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ESSENTIAL OILS AND SWINE REPRODUCTION: NEW FRONTIERS IN ANTIBIOTIC REPLACEMENT IN SEMINAL DOSES

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Abstract

The actual scenario regarding antimicrobial and especially antibiotic resistance is extremely worrying, and it is mandatory, also from an ethical point of view, that the scientific community invest time and efforts in the search for solutions. Essential oils (EOs) are products of the secondary metabolism of aromatic plants and are complex mixtures of several compounds. They show a wide variety of biological activities (antimicrobial, antioxidant, antitumor, insecticidal, etc.) highly employed in Human and Veterinary Medicine, and in pharmaceutical, food and cosmetic industries. Such properties may suggest interesting applications in the reproduction field, as cryo-preservatives or substitutes for antibiotics, but studies regarding their direct effects on spermatozoa are lacking.

The present research aimed at evaluating the opportunity to use essential oils in refrigerated swine seminal doses as a potential alternative to conventional antibiotics. The project included a preliminary step for the study of swine semen morpho-functional parameters and their impact on *in vivo* fertility in a farm setting. In addition to the standard spermatozoa evaluations, the work also considered bacteriological evaluation of the seminal doses. The outcome of the study was the set up of advanced statistical methods to better predict fertility on the basis of morpho-functional evaluations. In the first step of the project, some specific EOs were selected and chemo-characterizated by gas-chromatography, afterwards their cytotoxic effects on swine spermatozoa were evaluated to identify non-spermicidal concentrations. This part showed that low concentrations of *R.officinalis* and *M.alternifolia* Eos do not alter semen morpho-functinal parameters, thus may be used for several applications. Accordingly, the last step was the evaluation of antibacterial effects of the previously identified non-spermicidal concentrations on the liquid phase of refrigerated swine seminal doses. In order to obtained standardized results, an *in vitro* model with the addition of *E. coli* was set up. Analyses, performed at different time points, included optical density evaluation, bacterial DNA quantification by qPCR, and colony count. The results demonstrated that both *Melaleuca alternifolia* and *Rosmarinus officinalis* EOs, at the non-spermicidal concentration of 0.4 mg/ml, are capable of

delivering similar effects to ampicillin, used as control, on the experimental samples. Overall, these findings strengthen the hypothesis that phyto-complexes may be used as novel antimicrobial agents for reproductive biotechnologies.

In conclusion, this project showed the strong potential of Essential Oils in the field of swine artificial insemination, but also highlighted how it is necessary, before claiming natural compounds as future "miracle-workers", to investigate their safety and mechanisms of action.

Summary

Introduction

The fight against the antibiotic resistance

The end of the 1920s, due to the discovery of Penicillin by A. Fleming, marked the beginning of the golden age of antibiotics. Great optimism reigned during the following decades in light of the powerful therapeutic properties of such compounds. However, what could not be foreseen at that time was that the scenario would have rapidly changed during the following years. Indeed the first episodes of antibiotic resistance were described in the 40s, despite the problem being formally acknowledged as such only approximately forty years afterwards in 1980 (Figure 1) (Venter et al., 2017; Duval et al., 2019).

Nowadays, the antimicrobial resistance (AMR), and more specifically antibiotic resistance (AR), is considered as one of the biggest public health threats of the common age. Data from the 2016 report of the Government of the United Kingdom titled "Tackling drug-resistant infections Globally: Final report and Recommendations" (O'Neill, 2016) suggest that, in 2050, the number of deaths attributable to resistance to anti-infective drugs will approach the number of 10 million, way more than road accidents, diabetes and even cancer.

Antibiotic resistance, as the term suggests, occurs when microbes become resistant to antibiotics they were initially susceptible to, increasing the chance that common infections and minor injuries could once again become frequent killers (Carlet et al., 2012).

Obviously, AR is not a new phenomenon: it is intrinsic within bacterial evolution to adapt, develop and acquire mechanisms of resistance to survive (Duval et al., 2019). What is concerning is the growth rate of the AR events, characterized by rapid increase and dissemination of new mechanism of resistance and the development of multi-resistances (Carlet et al., 2012).

The principal cause for this phenomenon is the massive and irresponsible use of antibiotics in humans and animals medicine, but also in farming, agriculture and aquaculture (Venter et al., 2017), leading to a strong selective pressure on bacteria themselves.

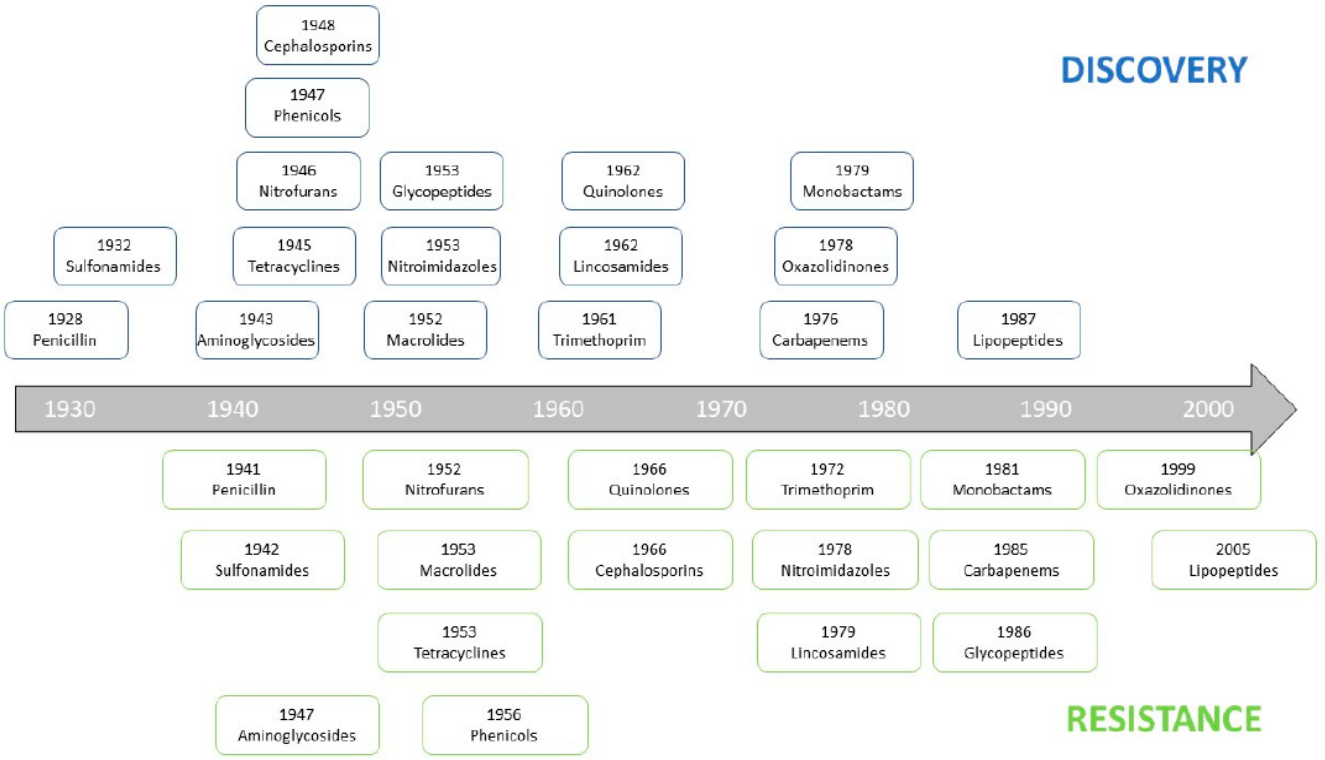


Figure 1: Antibiotics timeline from the end of the 1920s until today, indicating when the main antibiotic classes were discovered, and when the mechanism of resistance to these antibiotics were first described (Duval *et al*., 2019).

The highest concentrations of antibiotics and resistant bacteria have been recorded in effluents from hospitals and drug manufacturing sites. Moreover, also drinking water and the food chain have a pivotal role in the dissemination of resistant bacteria (Harbarth et al., 2015). The complex dynamics of transmission and exchanges between humans, animals and the environment (Figure 2) represent the roots of the "One Health" approach to fight AR. The transfer of AR between animals and from animals to humans has been deeply studied and analyzed, highlighting the principal role of the food chain in spreading resistances (Landers et al., 2012; Chantziaras et al., 2014).

Various strains of resistant *Escherichia coli* were isolated from pork food products and matched with etiologic agents of pathologies in humans (Shafiq et al., 2019; Venter et al., 2017).

The transmission of resistant pathogens from human to livestock has also been reported, as for the case of Methicillin Resistance *Staphylococcus aureus* strains (Price et al., 2012).

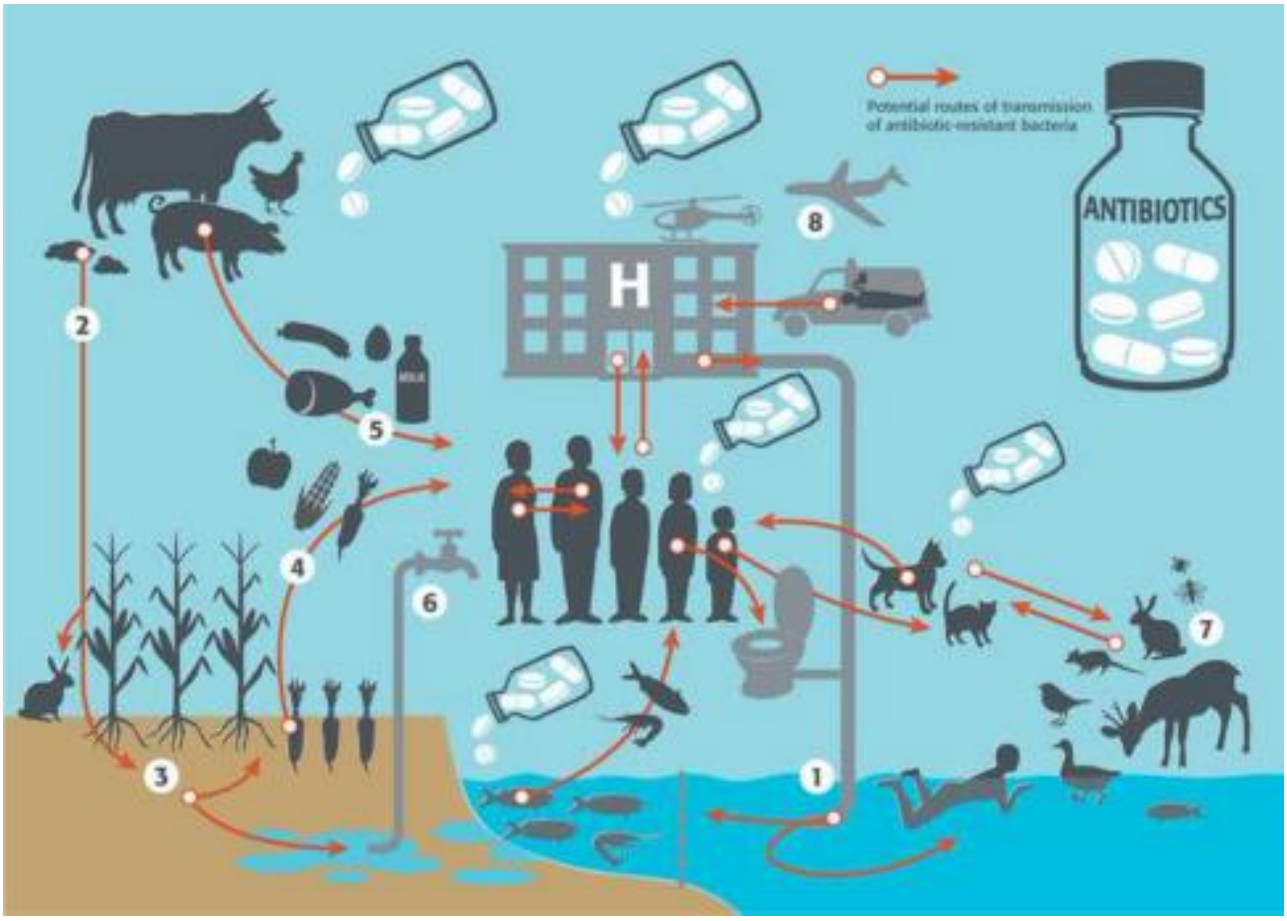


Figure 2: Potential routes of transmission of antibiotic-resistant bacteria. Legend: Humans in the community or in hospitals, pets, livestock and fish farms rely on similar classes of antibiotics to fight infectious diseases. Both pathogenic and non-pathogenic bacteria evolve or exchange the ability to survive when exposed to these antibiotics. They spread into the environment through different routes, such as water sanitation systems (1) , as wastewater treatment facilities do not entirely remove antibiotic resistent bacteria before releasing water into the environment. Another common route is through the application of manure to fields with cultivated crops (2), where antibiotic resistant bacteria can readily develop on the plants (3). The uptake of these resistant bacteria can then happen through the food chain, when humans later consume these plants (4) or the contaminated flesh of animals and fish harbouring resistant bacteria (5). As bacteria can easily reach water reserves, water distribution infrastructure is also a potential route for the spread of these germs (6). Even wildlife, insects and other bugs are potential carriers of antimicrobial resistance (7). Tourism, migrations and food imports (8) are nevertheless reported as the fastest way of spreading resistant strain of bacteria across borders. At the healthcare facilities level. Resistant bacteria can spread by contact between patients or with healthcare staff, or through contaminated surfaces and medical devices (Harbarth *et al*., 2015).

Recently, the World Health Organization (WHO) has published a priority list of bacteria (Table I) whose level of resistance to antibiotics is such that they represent a real threat to Health, with the aim to encourage pharmaceutical companies, universities and public institutions to invest in new antibiotics researches (Tacconelli et al., 2018).

Table I: WHO priority list for research and development of new antibiotics for antibiotic-resistant bacteria (Tacconelli et al., 2018).

Multidrug-resistant and extensively-resistant ***Mycobacterium tuberculosis***	
Other priority bacteria:	
Priority 1**: critical**	
	Acinetobacter baumannii, carbapenem resistant
	Pseudomonas aeruginosa, carbapenem resistant
	Enterobacteriaceae, carbapenem resistant, third-generation cephalosporin resistant
Priority 2: **high**	
	Enterococcus faecium, vancomycin resistant
	Staphylococcus aureus, methicillin resistant, vancomycin resistant
	Helicobacter pylori, clarithromycin resistant
	Campylobacter spp, fluoroquinolone resistant
	Salmonella spp, fluoroquinolone resistant
	Neisseria gonorrhoeae, third-generation cephalosporin resistant, fluoroquinolone resistant
Priority 3: ***medium***	
	Streptococcus pneumoniae, penicillin non-susceptible
	Haemophilus influenzae, ampicillin resistant
	Shigella spp, fluoroquinolone resistant

Despite the worrying scenario and the constantly increasing reports of AR, the war against antimicrobial resistance is not lost. It is a must for the scientific and regulatory communities to keep on focusing on such issue by promoting national and international program on the correct use and management of antibiotics, by forbidding their use unless clearly necessary, by educating and training the personnel involved, and, finally, by promoting and funding the research (Carlet et al., 2012; Harbarth et al., 2015; Venter et al., 2017; Duval et al., 2019). A pivotal point to succeed in this process is to also constantly search for new molecules with proven and reliable antimicrobial proprieties.

Use of antibiotics in the porcine industry

Pork is one of the commonly consumed meat in the world. The always-increasing market request has pushed the porcine industry within the years to a rapid growth, leading to extremely intense farming management to guarantee large-scale production. In such scenario, the routine use of antimicrobials for a wide variety of reason has become an integrated part of the production system (Aarestrup et al., 2008). Antibiotic in pigs are principally used in three main ways (Barton, 2014):

1) Growth promoters:

   Traditionally, antibiotics have been used at concentrations lower than therapeutic ones in order to determine increased growth rates and improve overall efficiency and product quality. This use has been controversial because the addition of low concentrations of antibiotic in pigs' feed creates the ideal situation for selection of antibiotic resistant bacteria and spread resistant-related genes. Since 1997, the EU started a battle against antibiotics as growth promotor (Maron et al., 2013), which was concluded in 2006, with the ban of the use of such compounds as additives for animal nutrition (European Commission, 2005)

2) Prophylactic or metaphylactic treatment to prevent diseases:

   This use indicates individual or group treatments with antibiotics aimed at avoiding or reducing the risk of diseases in pig. This practice relies on administering antibiotics via the feed (medicated feed) during all the productive cycle, especially during the weaning period when piglets are exposed to several challenges and stressors including changes in diet, separation from the sow and re-mixing. These stressor events compromise the immune system of the animals, making them more susceptible to infectious agents. EU proposed to ban also prophylactic in-feed antibiotics to reduce the use, by stimulating alternative approaches (vaccinations, good practice of management, high animal welfare condition) (Barton, 2014; Diana et al., 2019).

3) Therapeutic purposes to treat disease:

In case of diagnosed diseases, pigs are treated individually either orally or by injection, although in feed medication is also used. This last route of administration can be ineffective because sick animals usually show a lack in appetite, thus do not necessarily receive the appropriate dose of drug. The principal use of antibiotics is for gastro-intestinal and respiratory infections follow by reproductive/urogenital, skin/joints, nervous system and systemic diseases, depending on the productive category (sows, slaughter pigs; weaners; boars). The most common bacterial agents are summarized in table II. Precise information regarding the antibiotic agents used within the pig industry are difficult to obtain, but the main used compounds seem to be tetracyclines, macrolides, sulphonamides-trimethoprim, cephalosporins, fluoroquinolones, tylosin, penicillins and ceftiofur (Aarestrup et al., 2008; Barton, 2014; Yang et al., 2019).

As shown in several papers, some of these antibiotics classes are actually less used in light of their low efficacy. More recent antibiotics such as cephalosporins and fluoroquinolones seem to be, as of now, the most efficient, but their use should be extremely cautious in order to avoid influences when it comes to human treatments (Lekagul et al., 2019; Yang et al., 2019).

Table II: *Common bacterial infections and diseases in the pig (Aarestrup et al., 2008).*

Bacterium	Disease	Age groups
Enteric		
Escherichia coli	Neonatal scours	1-3 days
	Piglet scours	7-14 days
	Post-weaning diarrhea	5-14 after weaning
Clostridium perfrigens	Type C - necrotic enteritis	1-7 days
	Type A - diarrhea	10-21 days, weaned pigs
Clostridium difficile	Diarrhea, ill thrift	3-7 days
***Salmonella* spp.**	*Typhimurium* – occasional diarrhea, septicemia	Grower pigs 6-16 weeks
	Derby - occasional diarrhea	Grower pigs
	Choleraesuis – septicemia, diarrhea	Finishing pigs 12- 16 weeks
Lawsonia intracellularis	Porcine prolirative enteropathy (ileitis)	Grower pigs
	Regional/necrotic ileitis	Grower pigs
	Porcine hemorrhagic enteropathy	Finishing pigs and young adults 16-40 weeks
Brachyspira hyodysenteriae	Swine dysentery	Growers and finishers, 6-26 weeks; all ages in primary breakdown
Brachyspira pilosicoli	Intestinal spirochaetosis "colitis"	Grower pigs
Respiratory		
Pasteurella multicida (D) ***Bordetella bronchiseptica***	Atrophic rhinitis	1-8 weeks Nasal distortion lasts for life
Mycoplasma hyopneumoniae	Enzootic pneumonia	Grower and finisher pigs
Pasteurella multicida	Mycoplasma-induced respiratory disease (MIRD)	Grower and finisher – secondary invader
Actinobacillus pleuropneumoniae	Pleuro-pneumonia	Grower and finisher – MDA last for 10 weeks
Septicemic/bacteremic/other infections		
Echerichia coli	Bacteremia, arthritis, navel infections; Cystitis, nephritis	Post-weaning Sows
Streptococcus suis	Meningitis, endocarditis, arthritis and peritonitis	2-10 weeks
Haemophilus parasuis	Glässer's disease (arthritis, pericarditis, peritonitis)	2-10 weeks
Mycoplasma hyosynoviae	Mycoplasmal arthritis	16 weeks plus
Staphylococcus aureus	Bacteremia, arthritis, osteomyelitis, mastitis and metritis	All age groups
Staphylococcus hycus	Exudative epidermitis	Pre- and post-weaning piglets
Erysipelothrix rhusiopathiae	Erysipelas (dermatitis, arthritis and endocarditis)	Growers, finishers and sows

Swine artificial insemination, extenders and use of antibiotics

Artificial insemination (AI) is the predominant procedure for breeding commercial sows worldwide. The mostly used semen form is liquid-stored or extended because cryopreservation, successful in many mammalian species, is still inefficient in swine (Silva et al., 2015).

Conventionally, AI doses are set up with 2-3 billion of spermatozoa in 80/100 mL (concentration: $3x10^6$ spermatozoa/mL) of specific extenders and stored at 15-17°C (Fair and Romero-Aguirregomezcorta, 2019). The doses are usually deposited right after the cervix in uterine body, thus the need for a high number of spermatozoa and a large volume of extender. Nonetheless, this AI method is gradually being replaced by new strategies, which aim to deposit the semen closer to the site of fertilization using lower volume and less cells (Roca et al., 2016; Soriano-Úbeda et al., 2013). Several factors, both related to individual parameters (boar, ejaculate) and technical ones (storage time, temperature, pH), can influence the overall quality of the semen material, making the use of extenders necessary to preserve the functional lifespan of spermatozoa (Dziekońska et al., 2017; Pezo et al., 2019). From a physiological point of view, the spermatozoa find in the seminal plasma (liquid phase of the ejaculate) the necessary nutrients to maintain their high metabolism for a very limited amount of time (Gadea, 2003). To extend their survival time *in vitro*, it is necessary to reduce the metabolic activity by means of chemical inhibitors or by lowering the temperature, which also requires dilution (Johnson et al., 2000). In order to be efficient, the extender must contain substances that supply enough energy, protect the sperm from cold shock, regulate pH, control the osmotic pressure and inhibit bacterial growth and transmission of diseases. According to the commercial suppliers, extenders are classified based on their ability to preserve sperm as short- (1-2 days), medium- (3-4 days) or long-term (7-10 days) (Pezo et al., 2019).

Generally speaking, the main component is glucose, which is added in high concentrations likely to help maintaining adequate osmolarity (240/380 mOsm) and provide energy. Other energy sources

have been used, such as galactose, fructose, ribose or trehalose (Gadea, 2003). Another important component is citrate, which is used to maintain the osmotic pressure and pH, but is also used by the sperm through the Krebs cycle (Pezo et al., 2019). Special media components, such as bovine serum albumin (BSA) and other types of buffer, seem to reduce storage-dependent ageing processes and, therefore, a decrease in fertilizing capacity (Johnson et al., 2000).

The process of collecting semen from boars does not fall within sterile conditions. Although good hygienic practices for ejaculate sampling and managing can restrict bacteria contaminations, porcine semen still usually presents 10^4 up to 10^6 bacteria/mL (Althouse and Lu, 2005; Schulze et al., 2015). A wide variety of bacteria species can contaminate ejaculates: most of them are gram-negative bacteria, especially from the *Enterobacteriaceae*, *Alcaligenaceae* and *Xanthomonadaceae* family. table III summarizes the commonly isolated bacteria flora from boar neat and extended ejaculates (Kuster and Althouse, 2016). Aside from these contaminating bacteria, boars can also become infected with specific bacterial pathogens, that may be transmitted by semen, responsible for infectious diseases/zoonosis as Brucellosis, Chlamydophilosis, Leptospirosis, Tuberculosis and Mycoplasmosis (Maes et al., 2008).

Table III: Common bacteria isolated from neat and extended ejaculates (modified by Kuster and Althouse, 2016).

Bacteria isolated from:	
Boar neat ejaculates	**Boar semen extended**
Escherichia coli	*Escherichia coli*
Pseudomonas spp.	*Pseudomonas spp*
Bacillus spp.	*Bacillus spp.*
Staphylococcus spp.	*Staphylococcus spp.*
Klebsiella spp.	*Klebsiella spp*
Proteus spp.	*Klebsiella oxytoca*
Enterobacter spp.	*Proteus mirabilis*
Pasteurella spp.	*Enterobacter cloacae*
Citrobacter spp.	*Enterococcus spp.*
Corynebacterium spp.	*Corynebacterium spp.*
Streptococcus spp.	*Ralstonia picketti*
Actinomyces spp.	*CDC EF4*
Bacteroides spp.	*A xylosoxidans*
Lactobacillus spp.	*Ach (Acl) xylosoxidans*
Acinetobacter spp.	*Acinetobacter spp.*
Actinobacillus spp.	*Acinetobacter lwoffii*
Flavobacterium spp.	*Comamonas testosterone*
Micrococcus spp.	*Burkholderia cepacia*
Serratia spp.	*Serratia marcescens*
	Serratia liquefactions
	Providencia spp.
	Providencia rettgeri
	Stenotrophomonas maltophilia

The high number of environmental-associated species raises the question regarding the source of these contaminant bacteria, which can be arbitrarily classified as of animal or non-animal origin. Contamination of animal origin can be due to general or local infections within the boar. It can also originate from preputial cavity fluids, respiratory secretions, and feces produced during collection. Contamination of non-animal origin, originate from the person collecting the semen (hair, skin, respiratory secretions), from the water used during processing (e.g. water lines, holding tanks), the air/ventilation system, and from sinks/drains. Overall, the biggest source of opportunistic bacteria has been identified as the result of suboptimal-to-poor hygienic practices in the use of utensils in semen collection (bags, glasses, polystyrene cups, and so forth), in the transfer of ejaculates by air tube or

pass-through and in the manual operating elements, to the storage equipment, and to the disposal of the raw/extended ejaculates (Maes et al., 2008; Schulze et al., 2015).

Although the normal bacterial contaminant are not considered primary pathogens in swine, they can result in a decreased sperm quality as well as in reduced conception rates, early embryonic or fetal death, and contamination of female's reproductive tract (Bussalleu et al., 2011; Kuster and Althouse, 2016; Pinart et al., 2017). The main toxic effects of bacteria on spermatozoa appear to be related to direct perturbation of the cell (decrease in viability, alteration of the acrosome integrity) and are concentration-dependent. Additional mechanism have been described, including lipopolysaccharide exposure, leukocyte activation, anti-sperm antibody, cytolysin production, alteration of pH and inhibition of motility (Althouse and Lu, 2005).

