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Research Paper

Antibacterial Power of Lemon Essential Oil, Ginger And Their Synergy With Neem Vegetable Oil On Mastitis Pathogenic Germs Dairy Cows From The Poro Region In Ivory Coast

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Abstract: *Gas chromatography-mass spectrometry analysis of essential oils of (Citrus limon, Zingiber officinalis and Azadirachta indica vegetable oil), purchased, identified 32.80%, 42.40% and 47.10% of its constituents. This study consisted of evaluating the antimicrobial performance of some EOs obtained from three medicinal plants, considered to have a great bacterial effect. This is in order to standardize their use to apply them as an alternative remedy to antibiotics in the face of the advance of resistant bacteria and the failure of antibiotic therapy. The essential oils of these plants were tested alone or in combination on nine pathogenic microbial strains from cows suffering from mastitis and resistant to certain antibiotics, namely: four Gram (-) bacterial strains (Pseudomonas aeruginosa, E. coli ATCC25922, E. coli, Klebsiella spp) ; five Gram (+) bacterial strains (Staphylococcus aureus, Micrococcus spp, S. aureus ATCC19213, SCN (S. lentus and S. xylosus)); and E. coli ATCC25922, S. aureus ATCC19213 as reference strains. Reasonable antimicrobial activities of the studied EOs were observed on some of the germs tested. All the strains tested are sensitive to concentrations less than or equal to 100µg.ml $^{-1}$ of essential oil. Enterobacteria (Escherichia coli, Klebsiella spp) are resistant to standard antibiotics with the exception of Colistin, which have been shown to be sensitive to all essential oils studied. This observation applies to Gram-positive cocci (Staphylococcus aureus, Micrococcus spp, Staphylococcus lentus and Staphylococcus sylosus) which are very sensitive to the oils studied but resistant to three types of standard antibiotics (Ampicillin, Colistin and Penicillin). The CMB/MIC ratios of Zingiber officinalis and Citrus limon oils have a bacteriostatic action while that of the combination of EO (Zingiber officinalis, Citrus limon and Azadirachta indica oil) are bactericidal on all bacterial strains tested. There was no significant difference between the MIC and MBC values of the essential oil resistant strains. As a result of the study, an idea could be proposed for the development and upgrading of a new generation of combination of natural antimicrobial agents that can be used in humans and animals against infections with commonly used antibiotics.*

Keywords*: Mastitis, pathogenic germs, essential oils, antibiotics, Poro region.*

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I. INTRODUCTION

For a long time, the use of antibiotics to defeat infectious diseases has been a revolution in modern medicine, however it is obvious that each antibiotic has a limited lifespan at the end of which microorganisms develop resistance [1, 2]. The resurgence of this resistance to antibiotics represents an immense danger for global health and one of the greatest challenges facing public health. Among the agents involved in these pathologies, two bacteria Escherichia coli and Staphylococcus aureus are very frequently encountered and are at the forefront of the germs responsible for infections [3], and [4]. The fight against these multiresistant bacteria and the limitation of the risks of infections is important and the search for other therapeutic alternatives to stem certain infectious diseases is urgent. The search for biomolecules of plant origin with antimicrobial action is the subject of growing interest within the scientific community. Several previous studies have demonstrated that essential oils have significant antimicrobial activity due to the presence of high levels of secondary metabolites of the

terpene, alcohol, aldehyde or phenolic types [5]. Among these natural products, essential oils (EO) could be a credible alternative since they are known for their antimicrobial properties and diverse biological activities [6,7]. Some studies have concluded that Zingiber officinalis and Citrus limon have significant antibacterial activity on microbial strains [8,9,10]. Thus, the synergy of the components of an EO is therefore an essential factor in their antibacterial action [11]**.** Which brings us to were interested in this study, in three very widespread plant species used for their many therapeutic virtues. These are the essential oil of Zingiber officinalis, Citrus limon and the vegetable oil of Azadirachta indica. The objective is to verify the specificity of these species in terms of the antibacterial activity of these essential oils and their association of HE (Zingiber officinalis, citrus limon and vegetable oil of Azadirachta indica) according to the nine strains tested.

II. MATERIALS AND METHODS

Biological Material

In this work, the studied plant species of (Zingiber officinalis, Citrus limon and Azadirachta indica and (Azadirachta indica)) were purchased in March 2024 at the market (**Figure 1**). They are obtained by steam hydrodistillation. These plants were first dried for two weeks at most in the open air, protected from light and humidity. The extraction of essential oils was then carried out by steam distillation (hydrodistillation) by a Clevenger type device. For this, a plant mass of 100 g is immersed in distilled water in a flask with the addition of a few grains of pumice stone, the whole is then brought to the boil. The heating is maintained for three hours 30 minutes. The essential oils, less dense than water, float on the surface; They are then collected and stored in the refrigerator (+4˚C) in dark vials (bottles) in order to protect them from light and heat.

Figure 1: Essential oils of *(Lemon, Neem and Ginger)*

III. Method

Here, 2 to 5 mg of sample were diluted in 2 ml of dichloromethane and analyzed by gas chromatography-mass spectrometry (GC-MS). The analysis was carried out at the analysis and research center in the geochemistry department at PETROCI in Côte d'Ivoire. The GC-MS analysis was performed on a Perkin Elmer Clarus 680GC 600C MS instrument equipped with a 60 m long Restek Rtx-5ms column with an internal diameter of 0.25 mm and a stationary phase film thickness of 0.25 µm. Helium was used as the carrier gas at a fixed flow rate of 1 ml/min. The oven temperature program was 50°C for 5 min, then a gradient of 3°C/min was applied up to 250°C. This last temperature was maintained for 28 min, for a total analysis time of 100 min. The injector temperature was set at 250°C. The mass spectrometer was set to electron impact mode with an ionization source temperature of 200°C, an electron energy of 70 eV, a scan rate of 200 scans/min, and a scan range between 50 and 600 m/z.

Essential oil analysis

- **Lemon essential oil (*Citrus limon)***

The characterization of Citrus limon essential oil (Lisbon variety) was carried out by GC MS, 13 components were separated and identified (**Table 1**). The identification of the components showed that Citrus limon essential oil has five (5) major compounds; with a high rate of Tracetin (20.30%) followed by Trans-13-Octadecenoic acid (2.20%), 7-Methyl-Z-tetradecen-1-ol acetate (1.80%) and (1.50%), (1.40%) of Oleic Acid; α-Citral. And eight (8) of the compounds which are minor with a low rate of limonene and Linalool (0.30%) each, Linalyl acetate (0.40%), Glycerol 1,2-diacetate (0.50%), Neral (0.80%) and the other minor compounds **(Figure 2**).

- **Neem essential oil (Azadirachta indica)**

The characterization of Neem essential oil was carried out by GC-MS, 7 components were separated and identified (**Table 2**). The identification of the components showed that Neem essential oil has four (4) major compounds; with a high rate of Oleic Acid (21.50%) followed by Stearic Acid (9.20%), Palmitic Acid (7.90%) and Linoleic Acid (4.00%), (1.40%). And three (3) of the compounds which are in the minority with an average rate of Elaidic Acid and Acid-12-15-Octadecenoic (0.90%) each, of Acid 11-Octadecenoic (2.70%) (**Figure 3**).

