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In vitro study of the activity of essential oils of *Thymus capitatus* and *Thymus vulgaris* against enterobacteria of avian origin resistant to antibiotics

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Key words: Antibiogram, *Enterobacteriaceae*, *Thymus capitatus*, *Tymus vulgaris*, Poultry, MIC.

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Abstract

Bacterial resistance to antibiotics is a growing problem in veterinary practice. The purpose of this study is to find an alternative to antibiotics like thyme essential oil *in vitro*. The *Enterobacteria* are isolated from dead and sick chicken organs. An antibiogram is carried out on the latter strains in order to select the resistant strains. The essential oils of *Thymus capitatus* and *Thymus vulgaris* are extracted by hydrodistillation. By measuring the activity of the oils on agar MH has different concentration of 5 µl, 10 µl, and 15 µl. The MIC is determined by the macrodilution technique (macroscopic inhibition in a liquid medium). We noted a predominance of Escherichia coli 49%. The resistance to all antibiotic tested described is given for *E. coli* 52.78%, *Enterobacter* 64.29%, *Proteus* 66.67% and *Salmonella* 53.03%. The family *Enterobacteria* shows resistance better than 65.31% for Ampicillin, Ceftiofur, Neomycin and Flumequine. Test of aromatogram revealed inhibition diameters between 0 mm and 52.33±1.53 mm. The oil of *Thymus capitatus*, generates a MIC between 1.25 and 5 µL/mL with a bactericidal / bacteriostatic effect, whilst the oil of *Tymus vulgaris*, generates a lower MIC of 5 µl/mL, with a bactericidal effect. A statistically significant difference $p \leq 0.05$ in the effect of the essential oil of *Thymus capitatus* compared to CS 10µg and UB 30µg for concentrations of 5 µl and 10 µl. These results open up prospects for the use of essential oils *in vivo* to treat diseases in poultry that have not been treated by antibiotics.

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Introduction

The development of intensive livestock farming in the poultry sector has been accompanied by a massive use of antibiotics both for the treatment and prevention of infections and for the improvement of zootechnical performances(Hafed *et al.*, 2016). The emergence of antibiotic resistance is a major problem in animal health and human health, since the effectiveness of antibiotic treatments is reduced in the face of human or animal pathologies caused by resistant bacteria(Chazel *et al.*, 2009). Medicinal plants are a precious heritage for humanity(Salhi *et al.*, 2011), since the 1990s, herbal products, already used for their effects on zootechnical performance in poultry(Brenes and Roura, 2010). Essential oils and their components are known to possess antimicrobial activities(Mahboubi and Haghi, 2008). Several studies have been conducted to select essential oils with important antibacterial properties(Adrar *et al.*, 2016; Yahiaoui *et al.*, 2018). The *in-vitro* antimicrobial activity of the essential oils of several Thymus species has also been reported (Nabavi *et al.*, 2015; Ballester-Costa *et al.*, 2016; Bektas *et al.*, 2016; Boardman and Smith, 2016). A wide range of oils and bacteria have been tested (Yahiaoui *et al.*, 2018). The two plant species that were the subject of our study, namely the *Thymus capitatus* and *Tymus vulgaris*. Facing antibiotic resistance problems in pathogenic bacteria of poultry, a study is carried out.

The objective of this work is to solve *in-vitro* the problem of antibiotic resistance, the determination of the antimicrobinic activity of oils and characterize the effect of essential oil selected. *Enterobacteriaceae* are isolated from broilers in poultry farms in the region of Sidi Bel Abbes in Algeria and to test their resistance against different families of antibiotics used in the poultry industry.

The determination of the antimicrobial activity of oils. The biological effect of *Thymus capitatus* and *Tymus vulgaris* is determined, and their actions are characterized by Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). For the purpose also is to replace antibiotics with plant extracts.

Materials and methods

Location of the area of study

The wilaya of Sidi Bel Abbes is located in the north-west of Algeria at 80 km from Oran. Located at an altitude of 486 m, the region of Sidi Bel Abbes is characterized by a Mediterranean climate and it belongs to the semi-arid bioclimatic stage with a continental tendency: wet and cold winter, dry and hot summer, short intermediate seasons(Bennabi *et al.*, 2012) (Fig. 1).

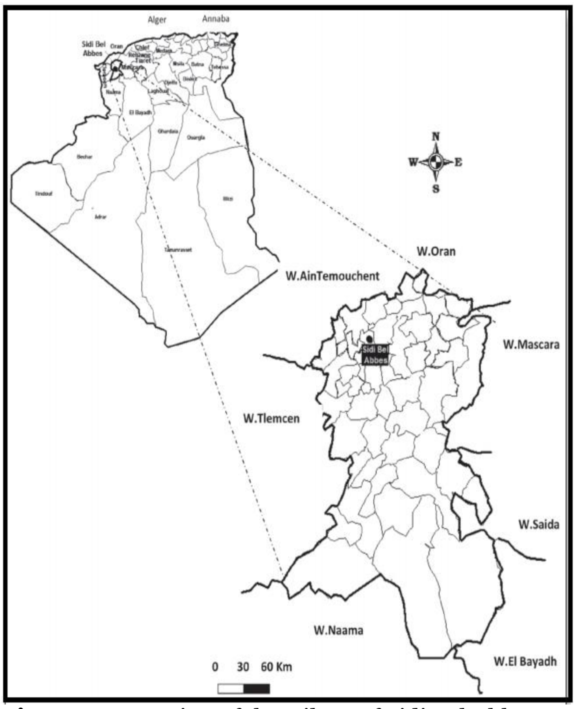

Fig. 1. Presentation of the wilaya of Sidi Bel Abbes.

Proceedings of our research

Visits were made to different livestock units where the chickens do not go out and are usually sold after 45 days of growth. Several chicken carcasses were recorded, 176 autopsies performed. The mortality is reported by the breeder, we note that some of them do not use the registration form. Autopsy is performed in the field to detect possible pathologies (Fig. 2).

Collection of the bodies

The autopsy of the various subjects concerned the examination of all organs located in the thoracic and abdominal cavity (Laanani *et al.*, 2015). Taking into account macroscopic changes of the organs that are the liver, heart, spleen and intestine. Samples are taken according to recommendations of the International Organization of Epizootics (O.I.E).

Isolation and identification of pathogenic bacteria

The culture media were chosen according to the bacterial groups sought(Dho and Mouline, 1983). For the Identification of *Enterobacterial*, Strains representative *Enterobacterial* colonies were randomly selected and streaked on appropriate media. Isolated bacterial strains were characterized using the API20 E galleries (Koffi-Nevry *et al.*, 2012).