To control bacterial growth and to prevent transmission of diseases, antimicrobial agents are essential components of semen extenders. The European Union proposes/requires the use of an effective combination of antibiotics, in particular against leptospires and mycoplasmas, to be added to the semen after final dilution. This combination must produce an effect at least equivalent to the following dilutions: not less than 500 IU per ml streptomycin, 500 IU per ml penicillin, 150 mg per ml lincomycin or 300 mg per ml spectinomycin (Council Directive 90/429/EEC, 1990).

Traditionally, penicillin and streptomycin represented the most common combination used in the extenders. Today, the most used antibiotics in porcine AI are aminoglycosides, especially gentamicin (Schulze et al., 2015), but B-lactams and lincosamides also seem to be adequate choices. Table IV reports the most used antibiotics in semen extenders and the respective spectra of action (Althouse and Lu, 2005).

Table IV: Characteristics of select antimicrobials commonly used in porcine semen extenders (modified by Althouse and Lu, 2005)**.**

Antibiotic	**Classification**	**Spectrum of activity**	
		Gram +	**Gram -**
Ampicillin	β-Lactam	+	+ +
Gentamicin sulfate	Aminoglycoside	+	+ +
Lyncomicin hydrochloride	Lincosamide	+ +	-
Neomycin sulfate	Aminoglycoside	+	+ +
Spectinomycin hydrochloride	Aminocyclitol	+	+ +

Alternatives to antibiotics in boar refrigerated seminal doses

The presence of antimicrobial agents in boar semen doses can be considered as a source of environmental exposition to these compounds: seminal reflux after AI or inadequate disposal of the unused doses can indeed lead to contamination of the facility and wastewater. It is therefore clear why new strategies have been and are currently being investigated to potentially avoid the use of antibiotic in seminal doses, thus minimizing the risk of developing antibiotic resistance (Barone et al., 2016; Schulze et al., 2018). One of the first approach that comes to mind when searching for alternative strategies is the obvious, but not so easy, physical removal of bacteria from the ejaculates. Morell and colleagues (Morrell and Wallgren, 2011; Morrell, 2019), for example, showed how single layer centrifugation of seminal material with species-specific colloid formulations could separate bacteria from spermatozoa (reduction up to 90% of bacteria), without any impact on cell quality. A more recent approach for physical bacteria removal was the microfiltration of seminal plasma: after the separation of the cellular component of ejaculates and re-suspension in an extender, the seminal plasma was filtered using a 1.2 μm and then a 0.22 μm syringe filter and subsequently re-added to create a standard AI dose (Barone et al., 2016). Again, no deleterious effects were notices on cell quality. The above-mentioned protocols proved to be effective but will hardly ever take off as real alternatives due to the laboratory standards required, the time needed and the high costs.

Another type of approach is represented by the identification and analysis of efficacy of different molecules with antibiotic properties such as, for example, antimicrobial peptides (Schulze et al., 2014; Speck et al., 2014; Fang et al., 2017; Sancho et al., 2017; Puig-Timonet et al., 2018; Shaoyong et al., 2019). Antimicrobial peptides are the host defense peptides, commonly present in various organisms (bacteria, fungi, animals and plants), mostly represented by cationic (positively charged) and amphiphilic (hydrophilic and hydrophobic) α-helical peptide molecules. These molecules are permeable towards the outer bacterial membrane (both gram negative and positive) and can bind and

alter the negatively charged bacterial inner cell membranes. This alteration induces cell membrane damage and the permeation of larger molecules such as proteins, capable of destroying cell morphology and membranes, eventually resulting in cell death (Lei et al., 2019). Finally, a preliminary study regarding the opportunity to use *Lactobacillus* spp. in boar extended semen to "select" the bacterial contamination was reported by Schulze and colleague (Schulze et al., 2018), but not competitive effect on other bacterial population was described.

Phytotherapy in Veterinary Medicine

Traditional medicine has relied on medicinal plants for millennia and the mankind has learnt, by experience and observation, to use plants correctly, building up an extensive traditional culture of their use. Over the last hundred years or so, scientific and technical progress and specifically the pharmacognosy studies performed of many plants, have allowed for the discovery of a large number of new molecules with peculiar mechanisms of action (Colalto, 2018). Phytotherapy in particular is the science-based use of plant-derived medications for treatment and prevention of diseases. It is distinguished from other more traditional approaches, such as medical herbalism, which relies on an empirical appreciation of medicinal herbs often linked to traditional knowledge (Capasso et al., 2011; Heinrich, 2019).

Phytotherapy applied to Veterinary Medicine is called also Ethnoveterinary medicine and exists wherever humans live in close relationship with animals, and it is especially relevant in societies where animal husbandry is the primary means of sustenance. It has been suggested that veterinary medicine has developed, to some extent, from the observation of animal self-treatment and in parallel with human medicine (Miara et al., 2019). Historically, many plants were used to treat cows, sheep, poultry, horses and pigs, and, in some areas, are still used (Viegi et al., 2003). In the last decades, public health issues (e.g. antibiotic/anthelmintic resistance) and the constant growth of organic production, have led to a new rise of ethnoveterinary medicine also in highly industrialized countries (Huffman, 2003; Mayer et al., 2014).

Phytotherapy mainly relies on natural complexes derive from plants that are mixtures of mainly low molecular weight chemical substances. Sources of phyto-complexes include components of fruits, vegetables, spices, and other parts of plants (e.g., pulp, bark, peel, leaf, berry, blossom). Essential oils, as of today, represent one of the most common natural compounds (Smith et al., 2005).

Essential oils

According to the European Pharmacopoeia, essential oils (EOs) are defined as: “Odorant product, generally of a complex composition, obtained from a botanically defined plant raw material, either by driving by steam of water, either by dry distillation or by a suitable mechanical method without heating. An essential oil is usually separated from the aqueous phase by a physical method that does not lead to significant change in its chemical composition”(“European Pharmacopoeia, 9th edition,” 2019).

EOs play an important role in plant protection as antibacterial, antiviral, antifungal and insecticide agents, and can also protect plants from herbivores in the light of the strong flavor (reduction of their appetite from such plant). They appear in liquid phase, volatile, limpid and rarely colored; chemically they are lipophilic and soluble in organic solvents with a generally lower density than that of water. They can be synthesized by all plant organs, i.e. buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, and are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes (Bakkali et al., 2008). Among the plant species, only 10% produce and contain EOs and are therefore, called aromatic plants (over 17,000 plant species). They are distributed all over the world and principally belong to the families of *Lamiaceae*, *Lauraceae*, *Asteraceae*, *Rutaceae*, *Myrtaceae*, *Poaceae*, *Cupressaceae* and *Piperaceae* (Asbahani et al., 2015).

The chemical profiles of EOs differ not only in the number of constitutive molecules, but also in the stereo-chemical type of molecules extracted, according to the type of extraction. The extraction products can also vary in quality, quantity and composition according to climate, soil composition, plant organ, age and vegetative cycle stage at which plant are collected (Angioni et al., 2006; de Macêdo et al., 2018; Chizzola, 2019).

EOs, from an industry point of view, can be obtained by several extraction methods, classified in traditional methods (hydrodistillation; steam distillation) and innovative technologies (ultrasound; microwave), the latter being often more efficient (Asbahani et al., 2015).

Some techniques:

- *Hydrodistillation (figure 3):* the plant material is immersed directly in water inside the alembic and the whole is brought to boiling. The set up also comprises a condenser and a decanter to collect the extract. The EOs are separated from the aqueous phase (hydrolates) by simple decantation. The principle of extraction is based on the azetropic distillation (Tongnuanchan and Benjakul, 2014; Asbahani et al., 2015).

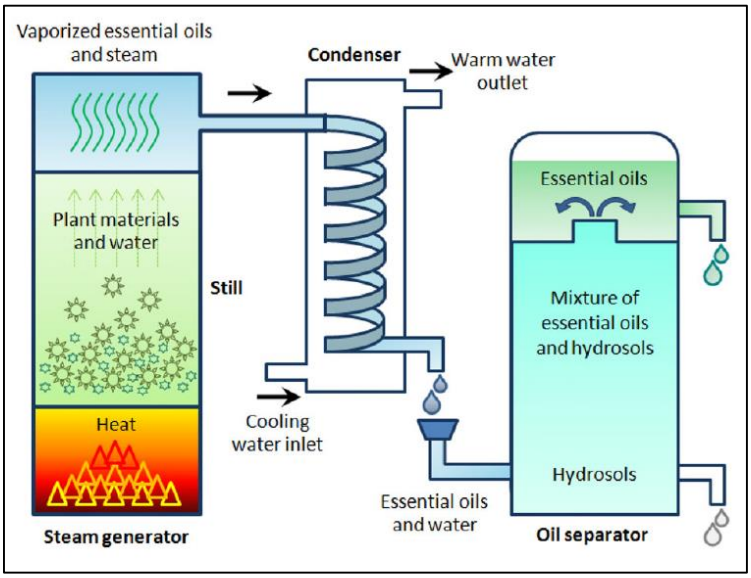


Figure 3: Diagrammatic illustration of hydrodistillation method (Tongnuanchan and Benjakul, 2014).

- *Entrainment by water steam (figure 4):* one of the official and most used methods to obtain EOs. It is similar to Hydrodistillation with the main difference being that there is no direct contact between plant and water. Extraction is shortened, reducing chemical alterations. There are some variants as vapor-hydrodistillation, vapor-distillation, hydrodiffusion (Masango, 2005; Tongnuanchan and Benjakul, 2014; Asbahani et al., 2015).

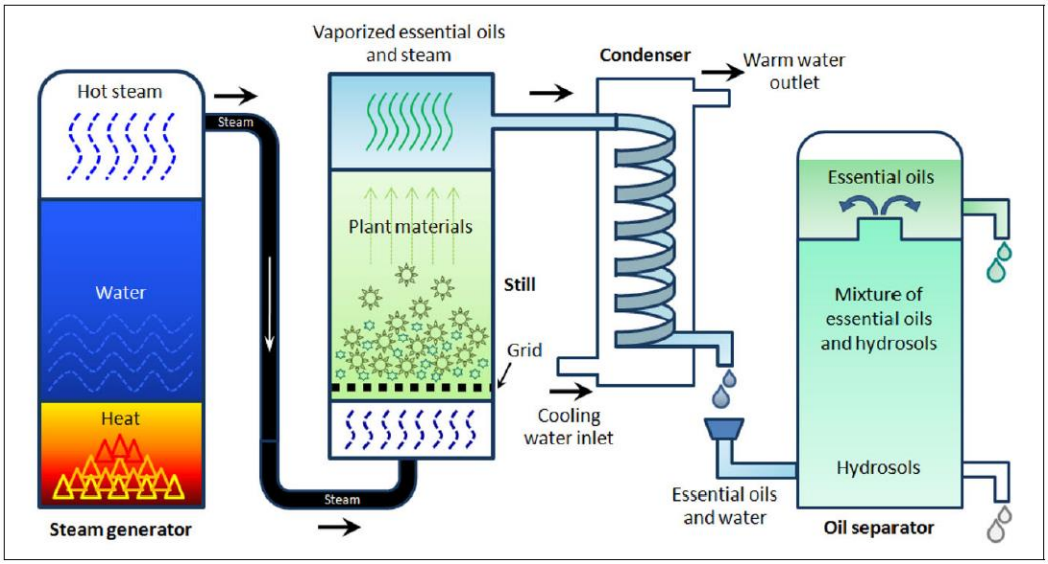


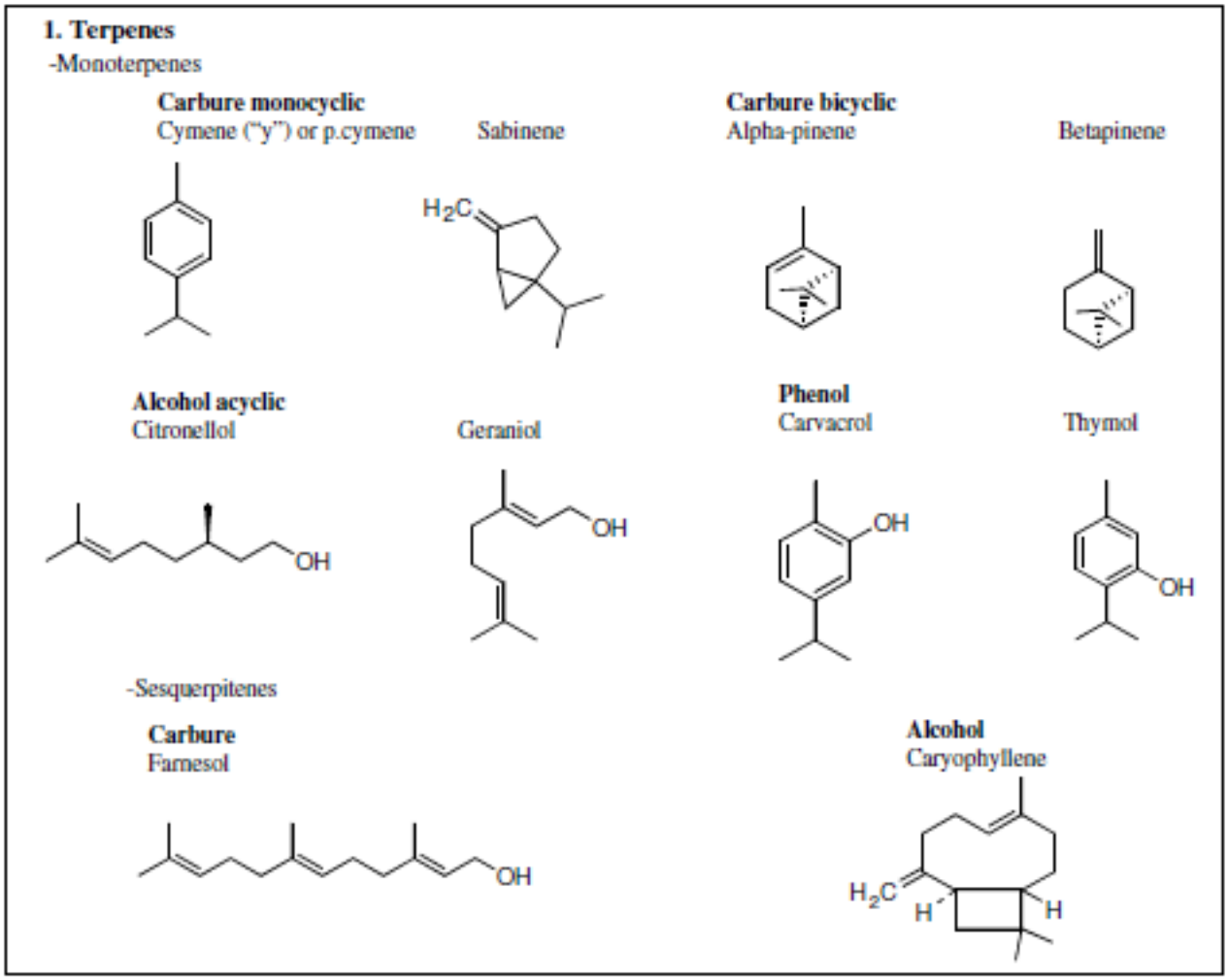
Figure 4: Diagrammatic illustration of steam distillation method (Tongnuanchan and Benjakul, 2014).

- *Cold pressing*: a traditional method to obtain EOs from citrus fruit zest. The oil is removed by cold mechanic pressing yielding a watery emulsion. Oil is recovered subsequently (Asbahani et al., 2015).
- *Organic solvent extraction*: a non-official method for EOs extraction. Conventional solvent extraction has been implemented for fragile or delicate flower materials, which are not tolerant to the heat of steam distillation. Different solvents including acetone, hexane, petroleum ether, methanol, or ethanol can be used for extraction. Generally, the solvent is mixed with the plant material and then heated to extract the essential oil, followed by filtration. Such method, unfortunately, can compromise the safety of products extracted (Asbahani et al., 2015; Tongnuanchan and Benjakul, 2014).
- *Innovative techniques*: the conventional methods have, as main limitation, the necessity of high temperatures that are critical for thermolabile compounds such EOs. The quality of the extracts, therefore, can be extremely damaged especially if the extraction time is long. New techniques aim at the reduction of extraction times, energy consumption, solvent use and CO_2 emissions, and the main reported are supercritical fluid extraction

(SCFE), subcritical extraction liquids (H_2 and CO_2), extraction with subcritical CO_2, ultrasound (UAE), microwave assisted extraction (MAE), solvent free microwave extraction (SFME), microwave hydrodiffusion and gravity (MHG) (Asbahani et al., 2015). These new methodologies seem to provide more stable products, therefore should help in the standardization of natural-based products.

Chemical composition of essential oils

EOs consist of a natural complex mixture derived by secondary metabolism of plants, and contain about 20-60 organic volatile components generally of low molecular weight below 300. Their vapor pressure at atmospheric pressure and at room temperature is sufficiently high so that they are found partly in the vapor state (Dhifi et al., 2016). Each EO is main constituted by 2-3 compounds, present in high concentration (20-70%). In general, the main constituents are terpenes (monoterpenes and sesquerpenes), aromatic compounds (aldehyde, alcohol, phenol, methoxy derivative, and so on), and terpenoids (isoprenoids) (figure 5). Compounds and aroma of essential oils can be divided into 2 major groups: terpene hydrocarbons and oxygenated compounds (Bakkali et al., 2008; Tongnuanchan and Benjakul, 2014).



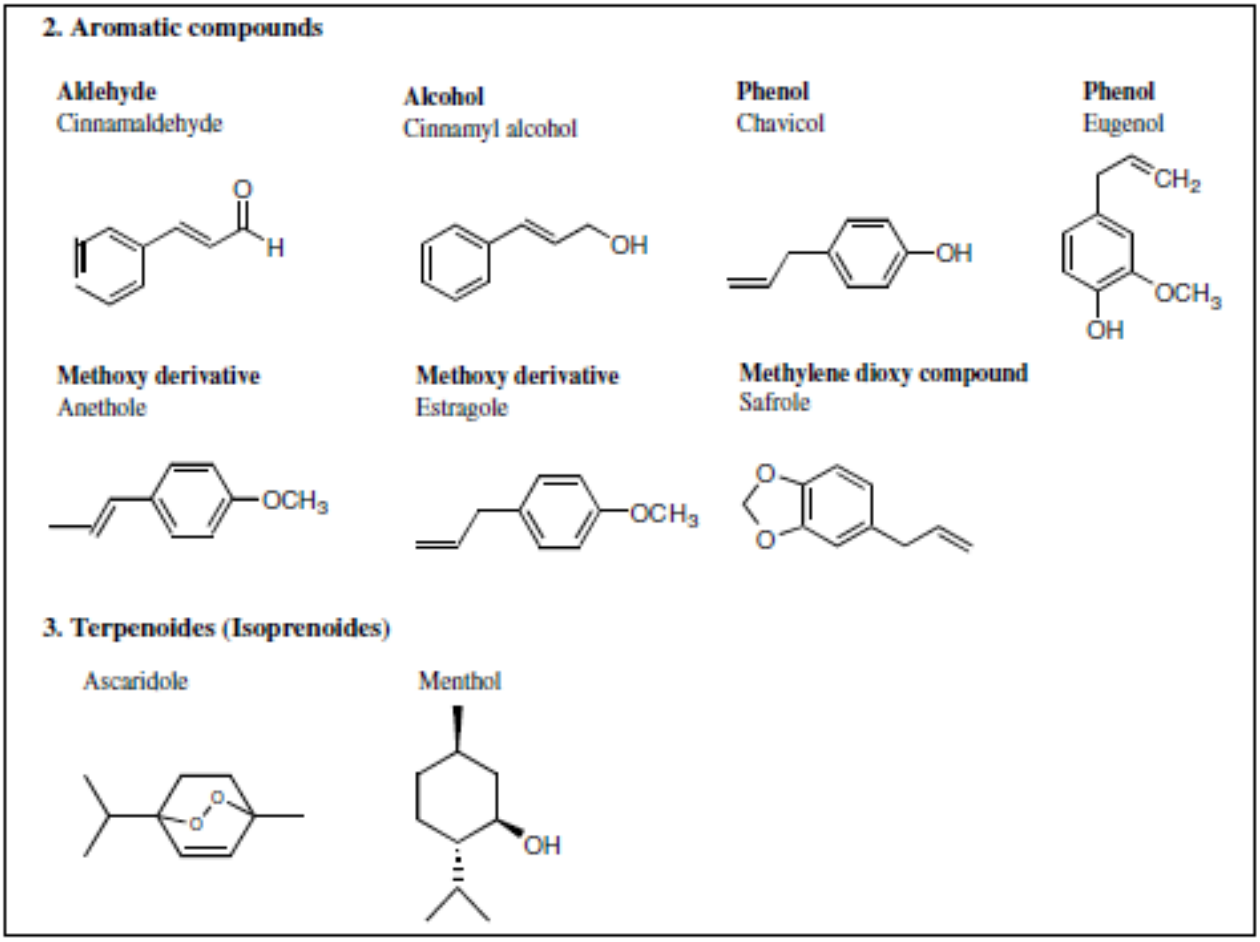


Figure 5: Chemical structures of principal components of essential oils (Bakkali et al., 2008).

- *Terpenes and terpenoids*: the most common class of chemical compounds found in essential oils. Terpenes are constituted by isoprene units (C5), which are the combination of 2 isoprene units (terpene unit). Terpenes are biosynthetically derived from the synthesis of isopentenyl diphosphate (IPP) precursor, and can be produced through the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase pathway, known as the mevalonate pathway. Another pathway for terpenes production is the 2-C-methyl-d-erythritol 4-phosphate/1-deoxyd- xylulose 5-phosphate pathway (MEP/DOXP pathway).

  EOs are mainly constituted by monoterpenes (C10) and sesquiterpenes (C15), which are hydrocarbons with the general formula $(C5H8)_n$. Diterpenes (C20), triterpenes (C30), and

tetraterpenes (C40) are also present in essential oils but at lower concentrations (Tongnuanchan and Benjakul, 2014; Jerković et al., 2019). When the molecule is optically active, the two enantiomers are very often present in different plants, but, in some cases, it is the racemic form which is the most frequently encountered (Bakkali et al., 2008). Terpenoids are the oxygenated derivatives of terpenes (de Matos et al., 2019).

- *Aromatic compounds*: phenylpropanoids (derivate from phenyl-propane) formed via the shikimic acid pathway leading to phenylalanine, and are usually less represented than terpenes. The aromatic compounds comprise aldehydes (cinnamaldehyde); alcohols (cinnamic alcohol); phenols (chavicol and eugenol); methoxy derivatives (anethole, estragole, and methyleugenols); methylenedioxy compounds (apiole, myristicin, and safrole) (Bilia et al., 2014).

EOs show a very high variability in their composition, both in qualitative and quantitative terms. Various factors are accountable for this variability and can be grouped into two main categories:

- Intrinsic factors: related to the plant, interaction with the environment (soil type and climate, etc.) and maturity of the plant.
- Extrinsic factors: related to the technological processes of extraction.

These parameters also include seasonal variations, plant organs, maturity of the plant, geographic origin, and genetics. In some cases, it is difficult to isolate these factors from each other as they are highly interrelated and influence each other (Dhifi et al., 2016).

Biological effects of essential oils

The complexity of the EOs is reflected in their wide biological proprieties that make them potentially useful bio-medical, pharmaceutical, agronomic, and food industry research fields. Several biological activities have been reported in literature, and depend on the type and peculiar composition of each oil. Sometimes the activities of EOs cannot be ascribed to single components but are the result of the synergistic combination of all the constituents. For example, it was proved how the inhibitory activity of *R. officinalis* EO against insects' larvae is due to combined effect of EO's compounds since individual molecules did not show any biological activity (Isman et al., 2008).

The majority of the literature reports that these phytochemicals possess antibacterial, antifungal, anti-parasitic and antiviral properties (Duschatzky et al., 2005; Moon et al., 2006; Freires et al., 2015; Tariq et al., 2019). The antimicrobial properties of EOs are mediated via several mechanisms depending of the peculiar chemical nature of the compounds. The most representative mechanism of action is the alteration of the cell membranes thanks to their lipophilic characteristic: the compounds can infiltrate lipid layers of the membrane with subsequent permeability alteration. Other proved mechanisms are the blockage of enzymes systems, alteration of ion permeability and respiratory metabolism, and structural changes of DNA (Lambert et al., 2001; Nieto, 2017; Cui et al., 2018; Tariq et al., 2019).

One of the proprieties that made EOs interesting at the eyes of the scientific community is the antioxidant capability. This is due to the fact that phenols are considered as chain-breaking antioxidants: they donate an H-atom from the phenolic hydroxyl group to peroxyl radicals (ROO which are responsible for the propagation of the oxidative radical chain). Therefore, they can slow down the peroxidation of unsaturated lipids (Amorati et al., 2013; Nieto, 2017). This opportunity to scavenge free radicals may also play a pivotal role in disease prevention such a brain dysfunction, cancer, heart disease and immune system decline (Dhifi et al., 2016).

Moreover, some EOs show clear anti-inflammatory and anti-cancer effects, mainly correlated to cytokines modulation and angiogenesis inhibition, suggesting potential applications in other medical fields (Nieto, 2017).

On the other hand, due their complex chemical compositions, EOs have no specific cellular ligands nor targets for pathogens, thus can exert cytotoxic effects regardless of the nature of the cell (Carson and Riley, 1995; Sieniawska et al., 2019; Guandalini Cunha et al., 2020). Indeed, as lipophilic mixtures, they are also capable of crossing the double membrane layer of eukaryote cells and degrade polysaccharides, phospholipids and fatty acid, increasing the overall permeability. Therefore, it is safe to say that EOs can have cytotoxic effects on every cell population, mediated by membrane and protein damage and cytoplasm coagulation.

This is why the use of such natural compounds needs to have a science-based approach aimed at the identification of efficacy as well as their toxic capabilities (Dhifi et al., 2016; Colalto, 2018).

Aim of the study

The present project aimed at evaluating the opportunity to use essential oils in refrigerated swine seminal doses as a potential alternative to conventional antibiotics. In the light of the increasing cases of antibiotic multi-resistance and the position taken by the European Community regarding antibiotics use in animal production, essential oils seem to be good candidates for such purposes.
Moreover, it has to be acknowledged that their antioxidant capabilities would provide an additional beneficial effects since spermatozoa are particularly affected by oxidative processes. This would also open new discussions in the field of cryopreservation of swine seminal doses.

