

# A comparative study of the antimicrobial activity of oregano, rosemary and thyme essential oils against *Salmonella spp*

Grammato Evangelopoulou<sup>1\*</sup>, Nikolaos Solomakos<sup>2</sup>, Anastasios Ioannidis<sup>1</sup>, Andreana Pexara<sup>2</sup> and Angeliki R Burriel<sup>1</sup>

<sup>1</sup>Department of Nursing, University of Peloponnese, Sparta, Greece

<sup>2</sup>Laboratory of Hygiene of Foods of Animal Origin, Faculty of Veterinary Medicine, University of Thessaly, 224 Trikalon Street, 43100 Karditsa, Greece

## Abstract

The emergence of *Salmonella* resistance to therapeutic agents against human infections is attributed to their excessive use in the treatment of food-producing animals. The spreading of resistance necessitates vigorous research on alternative therapeutic schemes in order to replace commonly used antimicrobials. Thus, the aim of the present study was to investigate the inhibitory activity of three commercially available essential oils (EOs) against fifty-nine *Salmonella* strains belonging to fourteen serovars isolated from pig carcasses at slaughter. Their antimicrobial activity was compared with that of twenty-four commonly used antimicrobials previously tested against the same strains. The three chosen EOs were oregano, rosemary and thyme. Oregano and thyme EOs showed high antimicrobial activity, compared to that of rosemary and a better antimicrobial effect compared to that of commercial antimicrobials. The results showed that oregano and thyme have the potential of an effective alternative treatment against *Salmonella spp.*, especially those strains resistant to synthetic antimicrobials. Thus, some EOs could be a safer alternative treatment against certain infections of food-producing animals or, perhaps, growth promoters, used for effective prevention.

## Introduction

Food-producing animals are a widely recognized source for *Salmonella* species causing human infections. Pork meat and products are major sources of foodborne salmonellosis in the European Union (EU) and around the world. Subclinically infected pigs, incriminated for carcass contamination at slaughter, are causing 10 to 20% of human cases of salmonellosis in the EU [1,2].

The most important threat to human health is the emergence of multidrug resistant *Salmonella* strains, mainly belonging to serovar Typhimurium. Pigs are recognized as a primary reservoir of such strains, necessitating control measures for public health protection [3-7]. Unregulated use of antibiotics in farming practices for many decades, causing selective pressure, is thought the main reason for this emergence [8-12]. The amounts of antimicrobials used and the ways those drugs are administrated affect the resistance of gut microflora [9]. Therapeutics, such as ampicillin, trimethoprim-sulfamethoxazole, third generation cephalosporins and fluoroquinolones, effective for treating human *Salmonella* infection, are also administered to food producing animals, implicated in the transmission of resistant strains [13,14]. Thus, the spreading of multi-resistant *Salmonella* through mobile genetic elements has become a global threat to effectively treating human cases, particularly in immunocompromised individuals with increased susceptibility to accidental *Salmonella* infection [15,16].

The increasing prevalence of antibiotic resistant bacterial strains promotes the investigation for new antimicrobial methods in order to control infections in animals. Among them is the application of plant essential oils (EOs) against a wide range of microorganisms [17]. EOs, also called volatile or ethereal oils, are aromatic oily plant liquid extracts recognized for their antimicrobial properties since ancient times and having recently re-emerged as safe, natural antimicrobials

[18-22]. Research has revealed that the EOs' high content of phenolic derivatives, such as carvacrol and thymol, target the bacterial membrane transport system, causing disruption at the cytoplasmic homeostasis, affecting finally cell respiration and the microbial enzyme system [23-25]. These properties of medicinal interest, in evidence of dangerous increases of microbial resistance to conventional treatments [26], has increased hope that EOs could become safe to public health when used as potential natural growth promoters (NGPs) for farmed animals. Specifically, their inclusion in animal diets for replacing antibiotics used as growth promoters (AGPs), could improve quality of gut microbiota thus, growth performance of animals and animal welfare [27], eventually contributing to consumer safety.