The strains were identified by comparing their characteristics with those of known taxa as described in Bergey's Manual for Determinative Bacteriology (Koffi-Nevry *et al.*, 2012).

Determination of antibio resistance

The purification of the strains was carried out by repeated cultures until obtaining a pure culture (Abeid *et al.*, 2015).

The susceptibility of strains to antibiotics was determined by the agar diffusion method as recommended by the Committee of the antibiogram of the French Society of Microbiology (CA-SFM, 2013). Antibiotic families have been selected for susceptibility tests are those used in avian according to the Algerian Network for Monitoring the Resistance of Bacteria to Antibiotics (RA-SRBA).

Plant material

The plant material consists of the aerial parts of the plants, which were harvested and dried away from light and moisture. By respecting the conditions and sampling period. Harvesting should be done in good weather, with no wind or rain(Jean and Jiri, 1983) (Fig. 3).

Essential oils

The essential oils are obtained by hydro distillation with a Clevenger type apparatus (Clevenger, 1928). The distillation was carried out by boiling for three hours 250 g of fresh plant material with 750ml of water. The yield of essential oil was determined in relation to the dry matter (Fig. 4).

Aromatogram

It is a method of in vitro measurement of the antibacterial power of chemotyped essential oils. Different types of aromatograms, in solid, liquid medium, are exploitable(Pibiri, 2006).

A bacterial suspension of density equivalent to 0.5 Mac Farland (10^8 CFU.mL^{-1}) is prepared(Guinoiseau, 2010) based on a well isolated colony, and then inoculation will be done on solid Petri dishes cast with solid Muller Hinton medium. Sterile filter paper disks (What man) with 6 mm diameter were impregnated with different volumes of essential oil 5 µl, 10 µl and 15 µl, and placed on the surface of Mueller-Hinton medium(El Amri *et al.*, 2014; Benbelaid *et al.*, 2017). Antibacterial activity is determined by measuring the diameter of the inhibition zone around each disc(Doughari *et al.*, 2007)

Statistical analysis

According to the test of variance (ANOVA) the mean differences were determined with significance at $p \leq 0.05$ with a 95% confidence interval. Using SPSS v 19.0 for windows. The difference was considered statistically significant when the value of p is <00.5.

Minimum Inhibitory Concentration (MIC)

For each the essential oils are diluted successively in cascade 1/2, 1/4 1/8, 1/16, 1/32, 1/50, 1/64. 1/80 and 1/ 128 (Oussou *et al.*, 2004; Etchike *et al.*, 2011). a sterile concentration range, ranging from 80 to 1.25 mg / ml with Tween 80. An inoculums whose turbidity is adjusted to 0.5 Mcfarland (or 10^8 ufc / ml) in Mueller-Hinton broth. In hemolysis tubes, 1 ml of each concentration and 1 ml of bacterial inoculum is mixed.

The concentration range of each extract is then diluted by half and is as follows: 80; 40; 20; 10; 5;

2.5; 1.25 and 0.625 mg / ml. After incubation, bacterial growth is examined in each tube by turbidity (Bolou *et al.*, 2011).

Minimum Bactericidal Concentration (MBC)

To determine the MBC, for each set of test tubes of the MIC, a loopful of broth was collected from those tubes which did not show any visible sign of growth and inoculated on sterile nutrient agar by streaking. Nutrient agar plates were streaked with the test organisms only to serve as control. The plates were then incubated at 37ºC for 24 h. After incubation the concentration at which no visible growth was seen was noted as the minimum bactericidal concentration (Doughari *et al.*, 2007).

The antibacterial effect was judged to be bactericidal or bacteriostatic depending on the ratio: MBC / MIC. Indeed, if MBC / MIC = 1 to 2, the effect is bactericidal and if MBC / MIC = 4 to 16, the effect is bacteriostatic(Berche *et al.*, 1991).

Results and discussion

Lesional diagnosis of chickens

Macroscopically the lesions were detected. There is a diversity of pathologies from one unit to another, and this is due to the different rearing conditions adopted by breeders and the non respect of hygienic and prophylactic practices (biosecurity) (Fig. 5).

Digestive diseases are recorded 75% for unit 4, hepatic 66.67% for unit 5, spleen 14.29% for unit 3, cardiac 21.43% for unit 3, respiratory 50% for unit 8 (Fig. 6).

The lesion is characterized by a green liver and congested pectoral muscles, pericarditis and peritonitis. The lesions observed correspond to intestinal inflammation, large thickened and edematous plaques containing blood and mucus(Jeffrey *et al.*, 2002).

Table 1. Distribution of *Enterobacteriaceae*.

Enterobacteriaceae	Number	Rate %
E. Coli	24	49%
Enterobacter	7	14%
Proteus	6	12%
Salmonella	11	22%
Serratia	1	2%
Somme	49	100%

Table 2. Critical values of the diameters of the inhibition zones.

Antibiotics	Critical diameters in mm		
	Resistant	Intermediate	Sensitive
AM 10 µg	13	14-16	17
TIO 30 µg	17	19-20	21
N 30 µg	13	14-17	18
UB 30µg	21	/	25
CS 10 µg	10	/	11
GM 10 µg	12	13-14	15

The most affected organs are the air sacs, the liver, the heart, the abdominal cavity. Organ damage is characterized by congestion, tissue thickening and fibrin deposition. This deposit is sometimes so important that the surface of the organ takes on the appearance of a pancake(Stordeur and Mainil, 2002).

Distribution of isolated bacteria

In a total of forty nine strains of *Enterobacteriaceae*, we record, *E. coli* (49%), *Enterobacter* (14%), Proteus (12%), *Salmonella* (22%), *Serratia* (2%) (Table 1).

Antibiogram of enterobacteriaceae

The resistance percentages of the forty-nine pathogenic *enterobacteriaceae* are summarized in (Fig. 7). All the microorganisms identified showed multiresistance of 55.44%, this percentage and distributed in the following form: ampicillin 75.51%, ceftiofur 65.31%, neomycin 71.43%, flumequine 89.80%, colistin 24.49% gentamicin 6.12%.

Table 3. Extraction rate of essential oils compared to other studies.

