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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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References

Aarestrup, F.M., Duran, C.O., Burch, D.G.S., 2008. Antimicrobial resistance in swine production. Anim. Health Res. Rev. 9, 135–148. https://doi.org/10.1017/S1466252308001503

Althouse, G.C., Kuster, C.E., Clark, S.G., Weisiger, R.M., 2000. Field investigations of bacterial contaminants and their effects on extended porcine semen. Theriogenology 53, 1167–1176. https://doi.org/10.1016/S0093-691X(00)00261-2

Althouse, G.C., Lu, K.G., 2005. Bacteriospermia in extended porcine semen. Theriogenology, Proceedings of the V International Conference on Boar Semen Preservation 63, 573–584. https://doi.org/10.1016/j.theriogenology.2004.09.031

Bag, A., Chattopadhyay, R.R., 2015. Evaluation of Synergistic Antibacterial and Antioxidant Efficacy of Essential Oils of Spices and Herbs in Combination. PLOS ONE 10, e0131321. https://doi.org/10.1371/journal.pone.0131321

Barone, F., Ventrella, D., Zannoni, A., Forni, M., Bacci, M.L., 2016. Can Microfiltered Seminal Plasma Preserve the Morphofunctional Characteristics of Porcine Spermatozoa in the Absence of Antibiotics? A Preliminary Study. Reprod. Domest. Anim. Zuchthyg. 51, 604–610. https://doi.org/10.1111/rda.12699

Brun, P., Bernabè, G., Filippini, R., Piovan, A., 2019. In Vitro Antimicrobial Activities of Commercially Available Tea Tree (Melaleuca alternifolia) Essential Oils. Curr. Microbiol. 76, 108–116. https://doi.org/10.1007/s00284-018-1594-x

Carson, C.F., Hammer, K.A., Riley, T.V., 2006. Melaleuca alternifolia (Tea Tree) Oil: a Review of Antimicrobial and Other Medicinal Properties. Clin. Microbiol. Rev. 19, 50–62. https://doi.org/10.1128/CMR.19.1.50-62.2006

Chikhoune, A., Stouvenel, L., Iguer-Ouada, M., Hazzit, M., Schmitt, A., Lorès, P., Wolf, J.P., Aissat, K., Auger, J., Vaiman, D., Touré, A., 2015. In-vitro effects of Thymus munbyanus

essential oil and thymol on human sperm motility and function. Reprod. Biomed. Online 31, 411–420. https://doi.org/10.1016/j.rbmo.2015.06.011

Chouhan, S., Sharma, K., Guleria, S., 2017. Antimicrobial Activity of Some Essential Oils—Present Status and Future Perspectives. Medicines 4. https://doi.org/10.3390/medicines4030058

Dávila, M.P., Bucci, D., Galeati, G., Peña, F.J., Mari, G., Giaretta, E., Tamanini, C., Spinaci, M., 2015. Epigallocatechin-3-Gallate (EGCG) Reduces Rotenone Effect on Stallion Sperm–Zona Pellucida Heterologous Binding. Reprod. Domest. Anim. 50, 1011–1016. https://doi.org/10.1111/rda.12628

de Oliveira, J.R., Camargo, S.E.A., de Oliveira, L.D., 2019. Rosmarinus officinalis L. (rosemary) as therapeutic and prophylactic agent. J. Biomed. Sci. 26, 5. https://doi.org/10.1186/s12929-019-0499-8

El Fawal, G.F., Omer, A.M., Tamer, T.M., 2019. Evaluation of antimicrobial and antioxidant activities for cellulose acetate films incorporated with Rosemary and Aloe Vera essential oils. J. Food Sci. Technol. 56, 1510–1518. https://doi.org/10.1007/s13197-019-03642-8

Elmi, A., Banchelli, F., Barone, F., Fantinati, P., Ventrella, D., Forni, M., Bacci, M.L., 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. Anim. Reprod. Sci. 192, 61–68. https://doi.org/10.1016/j.anireprosci.2018.02.014