The disinfectant and antibacterial properties of oregano EO were first recognized in ancient Greece, where it was often used for treating bacterial skin and wound infections. It was also used to protect food from the growth of bacteria. Oregano (*Origanum vulgare subsp. hirtum*, a herb of the *Labiatae* family) is a plant that thrives on mountainous terrain, from where it got its name meaning 'Delight of the Mountains' [28,29]. Carvacrol and thymol are the two main phenols, constituting about 78.85% of oregano EOs and responsible for the plant's antimicrobial activity [30]. Carvacrol in particular, has been

**\*Correspondence to:** Grammato Evangelopoulou, Department of Nursing, Faculty of Human Movement and Quality of Life Sciences, Laboratory of Integrated Healthcare, University of Peloponnese, Sparta, Greece, Tel: 30-694-584-7840, E-mail: [matinavet@hotmail.com](mailto:matinavet@hotmail.com)

**Key words:** antimicrobial resistance, essential oil, pig, *Salmonella*

**Received:** December 06, 2019; **Accepted:** December 20, 2019; **Published:** December 23, 2019

found by many as possessing remarkable antimicrobial properties [31-34]. In addition, other minor constituents, such as the monoterpene hydrocarbons g-terpinene and p-cymene are also reported as contributors to the antibacterial activity of the oil [35]. There are, also, reports on the chemical composition and the antimicrobial properties of the EOs of various oregano species, and their application in different commercial preparations, as antimicrobials and antioxidants [32,36].

Rosemary (*Rosmarinus officinalis*) or 'dew of the sea' (Latin ros for 'dew' and for 'sea'), is a member of the mint family *Lamiaceae* [37]. Its major constituents are 1,8-Cineole (26.54%) and  $\alpha$ -Pinene (20.14%) [38]. This fragrant evergreen herb has been known since ancient times for its medicinal properties. It was traditionally used to boost the immune system [39]. Among the pharmacologically validated medicinal uses of rosemary are anticancer, antidiabetic, anti-inflammatory and antinociceptive, antioxidant, antithrombotic, antiulcerogenic, improving cognitive deficits, antidiuretic, hepatoprotective and antibacterial effects against Gram positive and negative microorganisms [38,40,41].

Thyme belongs to the genus *Thymus* of the mint family (*Lamiaceae*) and is a relative of the genus *Origanum*. Its EO contains more than 60 ingredients, most of which possess important antioxidant and antimicrobial properties [42]. The essential oil of common thyme (*Thymus vulgaris*) contains 20–54% thymol, which is the compound [43] primarily responsible for its antioxidative activity, including *Salmonella* spp. [44], but similar activity have also carvacrol (2.2-4.2%), the monoterpene hydrocarbons p-cymene (18.5-23.5%) and g-terpinene (16.1-18.9%) [29,45]. These chosen to be investigated EOs are native to temperate Western and Southwestern Eurasia and especially the Mediterranean region [46] including Greece [47], with climatic conditions and geographical morphology promoting the growth of many species of aromatic plants. The antibacterial activity of oregano, rosemary and thyme has been studied against various multidrug resistant pathogenic bacteria, including foodborne pathogens, such as *Salmonella* spp. and *E.coli* [29,48].

Therefore, the objective of the present study was primarily to determine the activity of oregano, rosemary and thyme against 59 pig *Salmonella* spp. isolates and also compare it to the known antimicrobial activities of commonly used antimicrobials against the same isolates. The study focused on i) the investigation of the EOs' inhibitory effect in relation to the amount used ii) the comparison of the effectiveness of each EO used and iii) the investigation of each EO's bactericidal effect on the selected serovars as compared to commercial antimicrobials.

## Materials and methods

### Isolation, serotyping and antimicrobial susceptibility testing of *Salmonella* spp.

Fifty-nine (59) *Salmonella* strains, isolated from 615 pig tissue samples (ileum, mesenteric lymph nodes, faecal samples, gallbladder and neck muscle) collected from 123 pigs were investigated as described elsewhere [49-51].

The isolates belonged to fourteen serovars, which were 21 (35.6%) *Salmonella* Typhimurium, 9 (15.25%) *S. enterica* subsp. *enterica* ser. 4,12:i:-, 7 (11.86%) *S. enterica* subsp. *enterica* ser. 6,7:k:-, 6 (10.17%), *S. enterica* subsp. *enterica* ser. 4,5, 12:i:-, 3 (5.1%), *S. Bredeney* and from one isolate each (1.7%) for *S. Agona*, *S. Derby*, *S. Infantis*, *S. Meleagridis*, *S. Cerro*, *S. enterica* subsp. *enterica* ser. 6,14,25:-:1,2, *S. enterica* subsp. *diarizonae* 61:k:1,5, *S. enterica* subsp. *salamae* 38:b:1,2, *S. enterica* subsp. *houtenae* 40:g:t:-, while 4 (6.8%) were characterized as 'Rough'.

