Influence of Some Antibiotics and Essential Oils Used Alone or in Combination on the Vitality of Presumptive Probiotic Lactic acid Bacteria

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ABSTRACT

Aims: The aim of this study was to assess the *in vitro* antibacterial activity of selected antibiotics and essential oils alone or in combination, on selected presumptive probiotic lactic acid bacteria.
Study design: Experimental studies.
Place and Duration of Study: Department of Microbiology of the University of Yaounde I between August 2017 and December 2017 (5 months).
Methodology: The chemical composition of five essential oils was determined by gas chromatography coupled with Solid-phase micro extraction. Then the sensitivity of four lactic acid bacteria to the essential oils and four antibiotics was assessed by the well diffusion and macrodilution method. Subsequently, two essential oils active on these bacteria and broad spectrum antibiotics were combined according to the central composite design plan.
Results: In general, the chemical composition of essential oils is very diverse, with the example of carvacrol found only in *Origanum compactum* at 53.24% and thymol in *Thymus vulgaris* at 56.19% and in *Origanum compactum* at 15.28%. The antibacterial activity shows that the majority of antibiotics used are active on the bacteria in the study compared to the essential oils where two were active (*Origanum compactum* and *Cymbopogon winterianus*). The evaluation of the combinations of essential oils and antibiotics in terms of kinetics has given us three cases: the first case is the one with no acidity or no growth at all; the second is the one where growth is normal; the third where growth is delayed with a more pronounced latency phase.
Conclusion: This study suggest that the effect of essential oils and medicinal plant used alone or in combination to antibiotics on the gut microbiota have to be evaluated for validation as well as their toxicity activities before using them for human therapy.

Keywords: Essential oils, antibiotics, probiotics, vitality, combination

1. INTRODUCTION

The discovery of antibiotics and their use in therapy to control and limit the spread of pathogens raised hopes of eradicating all infectious diseases for many decades. The emergence of antibiotic-resistant bacteria has put an end to this wave of optimism. This resistance is due to excessive consumption, uncontrolled and often inappropriate use of antibiotics, as well as cross-transmissions due to the mobility of infected persons **[1,2].**Although antibiotics have brought a lot of benefits, there are proves of its negative impact on the microbiota. In fact they can influence the gastrointestinal tract, which consists of a complex microbial ecosystem that performs many biochemical and physiological functions **[3,4,5]**. The probiotics fraction of the microbiota is the most positive group because it plays a major role in the balance and stability of the intestinal microbiota contributing to infection control **[6]**. Antibiotic therapy kills not only the pathogenic bacteria responsible for infections, but also certain commensal bacteria and some probiotics leading to a temporary dysbiosis **[7]**. Faced with the ineffectiveness of antibiotics for treatment, populations are increasingly using hybrid treatments consisting of traditional products combined to antibiotics. Although several studies on the antimicrobial activity of antibiotics against probiotics have already been done **[8,9]**, there is little information on the behavior of probiotics in the presence of plant extracts such as essential oils alone or in combination with antibiotics. Antibiotics are used in first line for the treatment of some diseases such as oral and respiratory infections and these types of treatments are increasingly used in combination with essential oils **[10,11]**. It is hence becoming important to assess the impact these essential oils have on the probiotic fraction, especially when they are combined with antibiotics. This will help to better appreciate the consequences of therapies on the probiotic flora. Therefore, the aims of this study was to assess the *in vitro* antibacterial activity of selected antibiotics and essential oils alone or in combination, on selected presumptive probiotic lactic acid bacteria.

2. MATERIAL AND METHODS

2.1 Commercial plant extracts

The plant material used in this work consisted of five essential oils purchased from the PIERRE FABRE laboratory, Boulogne-France: *Cymbopogon winterianus* (panorome citronella, flowering tops, n° 501919), *Thymus vulgaris L. chemotype thymol* (thymol thyme, aerial parts, n° 403746), *Origanum compactum* (compact oregano, flowering tops, n°OF19950)*, Eucalyptus globulus labill* (eucalyptus, leaf and boughs, n°402124) and *Rosmarinus officinalis L. chemotype 1, 8- cineole* (rosemary, boughs and flowering tops, n°K00001).

2.2 Antibiotics and microorganisms

Ampicillin (AMP), Amoxicillin (AMOX), Streptomycin (STREP) and Ciprofloxacin (CPF) from Sigma-Aldrich, St Quentin Fallavier, France were used.

Microorganisms included in this study for antimicrobial activity were four lactic acid bacteria amongst which *Lactobacillus casei* LBLDL (LC), *Lactobacillus plantarum* ATCC 14197 (LP), *Lactobacillus rhamnosus* C1112 (LRH1) and *Lactobacillus rhamnosus* C24 (LRH2). All these strains are Gram+ bacteria kindly offered by the Laboratory of Food Microbiology, University of Bologna (Italy). Strains stored at -80°C were subcultured at 37°C for 24 hours twice in milk broth before being used in the tests.

2.3 Determination of the chemical composition of essential oils

The chemical composition of the essential oils was determined by using an Agilent Technology gas-chromatograph 7890N (Palo Alto, CA, US), equipped with an Agilent Network Mass Selective detector HP 5975C (Palo Alto, CA, US). The injector temperature

was maintained at 250°C while the detector was at 280°C with fragmentations carried out at 70 eV. The analysis was performed in conditions 1:10 split, using a capillary column SPB-5 30m length, 0.25mm ID and 0.25 µm film thickness (Supelco Park. Belfonte code number 24034). The following temperature programme: from 50 to 240 °C with a temperature increase of 3 °C/min, and a 1min hold at 240°C.

