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Research Paper

Antibacterial activity of *Citrus paradisi* essential oil

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ABSTRACT

The aim of this work was to determine the effectiveness of Grapefruit (*Citrus paradisi*) essential oil to inhibit the growth of wild food-borne spoilage and pathogenic bacterial strains. Additionally, the chemical composition and physical properties of this essential oil was evaluated. Essential oil was obtained as a by-product from agro-processing industry in the province of Corrientes, Argentina. Monoterpene hydrocarbon limonene representing 93% (v/v), quantified by gas chromatography, was the major component of essential oil. *Citrus paradisi* essential oil inhibited growth of *Escherichia coli*, *Staphylococcus aureus*, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *diacetylactis*, *Leuconostoc mesenteroides* subsp. *dextranicum* and *Lactobacillus plantarum*. The lowest concentration of essential oil (4.29ppm) was required to inhibit *Lactococcus lactis* subsp. *lactis* 207c VCOR (8.27±0.13 $\log_{10}$ CFU/ml) according to the Minimum Inhibitory Concentration. The effect of this oil on growth of wild strain 207c VCOR (9.17±0.024 $\log_{10}$ CFU/ml) was determined at different times by total count and spectrophotometric absorption to 560 nm. At the moment of essential oil injection, the number of microorganisms was $1.29 \pm 0.17 \times 10^9$ CFU/ml and, at 24 hours of contact, only $6.3 \pm 0.5 \times 10^6$ CFU/ml was detected. These results show that oil has a bactericidal effect if counts with and without addition of essential oil were compared. The loss of viability was 1.82×10^9 CFU/ml under these experimental conditions. This essential oil has very strong potential applicability as a natural antibacterial agent for food industry, particularly for pasta manufacture which is facing serious spoilage problems due to lactic bacteria activity.

Keywords*:* Natural preservative; Spoilage control; Essential oil; Lactic acid bacteria.

INTRODUCTION

Presence of undesirable microorganism in food and their multiplication is a problem for whole world. They can lead to spoilage and deteriorate the quality of food or cause illness. In 2011, 1.8 million child deaths were registered (WHO, 2013), most

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of them caused by contaminated water and food. In recent years, emergence of bacterial resistance against multiple antibiotics has accelerated dramatically. Community- and hospital-acquired pathogens and larger part of them are multi-drug-resistant bacteria (Lai et al., 2011; Solórzano-Santos and Miranda-Novales, 2012). So, food safety is a past, present and future public health concern all over the world.

Growing interest in substitution of synthetic antimicrobial agents by natural ones has fostered research on vegetable sources and screening of plant materials in order to identify new compounds or test natural chemicals already known for important activities that have not been discovered so far (Ait-Ouazzou et al., 2011; Lv et al., 2011; Badawy and Abdelgaleil, 2014).

Essential oils (EOs) extracted from different plant genera are, in many cases, biologically active. These aromatic compounds are relatively inexpensive and there is abundant raw material with practical applications for different industries.

Citrus is considered an important fruit in world production because its great value for human diet. Citrus is member of *Rutaceae* family from sub-tropical origin and is known for its semi-sweet taste.

Due to nutraceutical and economic importance, numerous investigations have been performed aiming at identifying chemical composition, antimicrobial activities of EOs from peel of different citrus species.

Citrus EOs has been recognized as safe due to their wide spectrum of biological activities, such as antimicrobial, antioxidant, anti-inflammatory and anxiolytic (Rehman, 2006; Chutia et al., 2009). These antimicrobial properties have shown to create a particularly interesting scope for application within food industry (Imran et al., 2013), for veterinary use (Fuselli et al., 2008; Roussenova, 2011), human medicine (Yin et al., 2012; Oliveira, et al., 2014) and plants for agricultural production (Badawy and Abdelgaleil, 2014).

Argentina is the world's eighth largest citrus grower and the first largest lemon grower. It has exported dry fruit, juices and EOs since 1970. Citrus production area covers 147.000ha, and has a total annual production of, almost, 3.000.000t. Main citrus fruit include lemon (47%), followed by orange (29%), mandarin orange (16%) and grapefruit (8%) (UIA, 2008).

Here we are try to determine physical characteristics and chemical composition of *Citrus paradisi* essential oil; to screen antibacterial properties of EO against wild food-borne spoilage and pathogenic bacterial strains and; to evaluate the effect of adding EOs to growth of sensitive microorganisms in dose according to the MIC.

MATERIALS AND METHODS

Essential oil: *Citrus paradisi* essential oil (EO) was obtained as a by-product in a citrus juice extraction plant. The method used for juice extraction was extruding or cold pressing in which follicular glands of citrus peel is mechanically ground to release its content. For this purpose, a Food Machinery Corporation (FMC) extractor was used. The objective of this process was to separate oil from fruit juice.