The same isolates had been previously tested against 24 antimicrobials, routinely used for animal and human treatments [49,50]. The commercially available (Thermo Scientific™ Oxoid™) antimicrobial susceptibility disks used were amoxicillin (30 µg), amoxicillin/clavulanic acid (20/10µg), ampicillin (10 µg), ampicillin/sulbactam (10/10 µg), aztreonam (30 µg), cefotaxime (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), ceftiofur (30 µg), ceftriaxone (30 µg), cefuroxime (30 µg), chloramphenicol (30 µg), colistin (50 µg), doripenem (10 µg), enrofloxacin (5 µg), erythromycin (15µg), gentamycin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), penicillin G (10 µg), rifampin (30 µg), sulfamethoxazole/trimethoprim (23.75/1.25 µg), tetracycline (30 µg) and tigecycline 15 (µg).

### Antimicrobial activity of EOs

The disk diffusion method was applied for the determination of the antibacterial activity of the three selected EOs: oregano, rosemary and thyme (ECO PHARM, Greece, <http://www.ecopharm.gr/index.php/products.html>). The inhibitory effect of each EO was examined against three graded undiluted commercial EOs, according to Skandamis et al. [52]. Each bacterial culture was standardized to the range of 0.5 McFarland standard and inoculated onto Mueller-Hinton agar (LMLAB 39). Sterilized Whatman paper discs 6 mm in diameter (Difco, Becton, Dickinson, Sparks, MD) were impregnated with 5, 15 and 30 µl of the EOs. Disks impregnated in absolute alcohol were used as negative controls. Disks with EOs and the negative control were placed on the surface of petri plates inoculated with *Salmonella*. The cultures were incubated at 37°C for 18-24 h and the zones of inhibition were measured and recorded in the same way as for commercial antimicrobials. Specifically, bacterial growth inhibition was the diameter of the zones (mm) without bacterial growth. For the interpretation of the results, four groups of inhibitory zones were examined: i) no inhibition, ii) < 12 mm (weak activity zone), iii) 12 mm  $\geq$  inhibition zone < 20 mm (intermediate activity) and iv) inhibition zone  $\geq$  20 mm (strong activity) [53].

### Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 17 was used for the statistical analysis of results. Statistical significance was set as  $p < 0.05$  or  $p < 0.001$ .

Quantitative data were presented as mean and standard deviation and qualitative as percentage. The normality assumption was tested with the Shapiro Wilk test, since the size of each group was small [54]. To compare the mean between quantitative variables the Mann-Whitney, T-test and one-way ANOVA were used [55].

## Results

### Antimicrobial susceptibility testing

The previously reported resistance of the examined isolates [49, 50] against commercially used antimicrobials is re-reported with table 1 for a comparison with the findings of each EO's activity. As has been reported the highest proportions of resistance were observed for penicillin G, rifampin, tetracycline, amoxicillin, ampicillin and sulfamethoxazole/trimethoprim (52-88%). Medium resistance was observed for kanamycin, chloramphenicol and colistin. Twenty- six isolates (44%) displayed resistance to at least three antimicrobial categories, while all *Salmonella* isolates were susceptible to cephalosporins, quinolones, doripenem and aztreonam.

### Antimicrobial activity of EOs

The *in vitro* antibacterial activity of EOs, against the *Salmonella* isolates, was qualitatively assessed by the presence or absence of

