alcohol (Trigo-gutierrez et al., 2021). Our experiments revealed that, at the concentrations tested, curcumin was entirely ineffective against Gram-negative bacteria. Conversely, activity was observed against MRSA, with MIC value of 208.33 ± 63 µg/mL. This result aligns with previous studies, which reported MIC values of curcumin against

MRSA ranging from 125 to 250 µg/mL, thus confirming the antimicrobial activity of curcumin against specific Gram-positive bacteria (Zorofchian Moghadamtousi et al., 2014).

The result regarding the antimicrobial activity suggests that geraniol possesses broad-spectrum activity, making it an interesting candidate for further investigation and potential applications in bacterial infection control. Nerolidol and curcumin, while demonstrating antimicrobial efficacy, exhibited a more selective activity compared to geraniol. For instance, curcumin was found to be primarily effective against Gram-positive bacteria, such as MRSA, consistent with data previously reported in the literature. Nerolidol, on the other hand, showed an intermediate profile, with a broader activity than curcumin but less extensive than geraniol.

One of the crucial aspects for the potential application of NICs in veterinary medicine concerns their cytotoxic activity. Indeed, evaluating cytotoxicity is essential to determine the safety of these compounds for potential therapeutic use. The results obtained from cytotoxicity tests on the MDBK cell line and hemolysis assays on defibrinated sheep red blood cells revealed a dose-dependent toxicity for all the tested NICs. Regarding cytotoxicity on the MDBK cell line, most of the NICs tested exhibited high cytotoxic activity, with the most toxic compounds identified as Nerolidol, P-cymene, and geraniol, at all tested concentrations. The cytotoxicity of nerolidol is known in literature on human cell lines (human hepatocellular carcinoma cells - HepG2) and model organisms such as *Saccharomyces cerevisiae* (Chan et al., 2016). This may be due to the hydrophobic nature of nerolidol, which allows it to easily cross the plasma membrane and directly interact with organelles and proteins, thereby interfering with cellular functionality (Ferreira et al., 2012; Chan et al., 2016). Also, geraniol is known in literature for its cytotoxic and genotoxic effects against human cells (HepG2 cells, MCF-7 cells and human lymphocytes) at low concentration (≤ 500 µg/mL; Queiroz et al., 2017; Chen and Viljoen, 2022). In contrast, α-pinene and R-limonene have been shown to have lower cytotoxic activity, leading to cell survival ≥ 80% at concentrations ≤ 250 µg/mL. Lastly, Eucalyptol was the compound with the lowest cytotoxic activity at all concentrations tested. Regarding the hemolysis essays on defibrinated sheep red blood cells, it was observed that β-caryophyllene, α-pinene, eucalyptol, and R-limonene showed the lowest hemolysis percentages, with minimal effect on red blood cell lysis. This

indicates that these compounds possess low hemolytic toxicity, which is a positive aspect for potential applications, as red blood cell lysis can cause tissue damage and increase risks associated with treatment. However, it is noteworthy that, despite showing low hemolysis and high survival of cell lines, these compounds did not exhibit antimicrobial activity, excluding them as option for application to prevent bacterial infections. On the other hand, among the compounds showing antimicrobial activity, curcumin exhibited the highest hemolysis percentage at all tested concentrations, indicating greater cytotoxicity compared to the other compounds examined. Nerolidol and geraniol, which showed great antimicrobial activity against various bacterial strains, exhibited a notable percentage of hemolysis up to concentrations of 500 µg/mL and 1000 µg/mL, respectively. For nerolidol, an absence of hemolysis was observed for concentrations ≤ 125 µg/mL, corresponding to the concentrations at which it was already active against *L. acidophilus* and *E. faecium*. This suggests that, while showing effective antimicrobial activity, nerolidol could be used at concentrations that are less cytotoxic, making it potentially safer and more effective, especially against Gram-positive bacteria. Considering both the cytotoxicity and antimicrobial activity data, this preliminary screening highlighted that many of the tested compounds were either inactive or highly cytotoxic at the concentrations analyzed. While compounds such as nerolidol, geraniol, and curcumin showed the best results in terms of antimicrobial activity; however, their high cytotoxicity at these concentrations suggests that they may not be suitable for direct use without further evaluation.

Second study: Assessment of Antimicrobial Activity and Cytotoxicity of Nanoliposome Formulation.

The results of the first study showed that not all NICs tested showed antimicrobial activity against the bacteria tested. Furthermore, although nerolidol, geraniol and curcumin showed the most interesting results in terms of antimicrobial activity, they were highly toxic. For this reason, it was decided to encapsulate the NICs within a nanoliposome system. However, given the technological difficulties in encapsulating Geraniol and the reduced spectrum of Curcumin, the only NIC encapsulated was Nerolidol. In literature, nanoliposomes are known for their excellent biocompatibility and low intrinsic toxicity; therefore, nanoliposomes represent an innovative system that allows both to increase the bioavailability of the bioactive compounds encapsulated within them and to reduce their toxicity.

In this study, nanoliposomes were produced using a technique a simil-microfluidic- technique; described in a patent (Barba et al., 2020). The preparation of the nanoliposome system involved two main phases. The first, referred to as the "ethanolic phase," consisted of a mixture of soy lecithin (rich in phosphatidylcholine) and cholesterol in a molar ratio of 5:1, into which Nerolidol was incorporated. The second phase, known as the "hydration phase," consisted simply of deionized water, used to hydrate the lipid mixture. It is important to note that, due to the intrinsic properties of nerolidol, a load ratio of nerolidol of 1% was used, since it is known that the terpene-loaded liposomes, also known as *invasomes,* cannot host large load of the guest molecule (Amnuaikit et al., 2018; Babaie et al., 2021; Vieira Nunes Cunha et al., 2023). Therefore, and in this case, the active molecule nerolidol is present as 1% of the total nanoliposomal concentration (5 g/L); further details will be report in "*Materials and Method section*".

After the preparation phase, the nanoliposome formulation was characterized to evaluate its physicochemical properties. The parameters analyzed included the Z-Average (305.08 ± 24.2 nm for the unloaded nanoliposomes versus 309.99 ± 9.0 nm for the loaded nanoliposome), the PDI (0.33 for the unloaded versus 0.44 for the loaded), and the Zeta Potential (-52.18 ± 6.4 mV for the unloaded

versus -42.36 ± 5.4 mV for the loaded). For the loaded nanoliposome, additional analyses determined an actual Nerolidol loading of 1.01 ± 0.1% and an encapsulation efficiency of 99.70 ± 0.42%.

Taking the above into account, in this second phase of the project we decided to evaluate the antimicrobial activity, expressed in terms of MBC (Minimum Bactericidal Concentration), by Time-kill study using four different time points: 2, 4, 6 and 24 h. The aim was to simulate the mean retention time of a monogastric animal to demonstrate a possible dose- and time-dependent antimicrobial effect. In addition, an analysis of the cytotoxic activity of the two nanoliposome formulations was conducted, using the same methods as reported in the first experiment. In addition, cytotoxic activity on intestinal porcine enterocyte cell line (IPEC-J2) was assessed; this latter evaluation was also extended on Nerolidol free form.

Materials and methods

Materials

L-a-Phosphatidylcholine (PC) from soybean, (CAS no. 8002-43-5) was purchased from A.C.E.F. (powder soybean lecitin E322, Fiorenzuola D'Arda, PC). Cholesterol (CHOL) (CAS no. 57-88-5) was purchased from CRODA (Cholesterol USP-PW (RB)LD 02210/SAMP; Mortara, PV). Ethanol of analytical grade (CAS no. 64-17-5) and Nerolidol (CAS no. 7212-44-4) were purchased from Sigma Aldrich (Milan, Italy).

Liposome production method

Unloaded nanoliposomes (UN) and loaded nanoliposomes (LN) have been prepared in according with the simil-microfluidic technique developed by the research group of University of Salerno, described in a patent (Barba et al., 2020) and elsewhere (Bochicchio et al., 2018; Bochicchio et al., 2018; Bochicchio et al., 2020; De Piano et al., 2023). It consists of two feed solutions (lipids/ethanol/Nerolidol and water) which are pushed through peristaltic pumps into the production section, a millimetric tubular device where the interdiffusion of the two flows leads to the formation of liposomes directly at nanometric scale. The lipids/ethanol solution were fed into a needle (0.6 mm

internal diameter) inserted into the production section tube, a 3 mm internal diameter silicon tube through which the water was fed. The ethanolic solution was prepared using a ratio of 5:1 between PC and CHOL (2.35 g of PC and 0.47 g of CHOL in 50 mL of ethanol for UN). To obtain LN, 0.0285 g of Nerolidol were added to this solution. In this way, the theoretical load ratio was 1% (Nerolidol divided by the sum of lipids and Nerolidol itself). The production process was carried out using a ratio between the volumetric flow rates of 10:1, i.e. 4.5 mL/min of ethanolic solution and 45 mL/min of water). By this way, roughly a flowrate of 3 L/hr of nanoliposome suspension was produced. The concentration of lipids is roughly 5 g/L (*nominal* concentration of 5000 µg/mL), and the Nerolidol concentration is roughly 0.05 g/L (load ratio of 1%, the tested encapsulation efficiency being close to 100%), giving a *nominal* concentration of 50 µg/mL.