Essential oil characterization: Grapefruit oil characterization was performed by determination of the following physical properties: refractive index, relative density and optical rotation using standardized methods.

Relative density method was performed according to recommendations of Argentine Institute of Standardization and Certification, IRAM-SAIPA Standard N° 18504 (2002), by pycnometer method. For this purpose, a 0.1 milligram-precision scale

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METTLER AJ150 (Germany) was used. Procedure consisted of determination of body of water (m_{H2O}) contained in the pycnometer, by weight difference of pycnometer with or without distilled water, and body of water using the same technique but with EO and using the same pycnometer. Both at reference temperature = 20±0.2°C.
Relative density of oil was calculated applying the following equation:

$$d^{20°C/4°C} = m_{aceite} / m_{H2O}$$

- $d^{20°C/4°C: oil}$ density at 20°C, relative to water to 4°C,
- m_{oil}: body of oil at full pycnometer at 20°C (g),
- m_{H2O}: body of water calculated at pycnometer calibration (g).

Additionally, refractive index was determined through IRAM-SAIPA Standard N° 18505 (2002) with an ABBE DR-M2 refractometer from ATAGO USA, Inc., with a temperature thermostatic control through water circulation sleeve among prisms and white light lamp. Tool precision is 0.0001, for a dynamic interval of $n^{D}_{20°C}$ = 1.300-1.700. The method is based on determination of critical angle of total reflection between oil and prism Flint glass, looking critical angle in observation field, visualizing as a clear separation between two fields (pale and dark) focused on two hair cruces. Using a 20ºC thermostatized oil sample (1 or 2 drops) reading in refractometer was performed. Determination of optical rotation $[\alpha]_t^D$ was done under IRAM-SAIPA Standard N° 18507 (2002), using a A&E type WXG-4 disc polarimeter from Shanghai, China, measuring range ± 180° and precision of 0.05°. Procedure was based on observation of angle of deviation for polarized plane light 589-nm wavelength (Sodium D line) caused by essential oil placed on a 10-dm-long sample holder at 20±0.2°C. For this purpose, oil sample was stabilized on a water bath at a temperature of 20±0.2°C. Angle observation was visualized in the observation field as the intermediate of two fields (a dark circle with a pale line in the middle and another pale circle with a dark line in the middle). Reading was done at external scale ($\alpha_{observed}$).
Optical rotation of oil was calculated applying the following equation:

$$[\alpha]_t^D = \alpha_{observed}\ 100/l$$

- $[\alpha]_t^D$: optical rotation to 20°C for Sodium D line (°), $\alpha_{observed}$: optical rotation angle observed (°), l: length of sample-holder flask (mm).

Chemical constitution of essential oil: identification of *Citrus paradisi* EO components was conducted by gas chromatography-mass spectrometry (GC/MS). A two-capillary column QP 5050 SHIMADZU equipment was used: one SE 52 (MEGA, Legnano, Italia) chemically bonded (25m x 0.25mm internal diameter; 0.25µm-thick stationary phase) column, covered by 5% phenil-polydimethylsiloxane (0.25µm-thick stationary phase) at a column temperature of 60ºC (8min), increasing up to 180ºC at a 3ºC/min speed, then up to 230ºC at a 20ºC/min speed. 250ºC injector temperature, split injection mode; 1:40 split ratio; 0.2µl oil injection volume. Mobile phase: Helium, 122.2kPa (51.6cm/sec), 250ºC interface temperature and 40-400m/z mass range acquisition. Another BP-20 (SGE, Australia) 25m x 0.25mm internal diameter fused silica capillary column covered by polyethyleneglycol 20.000Da (0.25 µm thick stationary phase). Column temperature at 40ºC (8min), increasing up to 180ºC (3 ºC/min), and up to 230ºC (20ºC/min). 250ºC, injector temperature, split injection mode; 1:40 split ratio; 0.2µl oil injection volume. Mobile phase: Helium, 92.6kPa (55.9cm/s), 250ºC interface temperature and mass range acquisition: 40-400m/z.

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Fragmentation patterns in each component were compared to those stored in the software library spectra (Mc Lafferty and Stauffer, 1991; Adams, 2001).

Microorganisms and growth conditions: forty autochthonous bacterial strains were obtained from "Institutional Collection of Wild Microorganisms" - Facultad de Ciencias Exactas y Naturales (School of Natural and Exact Sciences, Northeastern University of Argentina (Acronym: VCOR). In this study, the following were used as food spoilage bacteria: *Lactobacillus* (*Lb.*) *plantarum* (11 strains), *Leuconostoc* (*Leuc.*) *mesenteroides* subsp. *dextranicum* (1 strain), *Lactococcus* (*L.*) *lactis* subsp. *diacetylactis* (1 strain) and *L. lactis* subsp. *lactis* (11 strains). Additionally, *Staphylococcus aureus* (3 strains) and *Escherichia* (*E.*) *coli* (13 strains) were tested as pathogenic bacteria of food.