Elmi, A., Ventrella, D., Barone, F., Carnevali, G., Filippini, G., Pisi, A., Benvenuti, S., Scozzoli, M., Bacci, M.L., 2019. In Vitro Effects of Tea Tree Oil (Melaleuca Alternifolia Essential Oil) and its Principal Component Terpinen-4-ol on Swine Spermatozoa. Molecules 24, 1071. https://doi.org/10.3390/molecules24061071

Elmi, A., Ventrella, D., Barone, F., Filippini, G., Benvenuti, S., Pisi, A., Scozzoli, M., Bacci, M.L., 2017. Thymbra capitata (L.) Cav. and Rosmarinus officinalis (L.) Essential Oils: In Vitro

Effects and Toxicity on Swine Spermatozoa. Molecules 22, 2162. https://doi.org/10.3390/molecules22122162

Enumeration of β-glucuronidase positive Escherichia coli: pour plate method [WWW Document], 2014. . GOV.UK. URL https://www.gov.uk/government/publications/enumeration-of-glucuronidase-positive-escherichia-coli-pour-plate-method (accessed 5.23.19).

EUR-Lex - 31990L0429 - EN [WWW Document], n.d. . Off. J. 224 18081990 P 0062 - 0072 Finn. Spec. Ed. Chapter 3 Vol. 33 P 0180 Swed. Spec. Ed. Chapter 3 Vol. 33 P 0180. URL https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:31990L0429&from=it (accessed 4.30.19).

Giaretta, E., Bucci, D., Mari, G., Galeati, G., Love, C.C., Tamanini, C., Spinaci, M., 2014. Is Resveratrol Effective in Protecting Stallion Cooled Semen? J. Equine Vet. Sci. 34, 1307–1312. https://doi.org/10.1016/j.jevs.2014.09.011

Hussain, A.I., Anwar, F., Chatha, S.A.S., Jabbar, A., Mahboob, S., Nigam, P.S., 2010. Rosmarinus officinalis essential oil: antiproliferative, antioxidant and antibacterial activities. Braz. J. Microbiol. 41, 1070–1078. https://doi.org/10.1590/S1517-838220100004000027

ISO 4730:2017(en), Essential oil of Melaleuca, terpinen-4-ol type (Tea Tree oil) [WWW Document], n.d. URL https://www.iso.org/obp/ui/#iso:std:iso:4730:ed-3:v1:en (accessed 7.9.19).

ISO 16649-2:2001, 2001. Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of beta-glucuronidase-positive Escherichia coli -- Part 2: Colony-count technique at 44 degrees C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide [WWW Document]. ISO. URL http://www.iso.org/cms/render/live/en/sites/isoorg/contents/data/standard/02/98/29824.html (accessed 7.10.19).

Khezri, K., Farahpour, M.R., Rad, S.M., 2019. Accelerated infected wound healing by topical application of encapsulated Rosemary essential oil into nanostructured lipid carriers. Artif. Cells Nanomedicine Biotechnol. 47, 980–988. https://doi.org/10.1080/21691401.2019.1582539

Koch, A.L., 1970. Turbidity measurements of bacterial cultures in some available commercial instruments. Anal. Biochem. 38, 252–259. https://doi.org/10.1016/0003-2697(70)90174-0

Kuster, C.E., Althouse, G.C., 2016. The impact of bacteriospermia on boar sperm storage and reproductive performance. Theriogenology, Swine Reproduction 85, 21–26. https://doi.org/10.1016/j.theriogenology.2015.09.049

Lagha, R., Ben Abdallah, F., AL-Sarhan, B.O., Al-Sodany, Y., 2019. Antibacterial and Biofilm Inhibitory Activity of Medicinal Plant Essential Oils Against Escherichia coli Isolated from UTI Patients. Molecules 24, 1161. https://doi.org/10.3390/molecules24061161

Lopez Rodriguez, A., Van Soom, A., Arsenakis, I., Maes, D., 2017. Boar management and semen handling factors affect the quality of boar extended semen. Porc. Health Manag. 3. https://doi.org/10.1186/s40813-017-0062-5

Maes, D., Nauwynck, H., Rijsselaere, T., Mateusen, B., Vyt, P., de Kruif, A., Van Soom, A., 2008. Diseases in swine transmitted by artificial insemination: An overview. Theriogenology, Proceedings of the VIth International Conference on Boar Semen Preservation 70, 1337–1345. https://doi.org/10.1016/j.theriogenology.2008.06.018

