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Mentha piperita L. essential oil inactivates spoilage yeasts in fruit juices through the perturbation of different physiological functions in yeast cells

Erika Tayse da Cruz Almeida[a], Geany Targino de Souza[b], Jossana Pereira de Sousa Guedes[a], Isabella Medeiros Barbosa[a], Cristina Paiva de Sousa[c], Lúcio Roberto Cançado Castellano[d], Marciane Magnani[b], Evandro Leite de Souza[a,*]

[a] Laboratory of Food Microbiology, Department of Nutrition, Health Sciences Center, Federal University of Paraíba, João Pessoa, Brazil
[b] Laboratory of Microbial Processes in Foods, Department of Food Engineering, Center of Technology, Federal University of Paraíba, João Pessoa, Brazil
[c] Laboratory of Microbiology and Biomolecules, Department of Morphology and Pathology, Center for Biological and Health Sciences, Federal University of São Carlos, São Carlos, Brazil
[d] Laboratory of Culture and Cell Analysis, Technical School of Health, Health Sciences Center, Federal University of Paraíba, João Pessoa, Brazil

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ABSTRACT

This study evaluated the efficacy of the essential oil from *Mentha piperita* L. (MPEO) to inactivate cells of the potentially spoilage yeasts *Candida albicans*, *Candida tropicalis, Pichia anomala* and *Saccharomyces cerevisiae* in cashew, guava, mango and pineapple juices during 72 h of refrigerated storage. Damage in different physiological functions caused by MPEO in *S. cerevisiae* in cashew and guava juices were investigated using flow cytometry (FC). The effects of the incorporation of an effective anti-yeast MPEO dose on sensory characteristics of juices were also evaluated. MPEO displayed minimum inhibitory concentration of 1.875 μL/mL against all tested yeasts. A > 5 log reduction in counts of *C. albicans*, *P. anomala* and *S. cerevisiae* was observed in cashew and guava juices with 7.5 and 3.75 μL/mL MPEO. Tested MPEO concentrations (1.875, 3.75 and 7.5 μL/mL) were not effective to cause > 5 log reduction in counts of target yeasts in mango and pineapple juices during 72 h of exposure. Incorporation of 1.875 μL/mL MPEO in cashew and guava juices strongly compromised membrane permeability, membrane potential, enzymatic activity and efflux pump activity in *S. cerevisiae* cells. This same MPEO concentration did not affect appearance, odor and viscosity in fruit juices, but negatively affected their taste and aftertaste. These results show the efficacy of MPEO to inactivate potentially spoilage yeasts in fruit juices through disturbance of different physiological functions in yeast cells. However, the combined use of MPEO with other technologies should be necessary to decrease its effective anti-yeast dose in fruit juices and, consequently, the possible negative impacts on specific sensory properties of these products.

1. Introduction

The consumption of fresh, minimally processed and additive-free foods has been increasing in last years, imposing a challenge to industry to replace traditional preservation treatments (Schenk et al., 2011). However, fruit juices manufactured following this market trend are commonly spoiled by yeasts. *Candida*, *Pichia*, *Rhodotorula* and *Saccharomyces* are yeast genera involved in fruit juices contamination and spoilage (Tournas et al., 2006; Vantarakis et al., 2011; Aneja et al., 2014), causing alterations in taste and flavor in these products (Lawlor et al., 2009).