Bacterial strains were preserved on Milk-Yeast extract with glycerol (15%, v/v) at -20ºC. *Lactobacillus* strains were cultivated in MRS medium (Merck) at 30ºC; *Lactococcus lactis* strains were cultivated in Elliker medium (Biokar Diagnostic) at 35ºC; *Staphylococcus aureus* were cultivated in Brain Heart Infusion (Merck) at 37ºC and Nutrient medium (Britania) was used for proliferation of *E. coli* strains at 37ºC. Working bacterial cultures were transferred (2%, v/v) to a fresh broth three times prior to experiences.

***Lactic bacteria count*:** appropriate further decimal dilutions of bacterial suspensions were made in a peptone-saline solution (0.1-0.85%, w/v) for enumeration of lactic bacteria (LAB) according to Aerobic Plate Count (Maturin and Peeler, 2001). For growth, Elliker agar (Biokar Diagnostic) was used with incubation at 30°C for 48h.

Screening of antibacterial effect: antibacterial activity *in vitro* of EOs was assayed using Disk diffusion method (Ortez, 2005). Briefly, 50µl of EO were placed on sterile 0.55cm diameter filter paper discs (Whatman N° 1) located on the surface of adequate media in plates previously spread with 100µl of 10^8 CFU/ml overnight cultures. Plates have been allowed to dry for 15min in a sterile environment, inverted and incubated for 24h at optimal temperature of growth. Diameters of zones of inhibition (ZOI) were measured using Vernier caliper. Controls were bacterial cultures without EO exposure.

Minimum inhibitory concentration (MIC): according to Rankin (2005), an aliquot (5µl) of 10^8 CFU/ml overnight cultures was added to wells of sterile 96-well micro-titerplate, containing (135µl) adequate medium added of Bromocresol Purple to provide a final medium concentration of 0.16% (w/v). EO was diluted in sterile solution (0.5%, v/v) of Tween 80 and added (50µl) to wells to give final EO concentrations between 0.50 and 95.00% (v/v). Positive control wells contained adequate broth and cells without EO while negative controls wells contained, individually, medium, EO and Tween 80. Plates were incubated under normal atmospheric conditions at respective temperatures according to strain for 24h.

Inhibition of growth was made visible from change in color in pH indicator observed in a stereomicroscope (OLYMPUS C011-092879, Japan). The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of essential oil at which microorganism does not demonstrate any visible growth (Tao, et al., 2009).

Characterization of EO antagonistic effect on cell growth: considering MIC results, the effect of this essential oil on the growth of wild strain was determined at different times by total count and spectrophotometric absorption (JASCO spectrophotometer V-630, Japan) to 560nm (Abs_{560nm}). Active strains were transferred (2%, v/v) to two series of flasks containing Elliker broth, in sufficient number (30 tubes) for total monitoring of growth; third series of flasks without inoculum were used for blank test. Three series were incubated in water bath with low speed agitation (VICKING

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Thermostatic bath, Dubnoff model, Argentina) to 30°C during 120h. When cells reached exponential phase of growth, corresponding to 0.7-0.8 of spectrophotometric absorption, an aliquot (100μl) of EO dilution (5:95, EO: Tween 80) was added to each flask of one series and the other was used as positive control to normal growth. At different times, one flask from each series was used for determination of Abs_{560nm} and count of viable cells.

Statistical analysis: experiments were replicated three times. All results reported were expressed as Mean±SD. Data were analyzed using INFOSTAT Software by one-way ANOVA procedure (Di Renzo et al., 2008). Differences among means were detected by the Hotelling Test. Significance of all tests was set at $P<0.05$.

RESULTS

Essential oil characterization: the relative density value obtained (0.8573mg/ml) is within specifications of the given quality in IRAM-SAIPA Standard for *Citrus paradisi* oil (0.8520-0.8600mg/ml). Although it was greater than the value reported by other authors: 0.8433 (Pino, et al., 1999), 0.8500 (Viuda, et al., 2008) and a 0.8532-0.8508 mg/ml (Kesterson and MacDuff, 1948) range for cold-pressed EO of this same fruit; besides, it is within the range reported by (Güenther, 1961) from 0.8550 to 0.8600 mg/ml obtained under same conditions.

Refractive index measured (1.4723) was slightly under IRAM-SAIPA (1.4740-1.4790) and Güenther (1961) specifications (1.4745-1.4778), but resulted similar to those reported by Kesterson and MacDuff (1948) of 1.4714-1.4726. However, it was greater than those reported by other authors, 1.4692 (Pino, et al., 1999) and 1.4700 (Viuda, et al., 2008). Density values lower than 0.9000 mg/ml and refractive indexes close to 1.4700 indicate a high content of terpenes (Kesterson and MacDuff, 1948), consistently with ours chromatographic results.