Marini, E., Magi, G., Ferretti, G., Bacchetti, T., Giuliani, A., Pugnaloni, A., Rippo, M.R., Facinelli, B., 2018. Attenuation of Listeria monocytogenes Virulence by Cannabis sativa L. Essential Oil. Front. Cell. Infect. Microbiol. 8. https://doi.org/10.3389/fcimb.2018.00293

Morrell, J.M., Wallgren, M., 2011. Removal of bacteria from boar ejaculates by Single Layer Centrifugation can reduce the use of antibiotics in semen extenders. Anim. Reprod. Sci. 123, 64–69. https://doi.org/10.1016/j.anireprosci.2010.11.005

Nazzaro, F., Fratianni, F., De Martino, L., Coppola, R., De Feo, V., 2013. Effect of Essential Oils on Pathogenic Bacteria. Pharmaceuticals 6, 1451–1474. https://doi.org/10.3390/ph6121451

Nieto, G., 2017. Biological Activities of Three Essential Oils of the Lamiaceae Family. Medicines 4, 63. https://doi.org/10.3390/medicines4030063

Pinart, E., Domènech, E., Bussalleu, E., Yeste, M., Bonet, S., 2017. A comparative study of the effects of Escherichia coli and Clostridium perfringens upon boar semen preserved in liquid storage. Anim. Reprod. Sci. 177, 65–78. https://doi.org/10.1016/j.anireprosci.2016.12.007

Sancho, S., Briz, M., Yeste, M., Bonet, S., Bussalleu, E., 2017. Effects of the antimicrobial peptide protegrine 1 on sperm viability and bacterial load of boar seminal doses. Reprod. Domest. Anim. 52, 69–71. https://doi.org/10.1111/rda.13061

Satyal, P., Jones, T.H., Lopez, E.M., McFeeters, R.L., Ali, N.A.A., Mansi, I., Al-Kaf, A.G., Setzer, W.N., 2017. Chemotypic Characterization and Biological Activity of Rosmarinus officinalis. Foods Basel Switz. 6. https://doi.org/10.3390/foods6030020

Schulze, M., Ammon, C., Rüdiger, K., Jung, M., Grobbel, M., 2015. Analysis of hygienic critical control points in boar semen production. Theriogenology 83, 430–437. https://doi.org/10.1016/j.theriogenology.2014.10.004

Schulze, M., Grobbel, M., Riesenbeck, A., Brüning, S., Schaefer, J., Jung, M., Grossfeld, R., 2017. Dose rates of antimicrobial substances in boar semen preservation—time to establish new protocols. Reprod. Domest. Anim. 52, 397–402. https://doi.org/10.1111/rda.12921

Schulze, M., Junkes, C., Mueller, P., Speck, S., Ruediger, K., Dathe, M., Mueller, K., 2014. Effects of cationic antimicrobial peptides on liquid-preserved boar spermatozoa. PloS One 9, e100490. https://doi.org/10.1371/journal.pone.0100490

Shi, C., Zhang, X., Guo, N., 2018. The antimicrobial activities and action-mechanism of tea tree oil against food-borne bacteria in fresh cucumber juice. Microb. Pathog. 125, 262–271. https://doi.org/10.1016/j.micpath.2018.09.036

Touazi, L., Aberkane, B., Bellik, Y., Moula, N., Iguer-Ouada, M., 2018. Effect of the essential oil of Rosmarinus officinalis (L.) on rooster sperm motility during 4°C short-term storage. Vet. World 11, 590–597. https://doi.org/10.14202/vetworld.2018.590-597

WHO | Antimicrobial resistance: global report on surveillance 2014 [WWW Document], n.d. . WHO. URL http://www.who.int/drugresistance/documents/surveillancereport/en/ (accessed 7.13.17).

Zhang, X., Guo, Y., Guo, L., Jiang, H., Ji, Q., 2018. In Vitro Evaluation of Antioxidant and Antimicrobial Activities of Melaleuca alternifolia Essential Oil. BioMed Res. Int. 2018. https://doi.org/10.1155/2018/2396109

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.