- **Ginger essential oil (*Zingiber officinalis*)**

The characterization of Ginger essential oil was carried out by GC MS, 15 components were separated and identified (**Table 3**). The identification of the components showed that Ginger essential oil has six (6) major compounds; with a high rate of Oleic Acid (17.70%) followed by Palmitic Acid (9.20%), Linoleic Acid (4.70%) and Phthalic Acid mono-(2-ethylhexyl) ester (4.20%), Stearic Acid (1.90%); Erucic Acid (1.40%). And nine (9) of the minority compounds have a low level of Endo-Borneol and 7-epi-cis-sesquisabinene hydrate of (0.20%), Camphene, α-Terpinol, α-Curcumene and Beta-Sesquiphellandrene (0.30%) each, Eucalyptol (0.50%), Limonene and I-Zingiberene of (0.60%) each (**Figure 4**).

Table 1 : Chemical composition of Citrus limon essential oil (Lisbon variety) extracted by hydrodistillation

	Compound Name	Reten. Time [min]	Area [mV.s]	Area [%]
70	Limonene	11,593	186722701,361	0,3
96	Linalool	15,372	150753787,188	0,3
140	Glycerol 1,2-diacetate	22,258	264994072,000	0,5
143	Neral	22,662	433093432,105	0,8
147	Linalyl acetate	23,977	242974671,619	0,4
148	α-Citral	24,153	821762616,223	1,4
160	Triacetin	27,568	11530416311,690	20,3
206	Diethyl Phthalate	39,121	511274760,579	0,9
317	Linoleic acid	60,514	695125312,258	1,2
318	Oleic Acid	60,730	856393150,490	1,5
356	12- Methyl E-E-2,13-octadecadien-1-ol	69,842	696158293,726	1,2
357	trans-13-Octadecenoic acid	70,115	1227400576,104	2,2
392	7-Methyl-Z-tetradecen-1-ol acetate	76,950	1038954739,618	1,8
		Total	56683564358,280	100,0

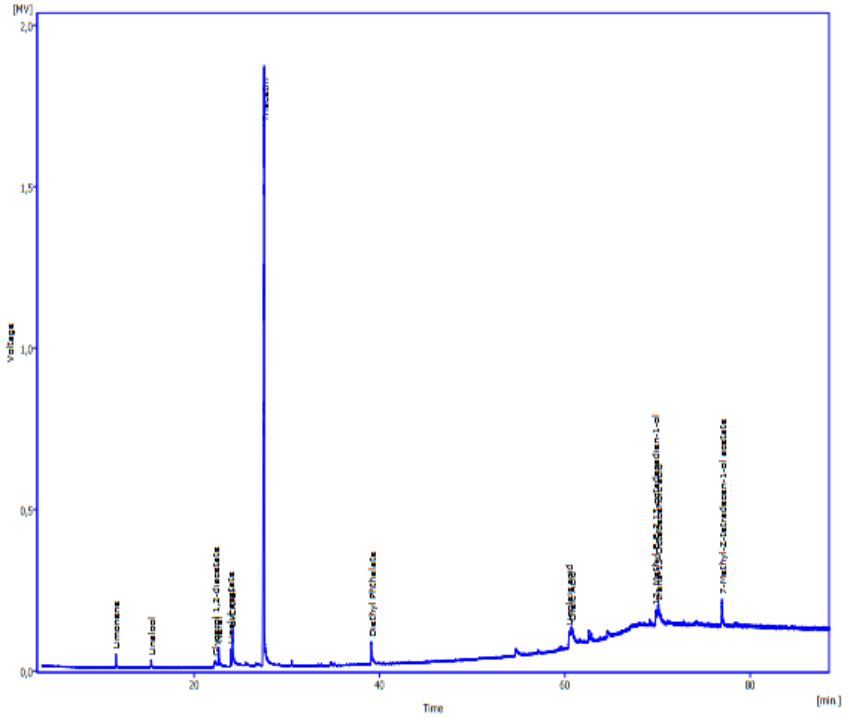

Figure 2: GC-MS of Lemon essential oil

Table 2 *:* Chemical composition of Ginger essential oil (variety) extracted by hydrodistillation

	Compound Name	Reten. Time [min]	Area [mV.s]	Area [%]
37	Camphene	7,004	98998514,391	0,3
63	Eucalyptol	11,592	186763493,679	0,5
64	Limonene	11,678	218753118,652	0,6
111	endo-Borneol	19,103	56579485,183	0,2
120	α-Terpineol	20,427	89899267,189	0,3
217	α-Curcumene	35,476	111367250,613	0,3
220	l-Zingiberene	36,215	217642234,919	0,6
223	7-epi-cis-sesquisabinene hydrate	36,886	75108551,699	0,2
227	β-Sesquiphellandrene	37,506	108891512,840	0,3
310	Palmitic acid	53,522	3285530424,756	9,2
344	Linoleic acid	59,298	1672111734,548	4,7
345	Oleic Acid	59,593	6308226173,103	17,7
347	Stearic acid	60,566	678412089,363	1,9
425	Erucic acid	72,874	491761114,432	1,4
429	Phthalic acid, mono-(2-ethylhexyl) ester	73,727	1512053632,467	4,2
		Total	35585663123,350	100,0

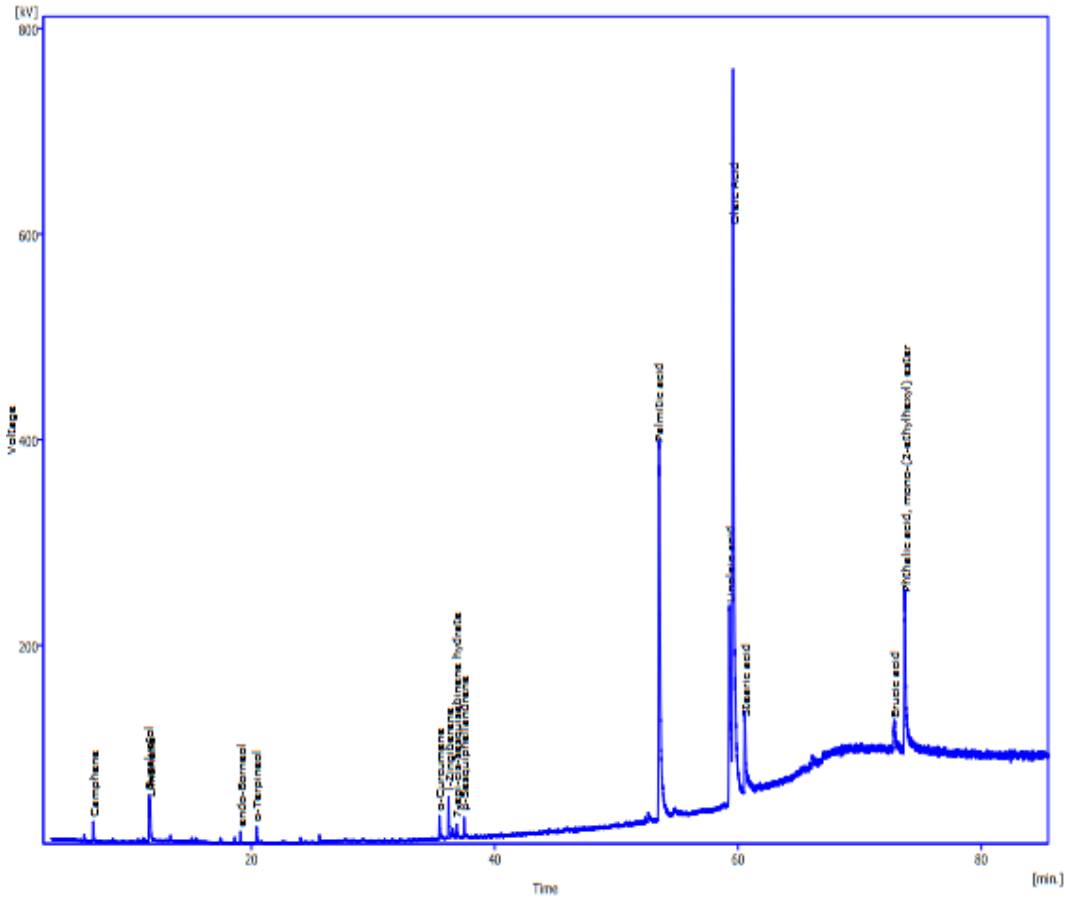

Figure 3 : *Zingiber officinalis* essential oil

Table 3 : Chemical composition of Neem essential oil (variety) extracted by hydrodistillation