Plants	Yield our work	% Yield			
Thymus vulgaris	2.75 %	4.0% (Guillén and Manzanos, 1998)	1.05 ± 0.1% (Lee *et al.*, 2005)	2,05 % (El Ajjouri *et al.*, 2008)	0,5% (El Ouali Lalami *et al.*, 2013)
Thymus capitatus	1.82 %	2,75% (Akrout *et al.*, 2004)	2,05 % (Amarti *et al.*, 2008)	0,45 à 1,46%. (Aafi et al., 2011)	0.4%(Attia *et al.*, 2012)

Table 4. MIC and MBC of essential oils on *Enterobacteriaceae.*

Souche	Essential oil of *Thymus capitatus*			Essential oil of *Thymus vulgaris*		
	MIC (μL/mL)	MBC (μL/mL)	MBC/ MIC	MIC (μL/mL)	MBC (μL/mL)	MBC/ MIC
Salmonella thyphi	5	5	1	ND	ND	ND
Proteus vulgaris	2,5	5	2	2,5	2,5	1
Enterobacter faecalis	1,25	5	4	2,5	5	2
Escherichia coli	2,5	5	2	5	5	1

ND : not determined.

In enterobacteriaceae, the enzymatic inactivation of antibiotic is the major mechanism of resistance, it concerns several families of antibiotics, mainly β-lactams and aminoglycosides, but also quinolones and chloramphenicol(Pantel, 2015).

Fig. 2. visits to production units.

Extended spectrum betalactamases represent resistance molecules encoded by different genes that confer resistance to beta lactamines and cephalosporins (Sanders *et al.*, 2011).

Quinolones represent the first generation of synthetic chemotherapeutic agents.

The broad spectrum, bactericidal activity of this class makes quinolones effective against many Gram-negative and Gram-positive pathogens important in human and veterinary medicine(Bager and Helmuth, 2001). *Enterobacteria* that colonize the digestive sphere (Brun-Buisson and Legrand, 1994) are in contact with antibiotics during medical treatment, and the digestive tract becomes a real reservoir of multidrug-resistant *enterobacteria.*

E. coli

All the identified germs showed multiresistance of 52.78%, this percentage is distributed in the following form: ampicillin 62.50%, ceftiofur58.33%, neomycin 66.67%, flumequine 91.67%, colistin 37.50%, gentamicin 0.00% (Fig. 8).

According to (Bi *et al.*, 2017) e. coli has a great resistance to colistin 100%, ampicillin 92%, gentamicin 56%. The high number of Escherichia coli isolated from carcasses was probably related to the natural presence of this bacterial species in the digestive tract (Maho *et al.*, 1997). Ampicillin has become the least active antibiotic on E. coli (Bouzenoune *et al.*, 2009).

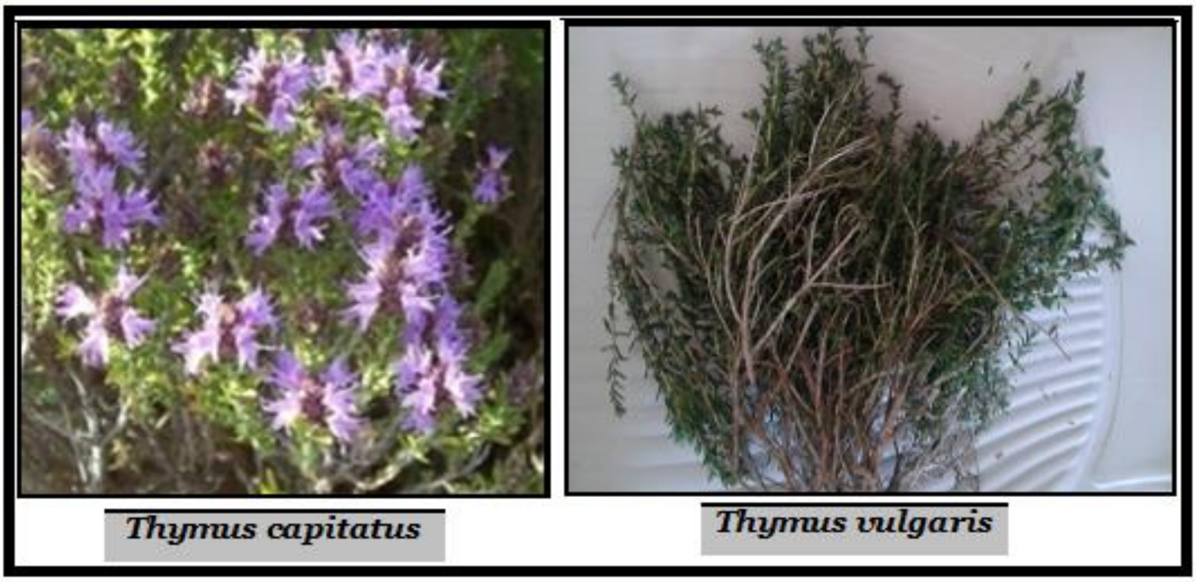

Fig. 3. Plant material.

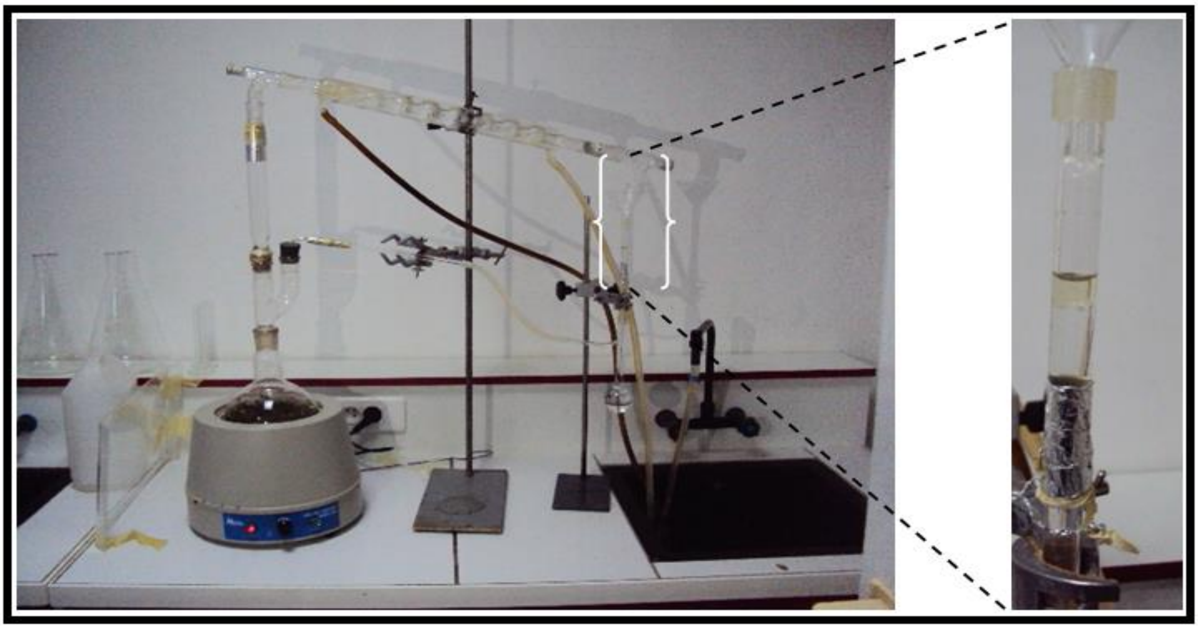

Fig. 4. Experimental setup.