For the essential oil head space analysis, 3 ml of the same dilution as previously indicated was introduced in a 10 ml vials and hermetically sealed. After heating the sample in water bath at 30°C for 10min with a SPME-DVB-carboxen/PDMS, 50/30 µm fiber (Supelco, Bellefonte, PA, USA) was exposed in the head space for 30min for absorption. Subsequently the fiber was then immediately inserted for desorption into the injector of a GC–MS for 5min. The identification of the volatile compounds was performed using the NIST (NIST/EPA/NIH Mass spectral Library, 1998, Version 1.6, USA) and WILEY (sixth edition, 1995, USA) and with the Kovach retention index in comparison with those of authentic samples or with published data in the literature **[12]**.

2.4 Evaluation of the antibacterial activity

Two methods were used to evaluate the antibacterial activity of the different essential oils and antibiotics: the well diffusion method and the serial broth macrodillution method.

The well diffusion method was carried out in accordance with CLSI recommendations **[13]**. Sample were dissolved in 10% DMSO then diluted to 2 final concentrations of 2000 ppm and 1000 ppm for the essential oils and of 1000 ppm and 500 ppm for antibiotics. These tested concentrations are different due to the fact that antibiotics are pure reference molecules and specific; therefore, the activity has already been proven. Briefly, 1 mL bacterial culture (10^5 cells/mL) were inoculated on a solidified Mueller Hinton agar with 5% glucose in a Petri dish; then circular wells (3 wells per dish sealed at the bottom with the same medium) of 6 mm were filed with a 50 µL of diluted samples. Wells filed with DMSO were used as negative control. The Petri dishes were then incubated at 37°C for 24 h. The growth inhibition zone diameter (IZ, mm) was measured to the nearest mm. Each experiment was performed in triplicate and the results presented in terms of the concentration that produced the highest inhibition diameter.

The serial broth macrodillution method was carried out in accordance with CLSI recommendations **[13]** in order to evaluate the antimicrobial activity of essential oils and antibiotics on selected lactic acid bacteria. A stock solution was first prepared by diluting the respective essential oils (150 000 ppm) and antibiotics (100 000 ppm) in 10% DMSO. Simultaneously, 10^5 cells/mL of bacteria inoculum was prepared in Mueller Hinton broth from an overnight milk broth culture. Subsequently, 40 µL of the stock solution was added to 3960 µL of bacteria inoculum to reach 1500 ppm and 1000 ppm as first test concentration for essential oils and antibiotics respectively. Then, from these concentrations, we proceeded to twofold dilution using bacteria inoculum to obtain concentrations ranging from 1500 ppm to 0.18 ppm for the essential oils and from 1000 ppm to 0.12 ppm for the antibiotics followed by incubation at 37°C for 24 h (after mixing with vortex). Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were defined as in **[13]**. The presence of viable bacterial after incubation was assessed through their capacity of acidifying the environment. This was done by adding two drops of bromocresol purple as a colored indicator: color change to purple indicated the metabolic activity of viable cells.

2.5 Evaluation of the effect of different combinations of essential oils and antibiotics on growth kinetics, assessed indirectly through growth medium titrable acidity

For this purpose, two strains (the most sensitive one and the most resistant one), two broad spectrum antibiotics and two essential oils were selected. The selection of the strains and antibiotics were done base on the means of inhibition diameter while for the essential oils, it was done using the MIC and MBC values.

To evaluate the effect of different combination made of essential oils and antibiotics on bacterial strains, a calibration curve was first developed to correlate the microbial growth to the titrable acidity as a function of time. Then, we evaluated the vitality of bacteria exposed to the combinations using a $3^{(k-1)}$ fractional design experimental plan.
The calibration curve was realized according to the **[14]** protocol. Briefly, 25 µL of a bacterial suspension was introduced in 250 mL of milk broth to obtain a final concentration of 10^5cells/mL. After 0, 1, 2, 4, 6, 8 and 24 hours, 1 mL of the solution corresponding to each time was used for titration. The operation was performed in duplicate. Three drops of phenolphthalein were added and a volume of NaOH (0.1 mol) until the pink titration solution turned. The volume of NaOH was used to calculate the mass concentration of lactic acid using formula as follows:

$$Cm = \frac{(Cb * Vb * Ma)}{Va} \qquad \text{Equation 1}$$

With Cm the mass concentration of lactic acid in g/L, Cb the NaOH concentration in mol/L, Vb the volume of NaOH in L and Ma the mass molar molecular in g/mol.

The microbial enumeration was performed according to [15]. 1ml of the previous batches was sampled and introduced into 9 mL of physiological water follow by serial dilution. The dilutions was sowed in Petri dishes and incubated at 37°C for 24 hours. The number of colonies count allowed to calculate the cellular concentration using the formula as follows:

$$C = (n * Fd)/V \qquad \text{Equation 2}$$

With C the cellular concentration in UFC/mL, n the number of colonies, Fd the dilution factor and V the sow volume in mL.

The evaluation of the combine effect of antibiotics and essential oils on the vitality of bacteria was assessed using a $3^{(k-1)}$ fractional design experimental plan **[16]** with two variables at three levels (Table 1).