	Compound Name	Reten. Time [min]	Area [mV.s]	Area [%]
305	Elaidic acid,	53,522	853051677,774	0,9
308	Palmitic acid	54,789	7851084984,680	7,9
332	12-15-Octadecadienoic acid	59,264	912257359,483	0,9
333	11-Octadecenoic acid,	59,571	2669468178,049	2,7
337	Linoleic acid	60,543	4036892135,213	4,0
338	Oleic Acid	60,918	21451124541,670	21,5
339	Stearic acid	61,720	9227513166,211	9,2
		Total	99939708180,690	100,0

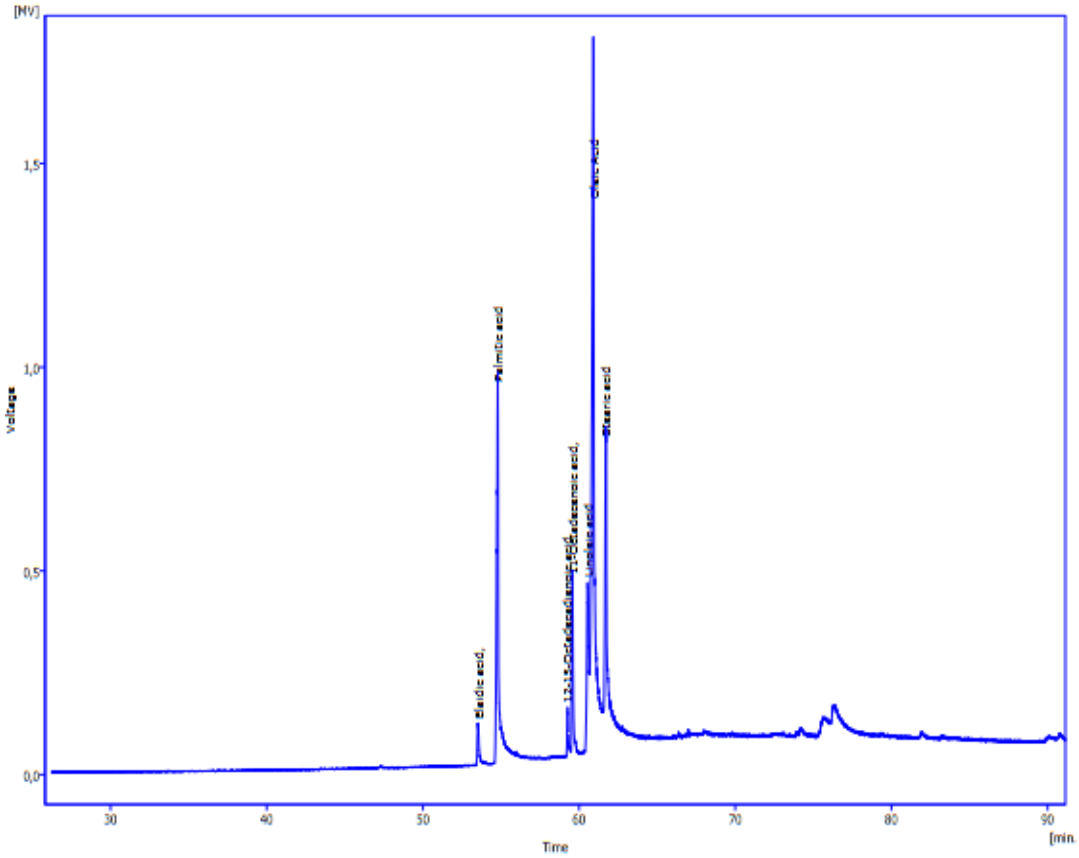

Figure 4 : GC-MS of Neem vegetable oil

Bacterial germs

To evaluate the antibacterial activity of our essential oils, we used nine (09) germs including eight (8) pathogens of GRAM negative Bacilli belonging to the Enterobacteriaceae, Pseudomonadaceae families and GRAM positive Cocci of the Staphylococcaceae family. The pathogenic strains were collected from milk samples from different quarters of dairy cows with mastitis from sites in the Poro region, except for the two reference strains which were purchased see **table 4.** The bacteria were identified by the classic microbiology method (API Staph gallery and API 20 ^{E} etc.). The table shows the origin of each selected microbial strain. The antibiotic sensitivity of the strains is determined by the antibiogram test according to the recommendations of the European Antibiogram Committee (EUCAST, 2023).

Antibacterial activity

To test the antimicrobial activity of the essential oils, we first diluted them in 95% acetone to obtain the following concentrations: 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.25 µg/ml, 6.125 µg/ml, 3.06 µg/ml for the three essential oils.

Evaluation of antimicrobial activity by diffusion method

It consists of making a well with the back of a sterile pipette and putting 75 µl of essential oil in the wells (three), on a microbial mat and then measuring the area where the microorganisms could not develop. This technique used is a transformation of the method of [12]. The inhibition diameter, which reflects the antimicrobial activity of the essential oil, is thus determined as a translucent halo around the well. A microbial suspension of density equivalent to the 0.5 Mac Farland standard (10^{8} CFU.ml $^{-1}$) is prepared by suspending a few bacterial colonies in a saline solution (0.9% Nacl) or in a physiological solution. The Petri dishes containing the agar culture medium (Mueller Hinton) are inoculated in a layer with the inoculum. On the surface of each dish, a well with a filter paper dimension (Wattman n°4) of 6 mm in diameter was filled with 75 µL of essential oil, a well filled with 75 µL of distilled water is used as a negative or sterility control on the same dish. The dishes are left for one hour at room temperature to allow the diffusion of the essential oil, then they are incubated at 37 ° C for 18 to 24 hours for each bacterium. After incubation, the inhibition diameter is measured in millimeters, well included.

Reading the results

Antimicrobial activity is manifested by the appearance of a halo of inhibitions of microbial growth around the wells containing the extract to be tested. The result of this activity is expressed by the diameter (D) of the inhibition zone indicated as below and can be symbolized by plus (+) or minus (-) according to [13].

- D< 8mm: Resistant strains (-).
-9mm≤D≤14mm: Sensitive strains (+).
-15mm≤D≤19mm: Very sensitive strains (++).
-D> 20 mm: Extremely sensitive strains (+++)

Measure with a tracer the diameters (D) of the inhibition zones from the outside of the closed box in order to avoid infections. The classification of the bacteria used in this test is done in one of the categories: sensitive or resistant according to the effectiveness of the extracted EOs.