The project is divided as follows:

1. **Preliminary step**: Study of swine semen morpho-functional parameters and their impact on *in vivo* fertility in a farm setting. In addition to the standard spermatozoa evaluation, the work also considered the bacteriological evaluation of the seminal doses. The outcome of the study was the set up of advanced statistical methods to better predict fertility on the basis of morpho-functional evaluations.

   - Elmi A., Banchelli F., Barone F., et *al.* (2018), "*Semen evaluation and in vivo fertility in a Northern Italian pig farm: Can advanced statistical approaches compensate for low sample size? An observational study"* Animal Reproduction Science, 192: 61-68.

2. **First step**: Selection of specific Essential oils, chemo-characterization by gas-chromatography and evaluation of the cytotoxic effects on swine spermatozoa. The overall aim was to identify non-spermicidal concentrations to be used for other applications.

   - Elmi A., Ventrella, D., Barone F., *et al.* (2017), "*Thymbra capitata (L.) cav. and rosmarinus officinalis (L.) Essential oils: in vitro effects and toxicity on swine spermatozoa"* Molecules, 22 (12), 2162.

   - Elmi A., Ventrella D., Barone F., *et al.* (2019), *"In vitro effects of tea tree oil (Melaleuca alternifolia essential oil) and its principal component terpinen-4-ol on swine spermatozoa"* Molecules, 24 (6), 1071.

3. **Second step**: Antibacterial effects of the previously identified non-spermicidal concentrations on the liquid phase of swine refrigerated seminal doses. In order to obtained standardized results, an *in vitro* model with the addition of *E. coli* was set up.

   - Elmi A., Prosperi A., Zannoni A., *et al.* (2019), *"Antimicrobial capabilities of non-spermicidal concentrations of tea tree (Melaleuca alternifolia) and rosemary (Rosmarinus officinalis) essential oils on the liquid phase of refrigerated swine seminal doses"* Research in Veterinary Science, in press.

Experimental section

Preliminary step

Study of swine semen morpho-functional parameters and their impact on *in vivo* fertility in a farm setting. In addition to the standard spermatozoa evaluation, the work also considered also the bacteriological evaluation of the seminal doses. The outcome of the study was the set up of advanced statistical methods to better predict fertility on the basis of morpho-functional evaluations.

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Semen evaluation and *in vivo* fertility in a Northern Italian pig farm: can advanced statistical approaches compensate for low sample size? An observational study

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Abstract

The evaluation of sperm functionality and morphology allows discerning between high and low quality ejaculates, but does not give detailed predictive information regarding *in vivo* fertility. The current developments in statistical modeling have helped in carrying out reproductive studies, but their biggest limitation is in the size of the dataset to be used. The aim of the present observational study was to evaluate whether advanced statistical approaches, such as mixed effects regression models and bootstrap resampling, can help in assessing the predictive ability of semen parameters in terms of *in vivo* fertility (farrowing rate and litter size), on a small/medium farm with a limited number of animals. Data regarding 33 ejaculates, including viability, subjective motility and acrosome reaction, were collected. Two hundred and thirty-five sows were inseminated with an outcome of 167 deliveries and 1734 newborn piglets. In order to evaluate the relationships among the parameters measured and fertility, mixed effects regression statistical models were used. Once the covariates to be included in the final models were identified, non-parametric bootstrapping was used. The results showed that the farrowing rate was highly associated with the total number of spermatozoa and subjective motility, while litter size was associated with percentage of acrosome reaction. In conclusion, the proposed statistical approach seemed to be suitable for studies regarding reproduction and fertility, even for relatively small sample sizes. Nonetheless, larger data sets are still preferable and required in order to achieve higher reliability.

Keywords: Swine semen parameter; in vivo fertility; farrowing rate; litter size; mixed effects regression model.

Introduction

The majority of the swine reproductive industry currently uses artificial insemination (AI) (Rodríguez-Gil and Estrada, 2013) in order to reduce disease transmission and increase zootechnical indices. To optimize AI, it is essential to evaluate the fertility of semen (Foote, 2003) since management errors, frequency of sampling, environmental temperature alteration, season and disease can influence spermatogenesis and thus fertility (Wolf and Smital, 2009). The evaluation of sperm functionality and morphology allows discerning between high and low quality ejaculates, without giving detailed predictive information regarding *in vivo* fertility (Jung et al., 2015; Schulze et al., 2013, 2014); it needs to be constantly performed since drops in quality are quite common. Several studies have investigated the ejaculate parameters in order to try and identify the best performing boars, but the conclusions are often in disagreement (Lee et al., 2014; McPherson et al., 2014; Popwell and Flowers, 2004; Tardif et al., 1999). Overall, sperm motility appears to be the most important quality index (Broekhuijse et al., 2012a) and one of the best *in vivo* fertility predictor when analyzed both subjectively (Tardif et al., 1999) and objectively (Ruiz-Sánchez et al., 2006). Other parameters, such as the concentration of the used insemination doses, related to both semen and boars, can influence pig field fertility (Broekhuijse et al., 2012a).

The current developments in statistical regression modeling have helped in carrying out reproductive studies, allowing for in-depth investigations regarding fertility, its predictive parameters, and their correlation (Broekhuijse et al., 2012b; Bucci et al., 2014; Didion, 2008; Gadea et al., 2004; Quintero-Moreno et al., 2004; Turba et al., 2007). In particular, appropriate specifications of these models can provide estimates of the joint effects of the characteristics recorded regarding *in vivo* fertility outcomes. Unfortunately, studies which

aim to analyze predictive values in the reproductive field have found their biggest limitation in the size of the dataset to be used, thus the need for specific, and accurate national and international databases (Broekhuijse et al., 2012a).

The aim of the present observational study was, therefore, to evaluate whether advanced statistical approaches such as mixed effects regression models and bootstrap resampling could help in assessing the predictive ability of semen parameters in terms of *in vivo* fertility (farrowing rate and litter size) on a small/medium farm with a limited number of animals.

Materials and Methods

Animals and sampling

Data regarding thirty-three ejaculates (n=33) were collected from 9 boars (6 Duroc and 3 Large White; age 11.5 to 31.2 months) bred according to the Italian Welfare laws at the Montrone breeding farm (Valsallustra; BO; IT).

All the samples included in the study derived from ejaculates collected using the hand-glove technique during the spring season (from March to May). The semen was routinely collected twice a week from each boar within the facility, but the samples analyzed in the present study were only the ones used for the insemination of the sows bred within the same facility (approximately every 21 days).

Health status was assessed on the basis of clinical examination and rectal temperature, carried out by the farm veterinarian together with a standard clinical chemistry panel carried out on the serum samples collected from the jugular vein at the beginning of March from each animal.

Semen preparation and insemination

Immediately after collection, total semen volume was measured with a sterile graduated cylinder at 28°C, and then diluted 1/1 (v/v) with Swine Fertilization Medium (SFM) (Lavitrano et al., 2002) at the same temperature.

Concentration was assessed in the farm laboratory using an optical densitometer accurately calibrated each morning using a Thoma hemocytometer. The total spermatozoa count (Tot spz) was subsequently calculated multiplying the concentration by the total volume of the ejaculate.

Subjective motility (SM) was immediately assessed by the farm veterinarian using contrast phase microscopy by loading 50 µl of each sample on a heated glass slide; ejaculates showing SM ≥85% were classified as optimal, those showing SM <85% as low motile ejaculates according to the literature (McGlone and Pond, 2003).

Insemination doses were prepared by diluting 3 x 10^9 spermatozoa in SFM for a final volume of 100 ml, and stored in a refrigerated thermostat at 16°C (±0.5) for a maximum of 72 hours. Multiparous Large White sows (n=235) were then inseminated by the farm personnel, upon post-weaning heat detection, twice within 12 hours.

Semen analyses and *in vivo* fertility data collection

For each ejaculate, an aliquot of 5 ml (diluted 1/1 v/v) was delivered to the laboratory of the Department of Veterinary Medical Sciences (University of Bologna) for morph-functional assessment consisting of viability (V) and acrosome reaction (AR).

Viability. An aliquot of 25 µl of semen was incubated with 2 mL of a 300 mM solution of SYBR green-14 and propidium iodide (PI) for 5 minutes at 37° C in dark conditions (Huo et al., 2002; Silva and Gadella, 2006). Ten microliters of suspension were then

placed on a microscope slide and analyzed using an epifluorescence microscope (Nikon Eclipse E600; Nikon Corporation, Tokyo, Japan) with a double-band-pass filter for green and red fluorescence. A minimum of 200 cells were counted and evaluated in order to obtain the percentage of viability; green heads were considered as live sperm and red ones as dead.

Acrosome reaction. Spermatozoa acrosomes were evaluated by Brilliant Blue G 250 (Sigma-Aldrich Corp. St. Louis, MO, USA) staining as described by Larson and Miller (Larson and Miller, 1999). Acrosomes stained blue were considered normal while the unstained ones were considered as reacted or lost. The percentage of AR was based on a minimum of 200 cells (Bacci et al., 2009). The percentage of AR was classified as appropriate when ≤ 5%, and as not appropriate when > 5% (Huo et al., 2002).

Bacteriological evaluation. The bacteriological evaluation was carried out by the General Diagnostic Section of the "Istituto Zooprofilattico Sperimentale of Lombardia and Emilia Romagna Bruno Umbertini (IZSLER)" by seeding a sample of each ejaculate on agar plates which were then incubated overnight at 37°C in normal air with 5% CO_2. The isolated microorganisms were identified using biochemical tests.

In vivo fertility. Data were collected directly at the farm. The number of deliveries and the number of newborn piglets were registered 114 days (± 2 days) after insemination. The two parameters analyzed were: farrowing rate (FR=number of deliveries/number of inseminated sows) and litter size (LS=total number of newborn piglets/number of deliveries).

Statistical analysis

Descriptive statistics of the recorded parameters were calculated. Continuous variables were expressed as mean and standard deviation, while the categorical variables as absolute and percentage frequencies.

In order to evaluate the relationships among the recorded characteristics and the fertility parameters, linear and generalized-linear mixed effects statistical models were used (Goldstein, 2011; Roy, 2013). The incorporation of a random effect term in the models gave the possibility of taking into account correlations among ejaculates of the same boar. Regarding FR analysis, mixed effects binomial regression models were used (Hosmer et al., 2013), setting the dependent variable as the log-odds of delivery (Eq.1). Here, the number of deliveries was viewed as the number of "successes" out of a given number of "trials" (inseminated sows), allowing for direct assessment of the probability of delivery, which is at the basis of FR calculation. The results of FR analysis were reported as Odds Ratio (OR) of delivery with associated 95% Confidence Interval (95% CI) and p-value.

Equation 1. Random effects binomial regression model used in FR analysis

$$logit(p_{ij}) = \left(X_{ij}\beta\right) + \varepsilon_{ij} + u_j$$

$$\mathrm{y}_{ij} \sim Bin\left(n_{ij}, p_{ij}\right);\ \varepsilon_{ij} \sim N(0, \sigma_\varepsilon^2);\ \ u_j \sim N(0,\ \sigma_u^2)$$

Notes to Eq.1:

β is a vector of regression parameters and X is the design matrix for the independent variables. For the i-th ejaculate related to the j-th boar: y_{ij} is number of deliveries, n_{ij} is number of inseminated sows, p_{ij} is the probability of delivery, ε_{ij} are error terms and X_{ij} is the ij-th row of X, while u_j is the random effect term related to the j-th boar.

With respect to Litter Size analysis, mixed effects linear regression models were used, excluding the two ejaculates which did not fertilize any sow, leading to a total number of 31 ejaculates included (n=31).Here, the dependent variable was the total number of born piglets (TNBP), while the other component of LS calculation - number of deliveries - was treated as a fixed covariate (Eq.2).Results of the LS analysis were reported as Mean Difference (MD) in TNBP with associated 95% CI and p-value.

Equation 2. Random effects linear regression model used in LS analysis

$$y_{ij} = \left(X_{ij}\beta\right) + \varepsilon_{ij} + u_j$$

$$\varepsilon_{ij} \sim N(0, \sigma_\varepsilon^2)\ ;\ \ u_j \sim N(0,\ \sigma_u^2)$$

Notes to Eq.2:

β is a vector of regression parameters and X is the design matrix for the independent variables. For the i-th ejaculate related to the j-th boar: y_{ij} is TNBP, ε_{ij} are error terms and X_{ij} is the ij-th row of X, while u_j is the random effect term related to the j-th boar.

In both FR and LS analyses, mixed models were fitted by using a variance components correlation structure, while estimation was performed with maximum likelihood method.

For each of the two analyses, a model selection procedure consisting of three steps was carried out. In the three phases, the following models were fitted:

- "basic" models: in FR analysis, all simple models (i.e. with one independent variable); in LS analysis, all the multivariable models including number of deliveries and one other independent variable;
- "multivariable" model: the multivariable model including all recorded variables;

- "final" models: two multivariable models, with covariates selected according to backward-stepwise ($p<0.05$ for staying in the model) and forward-stepwise ($p<0.05$ for entering in the model) variable selection procedures, respectively (Hastie et al., 2009).

Once the covariates to be included in the final models were identified, non-parametric bootstrapping was performed. Bootstrapping is a resampling technique which turned out to be robust and effective in estimating statistics of interest, giving more reliable estimates and confidence intervals (Efron and Tibshirani, 1993; Fox, 2002). In our case, two thousands cluster-bootstrapped samples (i.e. samples consisting of all ejaculates from nine boars randomly drawn with replacement from the original data) were generated and the structure of the final model was fit onto each of them. Bootstrapped regression estimates were the average estimated coefficients across bootstrapped samples. The confidence intervals for regression parameters were the 2.5^{th} and 97.5^{th} percentiles of the bootstrapped estimates, while p-values were calculated as described by Efron & Tibshirani (Efron and Tibshirani, 1993).

Finally, the Variance Partition Coefficient (VPC) index was calculated on the final models (Goldstein et al., 2002) in order to assess the rate of variance of the outcomes attributable to differences between the boars. The VPC for the linear model was calculated as the variance of random effects divided by the sum of the variances of random effects and error terms (σ_u^2 and σ_ε^2 in Eq.2, respectively) while, for the binomial model, it was calculated according to the latent variable approach described by Goldstein et al. (Goldstein et al., 2002) and already applied within the FR analysis by Iida & Koketsu (Iida and Koketsu, 2016).

Analyses were performed using R 3.0.3 statistical software (The R Foundation for Statistical Computing) at a confidence level equal to 95%.

Results

All the boars enrolled in the study were considered healthy. The clinical examinations did not show any alteration and the mean rectal temperature (38.42 ± 0.25°C) was within the physiological range (Reece, 2015).The blood work results (Supplementary Table S1) were normal.

The seminal, bacteriological and in vivo fertility parameters are reported in Table 1.

Semen evaluation. Sperm morphology analyses did not show any important alterations in the percentage of abnormal forms (20% ± 8.5), and only 5 samples showed the presence of few spermatic aggregates. Regarding subjective motility, 78.8% of samples (n=26) showed SM ≥85% (optimal), and the remaining 21.2 % (n=7) showed SM <85% but never asthenospermia.

Bacterial contamination was detected in 87.9% of the samples. The species isolated and their frequencies are reported in Table 1. Simultaneous isolation of two different species was only observed in two ejaculates; one sample showed the presence of E. coli and Proteus spp., and another showed E. coli and Polymorph bacteria flora. In 12.1% of the cases, none of the bacterial species investigated were detected.

In vivo fertility. Fertility data (Table 1) were collected at the farm: 167 sows, out of the 235 inseminated, gave birth to a TNBP of 1734. The average FR was 67.8 ± 27.8 and the born/delivery ratio (LS) was 9.7 ± 2.9.

Farrowing rate analysis.

The results obtained from mixed effect binomial regression models between the FR analysis outcome and the principal semen parameters are reported in Table 2. Sperm volume was not included in the models due to its high correlation with total spermatozoa count. Variables related to Pseudomonas spp., Proteus spp. and Staphylococcus spp. were also not considered due to the low number of positive samples (Table 1).

Both backward and forward selection procedures chose the same final model (Table 2); its results showed that FR was associated with the total number of spermatozoa (Tot spz) (OR= 1.7, *p value*= 0.0004, 95%CI = 1.2; 2.4) and also to subjective motility (OR= 0.21, *p value*= 0.0049, 95%CI = 0.09; 0.49), as was also observed in the basic and multivariable models. Acrosome reaction was associated with probability of delivery only in the basic model, but was not in the multivariable and final models. No other parameter showed association with probability of delivery.

According to our estimated final model, considering an ejaculate with average values of the random effect term and total count of spermatozoa parameters – zero and 101.4, respectively - the baseline odds (i.e. for an ejaculate with SM > 85% and Tot spz = 101.4) was 3.42 while the estimated delivery probability was 77.4%. Delivery probability was similarly calculated for ejaculates which showed low SM, and was equal to 41.9%.

Variability attributable to the boar within the final model, measured with the VPC index, explained 0.9% of the total variation in FR analysis.

Bootstrapped analysis gave similar results (Tot spz OR = 1.8, *p value*= 0.0158, 95% CI = 1.1; 3.7, SM<85% OR = 0.16, p value < 0.0001, 95%CI = 0.06; 0.46) and confirmed the findings of the final model.

Litter size analysis.

The results obtained from mixed effect linear regression analysis considering total number of born piglets and the principal semen parameters are reported in Table 3. As in the previous analysis, some of the parameters were not included (refer to the section on farrowing rate).

In light of the fact that the deliveries were treated as a fixed covariate in the LS analysis, as expected, its value was always highly significant (all, $p < 0.0001$).

In basic models, association was found only between TNBP and AR (MD=-6.1, *p value*= 0.0355, 95% CI = -11.4; -0.7) while, in the multivariable model, a relationship between TNBP and the age of the boar was also observed. Nonetheless, both our final models proved to be identical to the AR basic model.

Variability attributable to the boar was 7% of the total variation of the outcome.

Bootstrapped analysis confirmed the final model findings, in particular regarding the relationship between TNBP and AR (MD=-6.9, *p value*< 0.0001, 95% CI = -12.8; -3.1).

Discussion

In the present study, the farrowing rate and litter size of a small/medium Northern Italian pig farm were analyzed using statistical regression models. In order to compensate for the relatively small dataset, the models were improved by means of bootstrapping. Indeed, when the focus of the analyses was the prediction of the relationship between several variables, large sample sizes are required to achieve good reliability.

It should also be noted that the aim of the latest trends in reproductive and fertility studies is to discover new molecular biomarkers, using mostly proteomic and genomic approaches (Kwon et al., 2015; Rahman et al., 2013; Zannoni et al., 2017). These techniques can be extremely sensitive and accurate, but require time and economic

investment, making them more suitable for the research field and larger genetic facilities than for zootechnical production.

The present study only focuses on male performance, but the fertility outcomes also depend equally on the female reproductive status. The sows used in this study were healthy multiparous animals of proven fertility; in fact, the inseminations were performed upon post weaning heat detection. Overall, any analysis regarding *in vivo* fertility would be more complete if both males and females were taken into account, but it has to be stated that, within the reality of small pig farms, it is always easier to gain more in-depth information regarding boars since spermatozoa are, however, always collected and analyzed.

As previously stated, the boars were also considered healthy on the basis of a clinical chemistry panel, but despite the recent interest in setting specific age-related reference intervals for pigs (Ventrella et al., 2017), data regarding boars are still lacking. For the interpretation of the blood analysis results of the present study, non-age-specific reference intervals were used, and this might explain the small fluctuations between our data and the published standards. A good example representative of this issue can be find in the levels of Alkaline phosphatase (ALP), an enzyme directly related to osteoblast activity in younger, growing animals (Ventrella et al., 2017): the values of all the analyzed boars are indeed consistently and significantly lower than the reference interval ones. Moreover, it has to be acknowledged that the others parameters that slightly differ from the reference intervals, such as Albumin and Globulins concentrations, are coherent and similar within the entire group of animals, further validating the hypothesis of the authors rather than subclinical pathological statuses.

Several attempts to analyze the relationship between semen quality evaluation and in vivo fertility have already been carried out for the porcine species, with extremely variable results (Broekhuijse et al., 2012b; Didion, 2008; Gadea et al., 2004; Graham et al., 1990; Lee et al., 2014; Lovercamp et al., 2007; McPherson et al., 2014; Moretti et al., 2005; Schulze et al., 2015; Tardif et al., 1999).

Despite it being one of the most important parameter, our statistical analyses did not report any correlation between the outcomes and spermatozoa viability. This finding seemed to agree with what was reported by Gadea and colleagues (Gadea et al., 2004).

The microbiological analyses carried out in this paper demonstrated how the most representative bacterial species are consistent with those formerly described for swine ejaculates (Kuster and Althouse, 2016). The overall amount of bacterial contamination of the samples was extremely low, proving the good health state of the boars and that the specimens were sampled and handled in the best way possible. A negative correlation between the presence of bacteriospermia and semen quality has previously been reported in pigs (Schulze et al., 2015), but a direct correlation between FR and the presence of bacteria has never been demonstrated. On the other hand, LS seems to be affected by the presence of sperm agglutination, which is directly related to the amount of E. coli within the ejaculate (Maroto Martín et al., 2010). The results of the heretofore used statistical models did not demonstrate any association between the main species of bacteria isolated and either of the outcomes (FR and LS). One of the reasons why could be the fact that, despite its presence, the bacterial contamination found in the ejaculates used for this study was too low to influence any parameter in a significant manner.

Subjective (Tardif et al., 1999) or the objective (Broekhuijse et al., 2012a, 2012b) sperm motility in boars is the most important parameter for evaluation of the ejaculate quality

and one of principle predictors of *in vivo* fertility. Our results suggested that motility analysis, using a subjective method, may have a good predictive value for the farrowing rate, as has already been described and confirmed by the existing literature (Gadea et al., 2004). It has to be stated that an objective analysis of the motility by computer-assisted sperm analysis (CASA) is, in fact more reliable and reproducible, but not always possible; the instrumentation itself and slides still represent an important economic investment which not every farm can afford. Therefore, it is still quite common among smaller facilities to rely on well-trained experienced operators to subjectively analyze this parameter.

The multivariable binomial regression model (Table 2) highlighted how the only two parameters associated with FR are the total count of spermatozoa and subjective motility, with major emphasis on SM.

In fact, the data obtained showed that, the TotSpz being equal (referable to the mean value), the probability of completing the pregnancy for a sow inseminated with an optimal ejaculate (SM ≥85%) was 77.38%,which decreased to 42% for those inseminated with ejaculates having subjective motility <85%. The model and the statistical analyses were confirmed by the bootstrapping method.

This finding might have extremely important implications for the zootechnical swine industry since the analysis of subjective motility is simple and does not require advanced instrumentation as has previously been stated.

Regarding the analysis of litter size (Table 3), the only significant parameter in the final model was acrosome reaction, classified as appropriate when ≤5%, and not appropriate when >5%. This result seemed to confirm what has already been described by the existing literature for other species, including humans (Lee et al., 2014; Moretti et al., 2005). As

for farrowing rate analysis, bootstrapping did confirm the results of the final model, giving more strength and robustness to our findings.

Regarding intra-boar correlations, variations in FR and LS outcomes were only minimally dependent on the boar (respectively 0.9% and 7%, as measured by VPC), confirming the fact that the quality of semen is the result of a multifactorial interaction which depends minimally on the individual (Broekhuijse et al., 2012b).

The use of statistical models applied to our data highlighted some of their potentialities and limitations. In the former category, the ability of simultaneously controlling for the effect of several explicative parameters should be taken into consideration as well as flexibility in the specification of the model. Here, the specification of the model was suggested by the mathematical structure of both the FR and LS parameters. Similarly, the use of a binomial model, expressed in terms of log-odds of delivery, was also reported by Gadea et al. (Gadea et al., 2004) and Iida & Koketsu (Iida and Koketsu, 2016) while the use of linear regression within the LS analysis using TNBP as a dependent variable was also considered by Brooekhuijse et al. (Broekhuijse et al., 2012b). By using mixed effects models, researchers also have the possibility of accounting for differences among boars within model fitting, but also analytical variability partition indicators, such as VPC, can be derived.

Overall, despite the relatively low sample size, this study seemed to confirm how the most important parameters for the evaluation of *in vivo* fertility, were spermatozoa motility and appropriate acrosome reaction (i.e. < 5%) for the farrowing rate and litter size, respectively. Bootstrapping proved to be useful in improving the performance of the regression model used, by confirming the results of multivariable models and giving more strength to their findings

In conclusion, the proposed statistical approach seems to be suitable for studies regarding reproduction and fertility, even for relatively small sample sizes. Nonetheless, larger data sets are still required to achieve higher reliability.

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Conflict of interest statement

The Authors have no conflict of interest to declare.

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Table 1. Descriptive statistics for semen quality parameters and *in vivo* fertility (sample n=33).

***In vivo* fertility parameters**		
Inseminated sows (n)	mean (SD)	7.1 (3.7)
Deliveries (n)	mean (SD)	5.1 (3.5)
Born piglets (n)	mean (SD)	52.6 (36.6)
Farrowing rate (%)	mean (SD)	67.8 (27.8)
Litter size (born piglets/deliveries)	mean (SD)	9.7 (2.9)
Seminal parameters		
Volume (ml)	mean (SD)	218.1 (84.3)
Total number of spermatozoa (x 10^9)	mean (SD)	101.4 (37.3)
Concentration (spz x 10^6/ml)	mean (SD)	490.9 (161.7)
Viability (%)	mean (SD)	78.5 (11.6)
Acrosome Reaction, ≤5%	n (%)	21 (63.6)
Subjective Motility, ≥85%	n (%)	26 (78.8)
Bacteria Species		
Polymorph bacteria flora, positive	n (%)	12 (36.4)
Escherichia coli spp., positive	n (%)	11 (33.3)
Pseudomonas spp., positive	n (%)	4 (12.1)
Proteus spp., positive	n (%)	3 (9.1)
Staphylococcus spp., positive	n (%)	1 (3.0)

Table 2. Odds Ratio (95% CI) between the main semen parameters and the Farrowing Rate, calculated using mixed effects binomial regression models (Eq 1).