Enterobacter

All germs identified had multiresistance of 64.29%, this percentage is distributed in the following form: ampicillin 85.71%, ceftiofur 71.43%, neomycin 100%, flumequine 71.43%, colistin 42.86%, gentamicin 14.29% (Fig. 9).

E. aerogenes has a naturally inducible, low-level chromosomal cephalosporinase. E. aerogenes is therefore naturally resistant to first-generation aminopenicillins and cephalosporins (Meunier *et al.*, 1997). *E. aerogenes* can harbor, like all *enterobacteria* now, a plasmid encoding an ESBL(Philippon *et al.*, 1988; Bush, 1996; Meunier *et al.*, 1997).

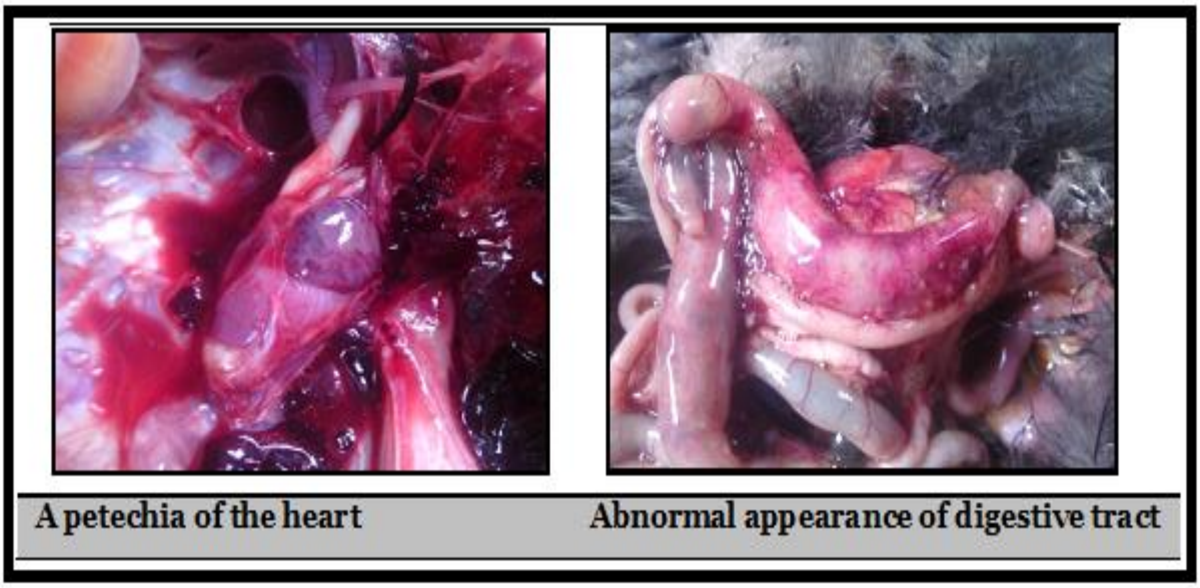

Fig. 5. Macroscopic examination of the abnormal appearance of some broiler organs on the spot.

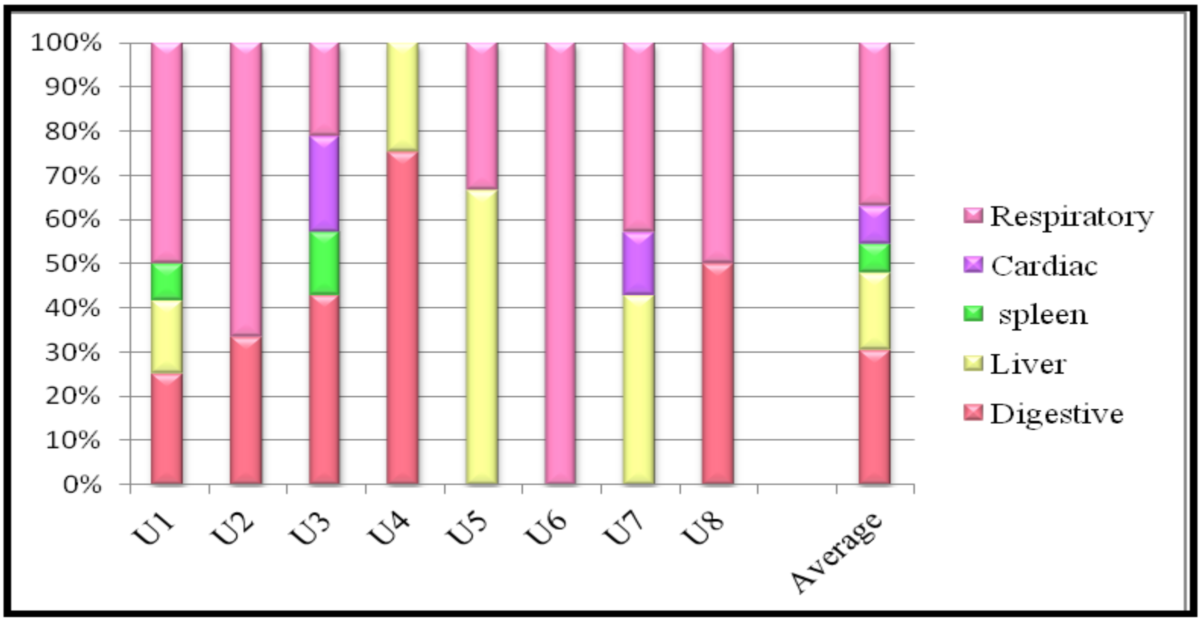

Fig. 6. Representation of pathologies encountered in poultry farms.

Proteus

All the identified microorganisms showed multiresistance of 66.67%, this percentage is distributed in the following form: ampicillin 100%, ceftiofur 100%, neomycin 100%, flumequine 100%, colistin 0%, gentamicin 0% (Fig.10).

Salmonella

All the identified germs showed multiresistance of 53.03%, this percentage is distributed in the following form: ampicillin 90.91%, ceftiofur 63.64%, neomycin 54.55%, flumequine 100%, colistin 0%, gentamicin 9.09% (Fig. 11).