Minimum inhibitory concentration (MIC)

One (1) ml of each dilution (200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.125 µg/ml, 3.06 µg/ml) of the oils to be tested are placed in tubes containing one ml of the bacterial inoculum. Before this experiment, tubes are inoculated from a bacterial culture in the exponential growth phase in order to obtain a final cell concentration of approximately 10^{6} to 10^{8} CFU/ml. The tubes that will serve as a control do not contain essential oil. The negative control is kept at 4°C while the positive control is incubated at the same time as the tubes containing the different concentrations of essential oil at 37°C for 18 hours. This technique consists of inoculating in a 5 cm streak on a box A, the bacterial inoculum, a decreasing concentration range in essential oil (1 ml of the concentration of EO plus 1 ml of the bacterial inoculum). After incubation, observation of the range allows access to the minimum inhibitory concentration (MIC), which corresponds to the lowest concentration of essential oil capable of inhibiting the growth of 90% of the microbial population.

Minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) corresponds to the lowest concentration of essential oil capable of killing more than 99.9% of the initial microbial inoculum (i.e. less than 0.1% of survivors); it defines the bactericidal effect of an essential oil [14]. The inoculation of a plate B starts from the 5 cm streaks from the MIC of plate A and the concentrations higher than the MIC; the reading of both plates A and B determines the MBC. Here, the bacterial suspension is subcultured from the tubes showing a complete absence of bacterial growth and then deposited in 5 cm streaks on agar appropriate for each germ (Mueller Hinton). The inoculated plates are incubated for 18 to 24 hours at 37 ° C. The MBC of the essential oil is deduced from the concentration of the experimental tube whose number of colonies on the streak (box B) is greater than or equal to the number of colonies of the lowest dilution on box A or devoid of bacteria. For each of the tests carried out; diffusion in solid medium, MIC and MBC the number of repetitions is three times. The antibacterial effect has been proclaimed bactericidal or bacteriostatic depending on the MBC/MIC ratio. Indeed, if MBC/MIC = 1 to 2, the effect is bactericidal and if MBC/MIC = 4 to 16, the effect is bacteriostatic [15].

IV. Expression of Results

Testing the sensitivity of germs to certain antibiotics

The results of the antibiograms carried out for the isolates (by the classic disk diffusion method) are shown in Table ***5. A significant number of*** *E. coli* strains (references and isolates), *Klebsiella spp*, are observed, which react differently to the antibiotics tested, which shows the mutated nature of these germs which gives them resistance to antibiotics. Among the germs studied, there are those which are resistant to several families of antibiotics (*Pseudomonas aeruginosa* and *Micrococcus spp.).*

Action of essential oils on isolates

In the samples of pathogenic bacteria, reference collected and used, we can note the high frequency of strains of Enterobacteriaceae (*E. coli, Klebsiella spp* and *E. coli ATCC25922)* which are a problem for the infection of cows. And are resistant to several families of antibiotics. The diameters of the inhibition halos and the minimum inhibitory and bactericidal concentrations of the essential oil of Zingiber officinalis, citrus limon and the combination of EO (Zingiber officinalis, citrus limon and Azadirachta indica oil) against the nine strains are grouped in Table 4.

Statistical analysis

The Excel 2016 spreadsheet was used to record and collect the data. The tests were carried out three times and the data were expressed as mean ± standard deviation. The statistical study was carried out by the **XLSTAT software version 2019.** Descriptive statistics were performed for all variables. This software also made it possible to calculate the means and standard deviations of the parameters analyzed. The differences are considered significant at the 5% probability threshold ($p < 0.05$). The determination of the effective concentration was carried out by determining the MIC and the MBC according to the concentrations studied.

Table 4 : Sources of the different tested

Group/Gram	Pathogenic germs	Family	Type of sampling
Gram-negative bacilli	*Pseudomonas aeruginosa*	Pseudomonadaceae	Milk of the neighborhoods
	E. coli ATCC25922 (reference strain)	Enterobacteriaceae	
	E. coli		
	Klebsiella spp		
Cocci Gram positives	*Staphylococcus aureus*	Staphylococcaceae	
	S. aureus ATCC19213 (reference strain)		
	Micrococcus spp		
	SCN (S. lentus et S. xylosus)		

Table 5 : Antibiotic susceptibility profile of the isolates

ANTIBIOTICS TESTED	Enterobacteriaceae			Staphylococcaceae				Pseudomonadaceae
	E. coli	Klebsiella spp	E. coli ATCC25922	S. lentus	S. xylosus	S. aureus	S. aureus ATCC19213	Pseudomonas aeruginosa
CEFALEXINE	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**R**
CHLORAMPHENICOL	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**R**
AMPICILINE	**R**	**R**	**R**	**R**	**R**	**S**	**S**	**R**
COLISTINE	**S**	**S**	**S**	**R**	**R**	**R**	**R**	**R**
NEOMYCINE	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**R**
PENICILLINE	**R**	**R**	**R**	**R**	**R**	**S**	**S**	**R**
GENTAMICILE	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**R**
SPIRAMYCINE	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**R**
TRETRACYCLINE	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**R**
TRIMETHOPRIME - SULFAMETHOXAZOLE	**R**	**R**	**R**	**S**	**S**	**S**	**S**	**R**

Antibacterial effects of essential oils

- **Citrus limon essential oil**

The study carried out by the Kirky-Bauer method shows an inhibition of microbial growth proportional to the diameter of the inhibition zone, as shown in **Table 6.** The inhibition diameters generated by essential oils are very variable depending on the oil used. Essential oils are considered inactive if they produce microbial growth inhibition diameters less than or equal to 8 mm [13]. With inhibition diameters greater than or equal to 9 mm,

the essential oils of Zingiber officinalis, citrus limon and the association of EO (Zingiber officinalis, citrus limon and Azadirachta indica oil) present a sensitivity on the strains of bacteria tested. On the other hand, the association of EO (Zingiber officinalis, citrus limon) and Azadirachta indica oil considerably inhibits the bacterial growths tested.

- **Zingiber officinalis essential oil**

Well aromatogram on agar medium, allowed us to highlight the antibacterial power of Ginger essential oil against the bacterial strains tested. According to Table **7**, we note that the bacterial strains tested are sensitive to the essential oil studied, we also note that the Gram + positive strains (Staphylococcus aureus, S. aureus ATCC19213, Staphylococcus lentus and Staphylococcus sylosus) have larger inhibition zones (in diameters) and are the most sensitive compared to Gram negative strains (Escherichia coli, E. coli ATCC25922, Pseudomonas aeruginosa, Klebsiella spp). And with the exception of Gram + positive Micrococcus spp which remains resistant.

- **Azadirachta indica essential oil**

Here, the aromatogram on agar medium, made it possible to profile the antibacterial power of Neem essential oil against the bacterial strains tested. According to Table **8**, we note that the bacterial strains tested are sensitive to the essential oil studied, we also note that Gram-negative bacilli (*Escherichia coli, E. coli ATCC25922, Klebsiella spp)* are the most sensitive strains compared to Gram-positive strains (*Staphylococcus aureus, S. aureus ATCC19213, Staphylococcus lentus* and *Staphylococcus sylosus*). And with the exception of *Pseudomonas aeruginosa* and *Micrococcus spp* which were shown to be resistant.