Parameters	Basic Models	Multivariable Model	Final Model (Backward)	Final Model (Forward)	Bootstrapped Final Model
Boar breed (LW vs. D)	0.76 (0.59; 1.42) p = 0.5560	0.49 (0.24; 1.02) p = 0.0568	/	/	/
Boar age (months)	0.91 (0.31; 1.89) p = 0.6900	0.93 (0.62; 1.38) p = 0.7056	/	/	/
Tot spz (standardized Gaussian scaled)	1.77 (1.20; 2.62) p = 0.0039**	1.86 (1.27; 2.72) p = 0.0013**	1.68 (1.17; 2.41) p = 0.0004***	1.68 (1.17; 2.41) p = 0.0004***	1.82 (1.10; 3.67) p = 0.0158*
Viability (%)	1.01 (0.98; 1.04) p = 0.4510	1.01 (0.98; 1.04) p = 0.6249	/	/	/
Acrosome Reaction (>5% vs. ≤5%)	0.39 (0.21; 0.74) p = 0.0040 *	1.01 (0.33; 3.07) p = 0.9865	/	/	/
Subjective Motility (<85% vs. ≥85%)	0.23 (0.09; 0.54) p = 0.0008***	0.23 (0.07; 0.80) p = 0.0201*	0.21 (0.09; 0.49) p = 0.0049**	0.21 (0.09; 0.49) p = 0.0049**	0.16 (0.06; 0.46) p = 0.0000***
Polymorph bacteria flora (positive vs. negative)	1.55 (0.82; 2.97) p = 0.1709	1.70 (0.84; 3.43) p = 0.1415	/	/	/
E. coli spp. (positive vs. negative)	0.73 (0.39; 1.35) p = 0.3140	1.02 (0.46; 2.30) p = 0.9458	/	/	/

Backward = backward-stepwise selection ; Forward = forward-stepwise selection ; LW= Large White; D= Duroc; Tot spz= Total spermatozoa count.

* = p < 0.05 ; ** = p < 0.01 ; *** = p < 0.001.

Table 3. Mean Differences in total newborn piglets (95% CI) between the main semen parameters calculated using mixed effects linear regression models (Eq 2).

Parameters	**Basic Models**	**Multivariable Model**	**Final Model (Backward)**	**Final Model (Forward)**	**Bootstrapped Final Model**
Deliveries (units)	10.30 (9.57;11.02) p = 0.0000***	9.80 (9.07;10.53) p = 0.0000***	10.03 (9.32;10.75) p = 0.0000***	10.03 (9.32;10.75) p = 0.0000***	10.04 (9.32;10.72) p = 0.0000***
Boar breed (LW vs. D)	3.63 (-1.44;8.70) p = 0.1479	2.1 (-2.51;6.70) p = 0.2637	/	/	/
Boar age (months)	-0.77 (-3.40;1.87) p = 0.3350	-0.02 (-0.04;-0.00) p = 0.0192*	/	/	/
Tot spz (standardized Gaussian scaled)	0.02 (-0.05;0.08) p = 0.3387	0.03 (-0.03;0.09) p = 0.2223	/	/	/
Viability (%)	0.18 (-0.02;0.37) p = 0.0848	0.06 (-0.14;0.25) p = 0.3332	/	/	/
Acrosome Reaction (>5% vs. ≤5%)	-6.08 (-11.37;-0.71) p = 0.0355*	-8.64 (-14.85;-2.42) p = 0.0128*	-6.08 (-11.37;-0.78) p = 0.0355*	-6.08 (-11.37;-0.78) p = 0.0355*	-6.91 (-12.76;-3.11) p = 0.0000***
Subjective Motility (<85% vs. ≥85%)	-2.89 (-10.04;4.26) p = 0.2868	3.35 (-3.94;10.64) p = 0.2613	/	/	/
Polymorph bacteria flora (positive vs. negative)	-0.49 (-5.32;4.35) p = 0.3878	1.44 (-3.04;5.92) p = 0.3225	/	/	/
E. coli spp. (positive vs. negative)	2.28 (-2.75;7.31) p = 0.2646	4.07 (-1.23;9.38) p = 0.1280	/	/	/

Backward = backward-stepwise selection; Forward = forward-stepwise selection; LW= Large White; D= Duroc; Tot spz= Total spermatozoa count. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Supplementary table S1. Clinical chemistry results expressed as means with standard deviations in brackets (SD).

Parameters	Reference Intervals*	Boar								
		I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**
Total Proteins	79 - 89	85.5	81.5	88.3	86.3	74.3	80	80.3	90.3	75.8
g/L		(6.7)	(2.1)	(4.9)	(5.5)	(8.6)	(7.4)	(3.9)	(2.5)	(2.6)
Albumin	19 - 39	44.5	44.5	44	45.3	42.5	43.8	44.8	41.8	48
g/L		(1.3)	(2.1)	(0.0)	(0.5)	(1.3)	(2.2)	(2.1)	(1.3)	(3.6)
Globulins	52.9 - 64.3	41	34	44.3	34	39	42	35.5	48.5	29
g/L		(6.7)	(0.0)	(4.9)	(0.0)	(0.0)	(0.0)	(4.0)	(2.4)	(4.0)
A/G	3.7 - 5.1	1.1	1.3	1	1.1	1.4	1.3	1.3	0.9	1.7
ratio		(0.2)	(0.2)	(0.1)	(0.2)	(0.4)	(0.3)	(0.2)	(0.1)	(0.4)
Urea	3.6 - 10.7	5.6	4.5	5.3	5.8	5.1	5.2	5.1	4.6	5.3
mmol/L		(0.9)	(0.3)	(0.2)	(0.6)	(0.4)	(0.4)	(0.2)	(0.7)	(0.2)
Creatinine	141 - 239	191.3	245.5	218.3	227	175.3	203.3	219.3	192.3	221.3
µmol/L		(13.8)	(20.5)	(24.8)	(44.6)	(28.8)	(45.6)	(18.7)	(14.6)	(21.9)
K^+	4.4 - 6.7	3.1	2.5	2.6	2.5	2.4	2.7	2.5	2.7	2.9
mmol/L		(1.1)	(0.7)	(0.9)	(0.8)	(1.0)	(0.7)	(0.5)	(0.7)	(0.6)
Na^+	135 - 150	141.7	139.5	142.3	134.3	133.3	139.8	143.8	144.8	139
mmol/L		(2.5)	(2.1)	(4.0)	(3.8)	(8.5)	(7.0)	(3.8)	(3.3)	(0.8)
ALP	118 - 395	28	42	25.7	38.7	41.3	42	30.5	25.3	53.3
U/L		(9.9)	(0.0)	(6.4)	(21.8)	(7.0)	(3.2)	(5.3)	(2.9)	(10.4)
ALT	31 - 58	37	27.5	20.5	41	46.5	43.5	33	38.7	34
U/L		(9.6)	(5.0)	(21.9)	(0.0)	(0.7)	(5)	(3.5)	(7.5)	(7.1)
AST	32 - 84	64.7	51	53	42	50	48.5	57.67	51	60
U/L		(16.3)	(21.2)	(9.9)	(0.0)	(18.4)	(6.4)	(5.7)	(10.4)	(14.1)
GGT	10 - 60	66	42	66	26	70	60	58	52	40
U/L		(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(22.6)	(11.3)	(0.0)

A= albumin, G=globulins, ALP= alkaline phosphatase, ALT= alanine transaminase, AST= aspartate transaminase, GGT= gamma glutamyl transferase.

* Kaneko JJ, Harvey JW, Bruss ML. Appendix VIII. In: Kaneko JJ, Harvey JW, Bruss ML, editors. Clinical Biochemistry of Domestic Animals. 6th ed. Philadelphia, PA: Elsevier; 2008. p. 882-888.

First step

Selection of specific Essential oils, chemo-characterization by gas-chromatography and evaluation of the cytotoxic effects on swine spermatozoa. The overall aim was to identify non-spermicidal concentrations to be used for other applications.

molecules

MDPI

Article

Thymbra capitata (L.) Cav. and *Rosmarinus officinalis* (L.) Essential Oils: In Vitro Effects and Toxicity on Swine Spermatozoa

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Abstract: Essential oils possess a variety of biological properties (i.e., antioxidant, antibacterial, and cytotoxic) that could possibly be applied in reproductive medicine, but their effects on spermatozoa are still partially unknown. The aim of the study was to describe the effects of *Thymbra capitata* (L.) Cav. and *Rosmarinus officinalis* (L.) essential oils on the main morpho-functional parameters of swine spermatozoa. Essential oils were preliminary characterized by gas chromatography and added with emulsifiers to facilitate diffusion. Experimental samples were prepared by suspending a fixed number of spermatozoa in 5 mL of medium with 10 different concentrations of essential oil (0.2–2 mg/mL, at intervals of 0.2). After 3 h of incubation, samples were analyzed for pH, viability, objective motility, and acrosome status. Results showed that the effects of the essential oils are concentration-dependent and that *R. officinalis* is well tolerated up to 0.6 mg/mL. *T. capitata* impaired the spermatozoa starting from the lowest concentration, with complete spermicidal effect from 0.4 mg/mL. The patterns of damage, confirmed by SEM, were different and quite distinct. As expected, spermatozoa proved to be sensitive to external stimuli and capable of showing different functional patterns, providing interesting insights to the action/toxicity mechanisms. The results of the present work represent the first step towards the systematic characterization of the effects of these compounds on spermatozoa. This kind of studies are necessary to strengthen the idea of future applications of essential oils in the reproductive field due to their antioxidant, antibacterial, or spermicidal properties.

Keywords: essential oils; *Thymbra capitata*; *Rosmarinus officinalis*; swine spermatozoa; in vitro cytotoxicity

1. Introduction

Looking back in history, it is safe to say that traditional medicine has used plants and their derivatives in order to cure and prevent diseases for centuries. During the last decades, the scientific community, in particular the medical one, has witnessed a considerably growing interest towards the application of essential oils (EOs) that are the products of the secondary metabolism of aromatic plants represented by complex mixtures of several compounds, including terpenoids and

phenylpropanoids [1,2]. Essential oils and their constituents are indeed effective against a large variety of organisms, including bacteria [3,4], viruses [5,6], and fungi [7]. Moreover, some constitutive components of EOs have been proven to have important antioxidant effects [8]. Nonetheless, it has been acknowledged that those substances show strong toxic activities when applied to different cell populations, including fibroblasts and epithelial cells, monocytes, neutrophils, and also spermatozoa [1,9,10], however without specific cellular targets [8,11].

All the above-mentioned properties, both positive and negative, initiate an extremely interesting discussion related with their application in the reproductive field, both human and veterinary. The toxic effects are probably more investigated and exploited in the human reproductive area, where spermicidal medical devices are constantly being designed [1,10,12]. On the other hand, molecules with proven antibacterial and antioxidant properties are necessary in veterinary artificial insemination, where refrigeration of the inseminating doses represents a zootechnical routine, for example in swine. This fact stems from the necessity to find alternatives to the use of antibiotics that is increasingly being limited by the European Commission [13] and to find molecules capable of ensuring improved quality and high fertility of the male gametes.

Spermatic cells represent a good model for in vitro toxicological evaluation of several compounds that can affect the reproductive process such as smoke, nicotine [14,15], and other substances [16]. The use of spermatozoa collected from humans unfortunately has several ethical and legislative limitations, thus, animals' sperm is often used as a translational model. As opposed to macaques, which are probably considered as the overall "gold standard" animal models, porcine spermatozoa are collected using non-invasive techniques (e.g., hand-gloved technique) from trained animals that do not require any sedation and provide high-quality samples [17]. To date, only few EOs, including *Trachyspermum ammi* [10] and *Thymus mumbianus* [1], have been tested on human spermatozoa showing spermicidal capabilities. The mechanism of actions of these compounds are still partially unknown and potentially extremely variable, thus, specific studies for each EO on different cell models are necessary. Concerning animal spermatozoa, in particular porcine ones, only preliminary data regarding the effects of a combination of two EOs has been reported [18].

The aim of the present study was to assess and describe the effects of *Thymbra capitata* (L.) Cav. [=*Coridothymus capitatus* (L.) Rchb.f., *Thymus capitatus* Hoff et Link.] and *Rosmarinus officinalis* (L.) essential oils on the main morpho-functional parameters of swine spermatozoa. These plants, belonging to the Lamiaceae family, are endemic and extremely common to the Mediterranean Basin and are indeed proven to show a variety of biological activities [19,20] with possible applications in zootechnical and reproductive medicine. Moreover, preliminary toxicological screening tests on spermatozoa may provide interesting information regarding the mechanisms of action of these compounds.

2. Results

2.1. Chemical Composition of the EOs

The chemical composition of *T. capitata* (*Tc*) and *R. officinalis* (*Ro*) EOs used in the present study are summarized in Tables 1 and 2, respectively. Carvacrol was the main component of *Tc* EO (65.2%), followed by p-Cymene (12.28%) and γ-terpinene (5.62%). This particular composition resembles what already described in literature [21].

Table 1. Composition of the essential oil of *Thymbra capitata* (L.) Cav.

Compounds	LRI [1]	Area %
α-Thujene	927	0.71
α-Pinene	933	0.96
Camphene	948	0.15
β-Pinene	976	0.13

Table 1. *Cont.*

Compounds	LRI [1]	Area %
β-Myrcene	993	1.37
α-Phellandrene	1006	0.16
α-Terpinene	1017	1.31
p-Cymene	1026	12.28
Limonene	1029	0.45
γ-Terpinene	1060	5.62
trans-sabinene hydrate	1067	0.09
α-Terpinolene	1089	0.21
Linalool	1102	2.37
Borneol	1167	0.18
Terpinen-4-ol	1179	0.7
Thymol	1296	3.49
Carvacrol	1312	65.2
β-Caryophyllene	1426	1.92
α-Humulene	1456	0.11
Caryophyllene oxide	1594	0.12
Total		97.54

[1] LRI: Linear Retention Index.

Table 2. Composition of the essential oil of *Rosmarinus officinalis* (L.).

Compounds	LRI [1]	Area %
α-Pinene	936	23.55
Camphene	949	10.16
β-Pinene	977	5.39
β-Myrcene	993	1.88
p-Cymene	1026	2.8
1,8-Cineole	1034	21.36
Linalool	1103	0.96
Camphor	1145	22.03
Borneol	1168	2.84
Terpinen-4-ol	1180	0.05
α-Terpineol	1193	2.45
Bornyl acetate	1290	1.38
β-Caryophyllene	1427	1.19
Caryophyllene oxide	1594	0.17
Total		96.22

[1] LRI: Linear Retention Index.

Regarding *Ro* EO, α-pinene (23.55%), camphor (22.03%), and 1.8-cineole (21.36%) were almost equally represented, followed by camphene (10.16%) and β-pinene (5.39%). When compared to the analyses of *Ro* EOs in the literature, the one used in this study seems to be similar with the exception of the relatively higher quantity of camphor [19].

2.2. Semen Morpho-Functional Evaluations

The descriptive statistics regarding the effects of the two tested essential oils on the main morpho-functional characteristics of semen are reported in Table S1 (*Tc*) and S2 (*Ro*).

The ANOVA outputs showed that the effects of both EOs were statistically significant with regard to sperm viability (V) (*Tc* $p < 0.0001$; *Ro* $p < 0.0001$), total motility (TotM) (*Tc* $p < 0.0001$; *Ro* $p < 0.0001$), progressive motility (ProgM) (*Tc* $p < 0.0001$; *Ro* $p < 0.0001$), and acrosome reaction (AR) (*Tc* $p < 0.0001$; *Ro* $p = 0.0036$). The analyses of variance for pH, for both EOs, did not show any difference (*Tc* $p = 0.9966$; *Ro* $p = 0.9999$).

The effects on sperm viability of the different concentrations of EOs compared to the control samples are represented in Figure 1. *Tc* EO determined a significant reduction in V starting from the lowest tested concentration of 0.2 mg/mL ($p = 0.005$), with stronger effects from 0.4 mg/mL up to 2 mg/mL ($p < 0.0001$). No significant differences were detected for V of spermatozoa treated with *Ro* EO at concentrations ranging from 0.2 mg/mL to 1.2 mg/mL, even if a decreasing trend was observed. Starting from 1.4 mg/mL of *Ro* EO, the effect on V was statistically evident ($p < 0.001$).

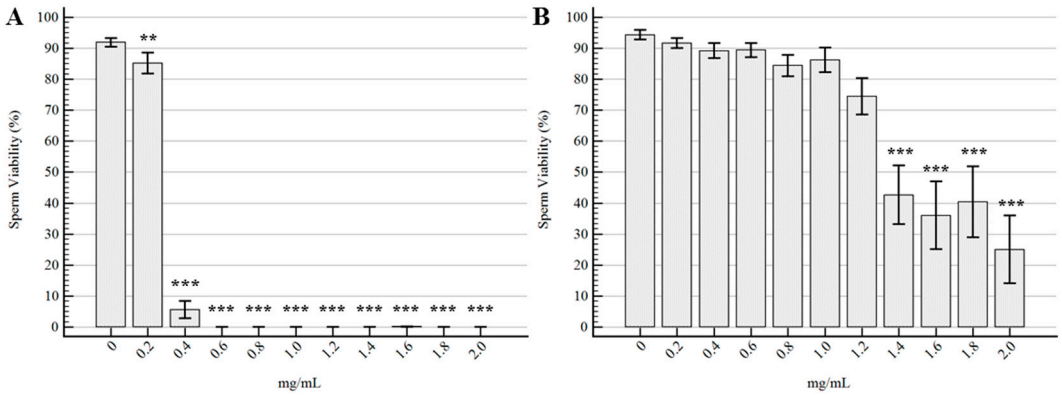

Figure 1. Effects of the EOs on sperm viability. (**A**) *Thymbra capitata*; (**B**) *Rosmarinus officinalis*. Data are expressed as mean ± standard error of the mean ($n = 6$). 0 mg/mL represents the control sample (only emulsifiers). ** = $p < 0.01$; *** = $p < 0.001$.

The trends of total motility for the different samples are represented in Figure 2. The *Tc* EO determined a strong reduction in total motility at 0.2 mg/mL, with an almost complete immobilization of spermatozoa at the other tested concentrations. On the other hand, *Ro* EO did not determine any significant difference in comparison to the control samples up to 0.6 mg/mL, although the concentration of 0.6 mg/mL showed a mild decreasing trend ($p = 0.08$).

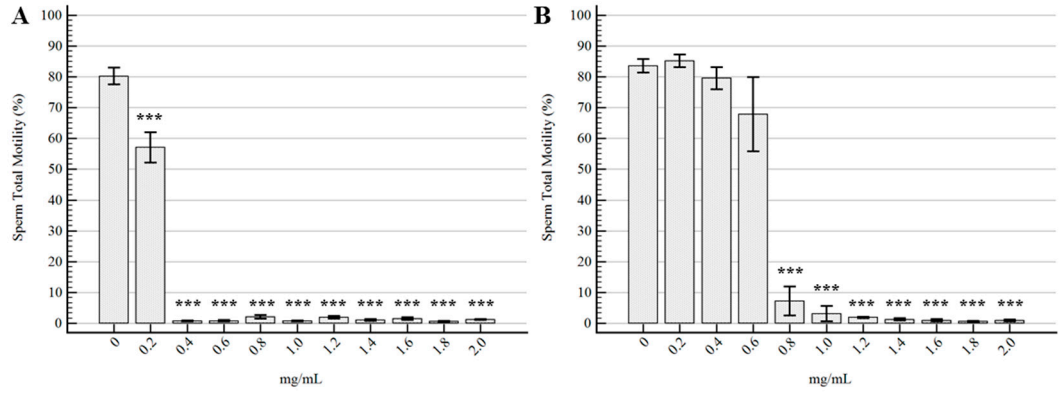

Figure 2. Effects of the EOs on total motility. (**A**) *Thymbra capitata*. (**B**) *Rosmarinus officinalis*. Data are expressed as the mean ± standard error of the mean ($n = 6$), and 0 mg/mL represents the control sample (only emulsifiers). *** = $p < 0.001$.

The statistical results for ProgM (Figure S1) showed exactly the same trends and differences of TotM for both EOs. The results of the analyses of the kinematic parameters of the samples with a TotM $\geq$ 20% are summarized in Table S3. It was not possible to accurately analyze the kinematic parameters of the other samples (from 0.4 mg/mL of *Tc* EO; from 0.8 mg/mL of *Ro* EO) because of the extremely low number of motile cells. The velocity (velocity average path: VAP; velocity curved line: VCL; velocity straight line: VSL) and distance (distance average path: DAP; distance curved

line: DCL; distance straight line: DSL) parameters showed the same behavior as TotM, with statistical differences starting from 0.2 mg/mL for *Tc* EO. Linearity (LIN), straightness (STR), and wobble (WOB) percentages, alongside with the amplitude of lateral head displacement (ALH) and the beat cross frequency (BCF), showed no significant differences among the analyzed samples.

Regarding the percentage of acrosome reaction, the results and statistical differences are represented in Figure 3: *Tc* EO, at the concentration of 0.2 mg/mL did not determine any difference compared to the control sample ($p = 0.93$), whereas all the other experimental concentrations showed important differences ($p < 0.0001$). On the other hand, it was evident that the effect of *Ro* EO on AR was significantly different only for 1.8 and 2 mg/mL, with respective *p*-values of 0.004 and 0.0003.

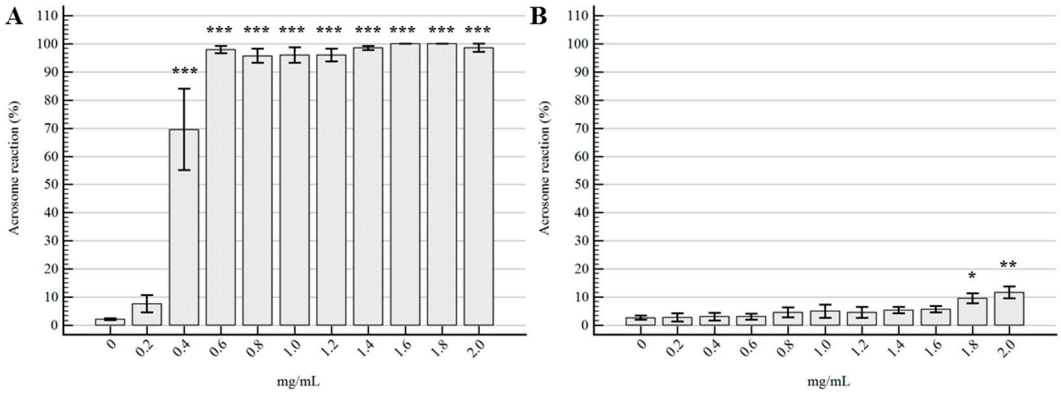

Figure 3. Effects of the EOs on acrosome status. (**A**) *Thymbra capitata*; (**B**) *Rosmarinus officinalis*. Data are expressed as mean ± standard error of the mean ($n = 6$). 0 mg/mL represents the control sample (only emulsifiers). * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

The angular coefficients (β) resulting from the simple linear regression models between EO concentrations and semen morpho-functional parameters are reported in Table 3.

Table 3. Simple linear regression models' angular coefficients (β).

Parameters	*T. capitata* EO	*R. officinalis* EO
	β (95% C.I.) ***p* Value**	**β (95% C.I.)** ***p* Value**
V %	−0.013 (−0.017; −0.009) $p < 0.0001$	−0.018 (−0.022; −0.015) $p < 0.0001$
TotM %	−0.015 (−0.020: −0.010) $p < 0.0001$	−0.014 (−0.017; −0.012) $p < 0.0001$
ProgM %	−0.027 (−0.037; −0.016) $p < 0.0001$	−0.026 (−0.031; −0.021) $p < 0.0001$
AR %	0.013 (0.010; 0.016) $p < 0.0001$	0.086 (0.052; 0.119) $p < 0.0001$
pH	0.508 (−1.168; 2.185) $p = 0.546$	−0.018 (−2.275; 2.238) $p = 0.987$

C.I. = confidence interval; V = Viability; TotM = total motility; ProgM = progressive motility; AR = acrosome reaction.

2.3. Morphological Evaluation by SEM

Scanning electron microscopy analyses highlighted great alterations of EO-treated spermatozoa compared to both the control sample and the additional capacitated sample, as shown in Figure 4.

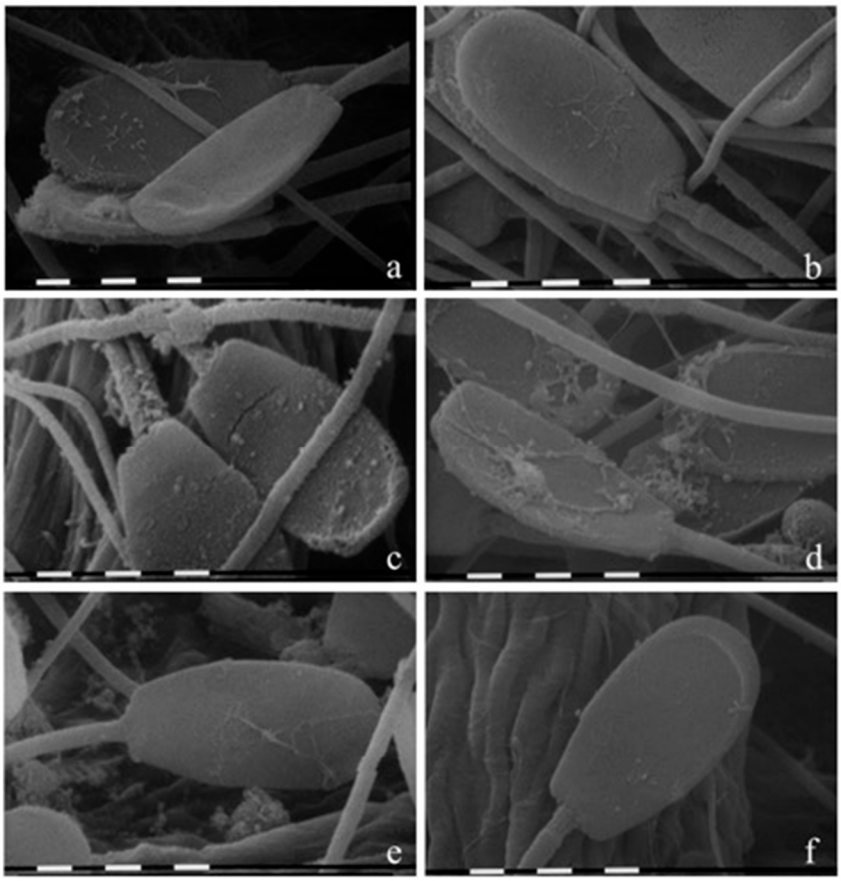

Figure 4. Scanning electron micrographs on the effect of EOs on sperm morphology. Semen samples (**a**) treated with 0.2 mg/mL of *Thymbra capitata*; (**b**) with 0.2 mg/mL of *Rosmarinus officinalis*; (**c**) with 2 mg/mL of *Tc*; (**d**) with 2 mg/mL of *Ro*; (**e**) capacitated spermatozoa; and (**f**) control sample (bars = 1 μm).