Different emerging technologies (e.g., pulsed electric field, UV-C light and ultrasound) have been studied to preserve fruit juices (Carbonell-Capella et al., 2017; Carrillo et al., 2018), including the use of essential oils, which are generally recognized as safe (GRAS) and considered "green" antimicrobials to use in beverages (USFDA, 2015). Essential oils from *Mentha* species (Lamiaceae family) are widely recognized because of their aromatic and medicinal properties. The essential oil from *M. piperita* L. (MPEO) has been commonly used as a flavoring substance in beverages, providing a "fresh-like" aroma and taste. Early studies have shown inhibitory effects of MPEO against spoilage and pathogenic microorganisms (Freire et al., 2011; Goldjil et al., 2015; de Oliveira et al., 2017; de Sousa Guedes et al., 2017), but no previous study exploited the efficacy of MPEO to inhibit potentially

* Corresponding author. Universidade Federal da Paraíba, Centro de Ciências da Saúde, Departamento de Nutrição, Campus I, 58051-900, Cidade Universitária, João Pessoa, Paraíba, Brazil.
E-mail address: evandroleitesouza@ccs.ufpb.br (E.L. de Souza).

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spoilage yeasts in cashew, guava, mango and pineapple juices, which are fruit largely cultivated in Brazil and consumed worldwide (FAO, 2015).

Flow cytometry (FC) coupled with specific fluorescent dyes has been considered a useful tool to measure viability and physiological functions of microorganisms (Ferrario and Guerrero, 2017), enabling fast and reliable detection of different cell responses (Pan et al., 2014). There are few investigations evaluating the effects of preservation techniques on juice-related microorganisms with the use of FC (Carrillo et al., 2018; Ferrario and Guerrero, 2017; de Sousa Guedes et al., 2017; Zhang et al., 2016). Only a previous study assessed the inactivation of spoilage yeasts in fruit juices containing *M. spicata* or *M. x villosa* essential oil, but with no investigation on the possible action mechanisms responsible for the reported killing effects (Almeida et al., 2018).

This study evaluated the efficacy of MPEO to inactive cells of *C. albicans*, *C. tropicalis*, *P. anomala* and *S. cerevisiae* in cashew, guava, mango and pineapple juices stored under refrigeration. The possible mechanisms underlying the anti-yeast effects of MPEO in cashew and guava juices were investigated through the measurement of damage in different physiological functions of *S. cerevisiae* cells using FC. The effects of the incorporation of an effective anti-yeast MPEO dose on sensory characteristics of juices were also evaluated.

2. Material and methods

2.1. MPEO and identification of MPEO constituents

MPEO (batch 185; density at 20 °C = 0.900; refractive index at 20 °C = 1.460; pH 5.21) obtained by steam distillation was purchased from Ferquima Ind. Com. Ltd. (São Paulo, Brazil). Composition of MPEO was investigated using a gas chromatograph coupled with mass spectrometer (CGMS-QP2010 Ultra Shimadzu, Kyoto, Japan) equipped with a RTX-5MS capillary column (30 m × 0.25 mm × 0.25 μm), operating with a program temperature: 60–240 °C (3 °C/min), injector temperature: 250 °C, detector temperature: 220 °C, carrier gas: helium adjusted to 0.99 mL/min, ionizing energy: 70 eV and mass range: 40–500. All compounds were identified by comparison of their mass spectra with the NIST/EPA/NIH Mass Spectral Database (National Institute of Standards Technology, Norwalk, CT) and FFNSC1.3 (Flavour and Fragrance Natural and Synthetic Compounds) libraries and Kovats index (Adams, 2001).