Optical activity of grapefruit EO was +90.25° being in the range +91.45-+94.36° reported by Güenther (1961) as well as in the range from +91.50° to +96.50° reported by Kesterson and MacDuff (1948). Instead, other reference (Pino, et al., 1999) reported a value greater than +94°. Values obtained from activity can be due to content in terpenes, mainly from limonene which is the greatest, as they have more optical and dextrorotatory activity compared to oxygenated components where optical activity is null and to those of sesquiterpenes where it tends to be levorotatory (Weast, 2010).

Chemical constitution of essential oil: 21 components were identified in the test by GC/MS: 9 terpenes, 3 sesquiterpenes, 2 aldehydes, 7 alcohols, and 1 ester (representing 99.10% of components).

In Table 1, identified components together with retention times (Tr), and composition average with its respective percentage values are shown. Limonene was the monoterpene present in greater proportion, for this compound 92.60% was obtained being in the upper limit of reported range (76.00-96.00%) for citrus EOs (Kirbaslar, et al., 2006; Espina, et al., 2011). Monoterpene in greater proportion following limonene was β-myrcene (1.20%), slightly under the percentage reported in literature for citrus EOs (Kirbaslar, et al., 2006). Other components are found in lower proportions (below 1.00%).

Screening of antibacterial effect: Assay results for inhibition tested of microorganism growth, indicated that EO exhibited varying levels of antibacterial activity against studied bacteria. Although a greater number of Gram (+) strains than

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Gram (-) strains was used for experiments (27 and 13, respectively), under identical assay conditions, 40.74% of Gram (+) and 100.00% of Gram (-) were sensitive to EO. All tested *E. coli* (13 strains) showed sensibility to this EO.

Of tested strains of Gram (+) lactobacillus, only 3 of them showed a minimum inhibition in the assay conditions (ZOI: 5.0-6.5mm). Among Gram (+) cocci tested strains, *Leuc. mesenteroides* subsp. *dextranicum* 14c isolated from caseario environment in the province of Corrientes (Argentina) presented a significant sensitivity (ZOI=11.0mm); *L. lactis* subsp. *diacetylactis* 166c showed less sensitivity to grapefruit EO (ZOI=9.1mm), and *L. lactis* subsp. *lactis* strains showed a variable response. Four strains can be highlighted (207c, 138c, 199c, and 140c, in descending order of importance) to have shown an interesting inhibitory response against essential oil. These results are shown in Fig. 1, *L. lactis* subsp. *lactis* 35c VCOR was used as negative control.

Table-1: Chemical composition of *Citrus paradisi* oil by GC/MS.

Peak Nº	TR (min)	Identified Components	%
1	12.62	α-pinene	0.60
2	15.38	Sabinene	0.60
3	16.71	β-myrcene	1.20
4	17.38	n-octanal	0.40
5	21.21	Limonene	92.60
6	2.46	Linalool oxide cis	0.10
7	23.72	Linalool oxide trans	0.10
8	24.19	Linalool	0.20
9	25.49	Mentha-2,8-dien-1-ol trans p	0.30
10	26.27	Limonene oxide (Z)+mentha 2,8-dien-1-ol cis p- +limonene oxide (E)	1.30
11	27.5	β-citronellal	Tr
12	30.17	Decanal	0.10
13	32.03	Carveol trans	0.30
14	32.87	Carveol cis	0.30
15	33.63	Carvone	0.30
16	35.33	Geranial + E-ocimenone	0.10
17	40.76	α-cubebene	0.10
18	41.23	Neryl acetate	0.10
19	43.77	β-caryophyllene	0.10
20	48.58	δ-amorphene	0.10
21	50.83	Spathulenol+caryophyllene oxide	0.20
Total			99.10

- TR: Retention time. Tr: Trace amounts.

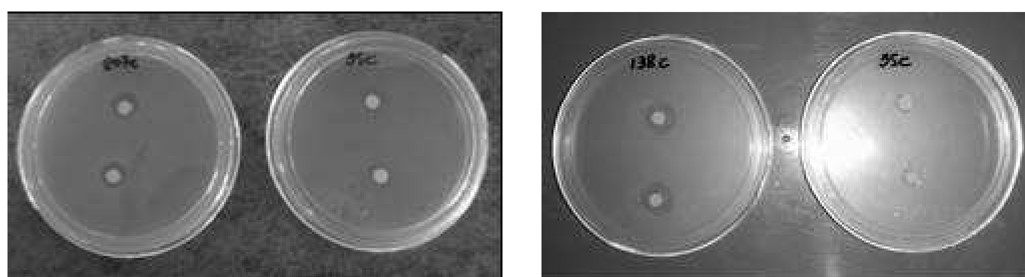

Figure-1: Inhibition in growth of *L. lactis* subsp. *lactis* 207c y 138c VCOR generated by *C. paradisi* EO.