Table 1. Coding of independent variables

Levels	-1	0	1
Essential oils	MIC/2	MIC	3/2 MIC
Antibiotics	MIC/2	MIC	3/2 MIC

For this realization, a milk broth was prepared in vials and antimicrobials (essential oils and antibiotics) were introduced at different concentrations according to a fractional design experimental plan so as to obtain a final volume of 100 mL; then 10 µL of a bacterial pre-culture were introduced to obtain in the broth a concentration of 10^5 UFC/mL of the various lactic acid bacteria. Each run was repeated 10 times, and the incubation was performed for 24 hours at 37°C. A series of samples during the incubation were titrated to determine the mass concentration of lactic acid produced in the presence of the different antimicrobial concentrations according to the above equation 1.

3. RESULTS AND DISCUSSION

3.1 Chemical composition of essential oils

Gas chromatography (GC) and solid-phase micro extraction (SPME) analyses of the essential oils allowed the identification of several components (Table 2). *Origanum compactum* showed the presence of thirteen components amounting to 82.62% of the total

chemical composition; the oil was characterized by three major monoterpenes compounds: carvacrol (53.2%), thymol (15.3%) and p-cymene (14.1%). *Thymus vulgaris* presented seven components amounting to 88.64% of the total chemical composition; the oil was characterized by two major monoterpenes compounds: thymol (56.19%) and m-cymene (32.45%). *Eucalyptus globulus* presented six components amounting to 95.89% of the total chemical composition; the oil was characterized by the monoterpenes eucalyptol (95.89%) as major compound. *Cymbopogon winterianus* presented eight components amounting to 89.66% of the total chemical composition; the oil was characterized by three major monoterpenes and one sesquiterpenes compounds: citronellal (38.34%), *trans*-geraniol (21.05%), beta-citronellol (18.58%) and elementol (11.69%). *Rosmarinus officinalis* presented eleven components amounting to 79.14% of the total chemical composition; the oil was characterized by two major monoterpenes compounds: eucalyptol (63.83%) and camphor (15.31%).

Analysis of the same EO obtained from leaves collected in Boulemane region **[17]** or leaves and stems in Cerrado region of Brazil **[18]** showed chemical composition dominated by eucalyptol (42.24-28.5%), camphor (10.81-27.7%) and α-pinene (16.31-21.3%) respectively. Thymol (56.191%) and m-cymene (32.455%) were obtained as major compounds for *Thymus vulgaris*. Analysis of the same EO obtained from flowering tops from France **[19]** or aerial plant from Romania **[20]** showed chemical composition dominated by thymol (36.58-45.5%), p-cymene (16.51-8.41%) and ß-terpene (13.70-30.90%) respectively.

Eucalyptol (95.830%) is the major compound for *Eucalyptus globulus*. Analysis of the same EO obtained from leaves in Brazil **[21]** or leaves in Haramaya University, Ethiopia **[22]** showed chemical composition dominated by eucalyptol (83.89%), limonene (8.16%) and α-pinene (4%); eucalyptol (55.29%), spathulenol (7.44%) and α-terpineol (5.46%) respectively. m-cymene (14.097%), thymol (15.288%) and carvacrol (53.242%) were obtained as major compounds for *Origanum compactum*. Analysis of the same EO obtained from 200g of powder from plant in Morocco **[23]** or leaves in Belgium **[24]** showed chemical composition dominated by carvacrol (58.1%), p-cymene (11.4%), thymol (9%) and α-terpinene (7.1%) ; carvacrol (30.53%), thymol (27.5%) and ß-terpinene (18.20%) respectively. Citronellal (38.348%), β-citronellol (18.581%), trans-geraniol (21.053%) and 11.689% elemental were obtained as major compounds for *Cymbopogon winterianus*. Analyses of the same EO obtained from leaves and stems in holzminden, Germany **[25]** showed chemical composition dominated by citronellal (27%), trans-geraniol (22.78%) and citronellol (10.9%).

209 **Table 2. Chemical composition of the five essential oils expressed as percentage of total compounds revealed by the GC-MASS**
210 **spectrum**

Great family	Compounds	Retention index (IR/SPB5)	*Origanum compactum*	*Thymus vulgaris*	*Rosmarinus officinalis*	*Eucalyptus globulus*	*Cymbopogon winterianus*
Monoterpenes	α-pinene	939	/	0.3	**5.9**	0.3	/
	Camphene	954	/	0.2	2.4	/	/
	β-pinene	979	/	/	4.7	0.2	/
	(+)-4-carene	1001	1.3	/	/	/	/
	β-carene	1011	0.8	/	/	/	/
	1,4-cineole	1014	/	/	/	0.2	/
	α –terpinene	1017	**9.4**	/	/	/	/
	p-cymene	1024	**14.1**	**32.4**	/	/	/
	Eucalyptol	1031	/	/	**63.8**	**95.9**	/
	α-thuyone	1102	0.5	/	/	/	/
	1,3,8-p-menthatrien	1110	/	/	4.1	/	/
	β-thujene	1114	0.6	/	/	/	/
	camphor, (1R.4R)-(+)-	1146	/	/	**15.3**	**/**	**/**
	(R)-(+)-citronellal	1153	/	/	**/**	**/**	**38.3**
	borneol acetate	1169	/	/	0.3	/	/
	p-cymen-8-ol	1182	0.1	/	/	/	**/**
	α-terpineol	1188	/	/	0.2	/	/
	(R)-(+)-β-citronellol	1225	/	/	/	/	**18.6**
	trans-geraniol	1252	/	/	/	/	**21.1**
	Thymol	1290	**15.3**	**56.2**	/	/	/
	Carvacrol	1299	**53.2**	**/**	/	/	/
	thymol acetate	1352	/	3.5	/	/	/
	isobornyl format	1239	0.500	/	/	/	/
	Eugenol	1359	**/**	**/**	/	/	1.8
Terpenes	D-limonene*	/	/	/	/	1.5	1.9
	α-phellandrene	1002	/	/	/	0.1	/
	Limonene	1029	/	/	0.5	/	/
	β-linalool	1096	1.3	3.7	/	/	/
	Borneol	1169	/	1.5	2.05	/	/