As expected, the control (Figure 4f) did not appear morphologically altered: the membranes were regular, smooth, and flattened, and every part of the cell was clearly distinguishable. The capacitated sample (Figure 4e) showed mild exfoliation and disruption of the head membranes in agreement with human literature [22]. On the other hand, the spermatozoa treated with the highest concentration of both EOs showed several morphology defects on the sperm membranes. As shown in Figure 4c, *Tc* (2 mg/mL) caused vesiculation, vacuolation, and lyses of membranes throughout the entire cell. At the same concentration, *Ro* (Figure 4d) only seemed to alter the head region with a different morphological aspect. Figure 4a,b report the spermatozoa treated with 0.2 mg/mL of *Tc* and *Ro*, respectively. In this case, the morphology did not seem to differ compared to the control spermatozoa.

3. Discussion

In the present study, different concentrations of *T. capitata* and *R. officinalis* EOs were applied to swine spermatozoa to investigate the effects on their main morpho-functional parameters. The rationale behind the work was the idea of better understanding the interactions between essential oils and male gametes in the light of the increasing interest toward natural substances and their future applications in veterinary and human reproductive medicine. Our data show clear concentration-dependent effects for both tested essential oils as confirmed by the simple linear regression models.

The results suggest that the essential oil derived from *T. capitata* induce more intense effects, even at the lowest tested concentration of 0.2 mg/mL. Viability and motility are, indeed, significantly reduced, resulting in semen deterioration below the accepted quality standards. At the same

concentration, the percentage of spermatozoa with reacted acrosomes show an increasing trend, but in a non-statistically significant manner. Starting at the concentration of 0.6 mg/mL, this EO induces a complete spermicidal effect, completely altering all the investigated parameters. The *Tc* EO used in this study is composed by carvacrol (65.2%) and thymol (3.49%), compounds that comprise the majority of its phenolic content. It has already been reported that the terpene phenols, in particular carvacrol and thymol, are the major compounds responsible for the antimicrobial effects of EOs [23]. They are, indeed, capable of altering the permeability of bacterial membranes by joining to the amine and hydroxylamine groups of the membrane proteins [20]. Our hypothesis is that such a high content in phenols of this particular EO has caused alterations in the spermatozoa membrane similar to that described in bacteria. This statement seems to be strongly supported by the findings regarding the viability and acrosome status. Spermatozoa viability, when assessed with the eosin-nigrosin staining technique, is actually a measure of the cytoplasmic membrane integrity [24] since the eosin only penetrates the head of spermatozoa with disrupted outer membranes. Therefore, this parameter can be considered as an indirect index of membrane deterioration. Likewise, the staining technique used to assess the percentage of the reacted acrosome, Comassie Blue, gives us information regarding the status of the acrosomal membranes. In the case of *Tc* EO, as previously reported, these two parameters were drastically affected, suggesting an extremely high level of membrane alterations. The above hypothesis seems to find a final iconographic validation in the SEM images, especially for the sample treated with 2 mg/mL, which display severe outer alterations on the entire cell. In the light of the results achieved by the present study, *T. capitata* seems to have a greater potential as a spermicidal agent for reproductive medicine. Nonetheless, analyses on spermatozoa treated with lower concentrations are necessary to identify a potentially harmless concentration.

As far as it concerns the analyses regarding the *R. officinalis* EO, the results are clearly different. Overall, this compound seems to be well tolerated by boar spermatozoa up to the concentration of 0.6 mg/mL. The membrane integrity, in terms of viability and acrosome reaction, is preserved up to concentrations of 1.2 mg/mL and 1.6 mg/mL respectively, never reaching an absolute spermicidal effect. In this case, the limiting parameter seems to be the motility, both total and progressive, which appears significantly altered starting from the concentration of 0.8 mg/mL. The inhibition of motility without any structural membrane alteration, represents a different pattern of damage when compared to the one induced by the *Tc* EO, and proves why the motility is one of the most important and sensitive parameter when analyzing spermatozoa [25] and spermicidal substances [12]. Bakkali and colleagues have reported that, in eukaryotic cells, EOs can depolarize mitochondrial membranes by decreasing membrane potential and affecting ionic cycling [8], and this might be the reason behind the motility inhibition. Due to its relatively wider "safety window", the EO of *R. officinalis* may be exploited, in reproductive medicine, for its antioxidant and antibacterial effects. For instance, Chaftar et al. [26] reported that the minimum inhibitory concentration (MIC) of this EO against several strains of Legionella pneumophila is <0.55 mg/mL, a concentration that, according to the present study, would be well tolerated by spermatozoa. Further studies are necessary to confirm its potential as an antibacterial agent, for example, in artificial insemination swine doses, where antibiotics are still mandatory [13].

4. Materials and Methods

The EOs of *Tc* and *Ro* used in this study were kindly supplied by APA-CT S.r.l. (Via Sacco Nicola, 22 47122, Forlì, Italy). For the experimental purposes, the EOs were reconstituted in 0.5% dimethylsulfoxide (DMSO) with Tween 80 (0.02% v/v) for easy diffusion [3].

4.1. Chemo-Characterization of the EOs

4.1.1. Gas Chromatography-Mass Detector (GC-MS) Analysis

Analyses were performed on a 7890A gas chromatograph coupled with a 5975C network mass spectrometer (Agilent Technologies, Waldbronn, Germany). Compounds were separated on an Agilent Technologies HP-5 MS cross-linked poly-5% diphenyl-95% dimethyl polysiloxane (30 m × 0.25 mm i.d., 0.25 µm film thickness) capillary column. The column temperature was initially set at 45 °C, then increased at a rate of 2 °C/min up to 100 °C, then raised to 250 °C at a rate of 5 °C/min, and finally held for 5 min. The injection volume was 0.1 µL, with a split ratio 1:20. Helium was used as the carrier gas, at a flow rate of 0.7 mL/min. The injector, transfer line and ion-source temperature was 250 °C, 280 °C, and 230 °C, respectively. MS detection was performed with electron ionization (EI) at 70 eV, operating in the full-scan acquisition mode in the m/z range 40–400. EOs were diluted 1:20 (v/v) with n-hexane before GC-MS analysis.

4.1.2. Gas Chromatography-Flame Ionization Detector (GC-FID) Analysis

Analyses were carried out on an Agilent Technologies 7820 gas chromatograph (Waldbronn, Germany) with a flame ionization detector (FID). Compounds were separated on an Agilent Technologies HP-5 crosslinked poly-5% diphenyl-95% dimethyl polysiloxane (30 m × 0.32 mm i.d., 0.25 mm film hickness) capillary column. The temperature program was the same as described above. The injection volume was 0.1 µL in split mode 1:20. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The injector and detector temperature was set at 250 °C and 300 °C, respectively. EOs and the reference standards were diluted 1:20 (v/v) with *n*-hexane before GC-FID analysis. The analyses were performed in duplicate.

4.1.3. Qualitative and Semi-Quantitative Analysis

Compounds were identified by comparing the retention times of the chromatographic peaks with those of authentic reference standards run under the same conditions and by comparing the linear retention indices (LRIs) relative to C8–C40 n-alkanes obtained on the HP-5 column under the above-mentioned conditions with the literature [27]. Peak enrichment by co-injection with authentic reference compounds was also carried out. Comparison of the MS-fragmentation pattern of the target analytes with those of pure components was performed. A mass-spectrum database search was carried out by using the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) mass-spectral database (version 2.0d, 2005).

Semi-quantification was calculated as the relative percentage amount of each analyte; in particular, the values were expressed as the percentage peak area relative to the total composition of each EO obtained by GC-FID analysis.

4.2. Boars and Ejaculate Collection

Two adult hybrid (Large White × Duroc) boars were enrolled as sperm donors for the experiments. Animals were housed in single pens, according to the national law (D.lgs n.122/2011). Semen was routinely collected by an experienced operator twice a week using the hand-gloved technique. The sperm-rich fraction (SRF) was immediately diluted 1:1 v/v with Swine Fertilization Medium (SFM) extender prepared as previously described [28], and an aliquot (2 mL) was analyzed to assess overall quality [29]. Each SRF was evaluated for spermatozoa concentration by a Thoma haemocytometer chamber, viability and total motility, as described later.

SRF inclusion criteria for the experimental protocol were set as sperm V > 85% and TotM > 80%, according to the common sperm quality standards. Twelve ejaculates, six from each boar, were included in experimental protocol.

4.3. Experimental Protocol

Each EO was tested on six different ejaculates (n = 6) (three from each boar). The experimental doses were prepared by suspending a fixed number of spermatozoa (15×10^7 spz) in 5 mL of SFM extender (final concentration = 3×10^7 spz/mL) with 10 different concentrations of EO (from 0.2 to 2 mg/mL, at intervals of 0.2). The SFM extender was prepared as described by Fantinati et al. [28], without any antibiotic. For each experiment, control samples were realized by only adding the emulsifiers (DMSO 0.5% v/v and Tween 80 0.02% v/v [3]). After preparation, the experimental doses were incubated for 3 h in a refrigerated bath at 16 °C (±1 °C), and subsequently evaluated for the principal morpho-functional parameters.

4.4. Semen Morpho-Functional Evaluations

Viability was assessed using the Eosin-Nigrosin staining method [29]. Briefly, 10 µL of staining solution were added to 10 µL of each dose, and 8 µL were immediately smeared on a glass microscope slide for the analysis. The percentage of live cells (undyed spermatozoa/all spermatozoa) was evaluated on a minimum of 200 cells.

Analyses regarding the acrosome status were performed using a modified Coomassie Blue staining protocol as previously described [30]. After two washings in phosphate-buffered saline (PBS), spermatozoa were fixed in 4% paraformaldehyde for 10 min. Sperm were then centrifuged and suspended with ammonium acetate (100 mM, pH 9). Twenty microliters were then smeared on a microscope glass slide, air dried, and incubated for 2 min with 0.22% Coomassie Blue G250 staining solution. The percentage of reacted acrosomes (AR) (undyed acrosomes/all acrosomes) was evaluated on a minimum of 200 cells. The slides prepared for the evaluation of viability and acrosome status were coded and analyzed by a blinded operator in order to avoid biases.

The objective motility of the spermatozoa, both total and progressive, and the kinematics parameters were assessed using a Computer Assisted Sperm Analysis (CASA; Hamilton Thorne CEROS II; Animal Motility II, Software Version 1.9, Beverly, MA, USA) unit. Prior to the analysis, an aliquot from each experimental sample was incubated for 10 min at 37 °C in a digital incubator (INCU-Line IL23; VWR International, Radnor, PA, USA). All samples were analyzed by the same blinded operator, and at least one thousand spermatozoa for each sample were tracked. Since the kinematics parameters derive from motile spermatozoa, we decided to only report the ones for samples with a minimum TotM of 20% (at least 200 motile cells).

The pH of each experimental sample was analyzed using a Medidor PH BASIC 20 (Hach Lange srl, Milan, Italy) after calibration according to the instrument's instructions.

4.5. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) studies were performed to visualize any possible membrane morphological change in the spermatozoa treated with *Tc* and *Ro* EOs. The samples treated with the highest and lowest concentration of each EO (0.2 and 2 mg/mL), one control with the emulsifiers, and an additional capacitated sample [31], were analyzed with SEM. For each sample, an aliquot of 500 µL was centrifuged at 800× g for 10 min, and the pellet fixed in 500 µL of 5% glutaraldehyde solution buffered at pH 7.2 with phosphate buffer 0.1 M. The mixture was dropped by a pipette on filter paper, washed with phosphate buffer 0.1 M pH 7.2, dehydrated through an increasing concentration of aqueous ethanol (10%, 20%, 30%, 50%, 75% and 95%) for 15 min and in 100% ethanol for 5 min. All the above steps were performed at 5 °C. The samples were then dried with an Emitech K850 (Emitech Ltd., Ashford, UK) critical point drier unit mounted on aluminum stubs with double-sided tape and coated with gold-palladium film using an Emitech K500 (Emitech Ltd., Strovolos, Cyprus) ion sputtering unit. Samples were observed with a Philips 515 SEM scanning electron microscope (Philips, Eindhoven, The Netherlands) at 10 kV, and pictures were taken with a Nikon 5400 Coolpix digital camera (Nikon, Tokyo, Japan).

4.6. Statistical Methods

The statistical analyses were performed using the software R 3.0.3 (The R Foundation for Statistical Computing). Descriptive statistics of the parameters were calculated and expressed as means and standard error of the mean. Normal distribution was assessed with the Shapiro-Wilk test ($p < 0.05$). To evaluate differences between the control doses and the others containing EOs, one-way ANOVA was performed with the significance level set at 0.05. Post hoc analyses were performed by means of Dunnett's test ($p < 0.05$) to assess the differences between the controls and the treatments. To evaluate the presence of a concentration-dependent effect of the EOs on the morpho-functional parameters, simple linear regression models were performed.

5. Conclusions

In conclusion, the proposed approach to evaluate the effects of the essential oils on spermatozoa seems to be capable of providing robust and repeatable results. As expected, these cells proved to be sensitive and susceptible to external stimuli and to be capable of showing a variety of different functional patterns, providing interesting insights to the toxicity mechanisms. Overall, the results of the present work represent the first step towards the systematic characterization of the effects of these natural compounds on spermatozoa. These kinds of studies are necessary to strengthen the idea of future applications of EOs in the reproductive field for their antioxidant, antibacterial, or spermicidal properties.

Supplementary Materials: The following are available online. Table S1: Descriptive statistics of the effects of *Thymbra capitata* EO on semen morpho-functional parameters. Table S2: Descriptive statistics of the effects of *Rosmarinus officinalis* EO on semen morpho-functional parameters. Table S3: Effects of *Thymbra capitata* and *Rosmarinus officinalis* EOs on spermatic kinematic parameters. Figure S1: Effects of the EOs on Progressive Motility.

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Author Contributions: M.L.B. and A.E. conceived and designed the experiments; A.E., D.V., and F.B. performed the experiments on the spermatozoa; S.B. performed the gas chromatographic analyses; A.P. and G.F. performed the electron microscopy analyses; A.E. analyzed the data; M.S. contributed reagents/materials; M.L.B., A.E., and M.S. interpreted the data; and A.E. and D.V. wrote the paper. All the co-authors approved the final draft.

Conflicts of Interest: The authors declare no conflict of interest. APA-CT did not play any role in the design of the study nor in the acquisition/interpretation of the results despite supplying the essential oils.

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Sample Availability: Not available.

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Supplementary materials: *Thymbra capitata* (L.) Cav. and *Rosmarinus officinalis* (L.) Essential Oils: *in vitro* effects and toxicity on swine spermatozoa.

Table S1. Descriptive statistics of the effects of *Thymbra capitata* EO on semen morpho-functional parameters. Data are reported as Mean (standard error of the mean), *n*=6.

	***Thymbra capitata* (mg/mL)**										
	0	**0.2**	**0.4**	**0.6**	**0.8**	**1**	**1.2**	**1.4**	**1.6**	**1.8**	**2**
V %	91.9 (1.4)	85.1 (3.4)	18.8 (2.8)	0	0	0	0	0	0	0	0
TotM %	80.2 (2.7)	57.1 (4.9)	0.7 (0.2)	0.8 (0.2)	2.1 (0.6)	0.8 (0.2)	1.9 (0.5)	1.0 (0.2)	1.4 (0.4)	0.5 (0.2)	1.2 (0.2)
ProgM %	46.8 (5.7)	24.0 (5.1)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0	0.1 (0.1)	0.1 (0.1)	0.3 (0.1)	0.1 (0.1)	0.1 (0.1)
AR %	2.1 (0.4)	7.6 (3.0)	69.5 (14.5)	97.8 (1.3)	95.7 (2.5)	95.9 (2.7)	96.0 (2.3)	98.5 (0.7)	100 (0.0)	100 (0.0)	98.5 (1.5)
pH	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.1)

V= Viability; TotM= Total Motility; ProgM= Progressive Motility; AR= Acrosome Reaction.

Table S2. Descriptive statistics of the effects of *Rosmarinus officinalis* EO on semen morpho-functional parameters. Data are reported as Mean (standard error of the mean), *n*=6.

	***Rosmarinus officinalis* (mg/mL)**										
	0	**0.2**	**0.4**	**0.6**	**0.8**	**1**	**1.2**	**1.4**	**1.6**	**1.8**	**2**
V %	94.3 (1.5)	91.7 (1.6)	89.2 (2.4)	89.4 (2.2)	84.4 (3.4)	74.0 (4.0)	74.5 (5.9)	42.6 (9.5)	36.0 (11.0)	40.4 (11.4)	25 (11.0)
TotM %	83.5 (2.2)	85.1 (2.1)	79.5 (3.6)	67.8 (12.1)	7.2 (4.7)	3.1 (2.5)	1.9 (0.2)	1.2 (0.3)	0.9 (0.4)	0.6 (0.2)	0.9 (0.3)
ProgM %	45.4 (5.2)	38.8 (7.0)	36.8 (5.0)	31.1 (7.3)	2.1 (0.9)	0	0.1 (0.1)	0.1 (0.1)	0	0	0.1 (0.0)
AR %	2.6 (0.7)	2.8 (1.5)	3 (1.4)	3 (1.1)	4.5 (1.8)	5 (2.3)	4.5 (2.0)	5.3 (1.1)	5.6 (1.2)	9.5 (1.8)	11.6 (2.1)
pH	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)	6.7 (0.0)

V= Viability; TotM= Total Motility; ProgM= Progressive Motility; AR= Acrosome Reaction.

Table S3. Effects of *Thymbra capitata* and *Rosmarinus officinalis* EOs on spermatic kinematic parameters. In the table are only reported the samples with a total motility ≥ 20%. Data are reported as Mean (standard error of the mean), *n*=6. Differences were calculated by means of Dunnett PostHoc test (* = p<0.05; ** = p<0.01; *** = p< 0.001).

	***Tc* (mg/ml)**		***Ro* (mg/mL)**			
	0	**0.2**	**0**	**0.2**	**0.4**	**0.6**
TotM (%)	80.2	57.1**	83.5	85.1	79.5	67.8
	(2.7)	(4.9)	(2.2)	(2.1)	(3.6)	(12.1)
VAP (μm/s)	90.0	48.8**	86.4	77.1	84.0	73.9
	(5.4)	(7.2)	(10.0)	(11.8)	(9.3)	(12.2)
VCL (μm/s)	197.4	106.6**	189.0	170.7	189.4	167.7
	(11.3)	(16.5)	(22.7)	(26.5)	(21.3)	(27.1)
VSL (μm/s)	49.7	26.7**	47.4	43.2	42.5	37.9
	(4.5)	(3.9)	(4.7)	(6.07)	(4.7)	(5.4)
DAP (μm)	52.3	29.3**	49.9	42.0	48.6	43.7
	(2.4)	(4.9)	(5.6)	(6.4)	(5.4)	(6.4)
DCL (μm)	117.1	65.7**	111.5	95.6	112.6	101.6
	(5.6)	(11.4)	(13.0)	(15.2)	(12.6)	(14.4)
DSL (μm)	27.1	15.2**	25.7	21.8	22.5	21.0
	(2.0)	(2.7)	(2.1)	(2.9)	(2.5)	(2.4)
LIN (%)	25.7	25.1	26.2	25.8	23.0	24.3
	(1.7)	(0.6)	(1.4)	(0.9)	(0.7)	(1.0)
STR (%)	54.9	52.8	55.4	54.9	50.7	52.8
	(2.5)	(1.6)	(2.2)	(1.4)	(1.2)	(1.8)
WOB (%)	45.6	45.8	46.1	45.2	44.2	44.6
	(1.0)	(0.6)	(0.6)	(0.6)	(0.3)	(0.7)
ALH (μm)	9.6	7.3	9.6	9.1	9.4	9.1
	(0.5)	(0.9)	(0.8)	(1.1)	(0.7)	(1.0)
BCF (Hz)	36.9	36.9	36.0	36.7	35.6	36.4
	(0.5)	(3.4)	(1.0)	(1.4)	(1.4)	(1.8)

TotM= Total Motility; VAP= velocity average path; VCL= velocity curved line; VSL= velocity straight line; DAP= distance average path: DCL= distance curved line; DSL= distance straight line; LIN= linearity (VSL/VCL); STR= straightness (VSL/VAP); WOB=wobble (VAP/VCL); ALH= amplitude of lateral head displacement; BCF= beat cross frequency.

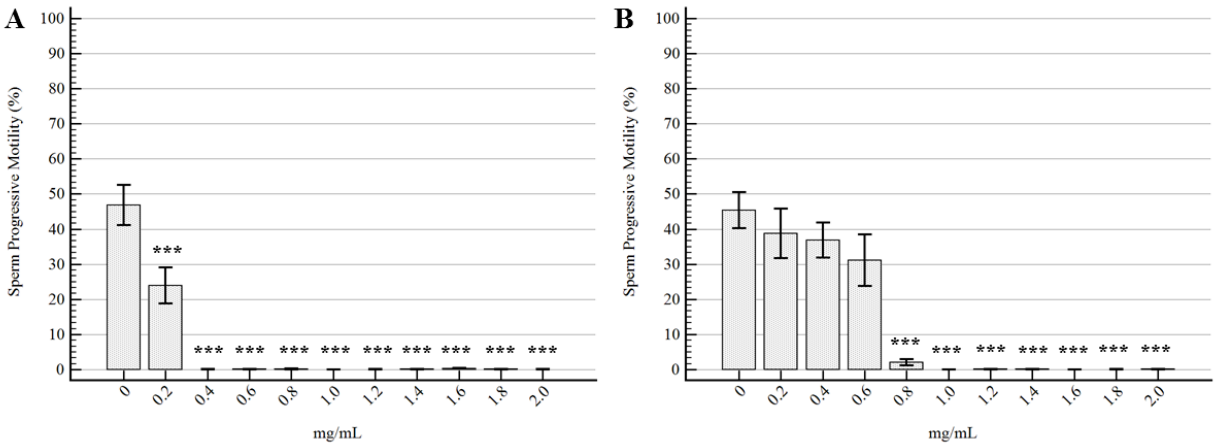

Figure S1. Effects of the EOs on Progressive Motility. (A) *Thymbra capitata*. (B) *Rosmarinus officinalis*. Data are expressed as mean ± standard error of the mean (*n*=6). 0 mg/ml represents the control sample (only emulsifiers). * = p<0.05; ** = p<0.01; *** = p< 0.001.

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In Vitro Effects of Tea Tree Oil (Melaleuca Alternifolia Essential Oil) and its Principal Component Terpinen-4-ol on Swine Spermatozoa

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Abstract: The growing interest towards essential oils stems from their biological capabilities that include antibacterial and antioxidant effects. Such properties may be extremely useful in the reproductive field; nonetheless essential oils show toxic effects that can lead to cell disruption. The present study aimed to evaluate and compare the effects of tea tree oil (TTO) and its principal component terpinen-4-ol (TER) on the morpho-functional parameters of swine spermatozoa. Experimental samples were prepared by suspending 15×10^7 spermatozoa in 5 mL of medium with different concentrations of the above-mentioned compounds: from 0.2 to 2 mg/mL at an interval of 0.2 for TTO, while TER concentrations were adjusted according to its presence in TTO (41.5%). After 3 h incubation at 16 °C, samples were analyzed for pH, viability, acrosome status, and objective motility. The results highlighted a concentration-dependent effect of TTO with total motility as the most sensitive parameter. TER was better tolerated, and the most sensitive parameters were related to membrane integrity, suggesting a different pattern of interaction. The study confirms the importance of evaluating the effects of natural compounds on spermatozoa before exploiting their beneficial effects. Spermatozoa seem to be good candidates for preliminary toxicological screenings in the light of their peculiar properties.

Keywords: tea tree oil; melaleuca alternifolia; terpinen-4-ol; essential oil; swine spermatozoa; toxicity

1. Introduction

The need for new and improved protocols for preservation of swine artificial insemination (AI) doses has led researchers toward the study of new active compounds with antibiotic and antioxidant potential [1]. Indeed, up to date, only 1% of porcine AIs around the world are conducted with frozen-thawed semen [2] because of the cellular damage that leads to low fertility in this species [3]. During cryopreservation protocols, antioxidants are necessary to improve cryotolerance [4], but synthetic ones do not always perform well in boars thus, lately, plant based ones have begun

to be taken into account [5]. Despite the efforts, liquid phase preservation (16 ± 1 °C) of boar AI doses is still the most common storage technique [3]. In this case, antibiotics are essential to limit bacterial growth [6], as this determines alterations to the midpiece, acrosome, and plasma membrane of the spermatozoa leading to worse quality parameters [7,8]. Moreover, the European Union (Council Directive 90/429/EEC) [9] dictates the use of antibiotics in swine seminal AI doses as mandatory to prevent the spreading of diseases. Nonetheless, it is important to acknowledge that antibiotics added to seminal doses may be potentially dangerous for both animal and human health as they can contribute to the phenomenon of antibiotic-resistance in the swine industry [10]. The scenario prospected by the EU regarding the risks of multi-resistant bacteria calls for ethical discussion regarding how actually necessary are antibiotics and what alternatives can be proposed especially in the zoo-technical world. All of the above leads, again, to the need for new molecules and substances with antioxidant and/or antibacterial capabilities.