The well diffusion method on agar medium allowed us to highlight the antibacterial power of the combination of EO (Zingiber officinalis, citrus limon and Azadirachta indica oil) against the bacterial strains studied. According to the table, the diameters of the inhibition zones (including the diameter of the wells) are indicated, we note that the bacterial strains studied are sensitive to the essential oil tested, we also note that the Gram-positive strains (Staphylococcus aureus, S. aureus

ATCC19213, Micrococcus spp, Staphylococcus lentus and Staphylococcus sylosus) are the most sensitive strains compared to Gram negative strains (Escherichia coli, E. coli ATCC25922, Pseudomonas aeruginosa, Klebsiella spp).

Table 6 : Diameters (mm) of inhibition zones of Citrus limon essential oil

Germs identified	Lemon HE inhibition diameter (mm)	Sensibility
Pseudomonas aeruginosa	8,33±0,33	Resistant
E. coli ATCC25922	9,33±0,33	Sensible
E. coli	10±00	Sensible
Klebsiella spp	10,66±1,22	Sensible
Staphylococcus aureus	13±1	Sensible
S. aureus ATCC19213	12±2,64	Sensible
Micrococcus spp	8±00	Resistant
Staphylococcus lentus	11±1,22	Sensible
Staphylococcus sylosus	12±2.44	Sensitive

NB : HE **(**Essential oil)

Table *7* : Diameters (mm) of Ginger Essential Oil Inhibition Zones

Identified germs	Ginger HE Inhibition Diameter	Sensitivity
Pseudomonas aeruginosa	8.66±0.90	Resistant
E. coli ATCC25922	10.66±00	Sensitive
E. coli	11.33±0.57	Sensitive
Klebsiella spp	11.66±1.22	Sensitive
Staphylococcus aureus	10.33±1	Sensitive

S. aureus ATCC19213	10±2.64	Sensitive
Micrococcus spp	8.33±1.15	Resistant
Staphylococcus lentus	9±1	Sensitive
Staphylococcus sylosus	10.66±0.57	Sensitive

Table 8 : Diameters (mm) of zones of inhibition of HE association (Zingiber officinalis, lemon citrus and vegetable oil Azadirachta indica

Identified germs	Inhibition diameter of Lemon HE + Ginger HE + Neem vegetable oil (mm)	Sensitivity
Pseudomonas aeruginosa	9.33±0.70	Sensitive
E. coli ATCC25922	11±1.22	Sensitive
E. coli	11.33±0.57	Sensitive
Klebsiella spp	12±1	Sensitive
Staphylococcus aureus	16±1.73	Very Sensitive
S. aureus ATCC19213	14±2.12	Sensitive
Micrococcus spp	9.66±0.90	Sensitive
Staphylococcus lentus	12.66±2.64	Sensitive
Staphylococcus sylosus	12.33±1.22	Sensitive

Comparison of the inhibitory activity of standard antibiotics and essential oils

This study allowed us to make a remarkable comparison between the inhibitory activity of the growth of germs by standard antibiotics and the antimicrobial effect of our essential oils. The results obtained showed an effectiveness, since most of the strains selected for this study present an almost total resistance to all the standard antibiotics tested (Table 5). Enterobacteria (Escherichia coli, Klebsiella spp) which are resistant to all the standard antibiotics tested with the exception of Colistin, were shown to be sensitive to all the essential oils studied. This observation also applies to Gram-positive cocci (Staphylococcus aureus, Micrococcus spp, Staphylococcus lentus and Staphylococcus sylosus) which are very sensitive to the oils studied while they are resistant to three types of standard antibiotics (Ampicillin, Colistin and Penicillin). This finding is consistent with the hypothesis we proposed earlier that essential oils can be used as an antibacterial alternative for germs at risk of resistance to standard antibiotics without therapeutic treatment. It is therefore necessary for us to determine the MICs for these oils as well as their MBCs in order to assess their antimicrobial efficacy and bactericidal power.

Evaluation of CMI and CMB

Determination of the minimum inhibitory concentration (MIC)

The results obtained are shown in Table 62. These results are clearly in agreement with the study of essential oil filtration carried out previously, by the agar diffusion method. Because it is the essential oils of Zingiber officinalis, citrus limon which present the highest MIC and the association of EO (Zingiber officinalis, citrus limon and Azadirachta indica oil) which present the lowest MIC for almost all the strains studied. Therefore, the essential oils of Zingiber officinalis, citrus limon remain less effective and the association of EO (Zingiber officinalis, citrus limon and Azadirachta indica oil) is therefore the most effective and interesting.

Determination of the minimum bactericidal concentration (MBC) in solid medium

It allows to determine the bactericidal effect of the essential oil studied. In this study the CMB is evaluated for the essential oils of Zingiber officinalis, citrus limon and the association of HE (Zingiber officinalis, citrus limon and Azadirachta indica oil) which presented the highest antimicrobial activity. Table **9** presents the MBCs for the germs tested. The MBC/MIC ratio is used to define the bacteriostatic or bactericidal character of an essential oil. When this ratio is less than 4 [16], the essential oil is considered bactericidal. In this study, the MBC/MIC ratios of Zingiber officinalis and citrus limon oils are equal to 4 for all the microbial strains studied while that of the association of EO (Zingiber officinalis, citrus limon and Azadirachta indica oil) is between 1 and 2. These associations of essential oils between 1 and 2 therefore have a bactericidal action against all the bacterial strains tested and that of Zingiber officinalis and citrus limon therefore exert a bacteriostatic action on the different bacterial strains.

Here we see that all strains were totally inhibited at 100µg.ml $^{-1}$, *Pseudomonas aeruginosa*, *Micrococcus spp* are the strains with high resistance compared to other strains tested (Escherichia coli, *E. coli ATCC2592***,**

Staphylococcus aureus, Klebsiella spp, Staphylococcus lentus, Staphylococcus sylosus). We also note that these Gram-positive and negative bacteria are resistant to the essential oil studied and the most resistant with MICs of 6.125 µg.ml $^{-1}$ each. According to the study, the CMB/MIC ratio is greater than 4 for lemon EO, so the essential oil studied has a bacteriostatic power against bacterial strains. Here, the table shows us that all the strains tested with Ginger essential oil were totally inhibited at 50µg.ml $^{-1}$. *Micrococcus spp and Pseudomonas aeruginosa* are the strains with high resistance compared to the other strains tested (Escherichia coli, *E. coli ATCC2592, Klebsiella spp, Staphylococcus lentus, Staphylococcus sylosus, Staphylococcus aureus, S. aureus ATCC19213*). We also note that Gram-positive bacteria in particular (Staphylococcus aureus, *S. aureus ATCC19213, Staphylococcus lentus, Staphylococcus sylosus*) and Gram-negative bacilli (Escherichia coli, *E. coli ATCC2592, Klebsiella spp*) are moderately resistant to the ginger essential oil studied compared to bacteria (*Micrococcus spp and Pseudomonas aeruginosa)* with MICs of 3.06 µg.ml $^{-1}$, 6.125 µg.ml $^{-1}$ respectively. According to the results, the CMB/MIC ratio is greater than 4 for ginger EO, so the essential oil studied has a bacteriostatic power against the bacterial strains tested. Referring to the table, we see that all strains were totally inhibited at 100µg.ml $^{-1}$. *Pseudomonas aeruginosa, Klebsiella spp and Staphylococcus aureus* are the strains with high sensitivity compared to the other strains tested (*Staphylococcus lentus, Staphylococcus sylosus , Staphylococcus aureus , S. aureus ATCC19213, E. coli ATCC2592, Micrococcus spp,* Escherichia coli). We also see that Gram-positive and Gram-negative bacteria are overall sensitive to the essential oil studied with MICs of 25 µg.ml $^{-1}$ to 100 µg.ml $^{-1}$.