Antimicrobial activity

Tested bacteria and bacterial inoculum preparation

The antimicrobial evaluation of LN, UN and Nerolidol was performed on five reference bacterial strains of veterinary interest: Methicillin-Resistant *Staphylococcus aureus* ATCC 43300 (MRSA), *Escherichia coli* ATCC 25922, *S. enterica* subsp. *enterica* serovar. Typhimurium ATCC 14028, *Enterococcus faecium* ATCC 19434 and *Lactobacillus acidophilus* ATCC 4356. All reference strains were purchased from ATCC® (USA). The bacterial inoculum was prepared following the CLSI method (2019), as described in the section Materials and Method of “First Step: Evaluation of Antimicrobial and Cytotoxicity activity of different NIC for veterinary applications”.

Time-Kill assay (TK)

TK of Nerolidol, UN and LN were performed according with literature (Makwana et al., 2014; Kumar et al., 2017). In brief, the starting point for the base dilutions of the two nanoliposome formulations was the stock solution at a concentration of 5 g/L (5000 µg/mL); while the final dilutions tested were between 2500 µg/mL to 9.765 µg/mL. To achieve this, a starting stock of 5000 µg/mL of each compound was diluted in a 2-fold with MHB medium. For LN, the tested concentration of Nerolidol

contained into the nanoliposomes ranged between 25 to 0.10 µg/mL. Subsequently, 2 mL of each tested concentration was mixed with 2 mL of a bacterial suspension containing 10^6 CFU/mL, prepared as previously mentioned, so that each tested concentration of UN and LN were added to 5×10^5 CFU/mL. Conversely, Nerolidol was initially dissolved in DMSO to prepare a stock solution at a concentration of 400 mg/mL. Serial two-fold dilutions of this stock solution were then made in DMSO. Further dilutions were carried out to achieve final test concentrations ranging from 4000 µg/mL to 7.81 µg/mL. Lastly, 40 µL of each tested concentrations were inoculated in a tube containing 3.960 mL of MHB and 5×10^5 CFU/mL of bacterial suspension.

Bacterial growth was quantified after 2, 4, 6 and 24 hours of incubation at 37 ºC in aerobic conditions for *E. coli*, *S.* Typhimurium, and MRSA, while in microaerophilia for *Enterococcus faecium* and *Lactobacillus acidophilus*. Ten microliters were plated on Mueller–Hinton Agar (MHA) for all bacteria, while for *Lactobacillus acidophilus* on MRS agar; the plates were incubated in aerobic/microaerophilic conditions at 37 °C for 24 h.

Finally, after incubation, for each experimental point and tested concentrations, colonies were counted, and inhibition percentages were calculated with the following formula:

$$Inhibition\ (\%) = \frac{\overline{CFU}_{GC} - \overline{CFU}_{TC}}{\overline{CFU}_{GC}} * 100$$

Where: $\overline{CFU}$= average colony forming unit; GC = growth control; TC = tested concentration

For each assay, three experiments with three replicates were performed.

Finally, for each experiment, growth and sterility controls were performed.

Cytotoxicity assay

MTT cell survival assay

The evaluation of cytotoxicity of UN and LN were performed by MTT cell survival assay, as reported in literature (Donofrio et al., 2008)(Donofrio et al., 2008), on Madin-Darby bovine kidney (MDBK) ATCC CRL-6071 and Intestinal Porcine Enterocyte cells IPEC-J2 (BS CL 205 purchased by Biobanking - of Veterinary Resources of Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna - Brescia-Italy); for Nerolidol, since it had already been evaluated on MDBK, only on IPEC-J2. Briefly, regarding UN and LN, 50 µl of stock solution (5 g/L- 5000µg/ mL) were added in a flat bottomed 96 wells sterile plate containing 10^5 cells/mL MDBK cells in MEM (Modified Eagle Medium) and FBS 10% (Fetal Bovine Serum) or $.5*10^5$ cells/mL IPEC-J2 cells in DMEM/F-12 (Dulbecco's Modified Eagle Medium, Hams's F-12 mixture), and scalar dilutions were performed ranging from 2500 µg/mL to 4.88 µg/mL. Regarding Nerolidol, the method was the same reported in Materials and Method- Cytotoxicity assay- "First Step: Evaluation of Antimicrobial and Cytotoxicity activity of different NIC for veterinary applications". The optical density was measured at 540 nm, using reading plates. For each essay, three experiments with three replicates each were performed; for each experiment, negative control was performed. Finally, Statistical differences with negative control were tested by Student's t test.

Hemolysis assay

Hemolytic activity on UN and LN was evaluated on defibrinated sheep blood (Thermofisher Diagnostics, lot 39997900), following the method reported in literature (Sala et al., 2018). In a sterile 96-well U-bottom microtiter plate, 50 µl of defibrinated sheep blood was placed and incubated with 50 µl of LN or UN, diluted at range between 2500 and 4,88 µg/mL for 24 h at room temperature. The positive control (100% hemoglobin release) and negative control (0% hemoglobin release) were set up with sterile water and sterile saline, respectively. After incubation, the plate was centrifuged at 1400 rpm for 15 min, and after transferring the supernatant to sterile plate, hemolysis was measured spectrophotometrically at 450 nm (Biophotometer plus, Eppendorf, Hamburg, Germany).

The percentage of hemolysis was calculated by the following formula:

[1- 〖(A〗 _comp-A_NC)/ (A_(PC)- A_NC)] x 100

Where:

- Acomp represents the optical density of the sample (NIC) at 450 nm
- APC the optical density of the positive control
- ANC the optical density of negative control.

For each assay, three replicates were performed for three independent experiments.

Statistical analysis

Statistical analysis and graph generation were carried out using Excel (Version 2410) and GraphPad Prism (version 8.0.2.263). Specifically, in the Time-kill study, a T-test was applied at each experimental point for each bacterial strain analyzed.

A significant level of 0.05 was chosen to determine the reliability of the observed differences.

Results

Time-kill (TK) assays

Nerolidol

TK assays with Nerolidol exhibited antimicrobial activity only on Gram-positive bacteria. In Figure 2 the results on all Gram positives are reported, and the strongest growth inhibition (> 90%), already after 4 h of incubation until the lowest tested concentration (7.81 µg/mL), was observed for *E. faecium*. Additionally, growth inhibition > 70% until a concentration of 31.25 µg/mL was reported for MRSA at the same time point. At 6 and 24 hours of incubation, Nerolidol on *E. faecium* showed a growth inhibition > 99.9% until a concentration of 15.63 µg/mL, while on MRSA was > 95% at 6 h only until 62.5 µg/mL and > 99% at 24 hours until 125 µg/mL. Regarding *L. acidophilus*, Nerolidol determined a strong inhibition (> 85%) until the lowest tested concentration (7.81 µg/mL) after 4 hours of incubation, and > 90% after 6 hours, while at 24 hours of contact only the three higher tested concentrations showed a complete inhibition of growth (99.99%).

Figure 2 Time-Killing (TK) assay of different concentrations (µg/mL) Nerolidol on Gram positive bacteria at different time points.

TK of Nerolidol against *Enterococcus faecium*

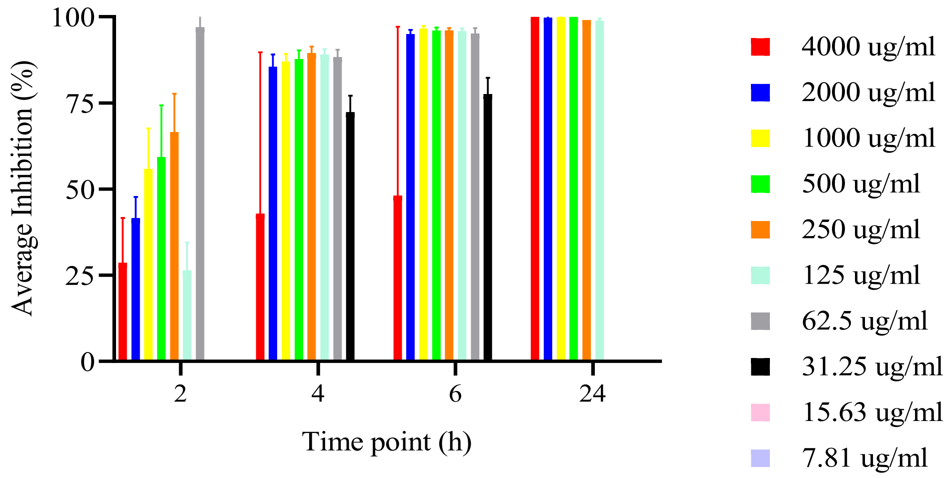
Average Inhibition (%)

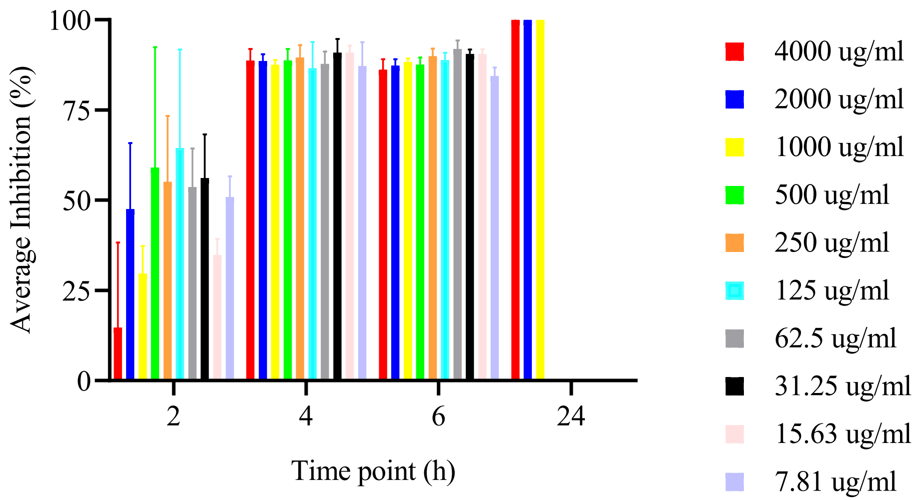
100
75
50
25
0

2
4
6
24

Time point (h)