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Minimum inhibitory concentrations: Minimum concentration of EO required inhibiting the growth was determined in 2 strains of *L. lactis* subsp. *lactis*: 138c and 207c. Cell reproduction of first strain, inoculated at 9.27±0.90 log_{10} CFU/ml density, was stopped by a 15% (v/v) concentration of EO:Tween 80. While second strain growth, 207c, at a cell density of 8.27±0.13 log_{10} CFU/ml, was inhibited with just 5% (v/v) of EO:Tween 80.
Considering the relation between volume and mass (mass = density/volumen), the experimental density (0.853mg/ml) and the EO volume used in the dilution that resulted inhibitory at the experiences (5 %, v/v), our results expressed that the lowest concentration of *Citrus paradisi* EO needed to inhibit *L. lactis* subsp. *lactis* 207c VCOR with a cellular density of 8.27±0.13 log_{10} CFU/ml, was 4.29ppm, according to the MIC using the Broth Microdilution testing.
Characterization of antagonistic effect of EO on cell growth: growth kinetics of 207c VCOR wild-type strain with and without EO is presented in Table 2. Test strain, at 9.17±0.024 log_{10} CFU/ml cellular density, was inoculated in two series of flasks. Once growth exponential phase was fully reached, 3 to 4 growing hours, showing an increasing spectrophotometric turbidity speed of 0.398 units Abs_{560nm}/h and a number of cells of 1.29± 0.17x10^9 CFU/ml, an aliquot of EO dilution was injected into flasks.

Table-2: Growth kinetics of *L. lactis* subsp. *lactis*.

Time (h)	Strain 207c		Strain 207c+EO	
	Abs_{560nm}	Viable count cell (log_{10} CFU/ml)	Abs_{560nm}	Viable count cell (log_{10} CFU/ml)
0	0.087 ± 0.05	9.17 ± 0.24	0.090 ± 0.05	9.17 ± 0.24
1	0.089 ± 0.01	ND	0.116 ± 0.01	ND
2	0.179 ± 0.03	ND	0.203 ± 0.00	ND
3	0.422 ± 0.02	9.11 ± 0.09	0.436 ± 0.03	9.11 ± 0.09
4	0.782 ± 0.00	9.11 ± 0.16	0.859 ± 0.02	9.11 ± 0.16
5	1.407 ± 0.02	ND	1.377 ± 0.02	ND
6	1.703 ± 0.02	ND	1.423 ± 0.01	ND
7	1.701 ± 0.08	9.11 ± 0.17	1.405 ± 0.08	9.11 ± 0.17
24	1.655 ± 0.07	9.43 ± 0.09	1.283 ± 0.05	7.11 ± 0.50
28	1.648 ± 0.02	9.26 ± 0.01	1.270 ± 0.01	6.80 ± 0.02
54	1.462 ± 0.02	7.95 ± 0.05	1.200 ± 0.03	4.48 ± 0.07

- ND: no determined.

Adding EO has no effects (P>0.05) on incremental speed of Abs_{560nm} reached by 207c VCOR strain with and without addition of EO (0.407/h, 0.492/h, respectively). However, smaller Abs_{560nm} was produced when reaching cryptic growing phase, showing a statistically significant difference (P<0.05) in the population measured by Abs_{560nm} of 0.280. Considering the methodology used for its determination, this difference might suggest a partial cell lysis.
Although kinetics has been tested for 120h, only the results of the first 54h are shown. No significant differences (P>0.05) were observed in absorbance measurements in each series over time, or major differences were seen in both series than those detected at 54h incubation at subsequent incubation periods.

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DISCUSSION

Chemical composition and values of grapefruit EO physical properties obtained were found near or within values reported in literature for this type of oil, and are consistent with extraction method used and its composition-high monoterpenic hydrocarbon content, especially limonene.

Reviewing opposing results about antibacterial effect, regarding differential sensitivity of bacteria (Gram + and Gram -) against EOs, some (Smith-Palmer, et al., 2001; Burt, 2004) support a greater Gram (+) sensitivity due to relative external membrane impermeability in Gram (-), and others (Tassou, et al., 2000; Fisher and Phillips (2006), reported similar sensitivity results both in Gram (+) and in Gram (-). Researchers postulated that differential sensitivity in both bacteria groups (Gram + and Gram -) does not depend exclusively from chemical or structural characteristics of cell wall, but there are other factors that influence response (Fisher and Phillips 2008; Bajpai et al., 2012). This postulate is consistent with our results.