According to the study, the CMB/CMI ratio is less than 4 for the combination of EO (Lemon, Ginger) and neem oil, so the essential oil studied has a bactericidal power against bacterial strains.

Table 9 : Minimum inhibitory concentrations (MIC) and bactericidal concentrations (MBC) of HE

Pathogenic germs	Lemon HE			Ginger HE			lemon HE + Ginger HE + Neem vegetable oil		
	MBC (µg/ml)	MIC (µg/ml)	MBC/MIC	MBC (µg/ml)	MIC(µg/ml)	MBC/ MIC	MBC (µg/ml)	MIC(µg/ml)	MBC/MIC
Pseudomonas aeruginosa	50	6,125	8	25	3,06	8	50	25	2
E. coli ATCC25922	50	12,25	4	25	6,125	4	100	100	1
E. coli	50	12,25	4	50	12,25	4	50	25	2
Klebsiella spp	50	12,25	4	50	12,25	4	100	100	1
Staphylococcus aureus	25	6,125	4	25	6,125	4	25	25	1
S. aureus ATCC19213	25	6,25	4	25	6,125	4	25	12,25	2
Micrococcus spp	50	6,125	8	50	6,125	8	25	12,25	2
Staphylococcus aureus	50	12,25	4	50	12,25	4	50	25	2
Staphylococcus sylosus	50	12.25	4	50	12.25	4	50	25	2

V. DISCUSSION

The essential oils of Zingiber officinalis, Citrus limon, and Azadirachta indica purchased, are extracted by a hydrodistillation technique, this extraction method is standardized for the extraction of essential oils [17]. Our results show an inhibition of microbial growth proportional to the diameter of the inhibition zone. The inhibition diameters, generated by essential oils, are very variable depending on the oil used. Essential oils are considered active if they produce microbial growth inhibition diameters greater than or equal to 9 mm [13. With inhibition diameters less than 9 mm, the essential oils of (Zingiber officinalis, Citrus limon, and the combination of EO (Zingiber officinalis, citrus limon and vegetable oil Azadirachta indica oil) present a non-sensitive or resistant bacterial activity on the bacteria tested. On the other hand, the essential oils of Zingiber officinalis, Citrus limon, and that of the combination of EO (Zingiber officinalis, citrus limon and Azadirachta indica oil) strongly inhibit the growth of the tested strains as well as bacterial if the inhibition halos are 9 to 30 mm and more. Even if some studies have concluded that Zingiber officinalis and Citrus limon have significant antibacterial activity on strains of Staphylococcus aureus Escherichia coli and Pseudomonas aeruginosa [8,9] ; then in accordance with the work of [10] who showed strong antibacterial activity of Zingiber officinalis, Citrus limon on different Gram+ and Gram- strains. The microbial response also varies from one strain to another; some strains; *Staphylococcus aureus , S. aureus ATCC19213 , Staphylococcus sylosus , Escherichia coli , E. coli ATCC25922, Klebsiella spp* showed notable sensitivity to most of the essential oils tested, while *Pseudomonas aeruginosa* and *Micrococcus*

spp showed resistance or even average sensitivity to all essential oils used, even those that showed a significant inhibitory effect such as the combination of EOs (Zingiber officinalis, Citrus limon and Azadirachta indica oil). This effect is less pronounced on reference strains (*S. aureus ATCC19213, E. coli ATCC25922)* and more on isolated strains, resistant to reference antibiotics with more efficacy on *Staphylococcus aureus, S. aureus ATCC19213, Staphylococcus sylosus , Escherichia coli , E. coli ATCC25922, Klebsiella spp* . Usually Gram-bacteria are known for their resistance while Gram+ bacteria have an inhibitory effect on EOs thanks to the particular structure of their external membrane [18]. The aromatogram allowed us to highlight the antibacterial power of Citrus limon essential oil according to the bacterial strains tested, the sensitivity of the strains is classified according to the scale of [13]. We also note that Gram+ positive strains (Staphylococcus aureus, S. aureus ATCC19213, Staphylococcus lentus and Staphylococcus sylosus) have larger inhibition zones (in diameters) and are the most sensitive compared to Gram-negative strains (Escherichia coli, E. coli ATCC25922, Pseudomonas aeruginosa, Klebsiella spp). And with the exception of Gram+ positive Micrococcus spp which remains resistant. This is due to the structure of Gram negative bacteria which has a lipopolysaccharide membrane which is considered a barrier to hydrophobic compounds [19]. Ginger essential oil with respect to the bacterial strains tested, showed the sensitivity of the strains and the diameters of the inhibition zones. We note that the bacterial strains tested are sensitive to the essential oil studied, we also note that Gram negative bacilli (*Escherichia coli, E. coli ATCC25922, Klebsiella spp*) are the most sensitive strains compared to Gram positive strains (Staphylococcus *aureus, S. aureus ATCC19213, Staphylococcus lentus* and *Staphylococcus sylosus*). And with the exception of *Pseudomonas aeruginosa* and *Micrococcus spp* which have shown resistance. And the combination of essential oil of (Lemon, Ginger and Neem vegetable oil) remains sensitive depending on the strains. We note that the bacterial strains studied are sensitive to the essential oil tested, we also note that the Gram positive strains (Staphylococcus aureus, S. aureus ATCC19213, Micrococcus spp, Staphylococcus lentus and Staphylococcus sylosus) are the most sensitive strains compared to the Gram negative strains (Escherichia coli, E. coli ATCC25922, Pseudomonas aeruginosa, Klebsiella spp). Our results clearly support the screening study of essential oils carried out previously, by the agar diffusion method. The essential oils of Zingiber officinalis and citrus limon have the highest MIC and the combination of essential oil of (Lemon, ginger and Neem vegetable oil (Azadirachta indica)) have the lowest MIC for almost all germs. These results are supported by the works [20], as well as those [9] which have also proven the antimicrobial activity of the essential oils of these two plants at very low MIC. The combination of essential oils of (Zingiber officinalis, citrus limon and neem oil) are therefore the most effective. It allows to determine the bactericidal effect of the combination of essential oils studied. In this study the MBC is evaluated for the essential oils of Zingiber officinalis and Citrus limon, of the combination of the three oils which have presented the highest antimicrobial activity. The CMB/MIC ratio is used to define the bacteriostatic or bactericidal character of an essential oil. When this ratio is less than 4 [20], the essential oil is considered bactericidal. In this study, the CMB/MIC ratios of Zingiber officinalis and Citrus limon oils are greater than or equal to 4 for all microbial strains, while that of the association of essential oils (Lemon, ginger and Azadirachta indica oil) is between 1 and 2. These associations of essential oils between 1 and 2 therefore have a bactericidal action against all the bacterial strains tested and that of *Zingiber officinalis* and *Citrus limon* therefore exert a bacteriostatic action on different bacterial strains. However, before using an antibacterial molecule as a preservative in a food, the minimum inhibitory concentration (MIC) must be estimated. It is intolerable when the effective antibacterial doses exceed the organoleptic acceptable levels. Therefore, these concentrations are determined in order to define the boundaries of sensory acceptability and antibacterial efficacy of essential oils [21]**.** According to [22], the smaller the inhibition zone, the lower the concentration of antimicrobial required to inhibit the growth of microorganisms. When the concentration of the antimicrobial becomes very diluted, it can no longer inhibit the growth of the tested bacteria, the inhibition zone is demarcated. The diameter of this inhibition zone is correlated with the minimum inhibitory concentration (MIC) for the particular bacteria/antimicrobial combination, the inhibition zone corresponds inversely to the MIC of the test [22]. On the other hand, all our bacterial strains were totally inhibited at 50µg.ml $^{-1}$ for lemon oil. *Pseudomonas aeruginosa, Micrococcus spp* are the strains with high resistance compared to the other strains tested (Escherichia coli, *E. coli ATCC2592, Staphylococcus aureus, Klebsiella spp, Staphylococcus lentus, Staphylococcus sylosus*). We also note that these Gram-positive and negative bacteria are resistant to the essential oil studied and the most resistant with MICs of 6.125 µg.ml $^{-1}$ each. The CMB/MIC ratio is greater than or equal to 4 for Lemon EO, so the essential oil studied has a bacteriostatic power depending on the bacteria [20]. The germs were totally inhibited at 50µg.ml $^{-1}$ for Ginger essential oil. *Micrococcus spp and Pseudomonas aeruginosa* are the strains with high resistance compared to the other strains tested (Escherichia coli, *E. coli ATCC2592, Klebsiella spp, Staphylococcus lentus, Staphylococcus sylosus, Staphylococcus aureus, S. aureus ATCC19213*). We also note that Gram-positive bacteria in particular (Staphylococcus aureus, *S. aureus ATCC19213, Staphylococcus lentus, Staphylococcus sylosus)* and Gram-negative bacilli (Escherichia coli, *E. coli ATCC2592, Klebsiella spp*) are moderately resistant to the ginger essential oil studied compared to bacteria (*Micrococcus spp and Pseudomonas aeruginosa)* with MICs of 3.06