Sesquiterpenes	(E)-β-caryophyllene	1424	2.5	/	0.4	/	/
	β-gurjunene	1433	/	/	/	/	0.6
	Elemol	1549	/	/	/	/	**11.7**
	caryophyllene oxide	1583	0.1	/	/	/	**/**
	Ledol	1602	/	/	/	/	**5.9**
	Total compounds identified (%)	**/**	**99.7**	**97.8**	**99.65**	**98.2**	**99.9**

211 * Compound identify from the mass spectrum

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3.2 Antimicrobial activities

The antimicrobial activities of these essential oils and antibiotics on the lactic acid bacteria were assessed by well diffusion method through the inhibition zone (IZ) diameter measurement and by macrodilution method determining the MIC and MBC values **[13]**. The IZ diameters expressed in mm are presented (Table 3). All the selected bacteria were not sensitive to the five essential oils at concentrations of 2000 ppm and 1000 ppm. However, for the antibiotics, the highest IZ diameters (2.63-4.07 mm) were observed with the ciprofloxacin at 1000 ppm for all the bacteria, the two more sensitive strains being *Lactobacillus casei* (4.07 mm) and *Lactobacillus rhamnosus* C24 (4.13 mm); the less sensitive strain was *Lactobacillus rhamnosus* C1112 (2.63 mm). Globally, the inhibition zone diameters were not proportional to the concentration of antibiotics. *Lactobacillus casei* was sensitive to the antibiotics, *Lactobacillus plantarum* was not sensitive to streptomycin. The two *Lactobacillus rhamnosus* strains were the less sensitive to amoxicillin and ampicillin.

Table 3. Sensitivity of bacteria to the essential oils and antibiotics expressed as inhibition zone diameter ± sd (mm)

Sample concentrations (ppm)		*LC*	*LP*	*LRH1*	*LRH2*
1000 ppm	Streptomycin	2.20±0.35	00±00	1.47±0.50	1.33±0.15
	Amoxicillin	2.73±0.31	3.27±0.23	0.00±0.00	0.00±0.00
	Ampicillin	3.60±0.53	3.53±0.50	0.00±0.00	1.60±0.17
	Ciprofloxacin	4.07±0.12	3.47±0.46	4.13±0.81	2.63±0.06
500 ppm	Streptomycin	2.73±0,31	0.00±0.00	1.10±0.14	1.33±0.06
	Amoxicillin	3.13±0.12	2.93±0.50	0.00±0.00	0.00±0.00
	Ampicillin	2.87±0.23	3.27±0.23	0.00±0.00	1.27±0.12
	Ciprofloxacin	4.07±0.12	3.00±0.20	3.40±0.92	2.57±0.21
2000 ppm	*Origanum compactum*	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Eucalyptus globulus	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Rosmarinus officinalis	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Cymbopogon winterianus	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Thymus vulgaris	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
1000 ppm	*Origanum compactum*	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Eucalyptus globulus	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Rosmarinus officinalis	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Cymbopogon winterianus	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Thymus vulgaris	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

LC (*Lactobacillus casei*), *LP* (*Lactobacillus plantarum*), *LRH2* (*Lactobacillus rhamnosus C24*), *LRH1* (*Lactobacillus rhamnosus C1112*).

The antibacterial activities of the essential oils and antibiotics were also evaluated using macrodilution method. The corresponding antibacterial activities (MIC and MBC) are presented (Table 4). The classification of the activity of essential oils was done based on

[26] in proposal. According to these authors, the antimicrobial activity can be high (MIC<100ppm), moderate (100<MIC<625ppm) or low (MIC>625ppm) depending on the MIC values. On the basis of this classification, most of the essential oils whose antimicrobial activity was evaluated showed low activity on all the bacteria studied.

Bacterial strains were more sensitive to antibiotics (ATB) compared to essential oils, *Lactobacillus plantarum* is the most sensitive strain (low value of MIC and MBC). The essential oils at the maximum concentration tested were less active on most bacterial strains.