**Table 1.** Cumulative results of antimicrobial resistance of *Salmonella* spp. isolates recovered from pigs.

Antimicrobial	<i>S. Typhimurium</i> (n=21)	S.I. 4,12;i:- (n=9)	S.I. 4,5,12;i:- (n=6)	S.I. 6,7;k:- (n=7)	S. Bredeney (n=3)	S. Agona (n=1)	S. Infantis (n=1)	S. Derby (n=1)	S. Meleagridis (n=1)	S. Cerrito (n=1)	S.I. 6,14,25;-:1,2 (n=1)	<i>S. enterica</i> subsp. <i>diarizonae</i> 61:k:1,5 (n=1)	<i>S. enterica</i> subsp. <i>salamae</i> 38:b:1,2 (n=1)	<i>S. enterica</i> subsp. <i>houstenae</i> 40:g,t:- (n=1)	Rough (n=4)	Total No (%) of resistant strains
AML	12	8	6	1	0	0	0	0	0	0	1	0	0	0	3	31(52.54)
AMC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1(I)	0
AMP	12	8	6	1	0	0	0	0	0	0	1	0	0	0	3	31(52.54)
SAM	1(I)	1	1	0	0	0	0	0	0	0	0	0	0	0	0	2(3.4)
AZT	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CTX	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FOX	0	1(I)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CAZ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
EFT	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRO	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CXM	4(I)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CT	2	2	0	1	0	0	0	0	0	1	0	1	0	0	0	7(11.86)
C	4	1	1	1	0	0	0	0	0	0	1	0	1	1	3	13(22)
DOR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ENR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E	21	9	6	7	3	1	1	1	1	1	1	1	1	1	4	59(100)
CN	1	2	1	0	0	0	0	0	0	0	0	0	0	0	0	4(8.1)
K	4	1	5	1(I)	3	0	0	0	0	0	0	0	1(I)	1(I)	1	14(23.73)
NA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P	18	9	6	6	2	1	1	1	0	1	1	0	1	1	4	52(88.13)
RD	15, 6(I)	7, 2(I)	6	6, 1(I)	3	1	1	1	1	1, (I)	1	1, (I)	0	1	4	49(83)
SXT	10	7	6	1	0	0	0	0	0	0	1	0	1	1	4	31(52.54)
TE	11	9	6	1	3	0	0	0	0	0	1	0	1	1	4	37(62.71)
TGC	0	0	0	0	0	0	0	0	0	0	1(I)	0	0	1(I)	0	0
<b>Total N.</b>	<b>21</b>	<b>9</b>	<b>6</b>	<b>7</b>	<b>3</b>	<b>1</b>	<b>1</b>	<b>1</b>	<b>1</b>	<b>1</b>	<b>1</b>	<b>1</b>	<b>1</b>	<b>1</b>	<b>4</b>	<b>59</b>

AML: Amoxicillin; AMC: Amoxicillin-Clavulanic Acid; AMP: Ampicillin; SAM: Ampicillin-Sulbactam; AZT: Aztreonam; CTX: Cefotaxime; FOX: Cefoxitin; CAZ: Ceftazidime; EFT: Ceftiofur; CRO: Ceftriaxone; CXM: Cefuroxime; C: Chloramphenicol; CT: Colistin; ENR: Enrofloxacin; E: erythromycin; CN: Gentamycin; K: Kanamycin; NA: Nalidixic Acid; P: Penicillin; G: RD Rifampin; SXT: Sulfamethoxazole/Trimethoprim; TE: Tetracycline; TGC: Tigecycline; I: Intermediate Resistance.

inhibition zones and quantitatively by the zone diameters. The zones of inhibition for each of the 3 amounts of the EOs are presented in table 2.

Oregano and thyme gave in all tested concentrations (5, 15 and 30µl) large inhibitory zones ranging from 20 to 60 mm for oregano and 11-70 mm for thyme, while rosemary exhibited smaller inhibition zones, or it did not show any activity. The inhibition zones ranged from 10 to 50 mm (table 3). The negative control (absolute alcohol) did not inhibit growth for any of the tested strains of *Salmonella* spp.

**Statistical analysis**

The findings are present in tables 3 and 4. All EOs showed a statistical significance between the Zone of Inhibition (ZOI) and the tested concentrations of 5-15, 5-30 and 15-30 µl (F=240,95, p<0,001 for oregano, F=188,592, p<0,001for thyme and F=15,79, p<0,001 for rosemary). Specifically:

i) oregano: mean ZOI for: 5 - 15 µl (t=-12.660, df=58, 2-tailed p<0.001), 5-30µl (t=-18.828, df=58, 2-tailed p<0.001) and 15-30 µl (t=-11.502, df=58, 2-tailed p<0.001).

ii) thyme: mean ZOI for: 5 - 15 µl ( t=-9.389, df=58, 2-tailed p<0.001), 5-30µl (t=-8.685, df=58, 2-tailed p<0.001) and 15-30 µl (t=-10.385, df=58, 2-tailed p<0.001).

iii) rosemary: mean ZOI for: 5 - 15 µl (t=-3.791, df=58, 2-tailed p<0.001), 5-30 µl (t=-4.201, df=58, 2-tailed p<0.001) and 15-30 µl (t=-3.397, df=58, 2-tailed p<0.001) (Tables 3,4).