µg.ml $^{-1}$, 6.125 µg.ml $^{-1}$ respectively. According to our results, the CMB/MIC ratio is greater than or equal to 4 for ginger EO, so the essential oil studied has a bacteriostatic power on bacterial strains [20]. At the level of the oil combination, we note that all strains were totally inhibited at 100µg.ml $^{-1}$. *Pseudomonas aeruginosa, Klebsiella spp and Staphylococcus aureus* are the strains with a high sensitivity compared to the other strains tested (*Staphylococcus lentus, Staphylococcus sylosus, Staphylococcus aureus, S. aureus ATCC19213, E. coli ATCC2592, Micrococcus spp,* Escherichia coli). We also note that Gram-positive and Gram-negative bacteria are overall sensitive to the essential oil studied with MICs of 25 µg.ml $^{-1}$ to 100 µg.ml $^{-1}$. According to the study, the CMB/MIC ratio is less than 4 for the combination of EO (Lemon, Ginger) and neem oil, so the essential oil studied has a bactericidal power against bacterial strains. Thus, the synergy of the components of an EO is therefore an essential factor in their antibacterial action [11]**.** It is well known that the antimicrobial activity of essential oil is generally attributed to monoterpenes [23,24] which are present in Citrus limon and Zingiber officinalis oil with a minority rate. The sensitivity of germs, even those resistant to antibiotics, to the essential oil and even to the combination of EO (Lemon, Ginger and Neem oil), suggests its possible use in therapy as a natural alternative. The antibacterial action observed by the essential oil of lemon, ginger and the combination of the three oils was revealed by GC-MS analysis and is related to the presence of monoterpenes (Linalool 0.30%, Neral 0.80%, limonene and Linalool 0.30%, Camphene 0.30%, α-Terpinol 0.30%, α-Curcumene 0.30%) and sesquiterpenes (Beta-Sesquiphellandrene 0.30%, Eucalyptol 0.50%, etc.), and volatile compounds. These compounds are known to have a great antibacterial action. Several previous works have demonstrated that essential oils have significant antimicrobial activity due to the presence of high levels of secondary metabolites of terpene, alcohol, aldehyde or phenolic types [5]. The characterization of the essential oil of Citrus limon (Lisbon variety) and ginger EO shows low characteristics in monoterpenes, sesquiterpenes and dominant in other unidentified compounds. Citrus limon essential oil has major compounds in Triacetin (20.30%) followed by Trans-13-Octadecenoic acid (2.20%), 7-Methyl-Z-tetradecen-1-ol acetate (1.80%) and (1.50%), (1.40%) Oleic Acid; α-Citral. And compounds that are minor in Limonene and Linalool (0.30%) each, Linalyl acetate (0.40%), Glycerol 1,2-diacetate (0.50%), Neral (0.80%) and other minor compounds. However, Ginger essential oil (variety) has six (6) major compounds; with a high rate of Oleic Acid (17.70%) followed by Palmitic Acid (9.20%), Linoleic Acid (4.70%) and Phthalic Acid mono-(2-ethylhexyl) ester (4.20%), Stearic Acid (1.90%); Erucic Acid (1.40%). And nine (9) of the minority compounds have a low rate of Endo-Borneol and 7-epi-cis-sesquisabinene hydrate of (0.20%), Camphene, α-Terpinol, α-Curcumene and Beta-Sesquiphellandrene (0.30%) each, Eucalyptol (0.50%), Limonene and I-Zingiberene of (0.60%) each. On the other hand, Neem essential oil has majority compounds; with a high rate of Oleic Acid (21.50%) followed by Stearic Acid (9.20%), Palmitic Acid (7.90%) and Linoleic Acid (4.00%), (1.40%). And compounds which are in the minority with an average rate of Elaidic Acid and 12-15-Octadecenoic Acid (0.90%) each, 11-Octadecenoic Acid (2.70%). And that the HE of (lemon and ginger) did not show a strong antibacterial activity on Staphylococcus *aureus, S. aureus ATCC19213, Staphylococcus sylosus , Escherichia coli , E. coli ATCC25922* and other germs involved in the contamination of milk from cow quarters; on the other hand, their association with Neem oil remains appreciable. For the test with transcutaneous application of a mixture of EOs, the waiting time was arbitrarily set at 4 days [25]. Other practitioners recommend a waiting period of 48 hours even for a transcutaneous application on the udder [26]. In the scientific literature, publications concerning EO residues in milk are few in number compared to antibiotic residues. Thus, the study made it possible to evaluate the antibacterial activity of essential oils (Zingiber officinalis, Citrus limon and their association with Azadirachta indica oil) on bacteria responsible for mastitis in dairy cows. The aim of this study was to investigate whether essential oils could contribute to the effective treatment of this pathology.

VI. CONCLUSION

Ultimately, the essential oils of Zingiber officinalis and Citrus limon have a bacteriostatic antibacterial activity on bacteria and have a low inhibition halo against most of the microbial strains studied, with the exception of the combination of EOs (Ginginer officinalis, Citrus limon and Azadirachta indica vegetable oil which are bactericidal) and have a broad inhibition halo on bacteria. From the antibacterial therapeutic activity evaluated, it appears that the combination of EOs (Ginginer officinalis, Citrus limon and Azadirachta indica vegetable oil) could have a significant antibacterial power on the resistant germs studied. The inhibition of growth varies according to the bacterial species and the concentration of the natural extract. Of the nine pathogenic germs tested, two of them (Pseudomonas aeruginosa and *Micrococcus spp)* showed ineffectiveness on the essential oils. These essential oils could therefore be a good alternative in the treatment and serve in the prevention of certain infectious diseases such as mastitis in dairy cows, in order to reduce bacterial resistance due to standard antibiotics. This study could give an idea for the development and upgrading of a new generation of combination of natural antimicrobial agents that can be used in humans and animals against infections with commonly used antibiotics. However, it is essential to evaluate the toxicities of the combination of oils, especially that of Neem vegetable oil, which will allow its use to be promoted and made safe. To increase the production of dairy cows and preserve the health of the consumer, an effective fight against mastitis is necessary. This fight will be focused on prevention,

because mastitis, once present, remains difficult to eradicate and its treatment is expensive. Health prophylaxis will therefore remain an important phase in prevention. We mainly recommend that farms in the Poro region focus on treating all dairy cows at drying off using essential oils (combination of Lemon, Ginger and Neem EOs) for which high sensitivity has been obtained on pathogenic strains.