Salmonella poses serious problems in both human and veterinary medicine and is all the more formidable as many its serotypes are currently resistant to many antibiotics. Some serotypes from the outset multiresistant (Chasseur-Libotte and Ghysels, 1983). Multi-resistant strains of S. typhi have also been reported in other countries (Anderson, 1975). According to the same author, *Salmonella* show resistance to gentamycin.

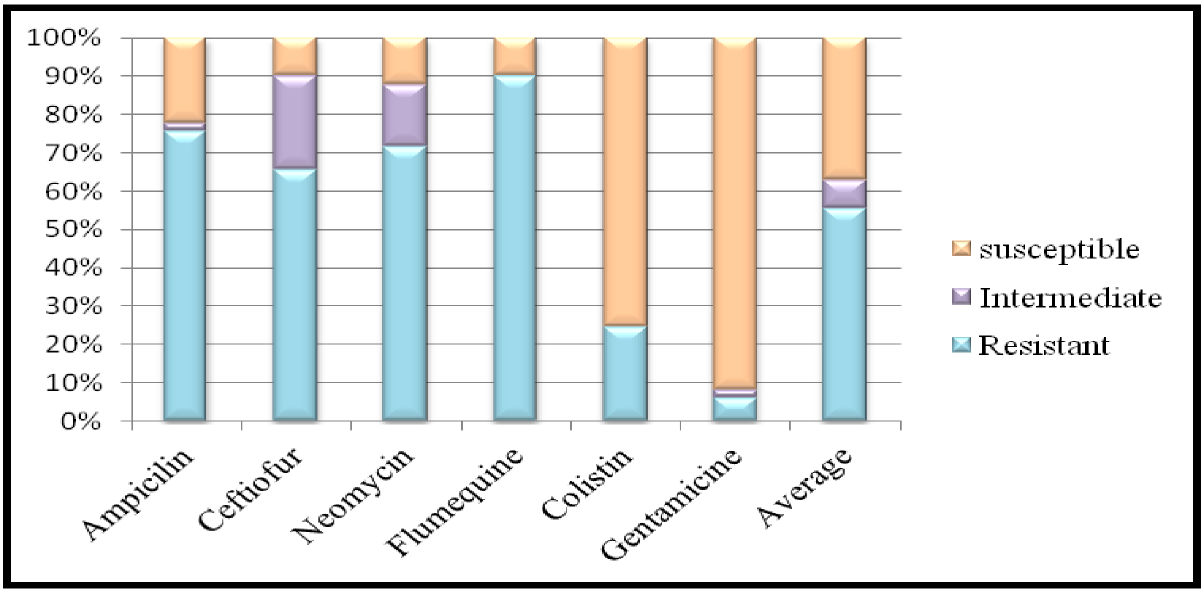

Fig. 7. The sensitivity of *enterobacteria* to each antibiotic used and in total. S-susceptible; I-intermediate; R-resistant.

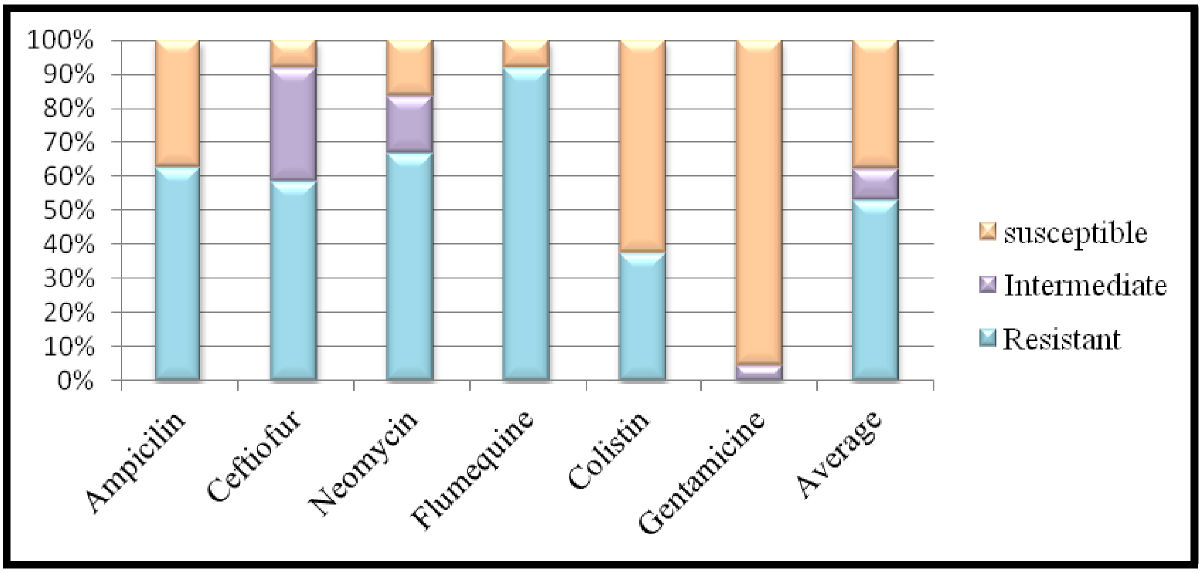

Fig. 8. The sensitivity of E. coli to each antibiotic used and in total.

Serratia

Serratia are presented with multiresistance of 16.67%, this percentage is distributed in the following form: ampicillin 0%, ceftiofur 0%, neomycin 0%, flumequine 0%, colistin 0%, gentamicin 100% (Fig. 12).

It is in intensively reared animals that the proportion of resistant bacteria, pathogenic or saprophytic, is the highest(Guillot, 1989).

Selection of the most resistant strains

Critical values of inhibition zone diameters are summarized in (Table 2).

The most resistant bacteria to many antibiotics were selected for an identification and aromatogram examination. The results are illustrated in the form of a histogram (Fig. 13).

Escherichia coli and *Enterobacter faecalis* show 83.33% resistance to all antibiotics tested except gentamicin. *Proteus vulgaris*, *Salmonella thyphi* have a resistance of 66.67% to colistin and gentamicin.