Repeated Measures ANOVA showed that the kind of the EO employed, affects significantly the width of ZOI (F=457,8, p<0,001) (Table 5). Analytically, the mean ZOI of 5 and 15 µl differed significantly between oregano and rosemary (t=40,558, df=58, 2-tailed p<0,001) and between rosemary and thyme (t=-24.612, df=58, 2-tailed p<0.001). No significance was observed between oregano and thyme (t=-0.883, df=58, 2-tailed p=0.381). However, a statistical significance was observed for the ZOI among the three pairs of the EOs (95% CI) at the concentrate of 30 µl.

Analysis of one-way variance showed a statistical significance among *Salmonella* serovars and the effect of oregano, rosemary and thyme (Sig.=0.009, Sig.<0.001, Sig.<0.001, respectively).

**Discussion and conclusion**

The use of antimicrobials, such as penicillin, tetracycline and their newer compounds is critical in guarding human and animal health [56]. However, their excessive use in veterinary medicine during the past decades has led, due to selection pressures, to the emergence of antibiotic-resistant bacteria transmitted to humans through the food chain [57].

The reported proportion [13,14,58-60] of *Salmonella* resistance to tetracycline, amoxicillin, ampicillin, sulfamethoxazole/trimethoprim and chloramphenicol, antimicrobials considered as Highly Important for Human Health [61], is reflecting the extensive use of such agents

**Table 2.** Inhibition zones (measured as mm) of the Essential Oils at different concentrations against *Salmonella* spp. isolates.