Traditional medicine, since the dawn of history, has exploited the knowledge about plants and natural compounds for medical treatments, laying the foundations of modern medicine and pharmacology. The growing interest towards the application of natural compounds, and in particular essential oils (EOs), stems from their potential multi-purpose functional use as antibacterial, antiviral, antifungal, and antioxidant agents [11–15]. The essential oil of *Melaleuca alternifolia,* (Maiden & Betche) Cheel, commonly known as tea tree oil (TTO), is a complex mixture of approximately 100 compounds, produced by the homonymous Australian plant, member of the Myrtaceae family [16,17]. TTO is highly employed in medicine and in the pharmaceutical, food and cosmetic industries due to several biological properties (antimicrobial, antioxidant, antitumor, insecticidal, etc.) [13,18,19]. Currently, the composition of *M. alternifolia* essential oil (terpinen-4-ol type) is regulated by the International Organization of Standardization (ISO) 2004, that sets the cut off value for the 15 main components of the TTO [20,21]. The composition of *M. alternifolia* EO is variable and depending to climate, age of leaves, leaf maceration, and duration of distillation [20]. Generally, the TTO contains numerous monoterpene and sesquiterpene as well as aromatic compounds, which the principal is the terpinen-4-ol (TER), also known for biological activities, especially for anti-bacterial effects [19,20,22,23]. Nonetheless, it has been acknowledged that EOs show toxic activities when applied to different cell populations, including fibroblasts, epithelial cells, monocytes, and neutrophils [12,24]. Only a few data have been reported about spermatozoa and EOs both in humans (*Trachyspermum ammi* [25]; *Thymus mumbianus* [26]) and animals (*Rosmarinus officinalis* [27]). On swine spermatozoa, some studies have already been performed about the effects of *Thymbra capitata* and *Rosmarinus officinalis* EOs [28].

The aim of this study was to evaluate and compare the effects of tea tree oil, and its principal component terpinen-4-ol, on the main morpho-functional parameters of swine spermatozoa, as the first step towards possible application in the reproductive industry.

2. Results

2.1. Chemical Composition

The chemical composition of the Melaleuca alternifolia EO used in the present study is shown in Table 1. As expected, the main component was terpinen-4-ol (41.49%) followed by other components as γ-terpinene (20.55%), α-terpinene (9.59%) and α-terpineol (4.42%).

Table 1. Composition of tea tree oil (TTO).

Compound	LRI [1]	Area%
terpinen-4-ol	1185	41.49
γ-terpinene	1061	20.55
α-terpinene	1018	9.59
α-terpineol	1194	4.42
α-pinene	933	4.4
p-cymene	1025	3.66
terpinolene	1089	3.18
1,8-cineole	1031	2.15
limonene	1029	1.78
aromadendrene	1446	1.38
caryophyllene oxide	1594	0.76
myrcene	992	0.72
allo-aromadendrene	1468	0.18
α-felladrene	1005	0.17
sabinene	973	0.03
β-pinene	975	0.03
α-humulene	1460	0.02
β-caryophyllene	1425	0.02
Total		94.53

[1] LRI = linear retention index.

2.2. Sperm Morpho-Functional Parameters

The descriptive analysis of the effects of TTO and TER on the principal sperm morpho-functional parameters is reported in the supplementary material: Tables S1 and S2.

The analysis of variance showed that both treatments statistically altered the majority of the analyzed parameters: viability (V) (TTO $p < 0.0001$; TER $p < 0.0001$), acrosome reaction (AR) (TTO $p < 0.0001$; TER $p < 0.0001$), total motility (TotM) (TTO $p < 0.0001$; TER $p = 0.0247$) and progressive motility (ProgM) (TTO $p < 0.0001$; TER $p < 0.0001$). The only unaltered parameter was pH (TTO $p = 0.9969$; TER $p = 0.7908$).

The results of the Dunnett's tests, comparing the different experimental samples to the control (CTR) for the morpho-functional parameters, are reported in Figures 1–4.

The V of spermatozoa treated with TTO (Figure 1A) was statically reduced starting from a concentration of 1 mg/mL ($p = 0.037$) and up to 2 mg/mL ($p < 0.0001$). Spermatozoa treated with TER showed a significant reduction in V ($p = 0.008$; Figure 1B) only for the three highest tested concentrations (equivalent to 1.6, 1.8, and 2 mg/mL of TTO).

Regarding AR, the results showed a statistical increase starting from 1.4 mg/mL for TTO ($p < 0.0001$; Figure 2A) and from 0.67 mg/mL for TER, equivalent to 1.6 mg/mL or TTO ($p = 0.0026$; Figure 2B).

Spermatozoa treated with TTO showed a decreasing trend for TotM (Figure 3A) already at 0.4 mg/mL, statistically appreciable only from 0.8 mg/mL ($p = 0.0003$). The treatment with TER (Figure 3B) did not determine significant alterations of sperm motility, with the only exception for the highest tested concentration of 0.83 mg/mL ($p = 0.05$), corresponding to 2 mg/mL of TTO.

On the other hand, the ProgM showed a slightly different behavior, with statistically significant alterations already starting from 0.4 mg/mL for TTO ($p = 0.003$; Figure 4A) and from 0.25 mg/mL for TER ($p = 0.043$; Figure 4B), equivalent to 0.6 mg/mL of TTO.

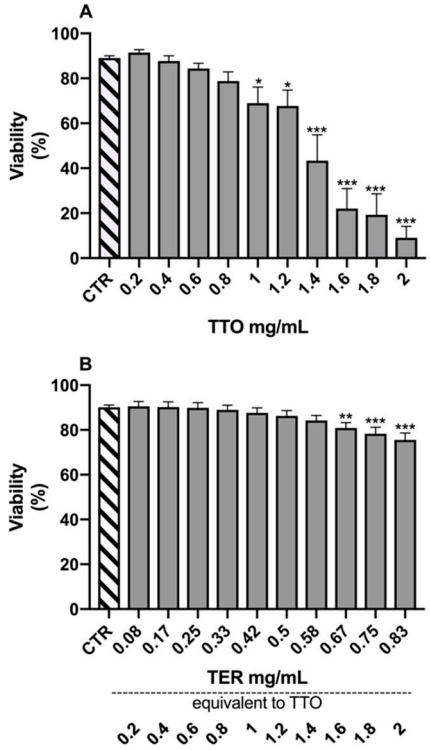

Figure 1. Effects of tea tree oil (**A**) and terpinen-4-ol (**B**) on sperm viability. Data are expressed as mean ± standard error of the mean. CTR = control samples (only emulsifiers). * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

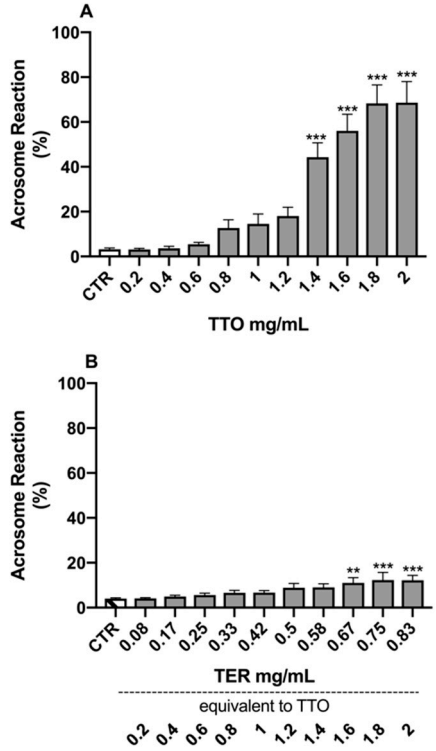

Figure 2. Effects of tea tree oil (**A**) and terpinen-4-ol (**B**) on sperm acrosome reaction. Data are expressed as mean ± standard error of the mean. CTR = control samples (only emulsifiers). ** = $p < 0.01$; *** = $p < 0.001$.

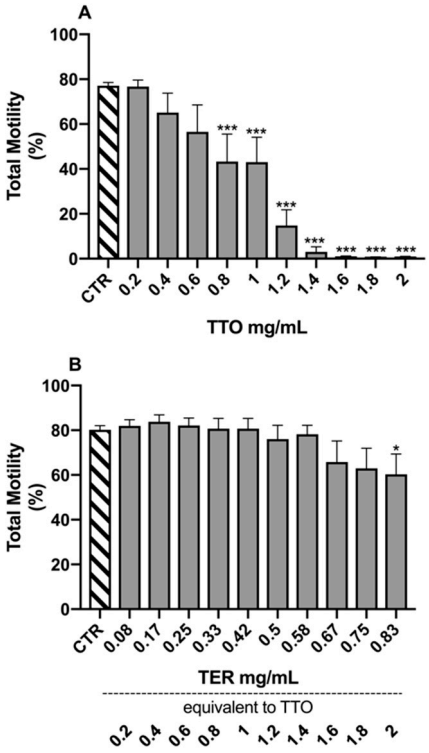

Figure 3. Effects of tea tree oil (**A**) and terpinen-4-ol (**B**) on total sperm motility. Data are expressed as mean ± standard error of the mean. CTR = control samples (only emulsifiers). * = $p < 0.05$; *** = $p < 0.001$.

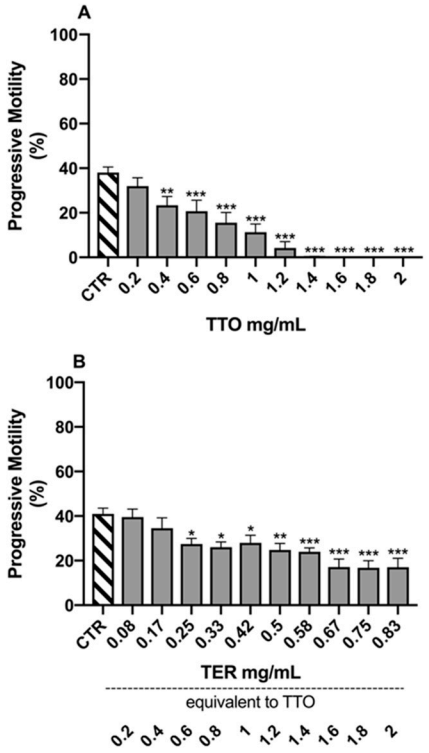

Figure 4. Effects of tea tree oil (**A**) and terpinen-4-ol (**B**) on progressive sperm motility. Data are expressed as mean ± standard error of the mean. CTR = control samples (only emulsifiers). * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Descriptive and inferential statistics of kinematics parameters are reported in Table 2 (TTO) and Table 3 (TER). The analysis of variance for the TTO group highlighted statistical differences for VAP, VCL, VSL, DAP, DCL, DSL, and STR; on the other hand, only VSL, DSL, LIN, and STR resulted statistically altered for the TER group.

Table 2. Effects of tea tree oil (TTO) on sperm kinematic parameters for samples with total motility ≥20%. Data are reported as Mean (standard error of the mean), $n = 9$. Differences were calculated by means of Dunnett PostHoc test (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

	TTO (mg/mL)					
	CTR	**0.2**	**0.4**	**0.6**	**0.8**	**1**
VAP (μm/s)	80.96 (2.63)	77.70 (3.89)	81.02 (5.63)	79.04 (4.37)	72.47 (3.15)	54.95 (7.87) ***
VCL (μm/s)	180.75 (5.62)	173.34 (8.61)	186.84 (12.73)	180.77 (6.22)	165.14 (6.14)	132.98 (16.83) **
VSL (μm/s)	41.10 (1.73)	37.30 (2.00)	34.35 (2.29)	33.33 (2.19)	32.02 (1.27)	25.36 (2.16) ***
DAP (μm)	48.19 (1.39)	47.37 (1.89)	49.14 (2.77)	48.08 (2.41)	44.78 (2.11)	36.71 (4.19) **
DCL (μm)	110.20 (3.49)	106.94 (4.83)	115.65 (6.59)	112.55 (3.62)	104.97 (5.06)	86.34 (9.86) *
DSL (μm)	22.71 (0.79)	20.88 (0.96)	18.45 (0.94) *	18.27 (1.12) *	18.11 (0.32) *	15.24 (0.83) ***
LIN (%)	22.74 (0.60)	22.58 (0.55)	19.54 (0.86)	20.07 (1.32)	21.03 (1.25)	21.41 (1.60)
STR (%)	50.90 (1.09)	48.67 (0.97)	43.19 (1.28) **	43.37 (2.16) *	46.48 (2.42)	47.43 (3.03)
WOB (%)	45.16 (0.32)	45.00 (0.35)	43.64 (0.89)	44.48 (1.02)	44.06 (0.83)	43.96 (0.97)
ALH (μm)	9.50 (0.26)	8.88 (0.38)	9.55 (0.69)	10.01 (0.44)	9.54 (0.66)	8.65 (1.01)
BCF(Hz)	37.75 (1.15)	37.34 (0.77)	38.15 (0.99)	36.60 (2.89)	34.94 (1.38)	35.11 (2.75)

VAP = velocity average path; VCL = velocity curved line; VSL = velocity straight line; DAP = distance average path: DCL = distance curved line; DSL = distance straight line; LIN = linearity (VSL/VCL); STR = straightness (VSL/VAP); WOB = wobble (VAP/VCL); ALH = amplitude of lateral head displacement; BCF = beat cross frequency.

Table 3. Effects of Terpinen-4-ol (TER) on sperm kinematic parameters for samples with total motility ≥20%. Data are reported as Mean (standard error of the mean), $n = 9$. Differences were calculated by means of Dunnett PostHoc test (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

	TER (mg/mL)										
	CTR	**0.08**	**0.17**	**0.25**	**0.33**	**0.42**	**0.50**	**0.58**	**0.67**	**0.75**	**0.83**
	Equivalent to TTO (mg/mL)										
		0.2	**0.4**	**0.6**	**0.8**	**1**	**1.2**	**1.4**	**1.6**	**1.8**	**2**
VAP (μm/s)	88.12 (2.26)	92.15 (4.46)	92.29 (4.22)	95.19 (5.92)	90.17 (5.92)	86.83 (6.27)	87.91 (5.09)	87.89 (5.61)	78.58 (5.71)	75.39 (6.40)	75.41 (4.92)
VCL (μm/s)	200.47 (5.82)	212.65 (12.22)	219.15 (7.81)	223.22 (8.89)	212.76 (9.48)	200.71 (12.55)	202.52 (11.31)	203.59 (11.60)	188.61 (13.59)	172.23 (12.16)	170.46 (10.83)
VSL (μm/s)	44.32 (1.63)	44.77 (1.56)	36.71 (3.73)	37.25 (1.88)	36.68 (3.25)	34.88 (2.86) *	35.54 (2.87) **	33.78 (1.90) **	27.72 (2.33) ***	28.15 (1.97) ***	29.19 (2.61) ***
DAP (μm)	51.32 (1.53)	54.01 (2.78)	54.23 (2.78)	55.39 (3.32)	53.45 (3.73)	51.58 (3.34)	51.15 (3.42)	50.77 (4.66)	48.48 (3.01)	44.37 (4.45)	45.79 (2.48)
DCL (μm)	119.94 (3.40)	127.73 (8.35)	132.63 (3.80)	134.08 (5.57)	129.96 (7.87)	123.33 (6.62)	122.08 (7.39)	124.67 (7.34)	121.17 (7.45)	108.95 (6.67)	107.95 (6.26)
DSL (μm)	23.62 (1.31)	24.27 (0.98)	19.08 (2.18)	19.36 (1.33)	19.28 (0.99)	18.06 (0.82) *	18.25 (0.94) *	17.88 (1.10) *	15.32 (1.25) ***	15.24 (1.10) ***	16.13 (1.41) ***
LIN (%)	22.93 (1.28)	22.67 (1.68)	18.15 (1.81)	18.16 (1.25)	18.74 (1.42)	20.09 (1.35)	17.03 (1.15) *	17.47 (0.50) *	15.95 (0.66) *	16.66 (0.69) *	16.23 (1.81) **
STR (%)	49.45 (2.11)	48.83 (2.65)	41.79 (3.25)	41.04 (2.60)	41.66 (2.51)	41.52 (1.71)	41.32 (0.91)	39.64 (1.20) *	36.99 (1.48) **	38.67 (2.19) **	39.01 (1.62) **
WOB (%)	10.31 (0.30)	10.57 (0.59)	174.74 (0.36)	11.38 (0.34)	11.08 (0.52)	10.95 (0.37)	43.55 (0.37)	42.98 (0.68)	42.28 (0.78)	43.54 (0.81)	44.29 (1.16)
ALH (μm)	10.31 (0.30)	10.57 (0.59)	11.25 (0.39)	11.38 (0.36)	33.08 (0.34)	10.95 (0.52)	11.44 (0.42)	11.30 (0.51)	12.34 (1.59)	10.67 (0.58)	10.67 (0.39)
BCF (Hz)	45.05 (3.30)	43.52 (3.95)	48.68 (7.60)	42.48 (4.63)	41.13 (5.56)	36.21 (1.82)	32.47 (1.01)	36.27 (1.82)	40.78 (5.74)	34.56 (2.00)	33.38 (2.29)

VAP = velocity average path; VCL = velocity curved line; VSL = velocity straight line; DAP = distance average path: DCL = distance curved line; DSL = distance straight line; LIN = linearity (VSL/VCL); STR = straightness (VSL/VAP); WOB = wobble (VAP/VCL); ALH = amplitude of lateral head displacement; BCF = beat cross frequency.

The angular coefficients (β) resulting from the linear regression models between concentrations and morpho-functional semen parameters are reported in Table 4.

Table 4. Simple linear regression models' angular coefficients (β).

	Tea Tree Oil β **(C.I. 95%)**	**Terpinen-4-ol** β **(C.I. 95%)**
V %	−0.091 (−0.104; −0.079) $p < 0.0001$	0.094 (0.010; 0.178) $p = 0.0279$
TotM %	−0.080 (−0.090: −0.070) $p < 0.0001$	0.049 (0.008; 0.091) $p = 0.020$
ProgM %	−0.163 (−0.185; −0.141) $p < 0.0001$	0.017 (−0.043; 0.077) p =0.564
AR %	0.100 (0.086; 0.114) $p < 0.0001$	−0.089 (−0.237; 0.060) $p = 0.239$
pH value	−2.071 (−10.308; 6.166) $p = 0.619$	4.348 (−8.203; 16.899) $p = 0.491$

C.I. = Confidence Interval; V = Viability; TotM = Total Motility; ProgM = Progressive Motility; AR = Acrosome Reaction.

2.3. Sperm Morphological Evaluation by Scanning Electron Microscopy

Images from scanning electron microscopy (SEM) are shown in Figure 5. Samples treated with the lowest concentrations of both substances (TTO Figure 5b; TER Figure 5f) did not show any morphological differences when compared to the control samples (Figure 5a,e), displaying a smooth and intact surface. On the other hand, the effects of the highest concentrations were easily appreciable: 2 mg/mL of TTO (Figure 5d) caused vesiculation, vacuolation, and lyses of membranes throughout the entire cell, 0.83 mg/mL of TER (Figure 5h) determined similar alteration of membranes, but mainly localized in the head region. The middle dosages (Figure 5c,g) determined milder alteration but with the same patterns.

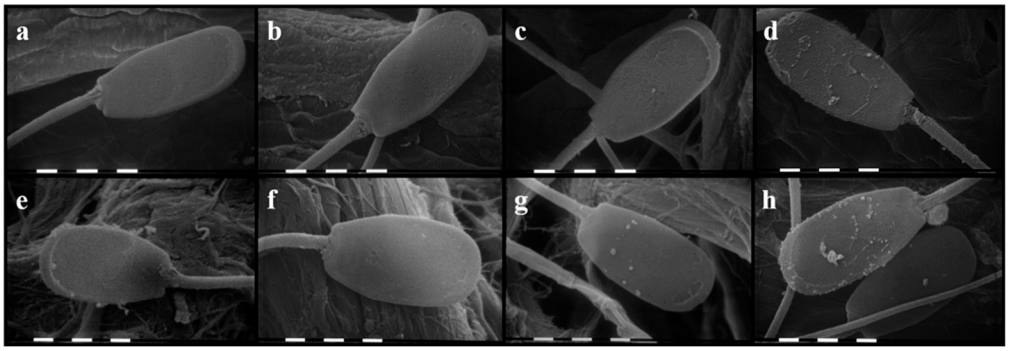

Figure 5. Scanning electron micrographs for the effects of the lowest, middle, and highest concentrations of tea tree oil (TTO) and erpinen-4-ol (TER) on sperm morphology. (**a**) control sample; (**b**) TTO 0.2 mg/mL; (**c**) TTO 1 mg/mL); (**d**) TTO 2 mg/mL; (**e**) control sample; (**f**) TER 0.08 mg/mL; (**g**) TER 0.42 mg/mL; (**h**) TER 0.83 mg/mL.

3. Discussion

The present study aimed at the evaluation and comparison of the effects of tea tree oil and its main constituent, terpinen-4-ol, on porcine spermatozoa, using the main sperm morpho-functional parameters as study variables. Nowadays, it is common opinion that natural substances, like EOs and or phytoextracts can represent an improvement of the standard cryopreservation protocols of swine AI, but their deleterious potential has to be taken into account and investigated in depth. Indeed, the antibacterial effects of essential oils have to be imputed to a mechanism of interaction with membranes, as highly lipophilic, that may not be selective towards bacteria, as opposed to

common antibiotics [12,13]. They can also impair mitochondrial activity by means of membrane depolarization [12,13], potentially leading to the loss of one of the most vital characteristics of spermatozoa represented by motility. This is why the characterization of the direct effects of each essential oil on the spermatic cell is necessary before considering their applications in the reproductive field. Moreover, the peculiar physiology of the spermatozoa, capable of generating motion, makes it a potentially useful tool for the in vitro characterization of general toxicity of exogenous compounds [29,30].

Melaleuca alternifolia EO (TTO) is defined by the international standard ISO-4730 that specifies certain characteristics of EO in order to facilitate assessment of its quality [21]. In this case, the used EO conforms to the ISO system: 1,8-cineol represents less than 15% (2.15%) and terpinen-4-ol, the main component, more than 30% (41.49%) of the overall composition.

Overall, TTO seems to be well tolerated by porcine spermatozoa up to a concentration of 0.6 mg/mL; higher quantities of this EO determined increasing impairment in a concentration-dependent manner as shown by the results of the linear regression models for all test parameters excluding pH. Such a pattern of concentration-dependent effects is in agreement with what has already been reported for two other essential oils (*R. officinalis* and *T. capitata*) [28]. Total motility, as already described by literature [27,28,31,32], was the most sensitive thus the limiting parameter with an early reduction, for TTO-treated samples, already at 0.4 mg/mL that became statistically significant at 0.8 mg/mL. On the other hand, membrane integrity seems to be less sensitive to the action of the EO as shown by the results of viability and acrosome status, and ichnographically confirmed by scanning electron microscopy. These findings may suggest an early functional impairment, probably due to interactions with the mitochondrial capability of producing motion [30,33], followed by a morphological one at higher concentrations. This scenario strengthens the necessity to test the toxic effects of exogenous compounds directly in spermatozoa, when the aim is to use them in the reproductive field, as motility impairment cannot be assessed on other cells. The overall findings regarding the effects of the essential oil of *Melaleuca alternifolia* are similar to the ones reported for *Rosmarinus officinalis* in porcine [28] and rooster spermatozoa [27].

The rationale for testing terpinen-4-ol by itself was driven by its strong presence in the used EO, accountable for the 41.49% of its whole composition. Indeed, the used concentrations of terpinen-4-ol were calculated on the ones chosen for the EO in order to be able to compare the results between the two groups. Overall, this molecule was better tolerated, as the first toxic effects started to appear at 0.67 mg/mL, the third highest tested concentration. The damage pattern seems opposite to the one determined by TTO, as the most sensitive parameters were, in this case, viability and acrosome reaction. Motility indeed was only significantly altered at the highest concentration, despite a decreasing trend starting from 0.67 mg/mL. Overall, all the tested parameters were less altered upon co-incubation with the single compound, as confirmed by SEM images, in contrast to that described by Hammer et al. [34], who stated that the single compounds of the *Melaleuca alternifolia* essential oil have higher cytotoxic activity when compared to the whole oil.

Several studies have already reported how terpinen-4-ol by itself still shows antibacterial activity [20], a feature that could be highly exploitable, for example, in swine AI. Nonetheless, the MICs (Minimal Inhibitory Concentrations) reported by literature against different bacteria are highly variable going from 0.6 up to 2.5 mg/mL [35], thus potentially toxic on the basis of the present study. The same statement seems to be true also for TTO, since the MICs available in the literature vary from very low concentrations (0.12–0.5 mg/mL [36]) up to extremely high ones (20 mg/mL [37]. Nonetheless, due to the singular nature of each batch of each essential oil, influenced by a wide variety of agricultural and industrial factors, the analysis of antibacterial capabilities through MICs can be very challenging. It is still important to acknowledge that synergistic effects of lower doses of different molecules, partially already confirmed [38], have to be explored and how recent studies have reported that sub-lethal doses of essential oils and natural compounds may still be effective in controlling bacteria by alteration

of peculiar virulence factors [39]. In such a scenario, if the aim is to exploit TTO and/or TER as antimicrobial agents in AI doses, further studies have to be performed to confirm such a possibility.

The results regarding toxicity upon membrane damage are also important since EOs, whether used as potential antimicrobial or spermicidal agents from an industrial point of view, should also be tested for their effect on the female reproductive apparatus. This context is even more delicate and complex to analyze as in vitro testing would not be sufficient. Indeed, the effects exerted by EOs on isolated cell will most likely change in in vivo conditions, where physiological defense mechanisms, such as mucus, are available.

For both tested substances, progressive motility follows the trend of total motility despite some "earlier" significant differences. Indeed, ProgM, is significantly altered already at "lower" concentrations. Nonetheless, from a biological point of view, these slight discrepancies between TotM and ProgM are only relatively important, as the actual number of progressive motile spermatozoa is directly related to the overall motile ones (ProgM is a small population of TotM).

The discussion regarding kinematic parameters can be quite difficult, as up to date, their biological meaning and correlation to semen quality and fertility still have to be definitively unveiled. TTO seems to alter velocity (VAP, VCL, and VSL) and distance (DAP, DCL, and DSL) parameters only when used at the highest studied concentration for kinematics; higher concentrations were not evaluated as TotM was not sufficiently relevant. These findings seem to suggest a quantitative impairment in speed, thus in distance, without alteration of the quality of movement as confirmed by the non-altered results of ALH, WOB, and BCF. Indeed, hyper-activated sperm subpopulation, showing abnormal movements, are characterized by increased ALH [33]. The same pattern of velocity–distance alteration was noticed starting from 0.42 mg/mL of TER; corresponding to 1 mg/mL of TTO, but only regarding the straight-line parameters (VSL and DCL). This is particularly interesting as TER-treated samples seem to maintain good TotM ($p > 0.05$) also at higher concentrations then the ones with kinematics alterations. Supposedly, modifications in kinematics parameters with maintained TotM may suggest early impairment, still allowing the spermatozoa to move but with less intensity. Overall, despite the relative lack of literature, different authors have reported how kinematics parameters, especially velocity-related ones, seem to have a good predictive potential when it comes to fertility [40,41]. This is why CASA analyses play a pivotal role when evaluating interactions between new molecules with potential in the reproductive field and spermatozoa [29,30].