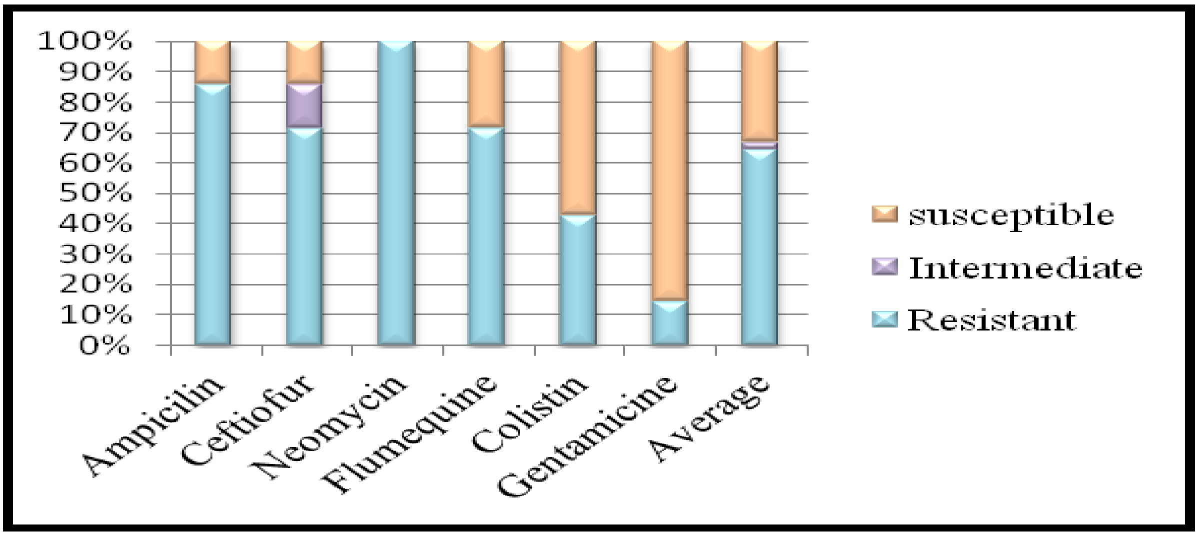

Fig. 9. The sensitivity of enterobacter to each antibiotic used and in total.

Extraction efficiency of essential oils

Essential oils of *Thymus vulgaris* and *Thymus capitatus* have characteristic color and odor (Fig. 14). Average yields of essential oils were calculated according to the dry plant matter (Table 3). For comparison the table summarizes previous investigations of the authors on the essential oils of several species from different countries. The yield of essential oil, depends on many factors (growth stage, pedoclimatic conditions, extraction technique, etc.)(Satrani *et al.*, 2007) of the harvest period(Aafi *et al.*, 2011). Our results are consistent with that of El Ouali Lalami *et al* (2013) when he confirms that *Thymus vulgaris* is less active against both *Enterobacteriaceae Salmonella sp* and *Escherichia coli*.

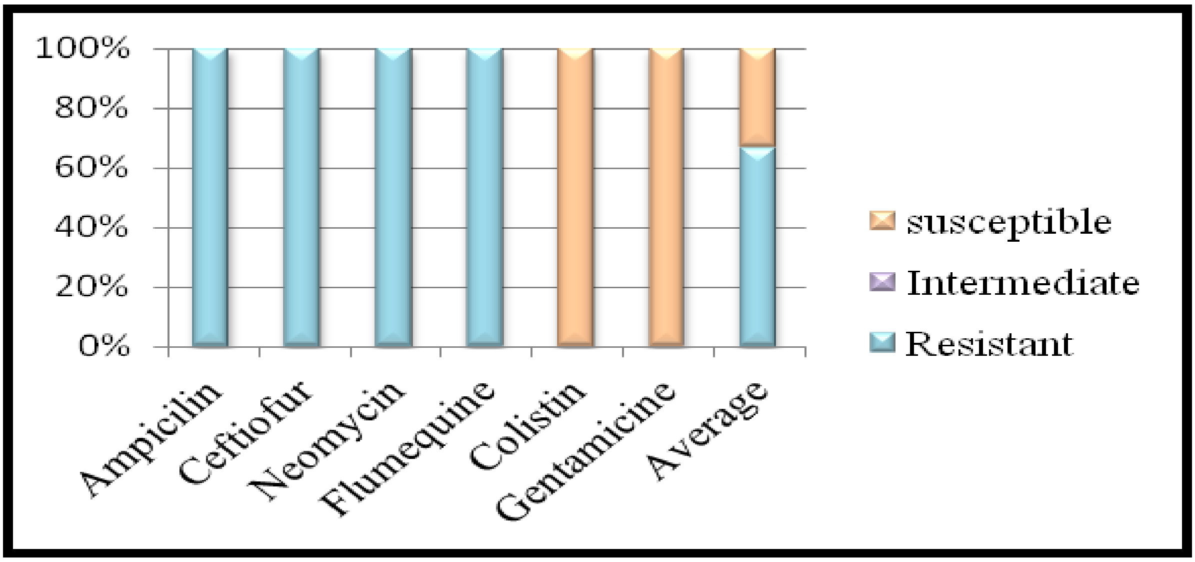

Fig. 10. The sensitivity of the proteus to each antibiotic used and in total.

Antibacterial activity of essential oils

The sensitivity character of the bacterial strains is appreciated by measuring the diameter of the inhibition zone in (mm) (expressed as means ± standard deviations). The results are shown by a histogram (Fig. 15).

Thymus capitatus generates 38.83 ± 1.63mm at 5 µl, 39.33 ± 1.05mm at 10 µl 30.83 ± 1.53mm to 15µl. *Thymus vulgaris* generates 26.33 ± 1.05 mm at 5 µl 31.00 ± 1.16 mm at 10 µl, 23.08 ± 0.83 mm at 15 µl. The two essential oils give inhibition diameters greater than 23 mm, around the 5 µl, 10 µl and 15 µl discs.

The essential oils of *Thymus capitatus* and *Thymus vulgaris* show a powerful effect, while there is a clear resistance of Salmonella thyphi to oils of *Thymus vulgaris*. Concentrations of essential oils expressed in means ± standard deviations.

There is a statistically significant difference in the effect of the essential oil of Thymus capitatus compared to CS 10 µg and UB 30 µg, for concentrations of 5 µl and 10 µl. While the essential oil of Thymus vulgaris at different concentrations have a non-statistically significant difference.