2.2. Yeast strains and culture preparation

Strains of four different yeasts species cited as potential spoilage agents in fruit juices (Tournas et al., 2006; Vantarakis et al., 2011; Aneja et al., 2014) were used as target organisms, to cite: *C. albicans* (INCQS 40277, origin ATCC 90028), *C. tropicalis* (INCQS 40096, origin ATCC 28707), *P. anomala* (INCQS 40101, ATCC 16763) and *S. cerevisiae* (INCQS 40001, origin ATCC 2601). These strains were gently supplied by Collection of Reference Microorganisms, National Institute of Quality Control in Health, Oswaldo Cruz Foundation (Rio de Janeiro, Brazil). Stocks were kept in cryovials containing Sabouraud dextrose broth (SDB; pH 5.6; Acumedia Manufacturers Inc., Michigan, USA) with glycerol (15 g/100 mL) stored at − 20 °C. Working cultures, obtained by streak onto Sabouraud dextrose agar (SDA; pH 5.6, Acumedia Manufacturers Inc., Michigan, USA) were maintained at 4 °C and transferred monthly to fresh SDA. Each yeast strain was cultured in SDB at 30 °C for 48 h (to reach stationary growth phase), harvested by centrifugation (4500 *g* × 15 min, 4 °C), washed twice and resuspended in sterile saline solution (NaCl 0.85 g/100 mL) to obtain cell suspensions with OD reading at 625 nm (OD_{625}) of 0.75 for *C. albicans*, *C. tropicalis* and *P. anomala*, and of 0.95 for *S. cerevisiae*, which corresponded to viable counts of approximately 7 log colony forming units per milliliter (CFU/mL) when plated onto SDA. Each yeast strain was tested alone as a single inoculum in assays.

2.3. Preparation of fruit juices

Cashew (*Anacardium occidentale* L.), guava (*Psidium guajava* L.), mango (*Mangifera indica* L.) and pineapple (*Ananas comosus* L. Merrill) fruit in commercial maturation stage with absence of mechanical damage and visible signs of infection were purchased from a local wholesale distributor (João Pessoa, Brazil). Each fruit surface was disinfected by immersion in a sodium hypochlorite solution (150 ppm, pH 7.2 adjusted using 1 M NaOH) for 5 min, washed with sterile distilled water and dried for 30 min in a biosafety cabinet. Fruit were aseptically peeled, chopped and mixed with sterile distilled water (50 g fruit in 100 mL sterile distilled water for guava juice and 60 g fruit in 100 mL sterile distilled water for cashew, mango and pineapple; Anonymous, 2003) using a domestic blender (for 3 min). Fruit juices were double filtered using a triple-cheesecloth layer and sterilized using Wattman® membrane filters nylon pore size 0.22 μm (Sigma Aldrich, St. Louis, USA). Juices samples were stored in 25-mL aliquots at − 20 °C, and, when necessary, aliquots were thawed under refrigeration (4 ± 0.5 °C) and used in assays (de Sousa Guedes et al., 2016).

2.4. Determination of the minimum inhibitory concentration (MIC) of MPEO

MIC of MPEO against each tested yeast strain was determined using a microdilution in broth assay (CLSI, 2008). A stock emulsion of 240 μL/mL MPEO was prepared in sterilized SDB containing Tween 80 (1%, v/v; Sigma Aldrich, Saint Louis, USA). Initially, 50 μL-aliquots of this MPEO emulsion were dispensed into wells (first line) of a 96-well microplate containing 50 μL of double concentrate SDB. Then, 50 μL-aliquots of the total volume (100 μL) contained in the wells of the first line were transferred to the following wells and homogenized. Through geometric dilutions of the reason two, the MPEO concentrations varied from 120 to 0.469 μL/mL. Subsequently, 50 μL-aliquots of the yeast suspensions were added to each well (approximately 7 log CFU/mL). Final MPEO concentrations in the wells varied from 60 to 0.234 μL/mL. Microplate also contained a positive (SDB inoculated) and a negative (SDB non-inoculated) control for each yeast strain tested. The system was statically incubated at 30 °C during 48 h. MIC was determined as the lowest MPEO concentration required to prevent visible yeast growth.