CONSENT

The agreement of the Ministry of Animal and Fisheries Resources of the Poro region was obtained, in order to facilitate exchanges between dairy farmers from different departments of the region and allow the collection of milk.

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COMPETING INTERESTS

The authors have declared that there are no conflicts of interest.

AUTHORS' CONTRIBUTIONS

This work was done in collaboration between all authors. Author FHK conceived and planned the experiments, collected milk samples, performed laboratory analyses, processed cases and prepared the article. Author AT helped with guidance in processing aspects, contribution to additional reference literature for improvement of the article and final formatting. Author ASG contributed to the literature and directed the article. All authors read and approved the final manuscript.

BIBLIOGRAPHICAL REFERENCES

[1]. **Kirby GC. "**Medicinal plants and the control of parasites", Transactions of the Royal Society of Tropical Medicine and Hygiene, Vol. 90, pp.605-609, 1996.

[2]. **Hostettmann K. and A. Marston**. "Twenty Years of Research into Medicinal Plants/ Results and Perspectives", Phytochemistry, Rev.1, pp.275-285, 2002.

[3]. **Hancock V., L. Ferrières, P. Klemm,** "Biofilm formation by asymptomatic and virulent urinary tract infections Escherichia coli strains", FEMS Microbial Letters, vol. 267, no. 1, pp.30-37, 2007.

[4]. **Horan TC., M. Andrus, MA. Dudeck**."CDC/NHSN surveillance definition of health care-associated infection and criteria for specific types of infections in the acute care setting", American Journal of Infection Control, vol. 36, no. 5, pp.309-32, 2008.

[5]. **Gianfranco Picone et al., (2013)**. Evaluation of the effect of carvacrol on the Escherichia coli 555 metabolome by usng ^{1}H-NMR spectroscopy. Food Chemistry 141, 4367-4374.

[6]. **Karaman S., M. Digrak, U.A. Ravid, A. Ilcim**. "Antibacterial and antifungal activity of the essential oils of Thymus revolutus Celak from Turkey. ", Journal of Ethnopharmacology, vol. 76, pp.183-186, 2001.

[7]. **Lamassiaude S.-Peyramaure**, "New therapies at the Homeopathy and aromatherapy pharmacy. Pharmaceutical News", Pharmaceutical News, vol. 47, no. 475, pp. 27-28.

[8]. **Nada Khazal Kadim Hindi, Zainab Khuduhur Ahmad AL-Mahdi, Zainab Adil Ghani Chabuck., (2014)**. Antimicrobial activity of the aquatic extract of fresh, dry powder ginger, apple vinegar extract of fresh ginger and crud oil of ginger (Zingiber officinale) against different types of bacteria in HILLA CITY, IRAQ. International Journal of Pharmacy and Pharmaceutical Sciences ISSN-0975-1491 Vol 6, Issue 5.

[9]. **Himed L., Merniz S., Bnbraham M**., **(2014)**. Evaluation of the antioxidant and antibacterial activities of Citrus limon essential oil (Lisbon variety) extracted by hydrodistillation. Algerian Journal of Natural Products. Algeria.

[10]. **Gayathri. G., S. Gomathi., V. Ambikapathyi., A. Panneeerselvam.,** (**2020**). Comparative Study of antimicrobial activity on fresh and dried Zingiber officinale Rosc, Asian Journal of Advances in

Medical Science 2(4): 16-23, 2020, Received: 20 October 2020 Accepted: 24 December 2020 Published: 11 January 2021.
[11]. **Fratini F. et al. (2014).** Antibacterial activity of essential oils, their blends and mixtures of their main constituents against some strains supporting livestock mastitis. Fitoterapia, 96, pp. 1-7.
[12]. **Fauchère JL, Avril JL (2002)**. General and medical bacteriology. Ellipses Editions Paris, 365 p.
[13]. **Ponce AG, Fritz R., Del Valle C. and Roura SI, (2004).** Antimicrobial activity of essential oils on the native microflora of organic Swiss chard. Lebensm. -Wiss.u.-Technol.36 (2003)679-684.
[14]. **Skandamis P, Koutsoumanis K, Fasseas K, et *al.*, (2001)** Inhibition of oregano essential oil and EDTA on E. coli O157: H7. ItalJ Food Sci 13:65–75.
[15]. **Berche P, Pezard C, Mock M, (1991)**. Contribution of individual Toxin components to Virulence of Bacillus anthracis. Infection and Immunity, 3472-3477.
[16]. **Eberln T, (1994)**. Antibotics classification, mode of action, therapeutic use. Nathan Pars, p1-35.
[17]. **Marie Elisabeth Lucchesi** ., **(2005).** Microwave-Assisted Solvent-Free Extraction Design and Application to the Extraction of Essential Oils: p 17; 23.
[18]. **Bonomo RA, Szabo D., (2006)**. Mechanisms of multidrug resistance in Acinetobacter species and Pseudomonas aeruginosa. Clin Infect Dis. 43(Suppl. 2): S49–S56.
[19]. **Inouye, S., Yamaguchi, H., Takizawa, T., (2001)**. Screening of the antibacterial effectsof a variety of essential oils on respiratory tract pathogens, using a modifieddilution assay method. J. Infect. Chemother.7 (4), 251–254.
[20]. **Canillac N., (2001)**. et Mourey A. Antibacterial activity of the essential oil of Picea excelsa on Listeria, Staphylococcus aureus and coliform bacteria. Food Microbiol. 18, 261-268.
[21]. **Tiwari B.K., Valdramidis V.P., O'Donnell C.P., Muthukumarappan K., Bourke P. et Cullen P. J., (2009).** Application of natural antimicrobials for food preservation. J. Agric. Food Chem. 57, 5987–6000.
[22]. **Pibiri P., (2006)**. Microbiological sanitation of air and ventilation systems in using essential oils. Doctoral thesis: Faculty of Natural, Architectural and Built Environment, EPFL (Switzerland). P161.
[23]. **Burt, S., (2004**). Essential oils : Their antibacterial properties and potential applications in foods: A review. International Journal of Food Microbiology, 94(3), 223–253.
[24]. **Tajkarimi, MM, Ibrahim, SA, and Cliver, DO, (2010).** Antimicrobial herb and spice compounds in food. Food Control, 21, 1199–1218.
[25]. **Harlet M., (2012).** Mastitis in dairy cows: study of the effectiveness of a mixture of essential oils by local cutaneous application in 34 Breton farms. Veterinary doctoral thesis. Lyon: Claude Bernard University, 144 p.
[26]. **Mauvisseau T., (2016)**. The Photograms®: a new tool for use in livestock farming. The veterinary point, special issue 47, pp. 76-83.