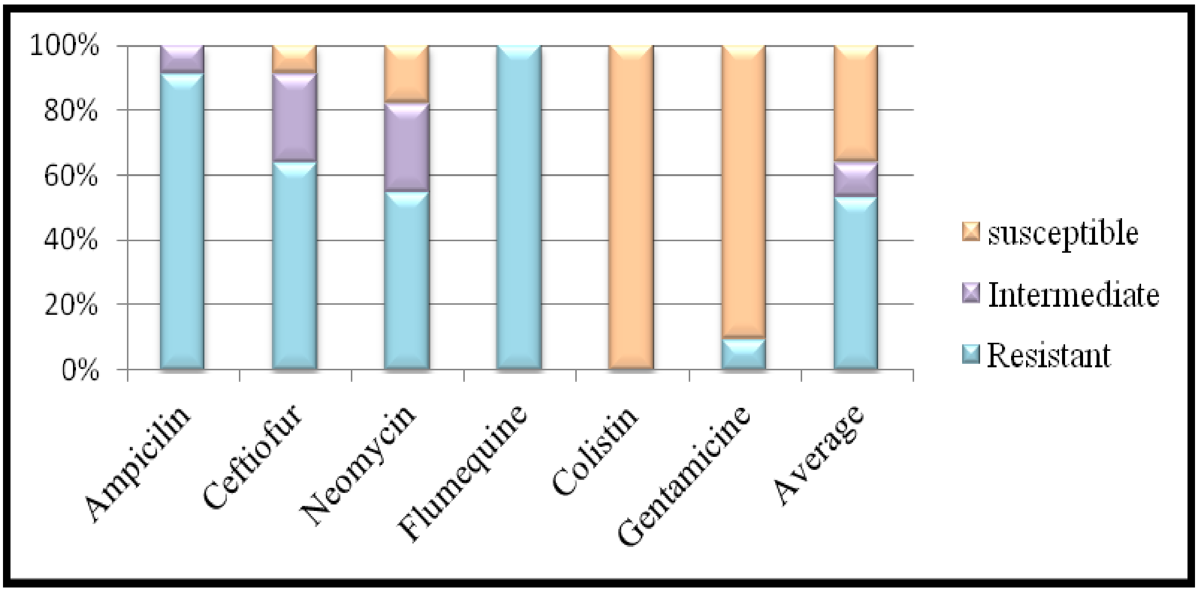

Fig. 11. Sensitivity of salmonella to each antibiotic used and total.

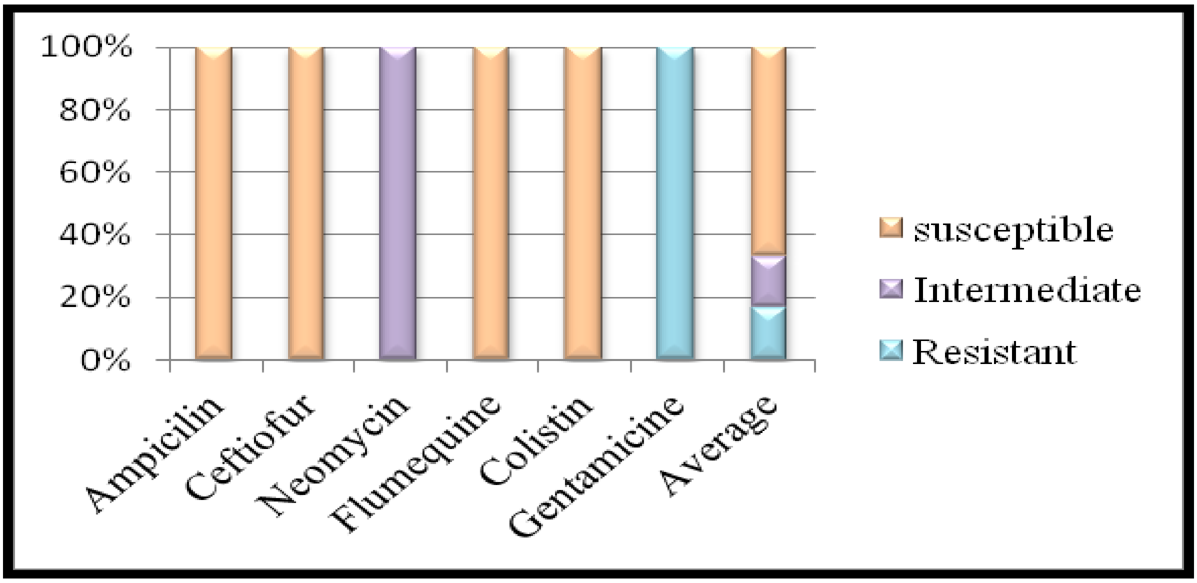

Fig. 12. The sensitivity of serratia to each antibiotic used and in total.

Evaluation of MIC and MBC

The results of the MICs and MBCs are summarized in (Table 4).

Oil of *Thymus capitatus* is very active because of its strong inhibition on all Gram- negative tested bacteria belonging to the *enterobacteriaceae* family, with a minimum inhibitory concentration between

1.25 and 5 (μL / mL) with a bactericidal / bacteriostatic effect.

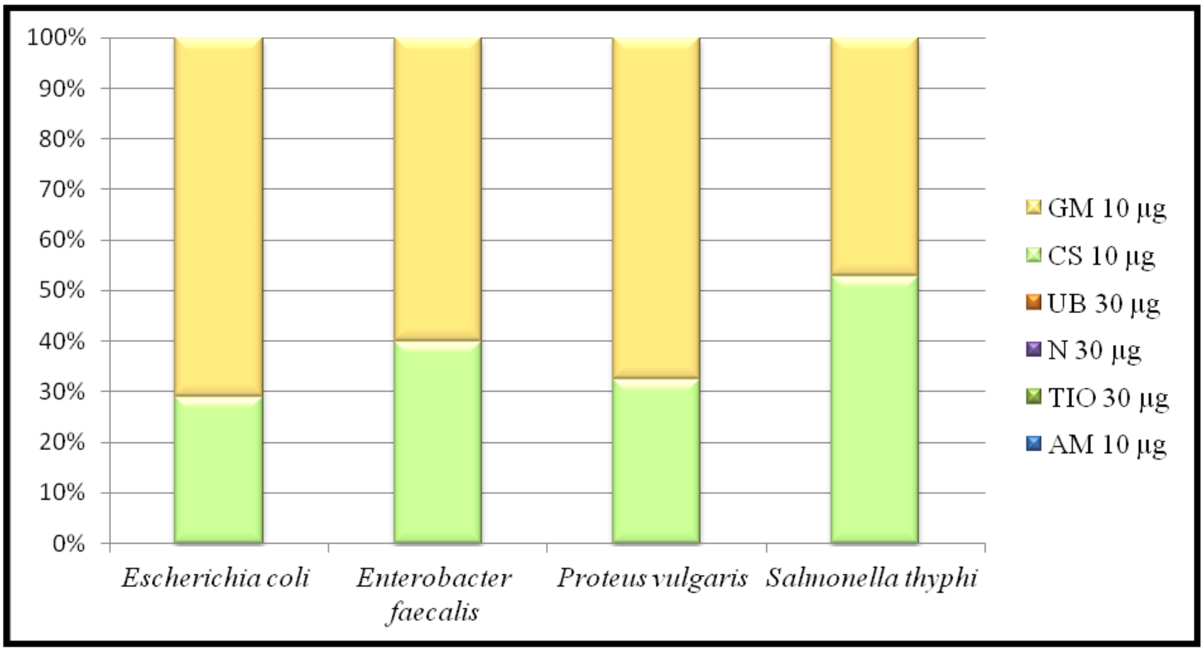

Fig. 13. The sensitivity profile of antibiotic resistant strains.