The results of the present study suggest that the toxic effects of the used EO are either to be imputed to other constituents rather than terpinen-4-ol or to the synergic interaction of all the components of the oil itself. This point is extremely critical when it comes to the application of phytocompounds and the study of their mechanism of action as the difference in chemotype may alter the outcome of the hypothesized synergistic capabilities of the single components.

4. Materials and Methods

All reagents, unless otherwise specified, were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Essential oil of *Melaleuca alternifolia* (TTO) used for the experiment was provided by APACT (Forlì, FC, Italy). For the experiment, the TTO was reconstituted in 0.5% dimethylsulfoxide (DMSO) and Tween 80 (0.002%) [14]. Terpinen-4-ol was purchased by Moellhausen (Vimercate, MB, Italy).

4.1. Chemo-Characterization of the M. alternifolia EO

4.1.1. Gas Chromatography-Mass Detector (GC-MS) Analysis

Analyses were performed on a 7890A gas chromatograph coupled with a 5975C network mass spectrometer (Agilent Technologies, Waldbronn, DE). Compounds were separated on Agilent Technologies HP-5 MS cross-linked poly-5% diphenyl-95% dimethyl polysiloxane (30 m × 0.25 mm i.d., 0.25 mm film thickness) capillary column. The column temperature was initially set at 45 °C, then increased at a rate of 2 °C/min up to 100 °C, then raised to 250 °C at a rate of 5 °C/min, and finally

held for 5 min. The injection volume was 0.1 µL, with a split ratio 1:20. Helium was used as the carrier gas, at a flow rate of 0.7 mL/min. The injector, transfer line and ion-source temperature was 250 °C, 280 °C, and 230 °C, respectively. MS detection was performed with electron ionization (EI) at 70 eV, operating in the full-scan acquisition mode in the m/z range 40–400. EOs were diluted 1:20 (v/v) with n-hexane before GC-MS analysis.

4.1.2. Gas Chromatography-Flame Ionization Detector (GC-FID) Analysis

Analyses were carried out on an Agilent Technologies 7820 gas chromatograph (Waldbronn, DE) with a flame ionization detector (FID). Compounds were separated by means of Agilent Technologies HP-5 crosslinked poly-5% diphenyl-95% dimethyl polysiloxane (30 m × 0.32 mm i.d., 0.25 mm film thickness) capillary column. The temperature program was the same as described above. The injection volume was 0.1 µL in split mode 1:20. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The injector and detector temperature was set at 250 °C, 280 °C, and 230 °C, respectively. EOs and the reference standards were diluted 1:20 (v/v) with n-hexane before GC-FID analysis. The analyses were performed in duplicate.

4.1.3. Qualitative and Semi-Quantitative Analysis

Compounds were identified by comparing the retention times of the chromatographic peaks with those of authentic reference standards run under the same conditions and by comparing the linear retention indices (LRIs) relative to C8–C40 n-alkanes obtained on the HP-5 column under the above-mentioned conditions with the literature ref. [42]. Peak enrichment by co-injection with authentic reference compounds was also carried out. Comparison of the MS-fragmentation pattern of the target analytes with those of pure components was performed. A mass-spectrum database search was carried out by using the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) mass-spectral database (version 2.0d, 2005). Semi-quantification was calculated as the relative percentage amount of each analyte; in particular, the values were expressed as the percentage peak area relative to the total composition of EO obtained by GC-FID analysis.

4.2. Boars and Ejaculates

Three adult boars (Large White × Duroc) housed in single pens, according to the National Law (D.lgs n. 122/2011) in compliance with good practice for animal welfare, were enrolled as ejaculate donors in the present work. Routinely, semen was collected twice a week by an experienced operator using the hand-gloved technique in a pre-heated (37 °C) thermos.

Eighteen sperm rich fractions (SRFs), six from each boar, were used for this work. Upon collection, the SFR was immediately diluted 1:1 v/v with an in-house prepared extender (Swine Fertilization Medium, SFM) as previously described [43] without any antibiotics.

To assess overall quality, each SFR was evaluated for spermatozoa concentration by a Thoma haemocytometer chamber, viability (V) by eosin-nigrosine staining, and total motility (TotM) by CASA following the later described protocols [28]. Only SFR with V >85% and TotM >80% were used for the experimental protocol.

4.3. Experimental Protocol

The experimental protocol was performed as previously described by the authors [28].

The TTO and TER were individually tested on three ejaculates from each boar (ejaculates N = 18; TTO $n = 9$; TER $n = 9$).

For each trial, 11 experimental samples were prepared by suspending 15×10^7 spermatozoa in 5 mL of SFM, with a final concentration of 3×10^7 spermatozoa/mL. Ten samples were added with increasing concentrations of either TTO or TER while one sample, the control (CTR), was only added with emulsifier (DMSO—0.5% and Tween 80—0.2% [14]). The TTO tested concentrations were from

0.2 to 2 mg/mL, at an interval of 0.2; the TER concentrations were adjusted according to the percentage of TER in the used TTO (41, 5%) for each concentration used (Table 5).

Table 5. Concentrations of tea tree oil (TTO) and Terpinen-4-ol (TER) used in the study. TER concentrations were calculated according to its presence in the used TTO (41.5%).

TTO (mg/mL)	TER (41.5 %) (mg/mL)
2	0.83
1.8	0.75
1.6	0.67
1.4	0.58
1.2	0.5
1	0.42
0.8	0.33
0.6	0.25
0.4	0.17
0.2	0.08

Upon preparation, samples were incubated at 16 °C (± 1 °C) in a refrigerated bath for 3 h and subsequently evaluated for the principal morpho-functional parameters.

4.4. Evaluation of Spermatozoa Morpho-Functional Parameters

Viability (V) was assessed using Eosin-Nigrosin staining [44], while the percentage of reacted acrosomes (AR) was calculated by means of a modified Coomassie Blue staining protocol [7,31]. The objective analysis of motility, including total (TotM) and progressive (ProgM) motility, was performed by a CASA unit (Hamilton Thorne CEROS II, Animal Motility II, Software version 1.9, Beverly, MA, USA) with a heated stage, after 5 min of incubation of the samples at 37 °C. Since the kinematics parameters can only be appreciated on motile spermatozoa, only samples with TotM ≥20% were used for their analysis as previously done [28]. Analyzed parameters included: average path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), distance of the average path (DAP), curvilinear distance (DCL), straight line distance (DSL), linearity (LIN, calculated from VSL/VCL), straightness (STR; calculated from VSL/VAP), wobble (WOB; calculated from VAP/VCL), amplitude of lateral head displacement (ALH), and beat cross frequency (BCF) [33]. The pH of each sample was analyzed using a Medidor PH BASIC 20 (Hach Large srl, Milan, Italy) after calibration according to the manufacturer's instructions.

4.5. Scanning Electron Microscopy

Observations by Scanning Electron Microscopy (SEM) were performed on samples treated with the lowest, middle, and highest concentrations of TTO (0.2 mg/mL; 1 mg/mL; 2 mg/mL) and TER (0.08 mg/mL; 0.42 mg/mL; 0.83 mg/mL) to visualize, if present, membrane morphological alterations.

Aliquots of 500 µL from the above-mentioned samples were centrifuged at 800× *g* for 10 min, and the pellet fixed in 500 µL of 5% glutaraldehyde solution buffered at pH 7.2 with phosphate buffer 0.1 M. The samples were resuspended and a drop of each was pipetted on a stapled filter paper bag and washed with phosphate buffer 0.1 M pH 7.2. Dehydration was performed by means of increasing concentrations of aqueous ethanol (10%, 20%, 30%, 50%, 75%, and 95%) for 15 min and in 100% ethanol for 5 min at 5 °C. Specimens were then dried with a critical point drier unit K850 (Emitech Ltd., Ashford, UK), mounted on aluminum stubs with double stick tape and coated with a gold–palladium film using an ion sputtering unit K500 (Emitech Ltd., Ashford, UK). The dry samples were then observed with a 515 SEM (Philips, Eindhoven, NL) at 10 kV, and the pictures were taken with a 5400 Coolpix digital camera (Nikon, Chiyoda-ku, Tokyo, Japan).

4.6. Statistical Analyses

The statistical analyses were performed using the software R 3.0.3 (The R Foundation for Statistical Computing) and graphically represented using the software GraphPad Prism v.8 (GraphPad Software Inc., San Diego, CA, USA). Descriptive statistics were calculated and expressed as means and standard error of the mean. Normal distribution was assessed by means of the Shapiro-Wilk test ($p < 0.05$). To evaluate differences between the treated samples and the control one, a one-way ANOVA followed by the Dunnett post-hoc test was performed (significance level set at 0.05). Linear regression models were set up to analyze the potential concentration-dependent effects.

5. Conclusions

In conclusion, this study highlights the importance of evaluating the effects of natural compounds on spermatozoa before suggesting and exploiting their beneficial effects in the reproductive field. As already reported, the analytical approach seems to provide robust and repeatable results that may also help to unveil the mechanism of interactions of such compounds. Motility proved to be the most sensitive parameter for the analysis of tea tree oil, strengthening the idea of future applications of spermatozoa in toxicological screenings, in the light of their mitochondria abundance and their capability to generate movement. The concentrations of TTO and TER that resulted as non-toxic on the basis of this study need further investigations for potential exploitation in the reproductive field.

Supplementary Materials: The Supplementary Materials are available online. Table S1: descriptive statistics of the effects of tea tree oil on semen morpho-functional parameters, Table S2: descriptive statistics of the effects of terpinen-4-ol on semen morpho-functional parameters.

Author Contributions: M.L.B. and A.E. designed the experiments; A.E., D.V., G.C., and F.B. performed the experiments on the spermatozoa; S.B. performed gas chromatography analyses; A.P. and G.F. performed scanning electron microscopy analyses; A.E. analyzed the data; M.S. contributed reagents/materials; M.L.B., A.E., and M.S. interpreted the data; and A.E. and D.V. wrote the paper. All the co-authors approved the final draft.

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Sample Availability: Samples of the compounds used in the present study are available from the authors.

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Supplementary file

In vitro effects of tea tree oil (*Melaleuca alternifolia* essential oil) and its principal component terpinen-4-ol on swine spermatozoa

Alberto Elmi, Domenico Ventrella, Barone Francesca, Giacomo Carnevali, Gianfranco Filippini, Annamaria Pisi, Stefania Benvenuti, Maurizio Scozzoli, Maria Laura Bacci

Table S1. Descriptive statistics of the effects of tea tree oil on semen morpho-functional parameters.

Data are reported as Mean (standard error of the mean). n=9.

	TTO (mg/mL)										
	CTR	**0.2**	**0.4**	**0.6**	**0.8**	**1**	**1.2**	**1.4**	**1.6**	**1.8**	**2**
V (%)	89.00	91.43	87.71	84.33	78.75	68.92	67.71	43.33	22.00	19.25	9.00
	(1.03)	(1.32)	(2.32)	(2.34)	(4.12)	(7.20)	(7.05)	(11.53)	(8.93)	(9.36)	(5.13)
TotM.(%)	78.38	77.76	74.92	65.88	56.48	43.04	14.79	3.03	1.00	0.71	0.86
	(1.46)	(3.19)	(4.99)	(8.94)	(11.81)	(11.09)	(7.02)	(2.29)	(0.20)	(0.07)	(0.21)
ProgM (%)	38.00	31.93	23.39	20.71	15.51	11.28	4.18	0.24	0.03	0.00	0.00
	(2.60)	(3.77)	(10.42)	(4.93)	(5.65)	(3.65)	(2.88)	(0.21)	(0.03)	(0.00)	(0.00)
AR (%)	2.83	3.13	3.64	5.44	9.36	10.42	18.00	44.25	56.06	68.31	68.63
	(0.39)	(0.51)	(0.92)	(0.85)	(1.78)	(1.73)	(3.97)	(6.43)	(7.42)	(8.24)	(9.44)
pH	6.69	6.68	6.67	6.72	6.68	6.68	6.68	6.69	6.68	6.67	6.69
	(0.01)	(0.03)	(0.03)	(0.05)	(0.03)	(0.02)	(0.02)	(0.02)	(0.03)	(0.03)	(0.04)

TTO= tea tree oil; V= Viability; TotM= Total Motility; ProgM= Progressive Motility; AR= Acrosome Reaction

Table S2. Descriptive statistics of the effects of terpinen-4-ol on semen morpho-functional parameters.

Data are reported as Mean (standard error of the mean), n=9.

	TER (mg/mL)										
	CTR	**0.08**	**0.17**	**0.25**	**0.33**	**0.42**	**0.5**	**0.58**	**0.67**	**0.75**	**0.83**
		equivalent to TTO (mg/mL)									
		0.2	0.4	0.6	0.8	1	1.2	1.4	1.6	1.8	2
V (%)	90.09	90.50	90.21	89.86	88.94	87.62	86.31	84.19	80.88	78.31	75.56
	(1.07)	(2.24)	(2.33)	(1.62)	(2.11)	(2.23)	(1.57)	(2.31)	(2.22)	(2.87)	(3.10)
TotM (%)	80.19	81.98	83.76	82.14	85.72	80.67	75.94	78.10	65.77	62.94	60.29
	(1.82)	(2.74)	(3.12)	(3.32)	(2.26)	(4.60)	(5.17)	(4.01)	(7.89)	(9.02)	(9.09)
ProgM (%)	40.94	39.52	34.52	27.46	31.75	27.98	24.77	24.20	17.14	16.80	17.04
	(2.64)	(3.62)	(4.69)	(2.53)	(3.96)	(3.38)	(2.47)	(1.79)	(3.13)	(2.99)	(4.01)
AR (%)	4.05	4.08	4.92	5.58	6.64	6.67	8.86	9.07	11.00	12.29	11.14
	(0.32)	(0.40)	(0.58)	(0.86)	(1.11)	(0.91)	(1.62)	(1.54)	(1.99)	(3.39)	(2.13)
pH	6.72	6.70	6.71	6.71	6.73	6.72	6.69	6.69	6.67	6.67	6.66
	(0.02)	(0.40)	(0.02)	(0.03)	(0.05)	(0.04)	(0.02)	(0.03)	(0.01)	(0.01)	(0.01)

TER= terpinen-4-ol; V= Viability; TotM= Total Motility; ProgM= Progressive Motility; AR= Acrosome Reaction

Second step

Antibacterial effects of the previously identified non-spermicidal concentrations on the liquid phase of swine refrigerated seminal doses. In order to obtained standardized results, an *in vitro* model with the addition of *E. coli* was set up.

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Antimicrobial capabilities of non-spermicidal concentrations of tea tree (*Melaleuca alternifolia*) and rosemary (*Rosmarinus officinalis*) essential oils on the liquid phase of refrigerated swine seminal doses.

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Abstract

Antimicrobial resistance is increasing within the porcine industry with consequential high impact on human health, leading to a need for new antimicrobials. Lately, the scientific community has turned its interest towards natural compounds, and different essential oils have been tested on spermatozoa for preliminary assessment of toxicity before considering them as good substitutes for standard antibiotics. The aim of the present work was to investigate the potential antimicrobial effect of *Melaleuca alternifolia* and *Rosmarinus officinalis* essential oils, already evaluated for toxicity, on swine artificial insemination doses deprived of spermatozoa and stored at 16 °C for 5 days. This was accomplished by setting up an *in vitro* model with a standardized quantity of *E. coli*. Essential oils, previously chemo-characterized by means of gas chromatography, were tested at 0.2 and 0.4 mg/ml. Analyses, performed at 24 and 120 hours, included optical density evaluation, bacterial DNA quantification by qPCR, and colony count. The results demonstrate that both *Melaleuca alternifolia* and *Rosmarinus officinalis* essential oils, at a concentration of 0.4 mg/ml, are capable of delivering similar effects to ampicillin, used as control, on the experimental samples. At the lower concentration, *M. alternifolia* essential oil seemed more effective when compared to *R. officinalis*. Overall, these findings strengthen the hypothesis of the potential use of phyto-complexes as antimicrobial agents for reproductive biotechnologies.

Keywords

Essential oil; *Escherichia coli*; antibacterial effect; swine reproduction; tea tree oil; rosemary.

1. Introduction

More than the 90% of the global porcine industry uses standard artificial insemination technologies in order to satisfy the growing request for porcine meat. This involves, inevitably, the constant necessity for the development of advanced and cost effective strategies, especially regarding sperm conservation. Although good hygienic practices for ejaculate sampling allow for controlled bacterial contamination (Schulze et al., 2015), porcine ejaculates still usually contain 10^4 and up to 10^6 bacteria/ml, mainly *Enterobacteriaceae, Alcaligenaceae,* and *Xanthomonadaceae* (Althouse and Lu, 2005). Sources of microbial contamination are various and range from the boar genital tract to environmental and laboratory pollution (Maes et al., 2008).

During the liquid phase storage of the seminal material, refrigerated at 16 ±1°C, the bacterial growth can alter spermatozoa quality, reducing the viability and motility, alongside a risk for female reproductive disorders and embryonic/foetal death (Kuster and Althouse, 2016; Pinart et al., 2017). Furthermore, the presence of pathogens such as *Brucella suis*, *Chlamydia* spp., *Leptospira* and *Mycoplasma* spp. cannot be excluded (Maes et al., 2008). In light of the aforementioned reasons, antimicrobials are routinely used in semen extenders, mainly penicillin, streptomycin and aminoglycosides, especially gentamicin (Schulze et al., 2017), and their presence is dictated by the European Directive 90/429 ("EUR-Lex - 31990L0429 - EN," n.d.).

Antimicrobial resistance is increasing within the porcine industry (Aarestrup et al., 2008) with high risks to human health ("WHO | Antimicrobial resistance," n.d.), leading to a necessity to identify alternative antimicrobial strategies, especially for reproductive biotechnologies, such as physical bacteria removal by colloid centrifugation (Morrell and Wallgren, 2011) and microfiltration of seminal plasma (Barone et al., 2016), or the use of antimicrobial peptides (Sancho et al., 2017; Schulze et al., 2014). During the last years, the scientific community has turned its interest towards natural compounds, and many phyto-extracts like essential oils (EOs) have been tested on spermatozoa of different species (Chikhoune et al., 2015; Dávila et al., 2015; Elmi et al., 2019, 2017;

Giaretta et al., 2014; Touazi et al., 2018). EOs and their components are capable of exerting a wide variety of antibacterial mechanisms of action, some of them still partially unclear. In particularly, EOs can affect the structure and the function of bacteria by, amongst others, interfering with cytoplasmic membranes and decreasing ATP synthesis (Nazzaro et al., 2013). The essential oil derived by the distillation of *Melaleuca alternifolia* (Maiden & Betche) Cheel (*Myrtaceae*) leaves, commonly known as tea tree oil (TTO), is capable of broad-spectrum antimicrobial activity thanks to its multiple components, including more than 100 terpenes and their related alcohols (Brun et al., 2019; Carson et al., 2006). The International Standard Organization regulates the concentration of the 15 principal components of TTO, of which the major compound is terpinen-4-ol ("ISO 4730:2017(en), Essential oil of Melaleuca, terpinen-4-ol type (Tea Tree oil)," n.d.). Likewise, *Rosmarinus officinalis* (L.) EO was reported to have antimicrobial properties (El Fawal et al., 2019; Khezri et al., 2019; Satyal et al., 2017). Rosemary, belonging to the *Lamiaceae* family, is endemic and extremely common to the Mediterranean Basin and is known for a variety of applications and biological activities (Hussain et al., 2010; de Oliveira et al., 2019; Nieto, 2017). The chemical composition of rosemary EO is quite variable, with different chemotypes based on the relative percentages of α-pinene, 1,8-cineole, camphor, borneol, verbenone, and bornyl acetate (Satyal et al., 2017).

Both *M. alternifolia* and *R. officinalis* EOs have already been evaluated for toxicity on porcine spermatozoa (Elmi et al., 2019, 2017) and were proved to exhibit different patterns of damage, either functional or morphological, with concentration-dependent effects. On the basis of such studies, objective motility assessed by CASA was the most sensitive parameter and was significantly altered starting from 0.8 mg/ml of both EOs. Concentrations lower than 0.6 mg/ml were proved as non-cytotoxic for porcine spermatozoa, thus potentially useful in the reproductive biotechnology field.

In light of the afore-mentioned points, the aim of the present work was to investigate the potential antimicrobial effectiveness of *Melaleuca alternifolia* and *Rosmarinus officinalis* EOs, at non-spermicidal concentrations, on swine artificial insemination doses deprived of spermatozoa and

stored at 16 °C up to 5 days. This was accomplished by setting up an *in vitro* model with a standardized quantity of *E. coli*.

2. Materials and methods

The EOs of *M. alternifolia* (Ma) and *R. officinalis* (Ro) used in the present study were supplied by APA-CT S.r.l. (Via Sacco Nicola, 22, 47122, Forlì, Italy) and their chemo-characterization was previously reported by the authors in the aforementioned toxicity studies (Elmi et al., 2019, 2017). For experimental purposes, the EOs were reconstituted in 0.5% dimethylsulfoxide (DMSO) with Tween 80 (0.02% *v*/*v*) to facilitate diffusion in water-based solutions (Bag and Chattopadhyay, 2015). All reagents, unless otherwise specified, were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

2.1. Boars and seminal plasma

Ejaculates from three adult commercial hybrid boars (Large White X Duroc) were included in the study. Animals were individually housed, according to national law (D.lgs n.122/2011), with 12:12 hour light:dark cycle and a minimum of 40 lux during the light period; wood, straw and ice cubes were used as environmental enrichment. Boars were fed with a custom formulated diet (Big Verri, Cooperativa Agricola "Tre spighe", Castel Guelfo, Italy) twice a day, with *ad libitum* water. Semen was routinely collected by an experienced technician twice a week using the hand-gloved technique and following standard hygienic practices (Elmi et al., 2018). In the present study, sperm poor fractions from 9 ejaculates (n=9; 3 from each boar) were used. Upon collection they were immediately centrifuged (3000 x g for 20 minutes) in order to obtain seminal plasma (SP).

2.2. Bacterial preparation

E. coli DH5 alpha strain (cod. C2007-1, Clontech Lab. Inc. CA, USA) were cultured in Luria Bertani (LB) medium (10g/l Tryptone, 5 g/L Yeast Extract, 5 g/l NaCl) for 16 hours at 37°C. The broth was supplemented with glycerol at a final concentration of 15%, aliquoted and stored at -80°C. Upon thawing, the number of colony forming units (CFU) of *E. coli* per ml was calculated using the method described below.

2.3. Experimental protocol and samples infection

Considering the normal proportion of medium/seminal plasma in swine seminal doses the experimental samples were prepared by mixing 20% of SP and 80% of Swine Fertilization Medium (SFM) (Barone et al., 2016) and adding *E. coli* (DH5 alpha strain) to a final concentration of 1×10^7 CFU/ml (Pinart et al., 2017). Ma and Ro EOs were tested at two different concentrations, 0.2 and 0.4 mg/ml, according to previous spermatozoa toxicity studies carried out by the authors (Elmi et al., 2019, 2017). Two control samples were prepared: one with Ampicillin (0.8 mg/ml; CTR +) and one without any antibiotic (CTR -). The experimental samples were incubated for 5 days in a refrigerated bath at 16°C (±1 °C), and optical density, bacterial DNA quantification and colony count were performed at 24 and 120 hours. At the same time points the pH of all samples was assessed using a pHmeter (SevenExcellence pHmeter, Mettler Toledo, Columbus, Ohio, US).

2.4. Optical Density measurements (OD600)

To monitor bacterial growth, 1 ml of each sample was collected at the 2 experimental time points and the optical density was immediately measured at 600 nm (Koch, 1970) using a spectrophotometer (GeneQuant 1300, GE Healthcare, Pittsburgh, PA).

2.5. Bacterial DNA quantification (BDQ)

The bacterial DNA quantification (BDQ) was performed as previously described (Barone et al., 2016). Briefly, 1 ml of each sample was centrifuged at 14000 x g for 10 minutes (Sigma 3-K30, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) to collect bacteria. QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used for the DNA extraction according to a modified "Isolation of DNA from stool for pathogen detection" protocol. The pellet was resuspended in 200 µL of ASL buffer and three glass marbles (ø 3 mm) were added. Subsequently, the sample was submitted to a mechanical lysis using Tissue Lyser (Qiagen) (50 Hz, 1 x 5 min and 1 x 3 min), placed on ice (3 min), incubated at 95°C for 5 min and placed again on ice. Proteinase K (15 µl) and then AL buffer (200 µl) were added to the sample and incubated at 70°C for 10 min. Absolute ethanol (200 µl) was added to the lysate and the whole sample was carefully applied to the QIAamp spin column. DNA was eluted with nuclease-free water (60 µl). The extracted DNA was spectrophotometrically quantified (DeNovix Inc.,Wilmington, DE, USA) and all samples were diluted 1:50 to fit in a range from 1400 to 6 pg. The bacterial DNA quantification was performed using the FemtoTM Bacterial DNA Quantification Kit (Zymo Research, Irvine, CA, USA), in accordance to the manufacturer's instructions and carried out in CFX96 Touch Thermal Cycler (Bio-Rad, Hercules,CA, USA). The amplification of DNA was done in duplicate (2 µl /well). Bacterial DNA standards comprised a 10-fold dilution series (provided by the kit) ranging from 20000 to 0.02 pg and were prepared in duplicates. Using standards values, the sample DNA was quantified (CFX Data Analysis Software, Bio-Rad) and expressed as DNA starting log quantity (mean ± SD).