4000 ug/ml
2000 ug/ml
1000 ug/ml
500 ug/ml
250 ug/ml
125 ug/ml
62.5 ug/ml
31.25 ug/ml
15.63 ug/ml
7.81 ug/ml


TK of Nerolidol against MRSA
Average Inhibition (%)
100
75
50
25
0
2
4
6
24
Time point (h)
4000 ug/ml
2000 ug/ml
1000 ug/ml
500 ug/ml
250 ug/ml
125 ug/ml
62.5 ug/ml
31.25 ug/ml
15.63 ug/ml
7.81 ug/ml


TK of Nerolidol against *Lactobacillus acidophilus*
Average Inhibition (%)
100
75
50
25
0
2
4
6
24
Time point (h)
4000 ug/ml
2000 ug/ml
1000 ug/ml
500 ug/ml
250 ug/ml
125 ug/ml
62.5 ug/ml
31.25 ug/ml
15.63 ug/ml
7.81 ug/ml

Unloaded Nanoliposome (UN) and Loaded Nanoliposome (LN)

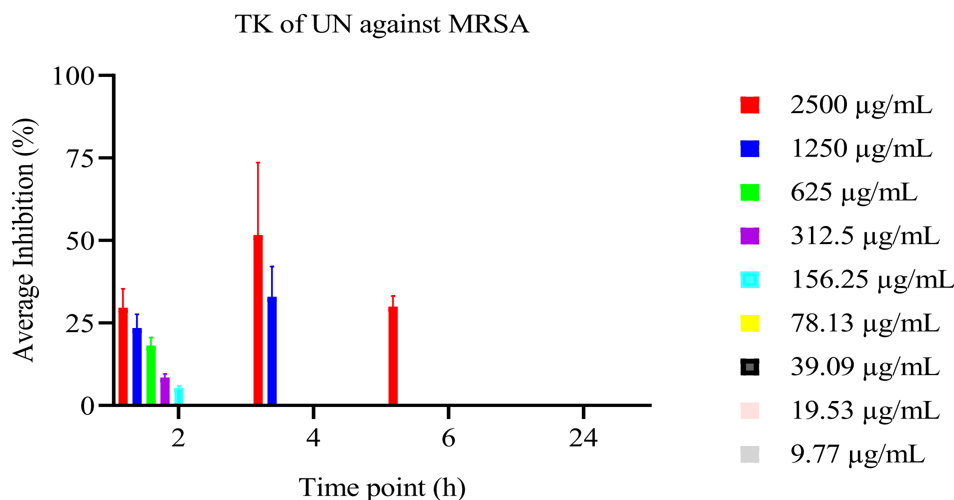
Gram positive bacteria

MRSA

In Figure 3 the results of the TK evaluation for both UN and LN against MRSA are represented. No statistical differences were observed between UN and LN at each time point and for all tested concentrations. For both the compounds, no antimicrobial activity was detected on MRSA after 24 hours of contact.

At 2 hours of incubation the inhibition percentages of both UN and LN resulted lower than 50%. At 4 hours of incubation LN showed a maximum inhibition percentage of 60.7% at the highest tested concentration, however showed 25%, also at 1250 and 625 µg/mL (42.09 and 33.91%, respectively). In addition, only the maximum tested concentration reported activity at 6 hours of incubation but decreased to 34%. Also, UN showed partial inhibition at the highest tested concentration at 4 and 6 hours of contact (51.55% and 29.91%, respectively).

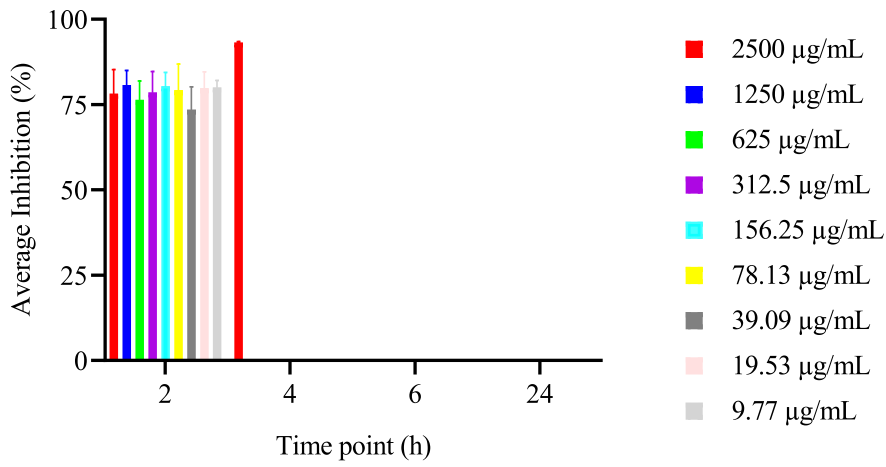
Figure 3. Time-Killing (TK) assay of different concentrations (µg/mL) of loaded nanoliposome (LN) with Nerolidol and uncharged nanoliposome (UN) on MRSA at different time points.

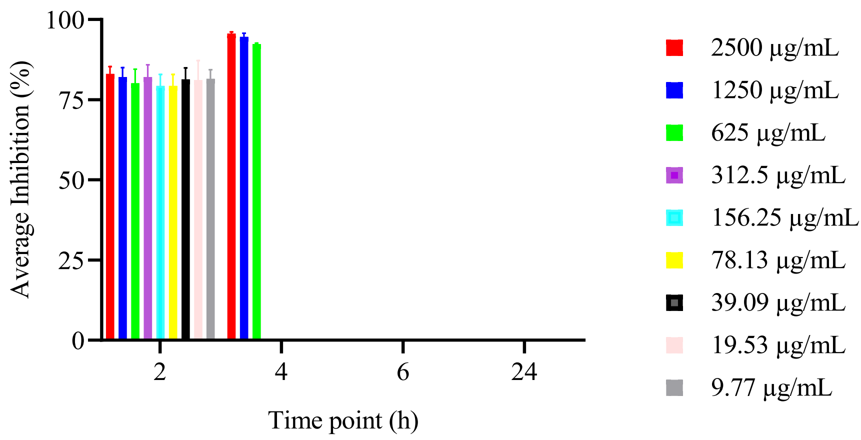


E. faecium

Figure 4 shows the Time-Kill results of LN and UN for *E. faecium*, where all tested concentrations displayed a partial antimicrobial activity already after 2 hours of incubation. However, while for LN only the highest tested concentration exhibited activity after 4 hours (93% at 2500 µg/mL), for UN at the same time point antimicrobial activity was reported both for 1250 and 625 µg/mL, showing a significative difference for these two concentrations respect to LN ($P < 0.05$). Finally, both formulations did not report antimicrobial activity after 6 and 24 hours of contact.

Figure 4. Time-Killing (TK) assay of different concentrations (µg/mL) of loaded nanoliposome (LN) with Nerolidol and uncharged nanoliposome (UN) on Enterococcus faecium at different time points.

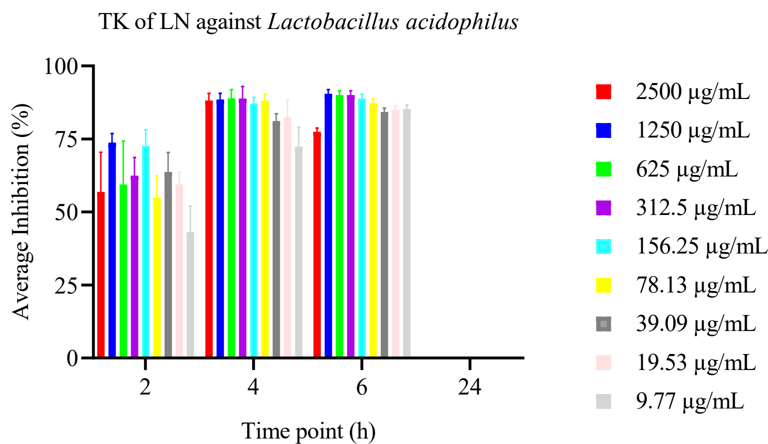

TK of LN against *Enterococcus faecium*
Average Inhibition (%)
100
75
50
25
0
2
4
6
24
Time point (h)
2500 µg/mL
1250 µg/mL
625 µg/mL
312.5 µg/mL
156.25 µg/mL
78.13 µg/mL
39.09 µg/mL
19.53 µg/mL
9.77 µg/mL

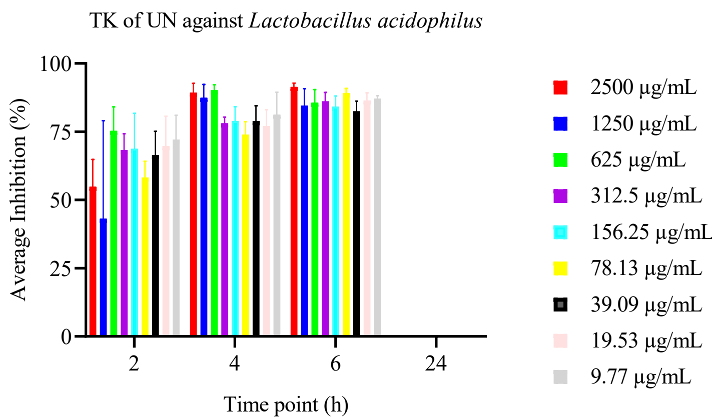

TK of UN against *Enterococcus faecium*
Average Inhibition (%)
100
75
50
25
0
2
4
6
24
Time point (h)
2500 µg/mL
1250 µg/mL
625 µg/mL
312.5 µg/mL
156.25 µg/mL
78.13 µg/mL
39.09 µg/mL
19.53 µg/mL
9.77 µg/mL

L. acidophilus

Figure 5 shows the TK results of LN and UN for *L. acidophilus*, where all tested concentrations of both compounds exhibited partial inhibition of growth at 2 and 4 hours of contact. Statistically differences among LN and UN were observed at 312.5, 156, 78.12, 19.53 and 9.765 µg/mL with greater antimicrobial activity for LN at all above mentioned concentrations, except for 9.765 µg/mL. No statistical differences were found at 6 hours for all tested concentrations. Lastly, no antimicrobial activity was observed at 24 hours for all tested concentrations.