2.5. Effects of the MPEO on the yeasts counts in fruit juices

Effects of different MPEO concentrations on yeast counts were evaluated in fruit juices (cashew, guava, mango and pineapple) along 72 h of refrigerated storage by enumerating the viable cells (de Souza et al., 2007). For this, 2 mL-aliquots of fruit juice samples with MPEO in an amount enough to provide the required final concentrations (i.e., 7.5, 3.75 or 1.875 μL/mL) were inoculated with 2 mL of the suspension of the respective yeast tested (approximately 7 log CFU/mL) and homogenized using a vortex for 30 s. Samples were maintained under refrigeration storage (4 ± 0.5 °C). At different storage time intervals (0 - just after homogenization, 5, 15, 30, 45 min and 1, 2, 4, 8, 12, 24, 48 and 72 h), a 100 μL-aliquot of each sample was collected and serially diluted (10^{-1} - 10^{-6}) in sterile saline solution (NaCl 0.85 g/100 mL) for inoculation onto SDA. Inoculated juices without MPEO were assayed similarly as negative controls. After incubation at 30 °C for 48 h, the visible colonies were counted and the results were obtained as the log of colony forming units per mL (log CFU/mL). Plates inoculated with aliquots collected from juice samples with MPEO were incubated for an additional period of 24 h at 30 °C compared with the samples collected from control juices. The results were expressed as change in log CFU/mL in respect to the initial yeast population in each measured storage time interval.

2.6. Staining procedure

Flow cytometry was used to monitor the physiological responses of *S. cerevisiae* cells (approximately 7 log CFU/mL) in cashew and guava juices with 1.875 μL/mL MPEO following a 45 min-exposure time period. For this, a 2 mL-aliquot of cashew or guava juices containing 3.75 μL/mL MPEO was inoculated with 2 mL of the test yeast suspension, resulting in a sample with a final MPEO concentration of 1.875 μL/mL. After exposure to MPEO in cashew and guava juices, yeast cells were harvested by centrifugation (4500 *g* × 10 min, 4 °C), washed twice and resuspended in phosphate-buffered saline (PBS; 8.0 g/L NaCl, 0.20 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4, pH 7.4) and immediately labeled with the fluorochromes: propidium iodide (PI, Sigma-Aldrich, St. Louis, MO, USA) for membrane integrity, bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$, Molecular Probes, Invitrogen, OR, USA) for membrane potential, fluorescein diacetate (FDA; ThermoFisher Scientific, Molecular Probes™, F1303) for enzymatic activity and ethidium bromide (EB; Sigma-Aldrich, St. Louis, MO, USA) for efflux activity. The same staining procedures were performed with positive (cells treated with ethanol 70% (v/v) for 45 min) and negative (inoculated juices without MPEO and PBS) controls (Carrillo et al., 2018; Kim et al., 2017; Silva et al., 2011). The exposure time used in positive control experiments, which was needed to cause sharp damage in measured yeast cell functions, directed the selection of the exposure time of yeast cells to MPEO in fruit juices used in FC analysis.

2.6.1. Membrane integrity and membrane potential

Cell pellets suspended in PBS were incubated with PI (1 μg/mL) and $DiBAC_4(3)$ (1 μg/mL) in a dark room for 30 min at 37 °C. After the staining exposure period, the samples were centrifuged (4500 *g* × 10 min, 4 °C) and washed with the equal volume of PBS to remove excess dye. Cell pellets were resuspended again in PBS and analyzed in flow cytometer (Kim et al., 2017).

2.6.2. Enzymatic activity

Cell pellets suspended in PBS were incubated with FDA (2.5 μg/mL) at 37 °C for 30 min in a dark room (Carrillo et al., 2018). Samples were centrifuged (4500 *g* × 10 min, 4 °C), the pellets resuspended in PBS and analyzed in flow cytometer.

2.6.3. Efflux activity

Cell pellets suspended in PBS with 1% (w/v) glucose were incubated with EB (5 μg/mL) for 5 min at 37 °C in a dark room (Silva et al., 2011). Samples were centrifuged (4500 *g* × 10 min, 4 °C) and washed with PBS. Cell pellets were resuspended in PBS and analyzed in flow cytometer.