SEROVAR	OREGANO			ROSEMARY			THYME		
	5µl	15µl	30µl	5µl	15µl	30µl	5µl	15µl	30µl
<i>S. Typhimurium</i>	30	40	44	R	R	R	20	21	35
<i>S. Typhimurium</i>	30	42	43	R	R	R	20	28	31
<i>S. Typhimurium</i>	30	40	40	R	R	R	20	25	32
<i>S. Typhimurium</i>	30	32	35	R	R	R	20	35	48
<i>S. Typhimurium</i>	27	30	35	R	R	R	42	45	58
<i>S. Typhimurium</i>	26	32	40	R	R	R	40	42	60
<i>S. Typhimurium</i>	25	30	35	R	R	R	30	42	47
<i>S. Typhimurium</i>	25	32	42	R	R	R	40	41	60
<i>S. Typhimurium</i>	28	33	45	R	R	R	40	42	60
<i>S. Typhimurium</i>	30	32	35	R	R	R	20	40	50
<i>S. Typhimurium</i>	30	32	41	R	10	11	20	28	31
<i>S. Typhimurium</i>	33	34	40	R	11	13	25	33	36
<i>S. Typhimurium</i>	28	35	35	R	R	R	16	34	35
<i>S. Typhimurium</i>	27	40	40	R	R	15	35	50	50
<i>S. Typhimurium</i>	21	23	37	R	R	R	21	40	51
<i>S. Typhimurium</i>	27	37	50	R	R	R	22	22	60
<i>S. Typhimurium</i>	25	31	35	R	R	R	16	26	34
<i>S. Typhimurium</i>	22	30	40	R	R	12	11	12	37
<i>S. Typhimurium</i>	36	38	37	R	R	R	22	23	32
<i>S. Typhimurium</i>	22	31	41	R	11	11	24	33	44
<i>S. Typhimurium</i>	25	30	35	R	R	R	30	41	46
S.I. 4,12:i:-	24	36	40	R	R	R	28	38	40
S.I. 4,12:i:-	30	45	53	R	R	R	38	47	55
S.I. 4,12:i:-	30	42	56	R	R	R	35	43	55
S.I. 4,12:i:-	30	50	60	R	R	R	35	45	60
S.I. 4,12:i:-	32	33	43	R	R	R	40	43	63
S.I. 4,12:i:-	24	30	40	R	R	R	20	28	30
S.I. 4,12:i:-	21	30	38	R	R	R	30	32	40
S.I. 4,12:i:-	25	27	41	R	10	11	24	40	40
S.I. 4,12:i:-	20	30	30	R	R	R	40	40	40
S.I. 4,5,12:i:-	40	42	46	R	R	R	40	44	47
S.I. 4,5,12:i:-	30	40	44	R	R	R	20	22	30
S.I. 4,5,12:i:-	30	30	45	R	10	15	28	32	42
S.I. 4,5,12:i:-	25	38	40	R	10	10	20	21	35
S.I. 4,5,12:i:-	27	32	45	R	R	R	25	42	45
S.I. 4,5,12:i:-	29	30	34	R	R	10	20	22	30
S.I. 6,7:k:-	22	31	35	R	R	R	20	40	50
S.I. 6,7:k:-	22	31	35	R	R	R	20	40	50
S.I. 6,7:k:-	20	32	34	R	R	R	22	41	52
S.I. 6,7:k:-	22	31	41	R	12	12	25	35	40
S.I. 6,7:k:-	20	31	34	R	R	R	22	41	50
S.I. 6,7:k:-	21	31	35	R	R	R	20	40	50
S.I. 6,7:k:-	22	32	38	R	R	R	40	45	58
<i>S. Bredeney</i>	21	30	40	R	R	R	40	42	65
<i>S. Bredeney</i>	21	29	42	R	R	R	40	41	70
<i>S. Bredeney</i>	30	32	35	R	R	R	20	40	50
<i>S. Agona</i>	31	45	47	R	R	R	25	30	40
<i>S. Infantis</i>	27	33	43	R	R	R	35	50	50
<i>S. Derby</i>	27	40	40	R	R	R	25	42	45
<i>S. Meleagridis</i>	38	47	55	R	R	R	38	48	60
<i>S. Cerro</i>	25	30	35	R	R	R	30	33	40
<i>S. enterica</i> subsp. <i>diarizonae</i> 61:k:1,5	38	48	55	13	21	28	38	45	60
<i>S. enterica</i> subsp. <i>salamae</i> 38:b:1,2	40	40	50	17	26	28	30	34	50
S.I. 6,14,25: - : 1,2	30	42	50	14	30	50	40	50	50
<i>S. enterica</i> subsp. <i>houtenae</i> 40:g:t:-	30	44	46	12	15	25	30	44	60
Rough	25	40	50	R	R	R	40	40	62
Rough	29	36	40	12	30*	40*	40	40	56
Rough	29	30	34	R	R	10	20	22	30
Rough	30	35	42	10	25*	38*	40	40	58

**Table 3.** Statistical analysis of Zone of Inhibition (ZOI) and the tested concentrations of 5-15, 5-30 and 15-30 µl for the antimicrobial activity of oregano, rosemary and thyme oils against *Salmonella* spp. Isolates.

Inhibitory activity	OREGANO			ROSEMARY			THYME		
	5µl	15µl	30µl	5µl	15µl	30µl	5µl	15µl	30µl
No Inhibition	-	-	-	53	46	42	-	-	-
<12 mm: weak activity zone	-	-	-	54	52	48	1	-	-
12 mm ≥ inhibition zone < 20 mm: intermediate activity	-	-	-	5	2	5	2	1	-
≥ 20 mm: strong activity	59	59	59	-	5	6	56	58	59
<b>Range Zone of Inhibition (mm)n ZOI</b>	20 – 40	23 - 50	30- 60	10-17	10-30	10-50	11 - 42	12 -50	30-70
<b>Mean Zone of Inhibition (mm)</b>	27.36	34.90	41.29	1.32	3.75	5.59	28.42	36,69	47,29
<b>Mode</b>	30	30	35	0	0	0	20	40	50
<b>Std. Deviation</b>	4,926	5,818	6,457	4,023	8,014	11,264	8,770	8,756	10,830

in pig production in Greece and across Europe [62]. Specifically, their use as growth promoters negatively affects the typical gut microflora, allowing the preferential colonization of the gut by organisms such as *Salmonella* spp. This colonization is making pigs an important risk for the contamination of pork carcasses and eventually pork products [63]. Among *Salmonella* serovars the monophasic S. Typhimurium has exhibited the highest antimicrobial resistance across Europe and in Greece [4,64,65].