Figure 5. Time-Killing (TK) assay of different concentrations (µg/mL) of loaded nanoliposome (LN) with Nerolidol and unloaded nanoliposome (UN) on *L. acidophilus* at different time points.



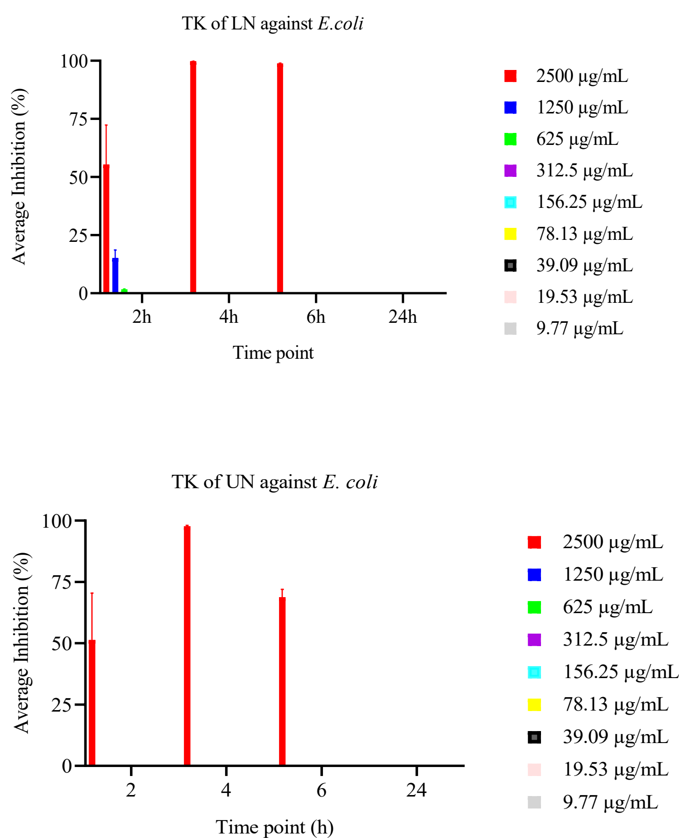


Gram negative bacteria

E. coli

Figures 6 illustrate the TK assay results for *E. coli*; in particular, both LN and UN demonstrated antimicrobial activity at 2 hours, with partial growth inhibition (> 50%) observed at a concentration of 2500 µg/mL; a significant difference (P < 0.05) was noted at 1250 µg/mL, with higher antimicrobial activity for LN. Additionally, no differences were reported after 4 hours and 6 hours. Lastly, none of the compounds showed antimicrobial activity after 24 hours of contact.

Figure 6. Time-Killing (TK) assay of different concentrations (µg/mL) of loaded nanoliposome (LN) with Nerolidol and uncharged nanoliposome (UN) on *E. coli* at different time points.



S. Typhimurium

Figures 7 illustrate the TK assay results for *S.* Typhimurium; at each point and for all tested concentrations, no significant differences were observed between UN and LN. Regarding *S.* Typhimurium, LN exhibited antimicrobial activity with a growth inhibition > 90% after 2 hours of

incubation at 2500 and 1250 µg/mL. The highest tested concentration showed an increase of grown inhibition with a tendency at values above 99% after 4 hours. However, after 6 h of contact, a decrease in growth inhibition was observed, reaching 89.33%. For the UN, antimicrobial activity was observed until 1250 µg/mL with a growth inhibition > 70% after 2 hours of incubation, but only the highest tested concentration maintained an inhibition > 99% at 4 and 6 hours of incubation. For both LN and UN, after 24 hours of contact a 40% of inhibition was retained by both at 2500 µg/mL.

Figure 7. Time-Killing (TK) assay of different concentrations (µg/mL) of loaded nanoliposome (LN) with Nerolidol and uncharged nanoliposome (UN) on S. Typhimurium at different time points.

Cytotoxicity assay

MTT cell survival assay

Figure 8 displays the cytotoxicity data of Nerolidol on IPEC-J2 cell lines. All tested concentrations of Nerolidol demonstrated significant cytotoxicity on both cell lines compared to their respective negative controls (P<0.001). None of the concentrations tested led to a survival rate of ≥ 30%.

The results of the cytotoxic activity of UN and LN are shown in Figure 9. Regarding the cytotoxicity activity of UN on the MDBK cell line, its maximum concentration tested (2500 μg/mL) showed the highest level of cytotoxicity with survival rate ≤ 10%. However, all remaining concentrations showed a survival rate close to 90%; in particular, concentrations ≤ 625 μg/mL had survival rate over 95%. A similar trend was reported for LN, in particular the concentrations ≤ 1250 ug/mL showed survival rate greater than 80%. On the IPEC-J2 cell line for both UN and LN the highest cytotoxicity was revealed at 2500 μg/mL. For both compounds at concentration of 1250 μg/mL cell survival was < 80%. At the concentrations ≤ 625 ug/mL LN and UN showed no difference in comparison to the negative control, showing a survival rate close to 100%.

Figure 8. Time-Killing (TK) assay of different concentrations (μg/mL) of loaded nanoliposome (LN) with Nerolidol and uncharged nanoliposome (UN) on S. Typhimurium at different time points.

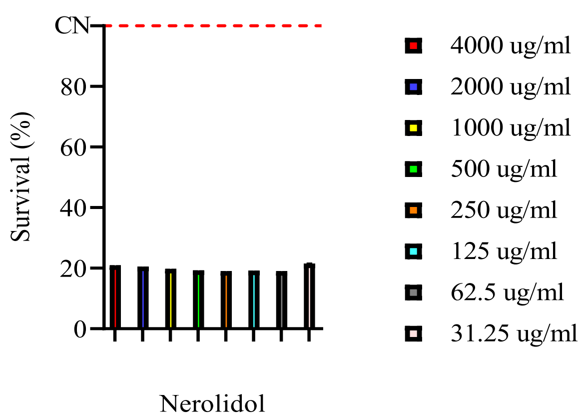
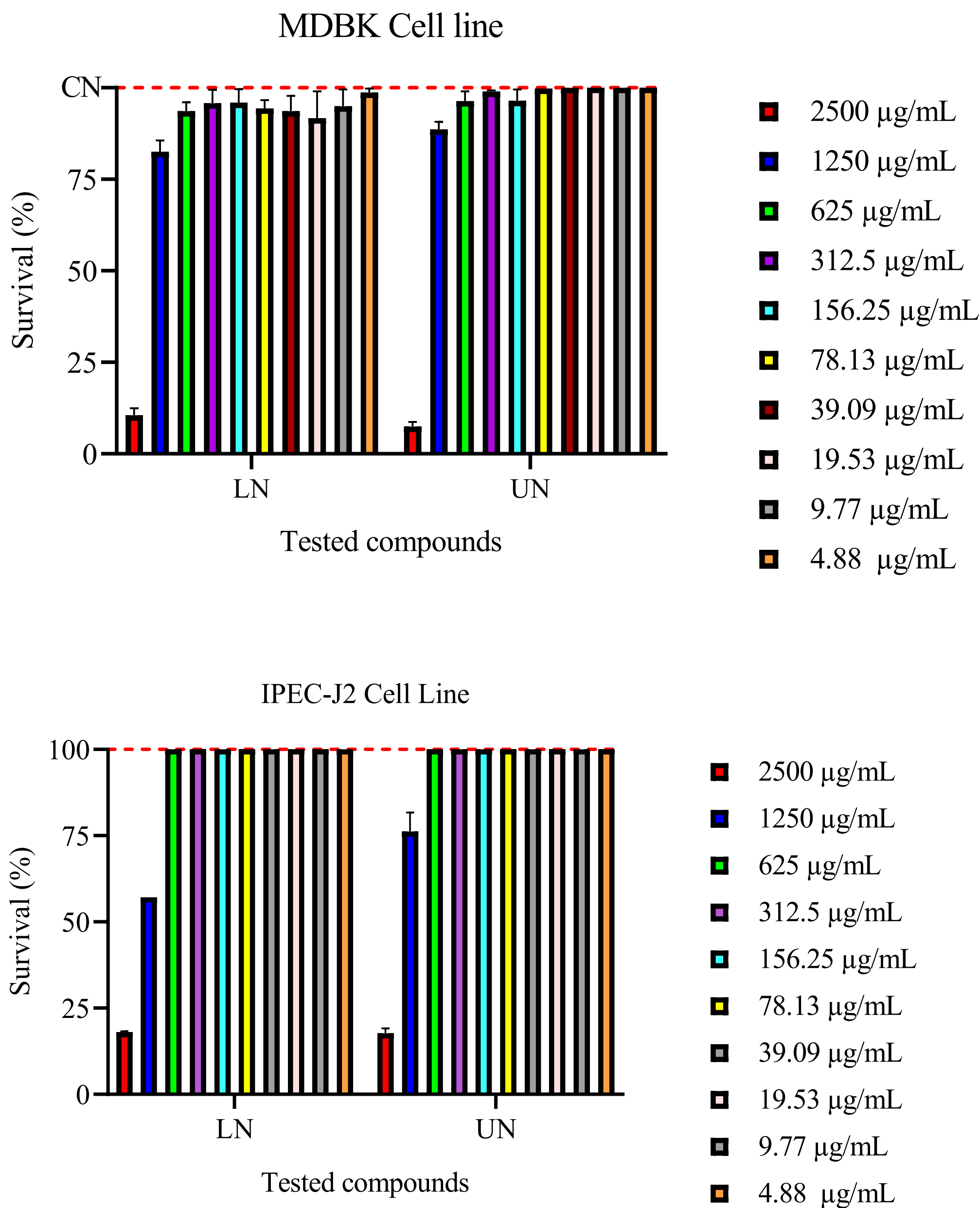