Grapefruit EO had an important spectrum of antibacterial activities against *E. coli* (Fig. 1) with zones of inhibition ranging from 8.3 to 17.2mm. These results are consistent with those reported by other authors (Akroum, et al., 2009; Uysal, et al., 2011; Imran, et al., 2013) for grapefruit EO antibacterial effect and other related species such as *C. union* (Sarmah and Kumari, 2013), *C. limettioides* (Vasudeva and Sharma, 2012) and *Citrus* spp. (Chanthaphon, et al., 2008; Fuselli, et al., 2008).

Lactic acid bacteria, particularly cocci, showed an interesting inhibition from this EO. In northeastern provinces of Argentina, there a great amount of fresh handmade pasta factories corresponding to classical Italian pasta in addition to those factories of traditional "chipa"-a tapioca-flour roll with cheese, eggs, and water, inherited from the Republic of Paraguay which, given its geographical position, borders Corrientes, Chaco, Formosa, and Misiones provinces. In these places, production and selling of "chipa" was so popular that nowadays it is sold baked for its direct consumption and as undercooked hard rolls or dough for preparation, refrigerated or frozen with different shelf life periods according to the conservation methodology. Considering that during spring and summer seasons high ambient temperatures are reached (42-46ºC), together with high humidity, commercial low temperature preservation systems do not bear this climate conditions minimizing its performance. As a consequence, this sector manufacturers face severe microbial reproduction problems, mainly because of lactic bacteria naturally present in raw material that do not involve a risk for consumer health since they are Generally Recognized As Safe (GRAS) according to American Food and Drug Administration (FDA) (FDA, 2013). However, their growth and proliferation can reach the order of 10^8-10^{10} CFU/g, causing acidification (50-60°Dornic), typical glycolytic metabolism of this microbial group (data not shown), obviously, with changes in the organoleptic characteristics of the product. This is why this modifying microorganism group gains importance in this food sector, especially for future transference of results to regional industry.

Although antagonistic effect to growth of this EO, with GRAS status (FDA, 2013), was determined against spoilage bacteria which are of interest for this study, we must consider that, for its potential application to food preservation, effective activity concentration shall not produce changes in its sensorial properties. Determined MIC for this EO (4.29ppm) against *L. lactis* subsp. *lactis* (1.86×10^8 CFU/ml) is low enough to be used in prevention of food spoilage with no cytotoxic effects for human health, or sensory changes in product. However, specific food system must be experimentally tested as factors like fat content, and pH contained, can reduce this beneficial effect

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determined *in vitro*.

There was a decrease ($P<0.05$) in the number of viable cells originally present when adding EO ($1.29\pm0.17x10^9$ CFU/ml). After 24h of contact, $6.3\pm0.50x10^6$ CFU/ml was detected, while cell density of control microorganism series remained in the order of 10^9 CFU/ml ($1.83\pm0.07x10^9$ CFU/ml). The antagonistic effect of EO lasted during all experimental incubation period, making only $4.48\pm0.07x10^4$ CFU/ml microorganisms keep their viability at 54h of contact and subsequently. These results verified EO bacterial effect on 207cVCOR strain under these assay conditions.

Grapefruit EO has undergone less studies than sweet orange, lemon and bergamot EOs and their main components respect to antimicrobial effect. Particularly, Fisher and Phillips (2006), reported absence of limonene antimicrobial effect against some pathogenic bacteria related to food-borne diseases. These results are in opposition to our experience.

However, it is necessary to consider the possibility of chemical interactions (positive or negative) between principal components and other components present at level of traces that could generate synergy.

Comparing series cell counts with and without addition of EO to this time, there was $1.82x10^9$ CFU/ml (99.29%) viability loss at 24h of contact and $8.95x10^7$ CFU/ml (99.97%) viability loss at 54h under these experimental conditions.

EO of *Citrus paradisi* can be used as safer and alternative means of food preservation to minimize microbial contamination and to improve quality of foods as a shift from synthetic chemicals to botanical antimicrobials is gaining popularity because of their environment safety and bio-rational mode of action. This essential oil has very strong potential applicability as natural antibacterial agent for food industry, specially, for pasta manufacture which is facing serious problems of spoilage due to lactic bacteria activity. EOs could have important implications for the development of antimicrobial strategies. It is likely that it will be more difficult for bacteria to develop resistance to the multi-component EOs than to common antibiotics or chemical preservers, generally constituted by a single molecular entity.

In our knowledge, this is the first time the Argentinean grapefuit essential oil is evaluated as control agent, for the growth of undesirable bacteria in food, from the viewpoint of their potential application in bio-preservation.

CONCLUSION

Essential oil of *Citrus paradisi* from Bella Vista, Corrientes, Argentina has limonene as main constituent (92.6%), inhibits spoilage and pathogenic bacterial growth, both Gram (+) and Gram (-), and has bactericidal effect on *Lactococcus lactis* subsp. *lactis* with a dose low enough to be used as bio-preservative in food where this microorganism causes spoilage.