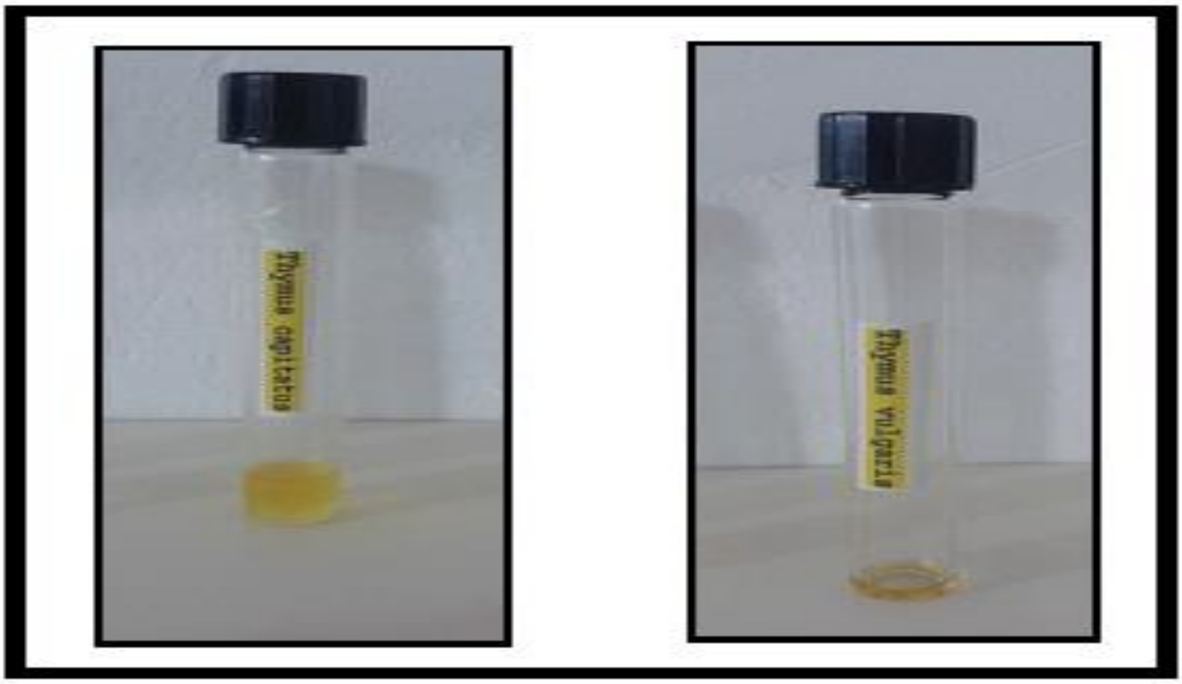

Fig. 14. The appearance of *Thymus vulgaris* and *Thymus capitatus*.

Oil of *Thymus vulgaris* is active by its strong inhibition on all bacteria tested with a minimum inhibitory concentration (MIC) of 2.5 to 5 (µL / mL) for *enterobacteriaceae*. This essential oil has a bactericidal effect.

The essential oil of thyme is the main oil for which the best effects are reported in zootechnical studies(Alleman *et al.*, 2013). Factors that may affect the results of the effectiveness of essential oil on animal performance are: the harvest time, plants ripening stage, the extraction methods of essential oils method and shelf life and storage and the possible synergistic or antagonistic effect of bioactive compounds (Brenes and Roura, 2010). Thymus vulgaris essential oil contains largely thymol and carvacrol, with percentages giving a strong antibacterial effect(Kaloustian *et al.*, 2008).

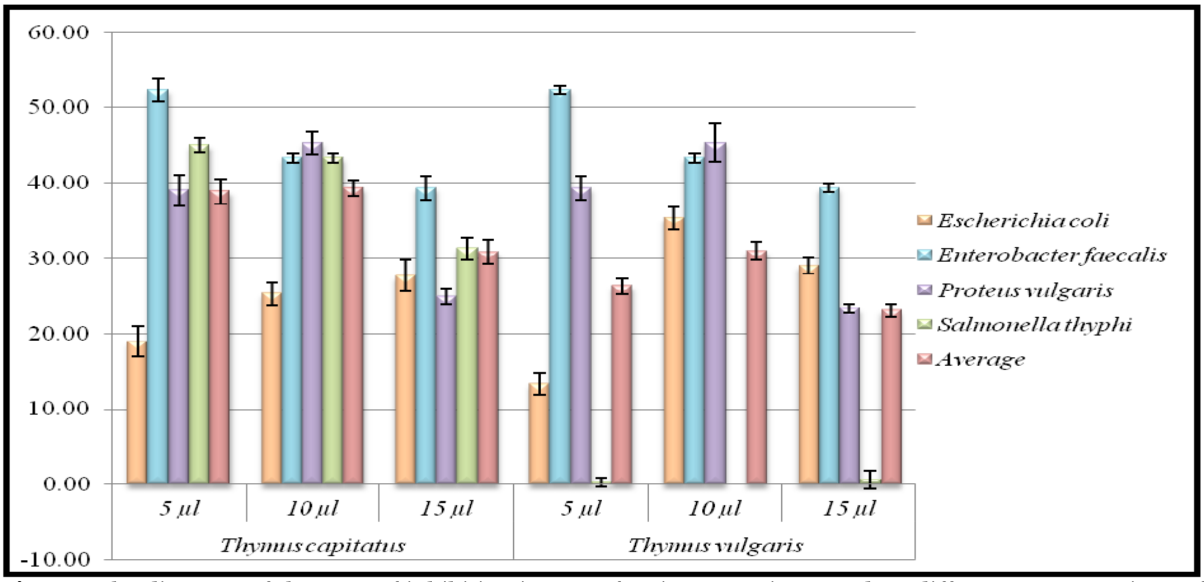

Fig. 15. The diameter of the zone of inhibition in mm of resistant strains tested on different concentrations of essential oils expressed in means ± standard deviations.

Conclusion

The in vitro results have shown the importance of the essential oil of Thymus capitatus and Thymus vulgaris, whose zone of inhibition, with a bactericidal and / or bacteriostatic effect. These oils have shown interesting antimicrobial properties.Thymus vulgaris oil showed bactericidal antibacterial activity against all microorganisms studied.

This allowed us to think about testing essential oils as an alternative to antibiotics (aromatherapy) in future research.

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