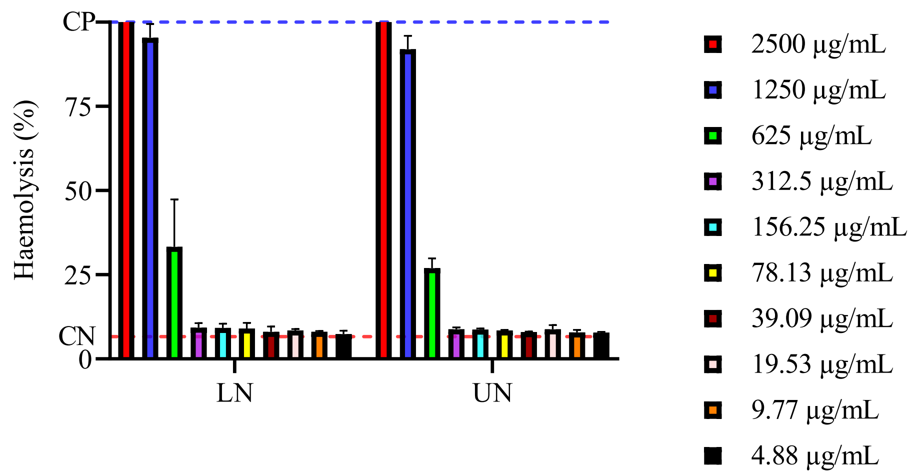
Figure 9. Survival of MDBK and IPEC-J2 cell lines after 24h contact with loaded nanoliposome (LN) with Nerolidol or unloaded nanoliposome (UN).



Hemolysis assay

The results of the hemolytic activity evaluation of LN and UN are shown in Figure 10. Specifically, for both compounds, the highest hemolysis percentages were observed at the first two concentrations. Hemolysis percentages below 10% were recorded for both compounds starting from a concentration of 312.5 µg/mL.

Figure 10. Percentage of hemolysis of blood cells after 24 h of contact with loaded nanoliposome (LN) with Nerolidol or unloaded nanoliposome (UN).



Discussion

The first part of the project highlighted the antimicrobial activity of various NICs, particularly curcumin, geraniol, and nerolidol. However, as previously mentioned, their high toxicity limited their direct application in veterinary medicine. For this reason, an attempt was made to encapsulate these NICs in nanoliposomes. However, successful encapsulation was achieved only by nerolidol. Therefore, the second part of the project aimed to evaluate the antimicrobial activity of nanoliposome formulations, both LN and UN with nerolidol, as well as to assess their cytotoxicity profiles. Additionally, the antimicrobial activity of free-form nerolidol was evaluated using time-kill (TK) methodology to study the behavior of this NIC over time. Experimental time points were selected to mimic the average retention time in the gastrointestinal tract of monogastric animals.

Based on this approach, the study results regarding the TK profile of nerolidol once again confirmed its antimicrobial activity in its free form. Notably, the findings demonstrate that nerolidol effectively inhibits bacterial growth within the initial moments of contact, even at lower concentrations, especially against *Enterococcus faecium*; however, it was completely ineffective against Gram-negative bacteria. Unfortunately, as expected, despite its significant antimicrobial activity against Gram-positive bacteria, nerolidol exhibited high toxicity at all tested concentrations towards the IPEC-J2 cell line. These results confirm the findings from the preliminary screening, highlighting the need to encapsulate nerolidol within a system capable of preserving its antibacterial properties while simultaneously reducing its cytotoxicity. For this reason, nerolidol was loaded into nanoliposomes. The nanoliposome suspension used for loading nerolidol had a concentration of 5000 μg/mL; however, due to the intrinsic properties of nerolidol, a load ratio of nerolidol of 1% was used, since it is known that the terpene-loaded liposomes, also known as *invasomes,* cannot host large load of the guest molecule (Amnuaikit et al., 2018; Babaie et al., 2021; Vieira Nunes Cunha et al., 2023). Therefore, and in this case, the active molecule nerolidol presents as 1% of the total nanoliposome concentration (5 g/L). This meant that, while it was possible to test concentrations of free-form nerolidol up to 4000 μg/mL, for the LN, a stock suspension containing 5000 μg/mL of nanoliposomes with 50 μg/mL of nerolidol was used.

Therefore, for both the antimicrobial and cytotoxicity evaluations, the maximum concentration tested was 2500 μg/mL of LN containing 25 μg/mL of nerolidol.

Taking all these considerations into account, the antimicrobial activity of LN was assessed using the TK assay, along with the evaluation of the UN formulation, to highlight the differences between the two formulations at the various experimental time points. Antimicrobial activity was evaluated for LN and UN by TK assay against bacterial pathogens commonly found in livestock and *L. acidophilus* as an indicator of compliance with the intestinal lactic microflora. In particular, the different time points were selected to simulate the average retention times within the gastrointestinal system of a monogastric animal, to hypothesize a potential *vivo* application by oral administration. It was observed that nanoliposome formulations demonstrated time and dose-dependent antimicrobial activity against all tested bacteria. However, similar behaviors were observed between the two nanoliposome formulations against MRSA and *S.* Typhimurium at all tested concentrations and time points. Conversely, slight differences were noted for the other bacteria only at 2 and 4 hours, and never at the highest tested concentration, maybe due to the intrinsic characteristics of the different bacteria (e.g. bacterial structure or time-replication). It is important to note that in this study, a nutrient-rich medium, such as MHB, was used, which allows bacteria to replicate over time. As a result, it is evident that nanoliposome formulations exhibited a growth inhibitory effect only at the first time points and not a bactericidal effect, except on S. Typhimurium, on which a partial inhibition was observed with the highest concentration at 24 h without differences among LN and UN. The same concentration was effective for 6 hours against MRSA, *E. coli* and *L. acidophilus*, with a similar effect between LN and UN. However, for the latter bacteria, both formulations showed activity until to the lowest tested concentration at the same point. Finally, the weakest antimicrobial activity was observed against *Enterococcus faecium*, where the formulations remained effective up to 4 h of contact.

These results indicate that, for LN and UN, the antimicrobial activity is exerted mainly by the nanoliposome, since the addition of nerolidol does not exert an improvement of the antimicrobial activity, except during the first time points (until 4 h), exclusively on *E. coli* and *L. acidophilus*. This is maybe due to the low concentration of nerolidol included in the nanoliposome formulation. In the

literature, it is commonly reported that the inclusion of EOs within encapsulation systems often enhances their antimicrobial activity compared to their free form, maybe due to a greater ability (Majeed et al., 2015; Zabihi et al., 2017; Zhu et al., 2021; Yousefi et al., 2024). In our study, we observed that LN exhibited activity even against Gram-negative bacteria, which free-form nerolidol did not affect. Moreover, LN demonstrated efficacy, using nerolidol at sub-MIC concentrations, suggesting that the nanoliposome itself may have facilitated its action. This is further confirmed by the absence of significant differences in most experimental points across various bacterial strains for LN and UN, highlighting the intrinsic antimicrobial properties of the nanoliposome platform itself. The antimicrobial efficacy of nanoliposomes could be due by non-specific mechanisms such as membrane fusion with the pathogens and subsequent destabilization of the bacterium inducing its death (Wang et al., 2020; Yousefi et al., 2024). Since liposomes are lipid-based vesicles, they can interact with bacterial membranes, disrupting their lipid bilayer structure. This disruption can impair vital membrane functions, including nutrient transport, respiration, and ion regulation. Furthermore, liposomes can induce oxidative stress, interfere with bacterial signaling, and enhance immune cell activity (even if these behaviors could not be of interest in present experiments- Lee et al., 2024).

These fundings suggest that the antimicrobial effect due to the delivery platform itself has an important role in the total efficacy of the substance. It was evidenced not only for pathogens bacteria but also commensal flora. This finding could represent a limitation of this study and these formulations, however it's important to consider *L. acidophilus,* is not the only indicator of healthy microbiota. Other bacteria, such as *Bifidobacterium*, *Firmicutes*, and *Bacteroidetes*, are also critical for intestinal health. Therefore, the antimicrobial effect on a single bacterium may not accurately reflect the broader impact the compound could have on the entire microbiota. The gut microbiota is a complex and interconnected ecosystem and targeting a single bacterium does not necessarily capture the overall effects on the balance and health of the intestinal environment (Fan et al., 2021). Additionally, if we compare the effects of natural molecules to those of traditional antimicrobials, it is widely recognized in the literature that antimicrobials disrupt the balance of the gut microbiota, promoting the onset of opportunistic infections and the development of antimicrobial resistance mechanisms. In contrast, the

risks of dysbiosis associated with nanoliposome formulations appear to be lower compared to conventional antibiotics (Ruzauskas et al., 2020; de Nies et al., 2023). This could be partly due to the short duration of action of these formulations (up to 6 hours), which reduces their impact on the gut microbiota, as seen with *Lactobacillus acidophilus*. However, further studies, particularly in vivo tests, will be necessary to obtain more precise information on the safety and efficacy of the compound. These studies will allow for a more comprehensive evaluation of its potential implications, especially regarding its effect on the gut microbiota, providing more detailed and reliable data for safe and effective use.