Table 4. Antibacterial activities (MIC and MBC) of the essential oils and antibiotics

		MIC (µg/ml)				MBC (µg/ml)			
Antimicrobials		***LC***	***LP***	***LRH1***	***LRH2***	***LC***	***LP***	***LRH1***	***LRH2***
Antibiotics	**Streptomycin**	31.25	3.90	31.25	31.25	62.50	15.62	31.25	31.25
	Amoxicillin	3.90	3.90	>1000	>1000	62.50	31.25	1000	>1000
	Ampicillin	3.90	3.90	>1000	500	125	31.25	>1500	>1000
	Ciprofloxacin	1000	500	62.50	1000	250	500	62.50	>1000
Essential oils	***Origanum compactum***	1500	1500	375	1500	>1500	>1500	>1500	>1500
	Eucalyptus globulus	>1500	>1500	>1500	>1500	>1500	>1500	>1500	>1500
	Rosmarinus officinalis	>1500	>1500	>1500	>1500	>1500	>1500	>1500	>1500
	Cymbopogon winterianus	750	>1500	>1500	>1500	1500	1500	>1500	>1500
	Thymus vulgaris	>1500	>1500	>1500	>1500	>1500	>1500	>1500	>1500

LC (*Lactobacillus casei*), *LP* (*Lactobacillus plantarum*), *LRH2* (*Lactobacillus rhamnosus C24*), *LRH1* (*Lactobacillus rhamnosus C1112*)

The sensitivity and inhibition parameters evaluated in this work revealed that the different essential oils had no antibacterial activity in general on our target probiotic germs for concentration lower or equal to 1500 ppm. Particularly, only the essential oil of *Origanum Compactum* and *Cymbopogon winterianus* had low activities on the tested strain. ***C. winterianus* could be bactericidal on *Lactobacillus casei* and *Lactobacillus plantarum* at 1500 ppm and inhibit *Lactobacillus casei* at 750 ppm. On the other hand, *O. Compactum* was not bactericidal but could inhibit the growth of all the selected strains at a concentration of 1500 ppm and 375 ppm for *Lactobacillus rhamnosus C1112*.** This antibacterial activity could be attributed to the presence in these oils of secondary metabolites with antimicrobial properties. Indeed, studies have reported that secondary metabolites such as tannins, phenols, flavonoids, saponins, phenolic compounds have antibacterial activity **[27]**.

The antibacterial activity varies from one oil to another; it could be explained by the difference in composition and concentration of secondary metabolite **[28]** present in each essential oil. In addition, **[29]** reports that several essential oils exhibit antimicrobial activity against many bacteria and fungi at high concentrations. However, the bactericidal activity detected in *Origanum compactum* in this study can be related to the presence of **carvacrol**, which is the major compound in this oil and is generally known for its antimicrobial properties **[30]**. In addition, bacterial strains were more sensitive to antibiotics than essential oils. This could be due to the fact that antibiotics are pure active compounds while essential oils are

mixtures of substances that contain in addition to active compounds; other substances such as polysaccharides, polypeptides that could bind to active compounds mask or decrease their activity **[31, 32]**.

Scientific investigations proved the application of major compounds carvacrol **[33, 34, 35, 36]** and thymol **[33, 35]** from *Origanum compactum* in pharmaceutical industries, in particular as antibacterial, antifungal and antileishmanial drugs but further clinical investigations need to be carry out for the development of anticancer, antimalarial, anti-inflammatory and antidiabetic drugs.

Citronellal, geraniol and citronellol from Cymbopogon winterianus possesses pharmacological activities such as antiobesity, antibacterial, antifungal, antinociceptive, antioxidants, antidiarrheal, antiparasitic, insect repellent and anti-inflammatory properties which enhance health **[37, 38, 39, 40]** (Wany et al., 2013; Avoseh et al., 2015; Sharma et al., 2019; Bayala et al., 2020).

3.3 Effect of different combinations of essential oils and antibiotics on the growth kinetics of the probiotic strains used

The determination of the inhibition zone diameters, MIC and MBC allowed the selection of two antibiotics: Ampicillin (AMP) and Ciprofloxacin (CPF), two essential oils: *Cymbopogon winterianus* (CW) and *Origanum compactum* (OC) and two bacteria: *Lactobacillus casei* (LC) and *Lactobacillus rhamnosus* C 24 (LRH2) based on the different averages obtained. For this evaluation, a calibration line was constructed in order to predict microbial concentration from the acidity data (Figure 1). From this calibration line, it could be observed that the increase of medium acidity could be correlated to the level of lactic acid bacteria growth.

From these figures we observe that both strains have a correlation coefficient more than 0.9 reflecting a satisfactory correlation between the titrable acidity and the microbial growth.

According to the fractional design experimental plan, we evaluated the effect of the combinations of essential oils and antibiotics after 24 hours of incubation by the expression of acidity increments (Table 5 and Table 6).