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REFERENCES

Adams, R.P., (2001): Identification of Essential Oil Components by Gas Chromatography, Quadrupole Mass Spectroscopy, Allured: C. Stream, IL.

Ait-Ouazzou, A., Cherrat, L., Espina, L., Lorán, S., Rota, C., Pagán, R., (2011): The antimicrobial activity of hydrophobic essential oil constituents acting alone or in combined processes of food preservation. *Innov. Food Sci. Emerg*., 12:320-329.

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Akroum, S., Satta, D., Lalaoui, K., (2009): Antimicrobial, Antioxidant, Cytotoxic activities & phytochemical screening of some Algerian plants. *Eur. J. Sci. Res.*, 31(2):289-295.

Badawy, M.E.I., Abdelgaleil, S.A.M., (2014): Composition and antimicrobial activity of essential oils isolated from Egyptian plants against plant pathogenic bacteria and fungi, *Ind. Crops Prod.,* 52:776-782.

Bajpai, V.K., Baek, K.H., Kang, S.C., (2012): Control of *Salmonella* in foods by using essential oils: A review, *Food Res. Int.*, 45:722-734.

Burt, S., (2004): Essential oils: their antibacterial properties and potential applications in foods, A review, *Int. J. Food Microbiol.*, 94(3):223-253.

Chanthaphon, S., Chanthachum, S., Hongpattarakere, T., (2008): Antimicrobial activities of essential oils and crude extracts from tropical *Citrus* spp. against food-related microorganisms, *Songklanakarin J. Sci. Technol.*, 30(1):125-131.

Chutia, M., Bhuyan, P.D., Pathak, M.G., Sharma, T.C., Boruah, P., (2009): Antifungal activity and chemical composition of *Citrus reticulata* Blanco essential oil against phytopathogens from North East India, *Food Sci. Technol.,* 42:777-780.

Di Renzo, J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L., Tabalda, M., Robledo, C.W., (2008): Statistical Software Infostat, Groip Infostat, Córdoba, Argentina.

Espina, L., Somolinos, M., Lorán, S., Conchello, P., García, D., Pagán, R., (2011):Chemical composition of commercial citrus fruit essential oil & evaluation of their antimicrobial activity acting alone/ in combined processes. *Food Cont.,* 22:896-902.

FDA, (2013): Food and Drug Administration. Department of Health and Human Service. Code of Federal Regulations, 21(3). Available online at: [http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr].

Fisher, K., Phillips, C., (2006): The effect of lemon, orange and bergamot essential oils and their components on the survival of *Campylobacter jejuni*, *Escherichia coli* O157, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* in vitro and in food systems, *J. Appl. Microbiol.*, 101(6):1232-1240.

Fisher, K., Phillips, C., (2008): Potential antimicrobial uses of essential oils in food: is citrus the answer? Review, *Trends Food Sci. Tech.,* 19:156-164.

Fuselli, S.R., García, S.B., Eguaras, M.J., Fritz, R., (2008): Chemical composition and antimicrobial activity of *Citrus* essences on honeybee bacterial pathogen *Paenibacillus larvae*, the causal agent of American foulbrood, *World J. Microbiol. Biotechnol.,* 24:2067-2072.

Güenther, E., (1961): The Essential Oils, Individual essential oils of the plant families *Rutaceae* and *Labiatae*, D. Van Nostrand Comp., Toronto, Canada, Vol 3:349-357.

Imran, K., Saeed, M., Randhawa, M.A., Rizwan Sharif, H., (2013): Extraction and applications of Grapefruit (*Citrus paradise*) peel oil against *E. coli*, *Pak. J. Nut.*, 12(6):534-537.

IRAM-SAIPA 18504, (2002): Products Flavorings. Determination of relative density at 20ºC, Argentina.

IRAM-SAIPA 18505, (2002): Products Flavorings. Determination of refractive index, Argentina.

IRAM-SAIPA 18507, (2002): Products Flavorings. Determination of optical rotation, Argentina.

Kesterson, J.W., MacDuff, O.R., (1948): Florida Citrus Oils. Commercial production methods and properties of essential oils (1947-1948 season). University of Florida, Gainesville, FL. *Agr. Exp. Sta. Tech. Bull.*, 452:1-44.

Kirbaslar, S., Boz, I., Kirbaslar, F., (2006): Composition of Turkish lemon and grapefruit peel oils, *J. Essent. Oil Res.*, 18:525-543.

Lai, C.C., Wang, C.Y., Chu, C.C., Tan, C.K., Lu, C.L., Lee, Y.C., (2011): Correlation between antibiotic consumption and resistance of Gram-negative bacteria causing healthcare-associated infections at a university hospital in Taiwan from 2000 to 2009, *J. Antimicrob. Chemoter*., 66:1374-1382.