To hypothesize potential *in vivo* action, it is important to consider the safety of these formulations, which is obviously related to their cytotoxicity. Analyzing the cytotoxicity results for UN and LN, a dose-dependent cytotoxicity was observed for both formulations on both cell lines; in particular, IPEC-J2 line was more sensitive to the cytotoxic activity of the nanoliposome formulations, with the highest survival rates observed at concentrations below 625 µg/mL. Whereas, for the MDBK cell line, both UN and LN showed survival rates greater than 80% at 1250 µg/mL.

Lastly, a higher cytotoxicity was observed with LN than UN, probably due to the presence of nerolidol, which, as mentioned above, exhibits high cytotoxicity. This therefore allows us to hypothesis a possible use of this nanoliposome formulation as feed additives for livestock. However, further studies will be necessary to understand how they can affect the livestock animal performances and efficiency production when included into feed formulations.

Third study: Investigation of the Effects of Nanoliposome Formulation on Animal Nutrition: An *In vitro* Study

Introduction

It is widely recognized in the literature that livestock farming contributes to GHG emissions, with a major impact on the environment. In recent years, the European Union has financed numerous studies to improve the environmental sustainability of this sector. Great interest has been given to CH_4 mitigation strategies to reduce the ecological footprint of livestock farming. The CH_4 is the second main contributor to global warming having a 21-fold greater global warming potential than that of CO_2 is produced also through emissions from livestock(Charles Nnaji et al., 2011). Thus, exploring strategies to mitigate these emissions is crucial for reducing the environmental impact of animal farming (Beauchemin et al., 2022). To mitigate CH_4 emissions, various strategies have been developed, among them the optimization of animal productivity and the use of production enhancing agents are the most known. In general, when animal productivity is improved through nutrition, management, reproduction or genetics, CH_4 production per unit of meat or milk is reduced. Several dietary strategies have been tested to reduce CH_4 emissions, including modifying the type and digestibility of carbohydrates used in diets or increasing feed intake. It has been observed that higher feed intake reduces fermentation due to a faster passage rate through the rumen, thereby decreasing the contact time between organic matter and microorganisms. Additionally, other nutritional strategies proposed over the years include reducing meal frequency, which lowers ruminal pH and inhibits methanogenesis. However, this approach could lead to reduced productivity, making it impractical for producers (Boadi et al., 2004). Other strategies involve the use of dietary fats and, most notably, alternative compounds as feed additives. In particular, different compounds have been tested *in vitro* and *in vivo* for their ability to influence methanogenesis directly by modulating the microbial population, and indirectly by acting on feed digestibility; for example, ionophores are known for their activity on gram-positive bacteria and protozoa, shifting fermentation pathways from acetate to propionate; whereas plant products are active both on gram-positive bacteria and, though less so, on gram-negative bacteria,

indicating a broader activity (Ku-Vera et al., 2020; Beauchemin et al., 2022). However, several *in vitro* studies have shown that different EOs and NICs have a dose-dependent effect on rumen bacterial flora, reducing the total count of viable bacteria and consequently inhibiting their activity (Calsamiglia et al., 2007; Benchaar et al., 2008). This effect may therefore explain the toxic and sperm-depressing effect reported in the literature with the use of these products on ruminal activity. Other important limitations related to the use of EO and their active principles, is related to their instability and high susceptibility that reduces their biological properties when used in free form; for this reason, several studies in recent years in the field of nutraceuticals have focused on the use of encapsulation systems (Turek and Stintzing, 2013; Rajendran et al., 2022). Encapsulation technology plays a crucial role in modern livestock production, offering a wide range of applications. It is used for immune modulation, probiotic delivery, improving growth performance, protecting the gut environment from rumen microbes (rumen bypass), improving nutrient digestibility and optimizing pharmacokinetics. This technology provides effective protection for active compounds, ensuring their stability and targeted release at specific sites, resulting in improved bioavailability (Rajendran et al., 2022). Nanoliposomal systems are widely used encapsulation methods capable of loading both hydrophobic and hydrophilic molecules. Despite their advantages, they are limited by their loading capacity and efficiency (Taylor et al., 2005; Nsairat et al., 2022). A few studies have explored nerolidol-loaded nanoliposomes, but for different purposes (e.g. food shelf-life; Ephrem et al., 2019), while to the best of our knowledge, there are no studies that have evaluated *in vitro* a possible application in veterinary nutrition for livestock use. For this reason, the aim of the third phase of the study is to evaluate the *in vitro* effects of nanoliposome loaded with Nerolidol (LN) and unloaded (UN) on dry matter (DM), crude protein (CP), and neutral-detergent fibre (NDF) digestibility of swine and bovine.

Materials and Method

Liposome

The materials and the protocols used to produce nanoliposome were previously reported in the section Material and Method in "*Step Two: Assessment of Antimicrobial Activity and Cytotoxicity of Nanoliposome Formulation*". Additionally, for the treatments LN and UN, the tested dosage was calculated as 0.1% of the substrate weight, equivalent to a hypothetical application of 100 g per 100kg of feed. To achieve this, considering the relative weights of both nanoliposome formulations, 1 mL of a stock solution containing 5 g/L of LN or UN was diluted in 1000 mL of deionized water.

Swine: *In vitro* dry matter and crude protein intestinal digestibility

Table 4. Chemical composition swine feedstuff expressed as percentage (%) of Dry Matter (DM).

Nutrients	% DM
Dry matter	91.0
Crude protein	17.5
Eher extract	3.9
Starch	/
Ash	5.11
Neutral detergent fibre	18.00
Acid Detergent fibre	7.42
Acid detergent lignin	1.79
Crude Fiber	5.09

In this study, 1kg of feedstuff sample of sample was ground to pass through a 1-mm mesh screen (Retch Bauknecht, Stuttgart, Germany. The chemical composition of the feedstuff was determined following the panel analysis described by Guerra et al. (2024), and is reported in table 4. In brief, DM, ash, and ether extract (EE) content were determined following European Commission Regulation No. 152/2009 (European Commission, 2009) recommendations. The N content determined by the combustion digestion of the sample at 900°C in an excess of oxygen by Dumatherm (Gerhardt GmbH &Co, Königswinter, Germany) as described by Mihaljev et al. (2015) and feedstuff CP content was calculated as percentage of N × 6.25. The starch content was analyzed by enzymatic method (method 2014.10; AOAC International, 2014). The amylase-treated NDF without residual ash (aNDFom), ADF and ADL were analyzed according to Mertens et al. (2002); for the boiling and filtering phase, a semi-automated system (FIWE Raw Fiber Extractor, VELP Scientifica, Usmate Velate, Italy) was employed.

The evaluation of the effect of three treatments: CONTROL (CTR), UN and LN on swine digestibility was carried out following the protocol reported in literature by Boisen and Fernhdez, (1997). In detail, 420 µL of LN and 470 µL of UN were added into each flask two analytical replicates were performed per run, for each treatment at each specific phase (gastric, small intestine and cecal). The whole experiment was replicated three times.

About 0.5 g of finely ground feedstuff for swine was weighed and placed into 125-mL conical flasks. Subsequently, 25 mL of phosphate buffer was prepared as follows: 3.72 g $NaHPO_4$ + 3.92 g $NaHCO_3$, + 0.19 g NaCl + 0.23 g KCL + 0.12 $CaCl_2$ + 2 in 1 L of distilled water were added to each flask (T^0). Subsequently, 10 mL 0.2M HCl was added, and pH was adjusted to pH 2.0 with 1M HCL or 1M NaOH solution. Subsequently, for the gastric phase, 1 mL of a freshly prepared pepsin solution containing 25 mg pepsin (porcine, P-7000, CAS no.9001-75-6, Sigma-Aldrich, St. Louis) was added in each flask. All the flasks were incubated into a water bath at 39°C for 2 h. After incubation, for the intestinal phase, 10 mL of phosphate buffer and 5 mL of 0.6 M NaOH were added to each flask and the pH of the solution was adjusted to 6.8 with a 1M HCL or 1M NaOH solution. Thereafter, 0.5 mL of fresh pancreatin solution (100 mg/mL Pancreatin 8 USP, P-7545, CAS no. 8049-47-6, Sigma-Aldrich, St. Louis) was added to the flask and incubated at 39°C for 4 h. Lastly, fFor the cecal phase, 10 mL of 0.2 M EDTA solution were added to the flask, and the pH was adjusted to 4.8 with 30 % acetic acid. Then, 0.5 mL of Viscozyme (V2010-multienzyme complex from *Aspergillus aculeatus* containing cellulase, β -glucanase, arabinase, xylanase, mannanase, and pectinase; Sigma-Aldrich, St. Louis) were added, and the flasks were incubated at 39°C for 18 h.

At the end of T0, gastric, intestinal and cecal phase, flasks were immediately removed, and their contents were filtered using a glass crucible (Robu Glass Filter – ROBU H3, Borosilicate 3.3, 30 mL – Por. 2, Hattert, Germany) containing 0.4 g of celite (CAS no. 68855-54-9 as filter aid; Sigma-Aldrich, St. Louis) to determine DMD and CPD for each phase. However, only for cecal phase, the residue was collected in a filtration unit containing 0.4 g celite and filled and let stand for 2 minutes two consecutive times with10 mL of ethanol (96%) and acetone (99.5%). The crucible was dried at 103 °C overnight,

and moisture was defined by calculation as 100 – DM. Subsequently, DMD was evaluated by taking account of initial DM of the feedstuff for each digestion phase.