2.7. FC analysis

FC measurements were conducted on a flow cytometer equipped with an argon-ion laser emitting at 488 nm (BD Accuri C6, New Jersey, USA). Green and red fluorescences were collected in the FL1 (533 nm ± 30 nm) and FL3 (> 670 nm) channels. Scatter and fluorescence signals of individual cells passing through the laser zone were collected as logarithmic signals. The fluorescence signal (pulse area measurements) was collected by FL1 ($DiBAC_4(3)$ and FDA) and FL3 (PI and EB) bandpass filters. Thresholds level for data acquisition was set on for FSC (30,000) in order to eliminate background and signals from debris considered much smaller than intact yeast. Yeast cells were gated per FSC/SSC parameters. Each sample acquisition was operated at the low flow rate setting and a total of 10,000 events were analyzed. All cytograms of fluorescence emissions were recorded using BD Accuri C6 Software (BD®, Becton Dickinson and Company, Franklin Lakes, NJ, USA).

Density plots indicating forward scatter light (FSC) vs. side scatter light (SSC) were obtained along measurements. FSC was analyzed in the plane of the beam and gave relative information on cell size. SSC was measured at 90° to the laser beam and provided information about cell granularity. Dot plot analysis of FL1 vs. FL3 was employed to establish fluorescence properties of the population. $DiBAC_4(3)^+$ PI^- cells (gate UL) correspond to depolarized and non-permeabilized cells; $DiBAC_4(3)^+$ PI^+ and $DiBAC_4(3)^-$ PI^+ cells (gate UR and LR) correspond to population of depolarized and permeabilized cells with different degrees of damage; and $DiBAC_4(3)^-$ PI^- cells (gate LL) correspond to unstained population of intact cells, polarized and non-permeabilized (Hammer and Heel, 2012). Density plot analysis of SSC vs. FL1 or FL3 was applied to determine the fluorescence properties of FDA^+ and EB^+ populations, respectively, indicating cells with altered enzymatic and efflux pump activities, respectively. These populations were gated into the right rectangles.

2.8. Sensory evaluation of fruit juices

Sensory evaluation of fruit juices was performed using an acceptability test with a previous approval from an Ethics Research Committee (protocol 1.125.993/2015). Juices were produced in the same day of the sensory tests and maintained under refrigeration storage (4 ± 0.5 °C) for a maximum time period of 4 h. Samples of fruit juices used in sensory evaluation were analyzed to certify compliance with the current Brazilian microbiological standards (Anonymous, 2001). Sixty untrained panelists (18–60 years old) were preselected according to interest in and frequency of fruit juice consumption. The tests were performed in individual booths with controlled temperature and lighting. Each panelist received three juice samples of each cashew, guava, mango and pineapple juices with or without 1.85 μL/mL MPEO. The different juice samples were served in 30-mL aliquots in white disposable cups coded with a randomized three-digit number. Immediately after removal from refrigerated storage, the samples were served simultaneously using a blind method of random sequence. The panelists were asked to use low-salt crackers and water to cleanse their palates between the samples. The appearance, odor, viscosity, taste and aftertaste attributes were evaluated on a 9-point hedonic scale ranging from 1 (dislike very much) to 9 (like very much) (Stone and Sidel, 1993; Leite et al., 2016).

2.9. Statistical analysis

Assays were performed in two independent experiments in triplicate. Different fruit juices batches (prepared using a pool of at least three different fruit) and standardized inoculum from a single yeast suspension prepared from two independent cultures of the test yeast were used in each independent experiment. MIC values are presented as modal values because the results were the same in all repetitions. For the yeast count assays, the statistical analysis was performed to determine significant differences ($p \leq 0.05$) based on Student t-test or ANOVA followed by post-hoc Tukey test. For sensory parameters, the statistical analysis was performed to determine significant differences ($p \leq 0.05$) based on Student t-test. The computational software Sigma Stat 3.5 software (Jandel Scientific Software, San Jose, California) was used for the statistical analysis. FC analyses were performed in two independent experiments in triplicate with consistent results.