Lv, F., Liang, H., Yuan, Q., Li, Ch., (2011): In vitro antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related

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microorganisms. *Food Res. Int*., 44:3057-3064.

Mc Lafferty, F.W., Stauffer, D.B., (1991): The Wiley/NBS Registry of Mass Spectral Data, Wiley, New York.

Maturin, L., Peeler, J.T., (2001): Aerobic Plate Count, Bacteriological Analytical Manual Food and Drug Administration, U.S. Available online at: [http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm063346].

Oliveira, A.C.S., Rabelo M.Z.J., Bispo Reis Di Iorio, F., Pereira, C.A., Cardoso, A.O., (2014): The antimicrobial effects of *Citrus limonum* and *Citrus aurantium* essential oils on multi-species biofilms, *Braz. Oral Res.,* 28(1):22-27.

Ortez, J.H., (2005): Test methods, Disk Diffusion Testing, In: Manual of Antimicrobial Susceptibility Testing, M.B. Coyle Coordinating, American Society for Microbiology, Panamerican Health Organization, pp. 39-52.

Pino, J., Acevedo, A., Rabelo, J., González, C., Escandón, J., (1999): Chemical Composition of Distilled Grapefruit Oil, *J. Essent. Oil Res.*, 11(1):75-76.

Rankin, I.D., (2005): Test methods. MIC Testing. In: Manual of Antimicrobial Susceptibility Testing, M.B. Coyle Coordinating (Ed). American Society for Microbiology, Panamerican Health Organization, pp. 53-62.

Rehman, Z., (2006): Citrus peel extract: A natural source of antioxidant. *Food Chem.,* 99:450-454.

Roussenova, N., (2011): Antibacterial activity of essential oils against the etiological agent of American foulbrood disease (*Paenibacillus larvae*), *Bulg. J. Vet. Med.*, 14(1):17-24.

Sarmah, N., Kumari, S., (2013): Comparative study of antibacterial activity of ripen &unripen Indigenous *Citrus union* of Assam India. *Int. J. Adv. Res. Technol.,* 2(9):25-31.

Smith-Palmer, A., Stewart, J., Fyfe, L., (2001): The potential application of plant essential oils as natural food preservatives in soft cheese, *Food Microbiol.*, 18(4):463-470.

Solórzano-Santos, F., Miranda-Novales, M.G., (2012): Essential oils from aromatic herbs as antimicrobial agents, *Curr. Opinion Biotechnol.*, 23:136-141.

Tao, N., Liu, Y., Zang, M., (2009): Chemical composition and antimicrobial activities of essential oil from the peel of bingtant sweet orange (*Citrus sinensis* Osbeck), *Int. J. Food Sci. Technol.*, 44:1281-1285.

Tassou, C., Koutsoumanis, K., Nychas, G.J.E., (2000): Inhibition of *Salmonella enteritidis* and *Staphylococcus aureus* in nutrient broth by mint essential oil, *Food Res. Int.*, 33(3):273-280.

UIA, (2008): National Agency for Science and Technology-Profecyt-Mincyt, Weaknesses and technological challenges of the productive sector. Citrus fruits (lemon, tangerine andorange). Available on line at: [http://www.cofecyt.mincyt.gov.ar/pciaspdfs/corrientes/UIAfrutas].

Uysal, B., Sozmen, F., Aktas, O., Oksal, B.S., Kose, E.O., (2011): Essential oil composition and antibacterial activity of the grapefruit (*Citrus paradisi* L.) peel essential oils obtained by solvent-free microwave extraction: comparison with hydrodistillation, *Int. J. Food Sci. Technol.,*46(7):1455-1461.

Vasudeva, N., Sharma, T., (2012): Chemical composition and antimicrobial activity of essential oil of *Citrus limettioides Tanaka*, *J. Pharm. Technol. Drug Res.* Available online at: [http://www.hoajonline.com/journals/pdf/2050-120X-1-2.pdf, doi: 10.7243/2050-120X-1-2].

Viuda, M., Navajas, R., Fernández, J., Pérez, J., (2008): Antifungal activity of lemon (*Citrus lemon* L.), mandarin (*Citrus reticulata* L.), grapefruit (*Citrus paradisi* L.) and orange (*Citrus sinensis* L.) essential oils, *Food Control,* 19:1130-1138.

Weast, R., (2010): Handbook of Chemistry and Physics, Ed. 90th. CRC Press, USA, pp. 318-324.

WHO, (2013): World Health Organization. Statistic. Available online at: [http://www.who.int/features/factfiles/food_safety].

Yin, X., Gyles, C.L., Gong, J., (2012): Grapefruit juice and its constituents augment the effect of low pH on inhibition of survival and adherence to intestinal epithelial cells of *S. enterica* serovar *typhimurium* PT193. *Int. J. Food Microbiol.*, 158:232-238.

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