Nitrogen, for each digestion phase, was determined following the Dumas method (AOAC 992.23) by the combustion digestion of the sample at 900 °C more than oxygen by Dumatherm® (Gerhardt GmbH & Co, Königswinter, Germany) as reported in literature (Mihaljev et al., 2015) , and feedstuff CP content was calculated as percentage of N × 6.25. CP digestibility was expressed by taking account the initial CP content of the feedstuff.

Swine: *In vitro* neutral detergent fiber cecal digestibility

For the *in vitro* swine cecal fermentation, 0.5 g of material underwent the same procedure previously described until the intestinal phase. The cecal phase step was carried out following the method described by Piva et al. (2002) using the dosage above (0.1 % of substrate). Briefly, three coelomic contents were removed from three swine at the slaughterhouse in accordance with European Union guidelines. The intestinal tracts were transported under anaerobic conditions into the laboratory, where the cecal fluids were collected from the cecum and colon. The cecal fluid of the three swine were mixed and then filtered and diluted (1:2) with McDougall's buffer solution (1948). The filtered cecal fluid was flushed with CO_2 and then inoculated into flasks from the previous second step of gastro-enteric digestion. The samples were then sealed and incubated at 39°C for 18 hours under anaerobic conditions. Then, their contents were filtered using a glass crucible (Robu Glass Filter – ROBU H3, Borosilicate 3.3, 30 mL – Por. 2, Hattert, Germany) containing 0.4 g of celite (CAS no. 68855-54-9 as filter aid; Sigma-Aldrich, St. Louis) and the residual aNDFom content was analyzed following the methods of Goering and Van Soest 1970) and Mertens et al. (2002), using α-amylase but omitting sodium sulfite, with corrections made for ash content. The boiling and filtration steps were conducted using a semi-automated system (FIWE Raw Fiber Extractor, VELP Scientifica, Usmate Velate, Italy). Analyses were conducted in triplicate in a single run.

Bovine: In vitro dry matter and crude protein ruminal digestibility

Table 5 Chemical composition bovine Tota-mixed-ratio expressed as percentage (%) of Dry Matter (DM).

Ingredient	% DM
Earlage	23.19
Corn silage	22.05
Feedstuff	21.08
Alfaalfa silage	16.07
Corn meal	8.33
Cottonseed	5.52
AlfaAlfa	3.75

One kilogram of fresh total mixed ration for lactating dairy cows was collected in a commercial farm in Piacenza (Italy). The sample was oven-dried at 55 °C for 48 h then ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass through a 1-mm screen, chemically analyzed and employed as a substrate for the digestions. The chemical composition of the substrate used in the experiment is presented in Table 5. To evaluate the effect of LN and UN on bovine digestibility (including at 0.1% of the substrate, as above mentioned in "*In vitro* swine digestion") the ruminal degradation was followed by a gastric and intestinal phase, two analytical replicates were performed per each treatment and phase in each run. The experiment was repeated three times.

For the ruminal digestion phase, the procedure followed the methodology described in literature (Goering and Van Soest, 1970). Briefly, 0.5 g of substrate were weighed and placed into each 125 ml flask, adding the treatment (420 µL of LN and 470 µL of UN, pre-diluted) and 40 ml of Van Soest buffer and incubated at 39 °C under CO_2 flushing. The rumen fluid was collected from four different cows at a slaughterhouse, stirred, and filtered through four layers of cheesecloth under continuous flushing with CO_2. Ten ml of filtered rumen fluid was then inoculated at a 1:4 ratio in each flask (T^0). For ruminal phase, flasks were incubated at 39 °C for 18h. The gastric and intestinal digestion phase were conducted according to the protocol described by Ross et al. (2013) on all flasks from the rumen phase. Specifically, for gastric phase 2–3 mL of 3M HCl was added to adjust the pH to 1.9; then 2 mL

of acid pepsin (224.1 Worthington U/mL) were added on all flasks. The flasks were then incubated at 39 °C for 1 h without CO_2 flushing. For the intestinal phase, the pH was adjusted to 5 using 2 mL of 2M NaOH. Subsequently, 10 mL of enzymatic solution containing a mix of digestive enzymes were added. The enzyme solution included: trypsin (PANREAC, code A4148, from porcine pancreas, 250 USP U/mg, 750 BAEE U/mg, Barcelona, Spain), chymotrypsin (MERCK, code 1.02307.0001, from bovine pancreas, activity 350 BTEE U/mg, Darmstadt, Germany), α-amylase (SIGMA, code 10069, from *Bacillus subtilis*, activity 380 U/mg, Sigma-Aldrich, St. Louis), bile salts (MILLIPORE, code B3883-100, from bovine, Darmstadt, Germania), and lipase (MP Biomedicals, code 102189-80, from porcine pancreas, activity 10 USP U/mg, Santa Ana, California). The flasks were then capped and incubated at 39 °C for 24 h.

After each phase, T0, ruminal, gastric and intestinal, flasks were immediately removed, and their contents were filtered using a glass crucible, as above-mentioned, to determine the DMD and CPD for the intestinal phase. two analytical replicates were performed per each treatment and phase in each run. The experiment was repeated three times. The DMD and CPD for each digestive phase were calculated as previously described in the section "In *Vitro* Swine Digestion."

B*ovine*: In vitro *neutral d*etergent fiber ruminal digestibility

The *in vitro* batch fermentation system, as described by Goering and Van Soest (1970) was used to evaluate the effects of UN and LN on NDF digestibility on the same substrate used for *in vitro* bovine digestibility, at two different fermentation points (24h and 48h; $NDFD_{24}$ and $NDFD_{48}$) using the dosage described in the section ("*In Vitro* Swine Fermentation"). Briefly, 0.5 g of substrate were weighed and placed into each 125 ml flask, adding the treatment (420 μL of LN and 470 μL of UN, pre-diluted) and 40 ml of Van Soest buffer and incubated at 39 °C under CO_2 flushing. The rumen fluid was collected from four different cows at a slaughterhouse, stirred, and filtered through four layers of cheesecloth under continuous flushing with CO_2. Ten ml of filtered rumen fluid was then inoculated at a 1:4 ratio in each flask and incubated at 39 °C at two different time points (24h and 48h). After incubation time, flasks were removed and analyzed for the determination of NDF; briefly each flask was transferred to a semi-automated system (FIWE Raw Fiber Extractor, VELP Scientifica, Usmate Velate, Italy) and

boiled with the addition of heat-stable amylase for 1 h; the residuals were then rinsed three times with boiling water and NDF was expressed on a DM basis including residual ash (Mertens et al., 2002). Then, $NDFD_{24}$ and $NDFD_{48}$ were calculated by difference and expressed as a proportion of supply. Analyses were conducted in triplicate and only one fermentation was performed.

Statistical analysis

All statistical analyses were performed using the SPSS for Windows software package (version 29.0; SPSS Inc., Chicago, IL, USA). The differences between treatments for DMD, CPD and NDFD were analyzed separately using the univariate procedure of the General Linear Model (GLM).

For swine digestion (DMD and CPD) a univariate GLM was applied, using the treatments as fixed factors. In contrast, for the bovine digestibility model (DMD and CPD), since different ruminal fluids were used among the experiment, a univariate GLM was employed where the factor "run" or "experiment" was treated as a random factor, while the various treatments were included as fixed factors.

Lastly, for preliminary evaluation of NDFD, because was performed only one experiment using, a GLM model without random factor was used for bovine and swine *in vitro* fermentation.

The LSD post hoc test was applied to evaluate the statistical significance between treatments. Differences were declared significant at $P \leq 0.05$. Results were reported as least squares means.

Results

Swine Result of *in vitro* dry matter and crude protein intestinal digestibility.

Table 6 shows the effects of the nanoliposome formulation on DMD. The addition of nerolidol or nanoliposome itself did not affect DMD through the whole gastro-intestinal tract (P > 0.05). Furthermore, no reduction in DMD was observed during the experiment. Additionally, the influence of UN and LN treatments on CPD is shown in Table 7. Also, in this case, no differences were due to the treatments (P > 0.05); thus depression in protein digestibility was observed.

Table 6. Results of dry matter digestibility (DMD) by loaded nanoliposome with Nerolidol (LN) and unloaded nanoliposome (UN) *in vitro* swine digestibility.

Phase	DMD %			P-value	SEM
	LN	UN	CTR		
T_0	20.70	24.81	19.62	0.622	2.19
Gastric	30.50	32.14	32.86	0.434	0.74
Intestinal	34.51	39.31	39.15	0.532	1.90
Cecal	76.50	70.80	73.31	0.489	1.91

Table 7. Influence of loaded nanoliposome with Nerolidol (LN) and unloaded nanoliposome (UN) on Crude Protein Digestibility (CPD) *in vitro* swine digestibility.

Phase	CPD %			P-value	SEM
	LN	UN	CTR		
T_0	62.97	71.76	67.09	0.477	2.79
Gastric	87.84	87.16	88.12	0.910	0.88
Intestinal	81.40	86.10	87.17	0.748	2.36
Cecal	94.05	93.05	93.55	0.924	1.03

Swine: Result of *in vitro* neutral detergent fiber cecal digestibility.