3. Results and discussion

3.1. Identification of MPEO constituents

Constituents identified in MPEO are shown in Table 1. The majority constituent in MPEO was menthol (45.58%), followed by menthone (24.87%), isomenthone (9.48%), eucalyptol (5.65%), menthyl acetate (4.62%), limonene (2.02%) and β-caryophyllene (1.02%). A wide variety of other constituents were identified in amounts ≤1%. Previous

Table 1
Constituents identified in the essential oil from *Mentha piperita* L.

Retention time	Kovats index	Constituent	Amount (%)[a]
5.894	932	α-Pinene	0.78
6.332	948	3-Methylcyclohexanone	0.12
7.003	972	Sabinene	0.28
7.131	976	β-Pinene	0.83
7.609	993	ethyl-Hexanol	0.23
8.664	1023	ρ-Cymene	0.20
8.818	1027	Limonene	2.05
8.921	1030	Eucalyptol	5.65
13.467	1144	Isopulegol	0.18
13.907	1154	Menthone	24.87
14.322	1164	Isomenthone	9.48
14.813	1176	Menthol	45.58
14.930	1178	(−)-Terpinen-4-ol	0.42
15.166	1184	Isomenthol	0.30
15.465	1191	α-Terpineol	0.21
17.600	1239	Pulegone	0.79
18.261	1254	Piperitone	0.11
19.155	1274	*cis*-Carvone oxide	0.10
20.012	1293	Menthyl acetate	4.62
24.049	1385	α-Bourbonene	0.15
25.560	1420	β-Caryophyllene	1.02
25.938	1429	β-gurjunene	0.10
27.023	1454	α-Caryophyllene	0.10
32.401	1584	Caryophyllene oxide	0.13

[a] Constituents detected in amounts ≥0.1%.

studies have also detected menthol and isomenthone as the prevalent constituents in MPEO (de Oliveira et al., 2017; de Sousa Guedes et al., 2016; Guerra et al., 2015).

3.2. Anti-yeast effects of MPEO

MPEO displayed MIC value of 1.875 μL/mL against *C. albicans*, *C. tropicalis*, *P. anomala* and *S. cerevisiae*. Early investigations reported MIC values of MPEO against *C. albicans*, *C. tropicalis*, *P. anomala* and *S. cerevisiae* varying from 1.13 to 6 μL/mL (Saharkhiz et al., 2012; Tyagi and Malik, 2011; Tyagi et al., 2013; Vuuren et al., 2009).

The effects of 1.875, 3.75 and 7.5 μL/mL MPEO on the counts of *C. albicans*, *C. tropicalis*, *P. anomala* and *S. cerevisiae* were studied in cashew (Fig. 1A–D), guava (Fig. 1E–H), mango (Fig. 2A–D) and pineapple (Fig. 2E–H) juices over 72 h of refrigerated storage. Incorporation of 1.875, 3.75 and 7.5 μL/mL MPEO decreased ($p \leq 0.05$) the counts of all tested yeasts in fruit juices over time.

Cashew juice with 7.5 μL/mL MPEO presented > 5 log reductions in counts of *P. anomala* after 72 h (Fig. 1C); the same reduction against *S. cerevisiae* was achieved in cashew juice with 3.75 and 7.5 μL/mL MPEO after 12 and 48 h, respectively (Fig. 1D). Cashew juice with 3.75 and 7.5 μL/mL presented > 5 log reductions in counts of *C. albicans* after 72 h (Fig. 1A). The lowest reductions (1.25–1.97 log) in *C. tropicalis* counts were observed in cashew juice with 1.875, 3.75 and 7.5 μL/mL MPEO (Fig. 1B).

Guava juice with 7.5 μL/mL MPEO presented 2.44 and 0.87 log reductions in counts of *C. albicans* (Fig. 1E) and *C. tropicalis* (Fig. 1F) after 72 h of storage, respectively. Otherwise, 1.875, 3.75 and 7.5 μL/mL MPEO in guava juice caused > 5 log reductions in counts of *P. anomala* after 24 h (Fig. 1G); this same reduction level in *S. cerevisiae* counts was observed in guava juice with 3.75 and 7.5 μL/mL MPEO after 72 h (Fig. 1H).