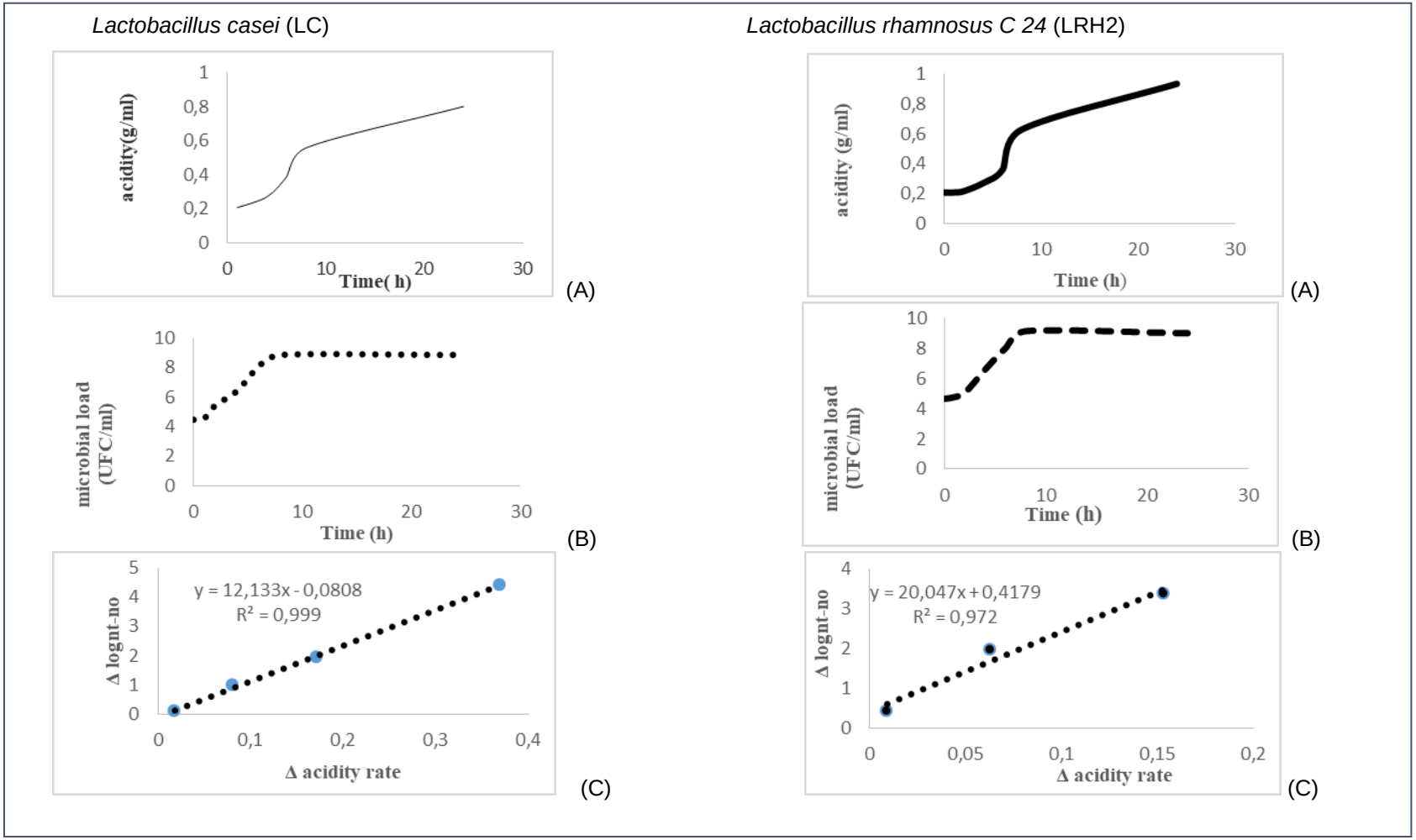

Fig. 1. Kinetics evolution of titratable acidity (A), bacterial load (B) and bacterial load calibration curve (C) for *Lactobacillus casei* and *Lactobacillus rhamnosus C 24*

Table 5. Acidity variation after 24 hours of the culture of *Lactobacillus casei* in the presence of combinations of essential oils and antibiotics

	Lactobacillus casei											
TEST	**AMP (ppm)**	**CW (ppm)**	**acidity**	**CPF (ppm)**	**CW (ppm)**	**acidity**	**AMP (ppm)**	**OC (ppm)**	**acidity**	**CPF (ppm)**	**OC (ppm)**	**acidity**
1	1.95	375	0.189	500	375	0.000	1.95	750	0.630	500	750	0.180
2	3.90	375	0.135	1000	375	0.000	3.90	750	0.630	1000	750	0.180
3	5.85	375	0.045	1500	375	0.000	5.85	750	0.270	1500	750	0.360
4	1.95	750	0.360	500	750	0.030	1.95	1500	0.540	500	1500	0.000
5	3.90	750	0.063	1000	750	0.036	3.90	1500	0.540	1000	1500	0.090
6	3.90	750	0.063	1000	750	0.036	3.90	1500	0.540	1000	1500	0.090
7	5.85	750	0.252	1500	750	0.090	5.85	1500	0.540	1500	1500	0.180
8	1.95	1125	0.333	500	1125	0.098	1.95	2250	0.630	500	2250	0.190
9	3.90	1125	0.243	1000	1125	0.036	3.90	2250	0.630	1000	2250	0.135
10	5.85	1125	0.000	1500	1125	0.126	5.85	2250	0.540	1500	2250	0.110

CW/AMP= *Cymbopogon winterianus* and Ampicillin, CW/CPF= *Cymbopogon winterianus* and Ciprofloxacin, OC/AMP= *Origanum compactum* and CW/AMP= *Cymbopogon winterianus* and Ampicilline, CW/CPF= *Cymbopogon winterianus* and Ciprofloxacin, OC/AMP= *Origanum compactum* and Ampicillin, OC/CPF= *Origanum compactum* and Ciprofloxacin.