WHO reports *Salmonella* spp. as one of the most prevalent bacterial species causing food-borne disease and a major health threat due to fast developing antimicrobial resistance [61]. The spreading of such resistant strains hampers the therapeutic possibilities of human *Salmonella* infections increasing the need of alternative treatments replacing commonly used antimicrobials in animal production. Thus, an increasing number of researchers give evidence of the EOs' potential inhibitory activity against pathogens, such as *Salmonella* spp. [18,27], reporting them as 'Generally Regarded As Safe' (GRAS) for both animals and humans, because they do not promote antimicrobial resistance, as observed with the long-term use of synthetic antibiotics [66,67]. In the present study, the evaluated EOs showed varying degrees of bactericidal ability against the tested *Salmonella* isolates at different treatment amounts. Rosemary was found less potential compared to oregano and thyme. Oregano and thyme showed high inhibitory effect, even for the very small amount of 5 µl (Table 2 and 3) against all the salmonellae tested. An example is thyme oil, which at the amount of 30 µl produced the largest ZOI in the study (70 mm) and showed the greatest mean ZOI (47.29 mm).

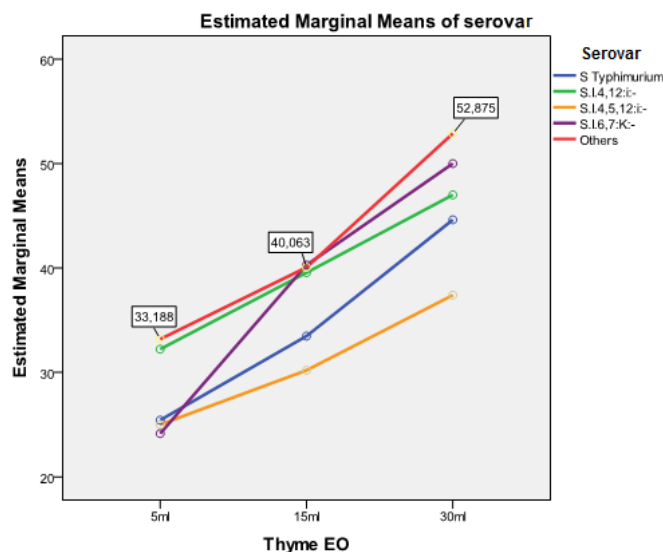
The obtained results showed that the inhibitory effect was dependent on the amount of the EO employed, showing that ZOI differed significantly only for the amount of 30 µl, with regards to the EO employed. This is of minor importance, because both oregano and thyme showed strong activity (>20mm) for this particular amount. The *Salmonella* serovar appearing dependent on the EO used, was the monophasic variant of S. Typhimurium, *S. enterica* subsp. *enterica* ser. 4,12:i:-. This serovar was more sensitive against oregano and thyme EOs (Table 2, Figures 1-3). A comparison of the antibacterial activity of these EOs with that of commonly used antimicrobials, showed that the EOs have a better killing ability against *Salmonella* isolates, independent of the level of resistance to commercially used antibiotics, an observation similar to that reported by Mayaud et al. [68]. The present findings, although *in vitro*, suggest that the tested EOs rich in thymol and carvacrol could control a wide range of microbes, becoming potential alternatives to commonly used antimicrobials. However, their effectiveness in animals, when administrated orally, must be experimentally demonstrated *in vivo* as to the maximum amount of EO administrated for avoiding toxicity or affecting the gut microflora.

**Table 4.** Statistical analysis of Zone of Inhibition (ZOI) and the tested concentrations of 5-15, 5-30 and 15-30 µl for the antimicrobial activity of oregano, rosemary and thyme oils against *Salmonella* spp. pig isolates.