The results of the influence of LN and UN on NDFD in an *in vitro* cecal fermentation model are presented in Table 8. No significant differences were observed.

Table 8. Influence of loaded nanoliposome with nerolidol (LN) and unloaded nanoliposome (UN) Neutral Detergent Fibre Digestibility (NDFD) *in vitro* swine fermentation.

Cecal	Treatmetns			P-value	SEM
	LN	UN	CTR		
NDFD %	33.40	45.04	30.68	0.234	0.235

Bovine: Result of *in vitro* dry matter and crude protein ruminal digestibility.

Table 9 shows the results of the effects of UN and LN on DMD of bovine. Specifically, no differences were observed between the treatments ($P > 0.05$) throughout the whole gastrointestinal digestion process. The treatments did not decrease the DMD. The effects of UN and LN on CPD are reported in Table 10, also in this case no differences were due to the treatments ($P > 0.05$); thus, no depression in protein digestibility was observed.

Table 9. Influence of loaded nanoliposome with Nerolidol (LN) and unloaded nanoliposome (UN) on Dry Matter-Digestibility (DMD) – *in vitro* bovine digestibility.

Phase	DMD %			P-value	SEM
	LN	UN	CTR		
T_0	31.04	33.50	32.37	0.354	2.36
Rumen	63.68	60.69	59.48	0.152	1.51
Gastric	62.75	67.03	65.29	0.594	1.95
Intestinal	71.87	67.35	71.78	0.124	2.24

Table 10. Influence of loaded nanoliposome with Nerolidol (LN) and unloaded nanoliposome (UN) on Crude Protein Digestibility (CPD) – *in vitro* bovine digestibility.

Phase	CPD %			P-value	SEM
	LN	UN	CTR		
T_0	36.71	37.53	35.28	0.897	0.20
Rumen	47.69	42.17	47.40	0.415	3.81
Gastric	63.14	69.76	65.67	0.692	3.09
Intestinal	87.58	86.37	87.67	0.081	1.13

Bovine: Result of *in vitro* neutral detergent fiber ruminal digestibility.

The results regarding the influence of LN and UN on NDFD at 24 and 48 hours are presented in Table 11. The NDFD was not altered by the addition of UN or LN after 24 h of fermentation ($p>0.07$). However, the fiber digestibility was numerically improved by nanoliposome supplementation, since both LN and UN show similar trend. After 48 h of fermentation the fiber digestibility was depressed by nerolidol addition ($P=0.008$). The addition of nanoliposome uncharged didn't affect the digestibility, however the values were still slightly higher than CTR.

Table 11. Influence of loaded nanoliposome with Nerolidol (LN) and unloaded nanoliposome (UN) Neutral Detergent Fibre Digestibility (NDFD) in rumen *in vitro* fermentation.

Phase	NDFD %			P-value	SEM
	LN	UN	CTR		
24 h	36.83	35.21	32.54	0.070	1.26
48 h	35.97^{b}	48.67^{a}	47.15^{a}	0.008	2.24

Discussion

Livestock, particularly ruminants, represent one of the major sources of CH_4 emissions in the livestock sector, both directly (enteric fermentation) and indirectly (manure management) (Yusuf et al., 2014; Lamb et al., 2021). Several strategies have been proposed to mitigate these emissions, including the use of EOs, and NICs, which, following the European Union's ban on the use of antimicrobial growth promoters in livestock in 2006, have gained significant attention and have been extensively studied. However, despite *in vitro* and *vivo* studies reporting a direct effect on methane mitigation using EOs and NICs, other research indicates that their application may reduce animal digestibility (Calsamiglia et al., 2007; Foskolos et al., 2015; Beauchemin et al., 2022). Specifically, a potential decrease in DMD, as well as NDFD, would result in reduced energy availability for the animal, leading to a decline in productive performance. Additionally, an increase in undigested material could be observed, potentially contributing to a subsequent rise in CH_4 emissions. As reviewed by Rajendran et al. (2022), numerous *in vivo* studies highlight the benefits of encapsulated EOs or NICS compared to their free forms. In cattle, encapsulation mitigates the negative impact of certain molecules on DMI and milk production, preserving rumen microbiome balance. However, at the best of our knowledge there are not *in vitro* studies reporting effects of loaded nanoliposome with nerolidol on DM, CD and NDF digestibility, using swine and ruminant *in vitro* digestion and fermentation system. Taking this into account, we evaluated the effect of LN and UN on DMD, CPD and NDFD using a dosage to simulate a hypothetical future *in vivo* use, with the amount calculated as 0.1% of the substrate's weight, corresponding to a hypothetical application of 100 g /100 kg of feed. No significant differences for DMD and CPD, across the various digestive phases analyzed, with no overall reduction in digestibility were observed. This indicates that the dosage used did not exert a depressive effect on the *in vitro* digestibility of DMD or CPD at the dosage hypothesized for potential *in vivo* application. Specifically, for the swine model, no cecal fluid was used; instead, a multi-enzymatic system was employed. Even under these conditions, the formulations showed no significant effects on digestibility of DM and CP, compared to the control, indicating that these concentrations did not interfere with the activity of digestive enzymes. Conversely, for the *in vitro* ruminant digestibility experiments, we used a ruminal fluid collected from slaughtered

cattle. Consequently, the absence of negative effects on DMD and CPD suggests that the formulations did not adversely affect the ruminal microbial population.

Regarding the evaluation of the influence of LN and UN on *in vitro* NDFD, ruminal and cecal fluid samples were collected from animals at slaughter for use in ruminal and swine fermentation systems, respectively. For swine fermentation, no significant differences in NDFD were observed among the different treatments. This result indicates that formulation does not have fiber digestibility. Whereas, in ruminant fermentation, did not induce significant differences between treatments at 24 h; conversely at 48 h, the LN formulation significantly reduced NDFD compared to the CTR and UN formulations, suggesting an effect on the ruminal microbiome, particularly on the microbial population involved in fiber digestion. This effect could be attributed to the presence of Nerolidol, which, once released in the rumen, led to a reduction in NDFD. Such a reduction, observed alongside unchanged DMD and CPD, may indicate a potential mitigating effect on the formation and subsequent emission of CH_4 by ruminants.

These results highlight how the effects of nanoliposome formulations vary depending on the digestive system considered. Specifically, for cattle, it was observed that the greatest influence was on Ruminal Neutral Detergent Fibre Digestibility (NDFD). This aspect, with no significant differences in DMD and CPD, could suggest a potential effect on reducing CH_4 emissions. For swine, the absence of significant differences in DMD and CPD indicates that the LN and UN formulations do not interfere with the enzymatic systems involved in the digestive processes. Furthermore, the absence of differences in NDFD suggests that the cecal microflora was not negatively affected by the inclusion of LN and UN. It is important to note that these results, and particularly those related to NDFD, should be considered preliminary, as they are based on a single experiment. Further evaluations will be necessary, using different substrates and, most importantly, cecal and ruminal fluids from different sources.

Nevertheless, encouraging results were observed regarding the potential indirect effect of LN and UN on methanogenesis, particularly in ruminants. Future studies, possibly using direct methodologies, will

be essential to understand whether the use of these compounds could represent an effective strategy for reducing CH_4 emissions in livestock farming.

Conclusions

Increasing consumer awareness, combined with new health and climate emergencies, has made it imperative to adopt a sustainability-oriented approach and an integrated view of One Health, in line with the One Health paradigm. This study was developed on these principles, with the aim of exploring alternative options to reduce the use of antibiotics in animal husbandry and improve the sustainability of the entire sector. In this perspective, the possibility of using new feed additives of plant origin was analyzed.

In the first year of the study, screening of different NIC revealed the potential of nerolidol which, however, showed significant limitations. In addition to solubility problems at higher concentrations, the compound proved to be highly toxic to several cell lines of veterinary interest. To overcome these limitations, nerolidol was encapsulated in nanoliposome systems. However, the loading process was limited, allowing nerolidol to be incorporated only up to 1% of the nanoliposome concentration (5000 µg/mL); 50 µg/mL.

Despite this restriction, results showed that both LN and UN showed interesting antimicrobial activity, with time-dependent effects against Gram-negative and Gram-positive bacteria. The antimicrobial activity of the nanoliposome formulations was attributed to their ability to interact with bacterial membranes. However, both formulations exhibited dose-dependent cytotoxicity, with greater toxicity for the LN, probably due to the presence of the compound. In particular, the IPEC-J2 cell line proved more sensitive, while MDBK cells showed greater tolerance up to concentrations of 1250 µg/mL.

In parallel, digestibility tests were conducted using both nanoliposome formulations. In a hypothetical 0.1% inclusion of nanoliposomes in a swine or bovine feed ration, *in vitro* results showed that DMD and CPD were not impaired. This indicates that the formulations did not adversely affect either the

enzyme activity or the ruminal microbiome. Furthermore, promising preliminary results emerged regarding the effect of the nanoliposome formulations on NDFD, suggesting a targeted action on bacteria involved in fiber degradation. This aspect could have a potential indirect effect on the methanogenesis process, with possible benefits in reducing CH_4 emissions, especially from ruminants. It is important to underline that all evaluations in this study were conducted *in vitro*.

In conclusion, although nerolidol has shown great potential as an antimicrobial agent, its high cytotoxicity represents a significant limitation for direct application in veterinary medicine, especially as a food additive. Nanoliposome formulations could offer a safer alternative, but further studies are needed to assess their vivo efficacy, particularly with respect to their impact on the gut microbiota of animals and their growth performance. Therefore, in future *in vivo* studies will be necessary to confirm the potential use of these nanoliposome formulations as feed additives in animal husbandry, with reference to swine and cattle species.

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