Table 6. Acidity variation after 24 hours of the culture of *Lactobacillus rhamnosus C 24* in the presence of combinations of essential oils and antibiotics

	Lactobacillus rhamnosus C24											
TEST	**AMP (ppm)**	**CW (ppm)**	**acidity**	**CPF (ppm)**	**CW (ppm)**	**acidity**	**AMP (ppm)**	**OC (ppm)**	**Acidity**	**CPF (ppm)**	**OC (ppm)**	**acidity**
1	250	750	0.009	500	750	0.000	250	750	0.495	500	750	0.000
2	500	750	0.036	1000	750	0.000	500	750	0.360	1000	750	0.000
3	750	750	0.000	1500	750	0.180	750	750	0.450	1500	750	0.090
4	250	1500	0.000	500	1500	0.000	250	1500	0.270	500	1500	0.000
5	500	1500	0.000	1000	1500	0.000	500	1500	0.315	1000	1500	0.090
6	500	1500	0.000	1000	1500	0.000	500	1500	0.360	1000	1500	0.090
7	750	1500	0.000	1500	1500	0.090	750	1500	0.090	1500	1500	0.180
8	250	2250	0.000	500	2250	0.000	250	2250	0.450	500	2250	0.540
9	500	2250	0.000	1000	2250	0.000	500	2250	0.360	1000	2250	0.000
10	750	2250	0.000	1500	2250	0.000	750	2250	0.000	1500	2250	0.360

CW/AMP= *Cymbopogon winterianus* and Ampicillin, CW/CPF= *Cymbopogon winterianus* and Ciprofloxacin, OC/AMP= *Origanum compactum* and Ampicillin, OC/CPF= *Origanum compactum* and Ciprofloxacin.

The acidity after 24 hours varies according to the different combinations of essential oils and antibiotics. The evaluation of the effect of combinations on bacteria has shown that acid accumulation synonymous of growth, varies according to the different concentrations of antimicrobials and the different combinations of essential oils and antibiotics. In addition, it is generally observed that when the values below the MICs of essential oils are combined with the MIC values of antibiotics, acid is produced indicating microorganism growth. According to **[41]**, it could be described as an antagonistic phenomenon of oil and antibiotics. Antibiotics generally act very specifically on certain structures of the bacterial cell; and this high specificity of action explains why they are active at very low concentrations **[42]**. On the other hand, some researchers have shown that the potency of essential oils varies according to their major constituents and that the mode of action is mainly related to the chemical profile of the constituents of each essential oil **[43]**. The presence of oils could therefore limit access to the site of action of antibiotics, thus reducing their effects. In situations where essential oils and antibiotics are combined with values lower than their MICs, no acid production is observed, example of OC/CPF combination on *Lactobacillus rhamnosus* C24.

Ampicillin is a broad-spectrum beta-lactam bacteria that acts on Gram+ bacteria and some Gram- bacteria it inhibits the enzymes of transpeptidation involved in the bridging of the polysaccharide chains of the peptidoglycan of the wall **[44]**. Ciprofloxacin is an ATB belonging to the fluoroquinolone family .it works by killing the bacteria responsible for infection by inhibiting bacteria DNA gyrase and therefore interferes with DNA replication transcription and other activities involving DNA (inhibition of nucleic acids) **[42]**.

Essential oils act on both Gram+ bacteria as well as Gram- bacteria nevertheless, Gram- bacteria seem less sensitive to their action and this is directly linked to the structure of their cell wall **[45]**. Several chemical components of EOs make it possible to modulate the intestinal flora and thus reduce the number of certain bacteria **[46]**. Carvacrol and thymol are able to form hydrogen bonds with the actives sites of microbial enzymes and thus may contribute to the antimicrobial effects of essential oils **[24, 47]**. This antibacterial efficacy of essential oils rich in carvacrol and thymol is explained by the position of the hydroxyl group on the phenolic structure of the molecules and hydroxylamine groups of the bacteria causing an ultracellular leak from the cells. All these alterations and changes lead to cell death **[48, 49]**.

It can be observed that the combination of CPF/CW and CPF/OC are not favorable to the growth of *L. casei* as the increase in acidity is very low independently oh the combination levels. This strain was more affected by the combination of AMP/OC while combining AMP/CW had irregular activity on the strain growth. Regarding *L. rhamnosus*, it can be observed that the combination of AMP/CW, CPF/CW and CPF/OC did not favor the strain growth as very low or no acid increase was observed. On the other hand, the combination of AMP/OC generally did not affect *L. rhamnosus* growth and acid production. Taking into consideration the *L. casei* and *L. rhamnosus* demonstrated to has almost the same acidification capacity (Figure 1) it can be observed that *L. rha*mnosus is more affected by the combination of AMP/OC than *L. casei.*

4. CONCLUSION

This work has showed that, no matter the composition of essential oils, they have in most of the cases, very low activities on presumptive probiotics when used alone. Antibiotics tested were generally active on all the bacteria tested. Combinations between essential oils at values below the MIC with antibiotics greater than or equal to the MIC reduced the effect of antibiotics on probiotics. However, combinations of essential oils and antibiotics at concentrations greater than or equal to their MICs lead in most cases to inhibition of the growth of the probiotics studied. Finally, our results suggest that the effect of essential oils and medicinal plant solvent extracts used alone or in combination to antibiotics on the gut

microbiota have to be evaluated for validation as well as their toxicity activities before using them for human therapy.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. Author GAK, AVNN, ANT and SLSK designed the study and wrote the protocol. Author GAK and AVNN did the bench work and author GAK wrote the first draft of the manuscript. Author GAK and SLSK performed the statistical analysis and managed the analyses of the study. Author GAK, AVNN, SLSK and JJEN managed the literature searches. All authors read and approved the final manuscript."