2.6. *E.coli* colony count (CFU count)

Upon sampling, aliquots were immediately transferred at 4°C to the Section of Parma of the "Istituto Zooprofilattico Sperimentale della Lombardia dell'Emilia-Romagna" for the colony count. Ten-fold dilutions (from 10^{-1} to 10^{-7}) in Buffered peptone water (BPW; peptone 10,0 g; NaCl 5,0 g; Na_2HPO_4 3,5 g; KH_2HPO_4 1,5 g; demineralised water 1 l; pH 7.0 ± 0.2 at 25°C) were performed and then

samples were tested using a previously described technique ("Enumeration of β-glucuronidase positive Escherichia coli," 2014; ISO 16649-2:2001, 2001), with slight modifications. Each diluted sample was pipetted aseptically into a sterile Petri dish, and supplemented with 15 ml of previously melted Tryptone Bile Glucuronide agar (TBX; Enzymatic digest of casein 20,0 g; Bile Salts No.3 1,5 g; 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid BCIG 144 µM; Dimethyl sulfoxide DMSO 3 ml; Agar 15,0 g; demineralised water 1000,0 ml; pH 7.0 ± 0.2 at 25°C). The inoculum and the medium were gently mixed and incubated at 44°C ± 1°C for 18-24 h. The typical colonies (blue coloured with several shapes) of two successive dilutions were counted, and the weighted mean was calculated in order to achieve the CFU *E. coli* per ml.

2.7. Statistical analyses

Statistical analyses were performed using the software R 3.0.3 (The R Foundation for Statistical Computing) and graphically represented using the software GraphPad Prism v.8 (GraphPad Software Inc., San Diego, CA, USA). Descriptive statistics were calculated and reported as mean ± SD. Normal distribution of the parameters was tested by Shapiro-Wilk test ($p<0.05$) and logarithmic transformations were performed for optical density and BDQ. Repeated measures ANOVAs were performed for optical density and BDQ. Data from the CFU analyses could not be normalized, therefore a Poisson repeated measures ANOVA was performed. Time, treatment and their interaction were set as factors in all ANOVAs. *Post hoc* Tukey tests were performed for the multiple comparisons. The significance level of all statistical analyses was set at $p<0.05$.

3. Results

The results of the descriptive statistics are reported in Table 1. Data of pH were not reported and were always within the expected range (6.8 ± 0.5). The repeated measures ANOVAs highlighted

significant differences for all the variables between times ($p<0.05$), treatments ($p<0.05$) and their interaction ($p<0.05$).

3.1. Optical Density measurements

The results of the *post hoc* Tukey tests are represented in Figure 1.

The effect of time within each treatment (24 vs 120 h) showed a statistically significant increase for CTR- ($p<0.001$), 0.2 Ro ($p<0.001$) and 0.2 Ma ($p=0.002$). No statistical differences were found for the other samples (0.4 Ro $p=1.000$; 0.4 Ma $p= 0.545$; CTR+ $p=1.000$). When comparing the effects of the different treatments within the same time point, 0.4 Ro and CTR+ result statistically lower than CTR- at 24h, whereas the other treatments showed intermediate results. At 120 hours, all samples resulted in statistically differences from CTR-, with 0.4 Ro and 0.4 Ma statistically similar to CTR+.

Fig. 1. Box plots for Optical Density (OD600) of the different treatments at 24 and 120 hours.

CTR- = control without antibiotics; CTR + = control with antibiotic; 0.2 = 0.2 mg/ml; 0.4= 0.4 mg/ml; Ma= *Melaleuca alternifolia*; Ro= *Rosmarinus officinalis*. Lowercase and uppercase letters indicate differences ($p<0.05$) between treatments at 24 and 120h respectively. Differences ($p<0.05$) for the same treatment between the 2 time points are indicated by connecting lines above boxplots.

3.2. Bacterial DNA quantification

The results of the *post hoc* Tukey tests are represented in Figure 2.

The effect of time within each treatment (24 vs 120 h) did not show any statistical difference.

Comparison between the treatments at 24 hours showed that 0.4 Ro, 0.4 Ma and CTR+ were statistically different from CTR-; 0.4 Ma, moreover, was not statistically different when compared to CTR+. At 120 hours, only 0.4 Ma and CTR+ were different from CTR -.

Fig. 2. Box plots for bacterial DNA quantification (BDQ) of the different treatments at 24 and 120 hours.

CTR- = control without antibiotics; CTR + = control with antibiotic; 0.2 = 0.2 mg/ml; 0.4= 0.4 mg/ml; Ma= *Melaleuca alternifolia*; Ro= *Rosmarinus officinalis*. Lowercase and uppercase letters indicate differences ($p<0.05$) between treatments at 24 and 120h respectively.

3.3. Colony-count analyses

The results of the *post hoc* Tukey tests are represented in Figure 3.

When comparing the individual treatments between the 2 time points, only CTR- showed a statistically significant difference.

At 24 hour CTR- showed the highest colony count, statistically different from 0.2 and 0.4 Ma, 0.4 Ro and CTR+. The sample treated with 0.2 mg/ml of Ro showed statistically different counts in comparison to 0.4 Ro, 0.4 Ma and CTR+. At 120h, all the experimental samples, with the exception of 0.2 Ro, were statistically different when compared to CTR-.

Fig. 3. Box plots for colony counts (CFU) of the different treatments at 24 and 120 hours.

CTR- = control without antibiotics; CTR + = control with antibiotic; 0.2 = 0.2 mg/ml; 0.4= 0.4 mg/ml; Ma= *Melaleuca alternifolia*; Ro= *Rosmarinus officinalis*. Lowercase and uppercase letters indicate differences ($p<0.05$) between treatments at 24 and 120h respectively. Differences ($p<0.05$) for the same treatment between the 2 time points are indicated by connecting lines above boxplots.

4. Discussion

The present work highlights the antimicrobial effect of *M. alternifolia* and *R. officinalis* essential oils, at non-spermicidal concentrations, using an *in vitro* model that mimics swine seminal doses infected with 10^7 CFU/ml *E. coli* and stored at 16°C up to 5 days.

The boars enrolled in the present study, housed in an experimental facility with good biosafety measures, proved to have a relatively low bacterial contamination in preliminary studies. Moreover,

semen collection procedure was performed by trained staff with particular attention towards environmental contamination (Kuster and Althouse, 2016; Lopez Rodriguez et al., 2017; Schulze et al., 2015). Such conditions led to the decision to add *E. coli* to the samples in order to create an *in vitro* model capable of showing the potential efficacy of EOs on bacterial growth (Pinart et al., 2017). Spermatozoa were not included in the experimental samples in order to be sure to only evaluate bacteria without any bias to the interpretation of the results. This was possible since the two EOs used in the study were preliminary assessed for spermatozoa toxicity and were proved not to alter morph-functional parameters at concentrations lower than 0.6 mg/ml (Elmi et al., 2019, 2017).

The chosen analytical methodologies aimed to quantify the bacteria present in samples, both live and dead, by means of qPCR and spectrophotometry, and to quantify bacteria with maintained replication capabilities by means of classical bacteriology (colony count). The latter can be challenging when the bacterial population is partially unknown, thus the addition of a standardized quantity of a single bacterial strain makes the experimental conditions more reproducible.

The spectrophotometry analyses and bacterial DNA quantification, indicative of all bacteria present in experimental samples, showed interesting results. Indeed, both essential oils at a higher concentration (0.4 mg/ml) displayed similar effects to a conventional antibiotic throughout the entire experimental protocol (Fig. 1 and 2) by two analytical methodologies. The lower concentration, on the other hand, seems to induce milder effects since the samples, at 24 h, do not show any differences when compared to both controls. This finding was partially expected as it is already known that the majority of effects of natural compounds, and in particular essential oils, are concentration- dependent (Chouhan et al., 2017). Nonetheless at 5 days, for the OD600 analysis, lower concentration of both essential oils started exhibiting differences when compared to controls. Both the DNA quantification and the spectrophotometric analyses are not capable of discriminating between alive and dead bacteria, thus the relatively higher values at 5 days post incubation were expected to be a representation of both. Therefore, it can be deduced by these methods that to add

both *M. alternifolia* and *R. officinalis* essential oils to the samples had an influence on bacterial populations.

The results of the colony count, only evaluating *E. coli*, strengthened and further confirmed prior results. At each time point, the highest concentration of both oils did not show any statistical difference when compared to CTR+ (ampicillin). The samples containing 0.2 mg/ml of Ro showed intermediate colony counts between the two controls at both time points, again highlighting milder concentration-dependent effects. The same concentration of Ma essential oil determined a relatively similar situation at 24h, even if in this case a statistical difference against CTR- could already be observed. On the other hand, at 5 days 0.2 mg/ml exhibited the same effects as the conventional antibiotic and the higher concentration of both oils. These findings seem to suggest a stronger overall effect of the *M. alternifolia* essential oil, capable of inhibiting *E.coli* growth earlier and at a lower concentration. The colony count proved to be more accurate compared to the other methodologies applied, potentially because of selectivity towards *E. coli* and not other bacteria present in the boar ejaculates between 24 hours and 5 days. The control without antibiotic showed a reduction in colony count and an increase in DNA and optical density. In the light of this data, it can be hypothesized that, during storage, a decrease in nutrients and the incubation temperature were not favorable for the *E.coli* thus suggesting bacterial death or growth limitation (Althouse and Lu, 2005). Nevertheless, it cannot be excluded that other bacterial species, more robust at the chosen storage temperature or/and selection by the substrates, increased during the 5-day storage. Overall, when looking at the experimental design of the study, this phenomenon would apply to all samples and, therefore, would not represent a bias to the results. Moreover, the samples containing the standard conventional antibiotic (CTR+) were always statistically different when compared to the ones without any antimicrobial (CTR-), further confirming this *in vitro* model itself. Generally, the presence and replication of bacteria in seminal doses can induce acidification (Althouse et al., 2000), which was not found in the experimental samples, including the control without any antibiotic. The chosen experimental concentrations of the EO were not cytotoxic on swine

spermatozoa, as previously published by the authors (Elmi et al., 2019, 2017), but were also lower than the reported Minimal inhibitory concentration (MIC) for *E.coli*: between 1.52 and 3.125 mg/ml for *R. officinalis* (Hussain et al., 2010; Lagha et al., 2019), and between 2 and 8 mg/ml for *M. alternifolia* (Shi et al., 2018; Zhang et al., 2018). This leads to the hypothesis, somehow already formulated and partially demonstrated by other authors (Marini et al., 2018), that essential oils can have effects on bacteria also at sub-lethal concentrations. It still has to be acknowledged that MIC evaluation for phyto-complexes can be challenging and somehow misleading, as any batch of each essential oil can exert highly variable effects based on their specific compositions.

5. Conclusions

In conclusion, the results of the present work show that both *Melaleuca alternifolia* and *Rosmarinus officinalis* essential oils, at the concentration of 0.4 mg/ml, are capable of exerting similar effects to ampicillin on swine artificial insemination doses deprived of spermatozoa and spiked with *E. coli*. At lower concentration, *M. alternifolia* essential oil seemed more efficient than the other tested essential oil. Such findings strengthen the hypothesis of the potential use of phyto-complexes as antimicrobial agents for reproductive biotechnologies.

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Table 1

Descriptive statistics for optical density (OD600), bacterial DNA quantification (BDQ) and colony count (CFU) reported as mean (SD) (n=9).

	24 h						120 h					
	CTR -	**0.2 Ro**	**0.4 Ro**	**0.2 Ma**	**0.4 Ma**	**CTR +**	**CTR -**	**0.2 Ro**	**0.4 Ro**	**0.2 Ma**	**0.4 Ma**	**CTR +**
OD600	0.291 (0.034)	0.228 (0.044)	0.178 (0.024)	0.231 (0.046)	0.195 (0.049)	0.145 (0.065)	0.537 (0.046)	0.379 (0.062)	0.217 (0.111)	0.367 (0.119)	0.263 (0.092)	0.130 (0.073)
BDQ ng/µl	15.94 (6.58)	12.23 (3.94)	7.53 (3.70)	11.13 (3.76)	6.18 (2.69)	7.80 (2.31)	34.39 (12.50)	28.07 (15.68)	24.86 (20.66)	25.33 (13.49)	16.93 (16.29)	10.28 (4.08)
CFU count CFU x 10^6/ml	2.117 (1.442)	1.417 (0.989)	0.334 (0.557)	0.781 (0.538)	0.0912 (0.092)	0.000 (0.000)	1.134 (0.592)	0.620 (0.537)	0.043 (0.084)	0.061 (0.061)	0.004 (0.006)	0.000 (0.000)

CTR- = control without antibiotics; CTR+ = control with antibiotic; 0.2 = 0.2 mg/ml; 0.4= 0.4 mg/ml; Ma= Melaleuca alternifolia; Ro= Rosmarinus officinalis.

Figure 1

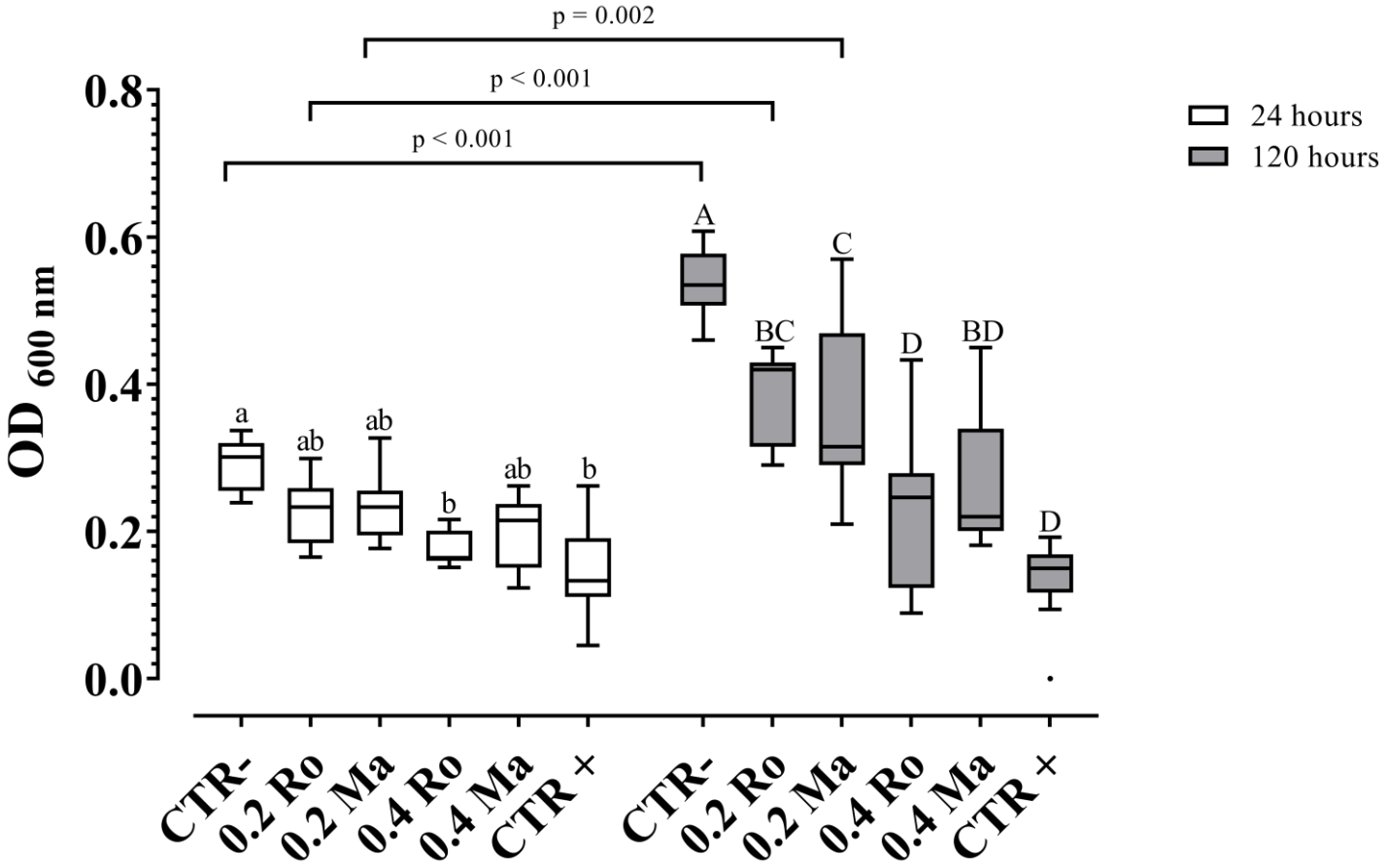

p = 0.002
p < 0.001
p < 0.001
0.8
0.6
0.4
0.2
0.0
OD 600 nm
a
ab
ab
b
ab
b
A
BC
C
D
BD
D
CTR-
0.2 Ro
0.2 Ma
0.4 Ro
0.4 Ma
CTR +
CTR-
0.2 Ro
0.2 Ma
0.4 Ro
0.4 Ma
CTR +
24 hours
120 hours

Figure 2

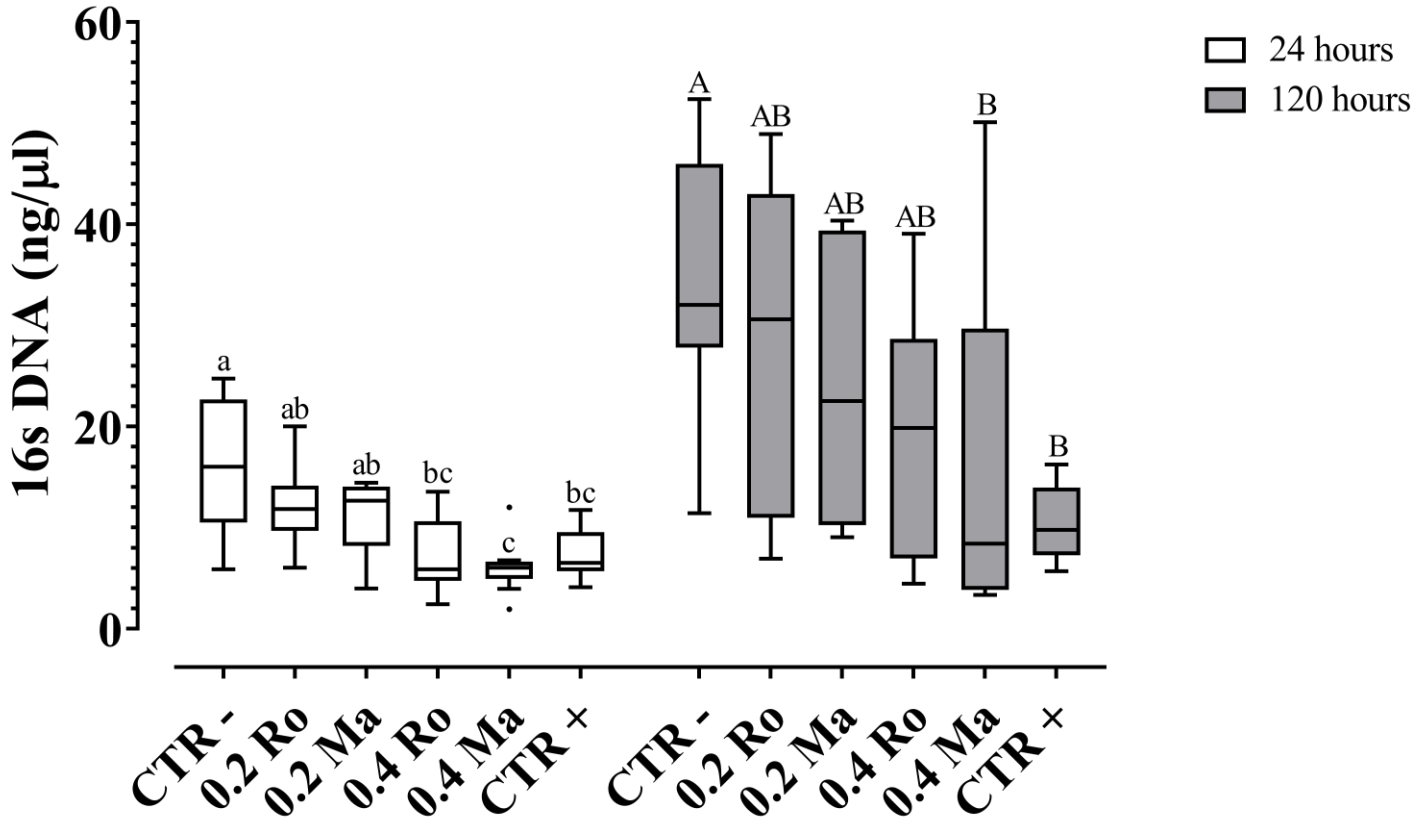
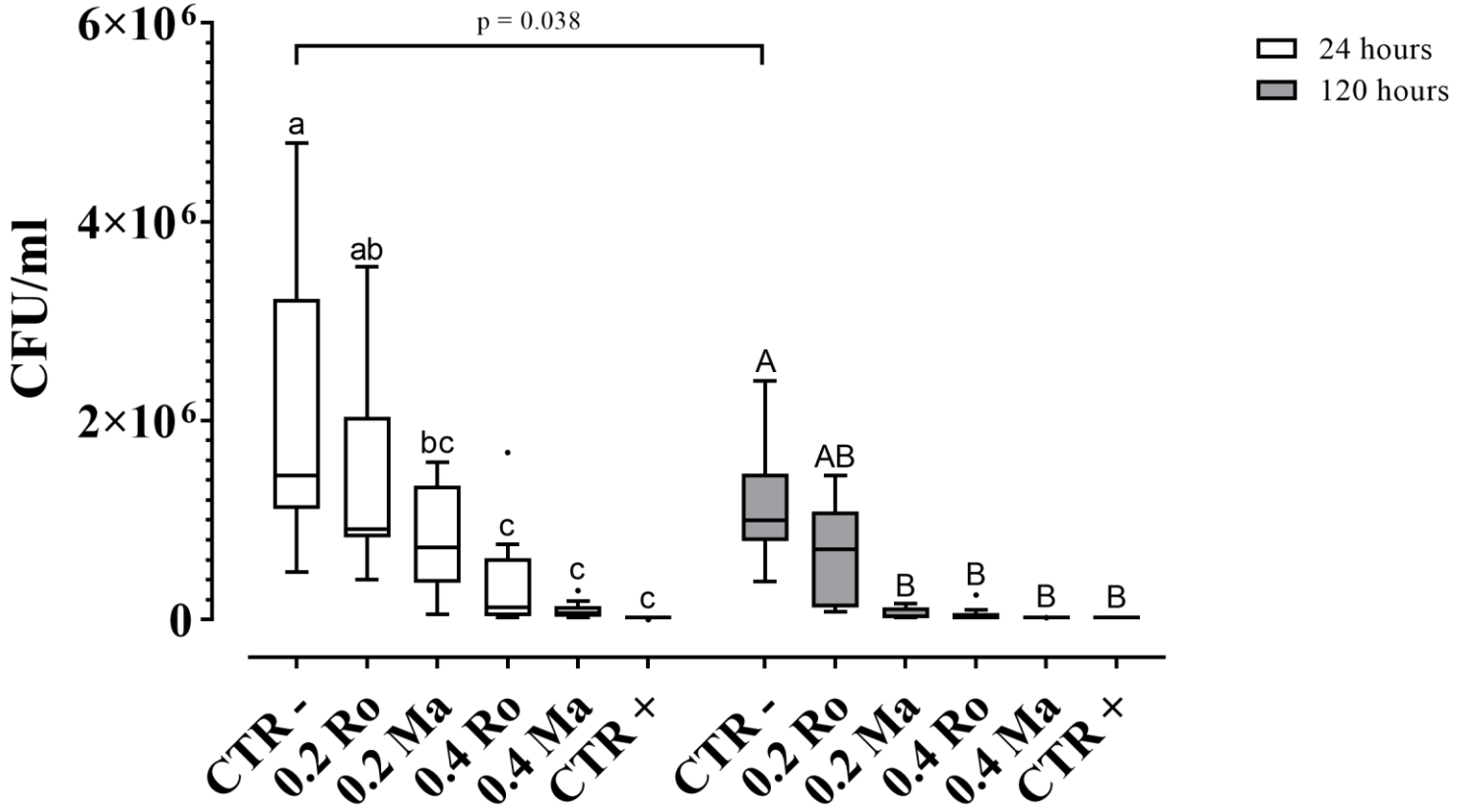

16s DNA (ng/µl)
60
40
20
0
24 hours
120 hours
a
ab
ab
bc
c
bc
A
AB
AB
AB
B
B
CTR -
0.2 Ro
0.2 Ma
0.4 Ro
0.4 Ma
CTR +
CTR -
0.2 Ro
0.2 Ma
0.4 Ro
0.4 Ma
CTR +

Figure 3


p = 0.038
24 hours
120 hours
CFU/ml
6×10⁶
4×10⁶
2×10⁶
0
a
ab
bc
c
c
c
A
AB
B
B
B
B
CTR -
0.2 Ro
0.2 Ma
0.4 Ro
0.4 Ma
CTR +
CTR -
0.2 Ro
0.2 Ma
0.4 Ro
0.4 Ma
CTR +

Conclusion

According to the author's opinion, this PhD project represents a first step toward the potential application of Essential Oils as conventional antibiotic substitutes in the field of swine reproduction.

The actual scenario regarding antimicrobial and especially antibiotic resistance is indeed extremely worrying, and it is mandatory, also from an ethical point of view, that the scientific community invests time and efforts in the search for solutions. Veterinary medicine, and in particular food production, has always been considered as a major player in the development of resistances due to the often exaggerate and inappropriate use of antibiotics. Such molecules are, and potentially always will be, pivotal in the treatment of several pathologies, this is why it is extremely important to "preserve" their efficacy as much as possible. Therefore, alternatives have to be found especially when the use is mainly technological rather than directly therapeutic/preventive. Swine artificial reproduction perfectly seems to fit the case.

According to the results obtained, *R. officinalis* and *M. alternifolia* essential oils, at the right concentrations, seem to be good candidates in replacing conventional antibiotics in swine A.I. doses without affecting sperm quality. Next step, before moving to *in vivo* trials, will be the analysis of the *in vitro* fertility and of the direct effects on the genital tract of sows.

In conclusion, the work hereby presented focusses on the strong potential of Essential Oils in the field of swine artificial insemination, but also highlights how it is necessary, before claiming natural compounds as future "miracle-workers", to investigate their safety and mechanisms of action. As of today, unfortunately, a lot of work still needs to be done for an extensive characterization of what natural compounds can actually do and how they do it, before safely and effectively using them in different areas of Veterinary and Human medicine.

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