Quantity	Essential Oil	t	df	p
5 – 15 µl	oregano	-12.660	58	<0.001
	thyme	-9.389	58	<0.001
	rosemary	-3.791	58	<0.001
5 – 30 µl	oregano	-18.828	58	<0.001
	thyme	-8.685	58	<0.001
	rosemary	-4.201	58	<0.001
15 – 30 µl	oregano	-11.502	58	<0.001
	thyme	-10.385	58	<0.001
	rosemary	-3.397	58	<0.001

**Table 5.** Repeated Measures ANOVA comparing in pairs the antimicrobial activity of oregano, rosemary and thyme oils against *Salmonella* spp. isolates recovered from pigs

quantity	pairs	t	df	p
5ml	oregano - rosemary	40,558	58	<0.001
	oregano - thyme	-0,883	58	0,381
	rosemary- thyme	-24,612	58	<0.001
15ml	oregano - rosemary	27,006	58	<0.001
	oregano - thyme	-1,414	58	0,163
	rosemary- thyme	-22,642	58	<0.001
30 ml	oregano - rosemary	23,544	58	<0.001
	oregano - thyme	-4,486	58	<0.001
	rosemary- thyme	-21,161	58	<0.001



**Figure 1.** Estimated marginal means of *Salmonella* serovars with regards to thyme EO

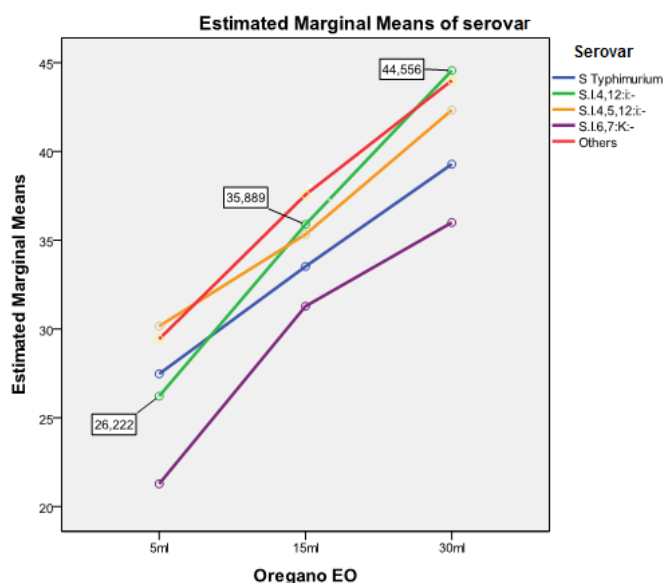


Figure 2. Estimated marginal means of *Salmonella* serovars with regards to oregano EO

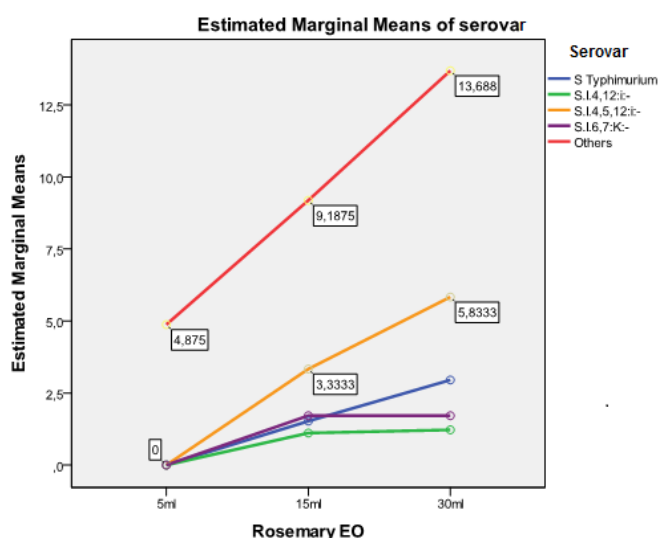


Figure 3. Estimated marginal means of *Salmonella* serovars with regards to rosemary EO.

In addition, a standardization of the methodology to evaluate their inhibitory activity should be established.

The unnecessary use of antibiotics has been a major promoter of the emergence and transmission of drug-resistant bacterial strains. Resistance to antimicrobials, whether attributed to animal or human excessive use, demonstrates the dynamic evolution of microorganisms' response to the hostile environment of antibiotics becoming a threat to human health. The food animal reservoir of such strains is an important source for human infections. By eliminating the use of drugs important for human medicine in animals, through the administration of EOs, the prevalence of resistant isolates could be reduced for the benefit of consumers.

In conclusion, EOs should be considered possible alternatives to synthetic drugs, administrated as growth promoters. The exploitation of

EOs as potential growth promoters in animals is the new hope of using phytopharmaceuticals against multidrug-resistant microorganisms. The present results add to the knowledge of previous studies, encouraging for more research into establishing EOs as reliable curative agents.

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