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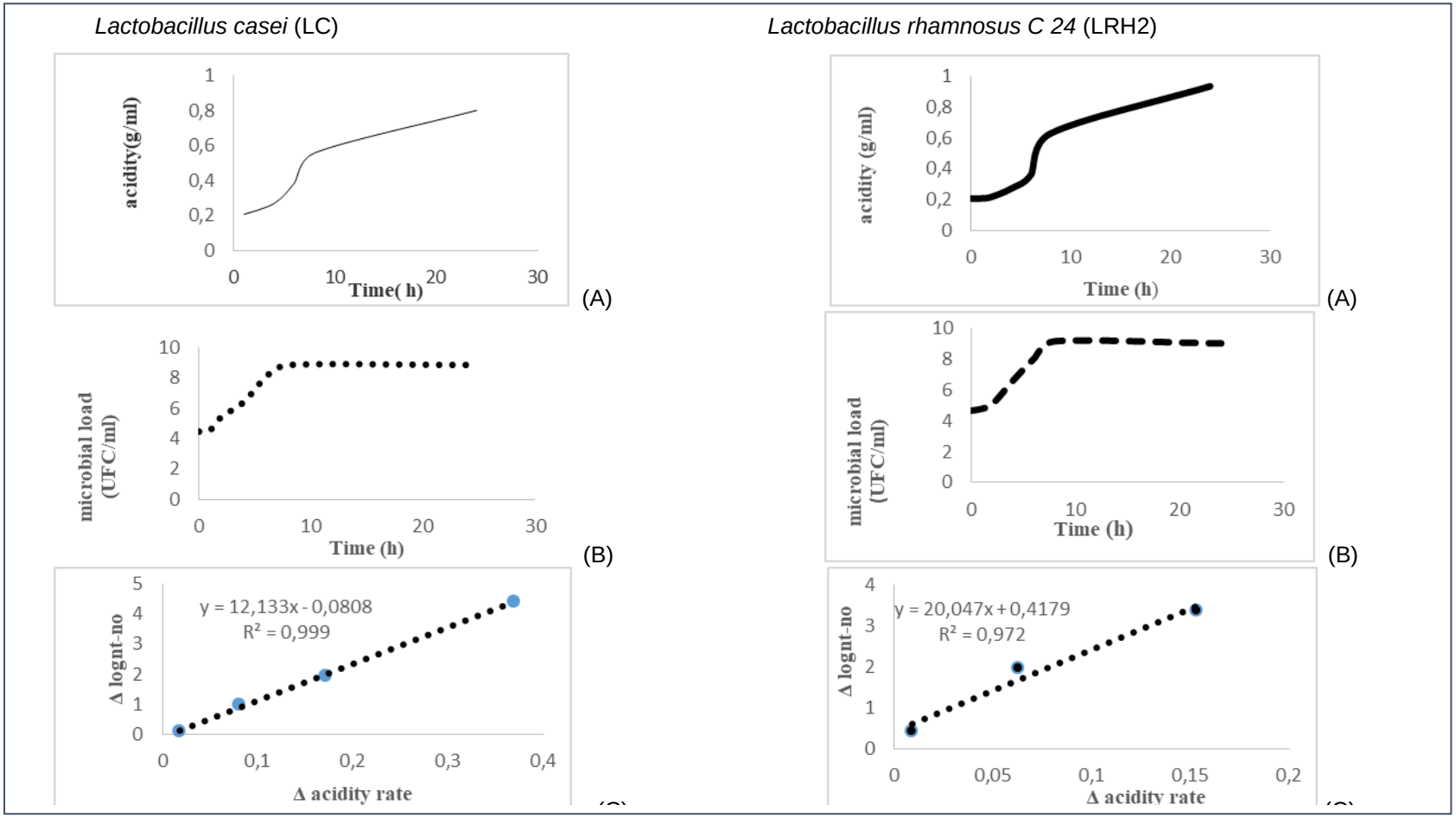
ABBREVIATIONS

MIC: Minimum inhibitory concentration
MBC: Minimum bactericidal concentration
UFC: Units Forming colony

Cm: Mass concentration of lactic acid (g/L)
Cb: NaOH concentration (mol/L)
Vb: Volume of NaOH (L)
Ma: Mass molecular (g/mol)
C: Cellular concentration (UFC/mL)
n: number of colonies
Fd: Dilution factor
V: Sow volume (mL)
GC: Gas Chromatography
SPME: Solid-phase micro extraction
EO: Essential oil
ATB: Antibiotic
IZ: Inhibition zone
OC: *Origanum compactum*
CW: *Cymbopogon winterianus*
AMP: Ampicilin
AMOX: Amoxicillin
STREP: Streptomycin
CPF: Ciprofloxacin
LC: *Lactobacillus casei* LBLDL
LP: *Lactobacillus plantarum* ATCC 14197
LRH1: *Lactobacillus rhamnosus* C1112
LRH2: *Lactobacillus rhamnosus* C24

FIGURE CAPTION

Fig. 1.



Kinetics evolution of titratable acidity (A), bacterial load (B) and bacterial load calibration curve (C) for *Lactobacillus casei* and *Lactobacillus rhamnosus C 24*