Incorporation of 1.875, 3.75 and 7.5 μL/mL MPEO in mango and pineapple juices caused lower reductions in yeast counts when compared to cashew and guava juices during 72 h of storage. In both mango and pineapple juices, the highest reductions in counts of *C. albicans* (1.44 and 3.63 log, respectively), *C. tropicalis* (0.66 and 1.13 log, respectively), *P. anomala* (4.81 and 3.70 log, respectively) and *S. cerevisiae* (3.68 and 3.92 log, respectively) after 72 h were caused by 7.5 μL/mL MPEO (Fig. 2A–D and Fig. 2E–H, respectively). Reductions in yeasts counts caused by 1.875 and 3.75 μL/mL MPEO in mango and pineapple juices were in the range of 0.33–3.60 and 0.72–3.76 log, respectively.

Data of time-kill studies in fruit juices showed the efficacy of MPEO to inactivate cells of potentially spoiling yeast over time, although the inactivation rates varied with the essential oil concentration and target yeast strain. The following general ranking of sensitivity to MPEO was observed: *S. cerevisiae/P. anomala* > *C. albicans* > *C. tropicalis*. There are few reports on the anti-yeast effects of *Mentha* essential oils in fruit juices. Previous studies detected 2-log reductions in *S. cerevisiae* counts in a mixed fruit juice (orange and apple) with 1.13 mg/mL MPEO after eight days of storage at room temperature (Tyagi et al., 2013). Another study observed that 1 μL/mL MPEO in apple juice caused ≥5-log reductions in *Zigosaccharomyces rouxii* and *Z. bailii* counts after 15 days of refrigerated storage (Karaman et al., 2016). The incorporation of *Mentha spicata* L. (0.937–3.75 μL/mL) and *M.* x *villosa* Huds essential oils (3.75–15 μL/mL) in cashew, guava, mango and pineapple juices reduced the counts of *C. albicans*, *C. tropicalis, P. anomala* and *S. cerevisiae* in the range of 1 – ≥5 log CFU/mL. Similar to our findings, *S. cerevisiae* and *C. tropicalis* were the yeast most and less sensitive, respectively, to the action of the tested essential oils (Almeida et al., 2018).

3.3. Flow cytometry study

In order to investigate possible mechanisms underlying the inactivation of yeast cells caused by MPEO in fruit juices, four fluorescent probes (PI, $DIBAC_4(3)$, FDA and EB) were used to measure populations of *S. cerevisiae* cells with altered physiological functions after a 45-min exposure to 1.875 μL/mL MPEO in cashew and guava juices. These juices were selected because overall the highest yeast inactivation rates were observed when MPEO was incorporated into them. *S. cerevisiae* was used as target organism because this yeast species has been used as a model organism to study the anti-yeast action modes of different antimicrobial compounds and procedures (Ferrario and Guerrero, 2017; Ling et al., 2013; Schenk et al., 2011). The dose of 1.85 μL/mL MPEO was selected to use in FC analysis because it was the MIC value of MPEO against all the tested yeast strains.

Membrane potential is generated due to differences in electrical state of internal and external sides of cell membrane (Comas-Riu and Rius, 2009). $DiBAC_4(3)$ is an anionic molecule and fluorescent dye capable of indicating membrane potential alterations. Anionic molecules are typically excluded by polarized cells, while they are accumulated by depolarized cells (Silva et al., 2011). PI is an impermeant DNA dye that penetrates only damaged membrane cells and binds to nucleic acids. Fluorescence density plot using dual-parameter of green fluorescence (y-axis; $DiBAC_4(3)$) and red fluorescence (x-axis; PI) demonstrated different magnitude of membrane damage and membrane potential alteration in *S. cerevisiae* cells exposed to the different treatments. For both juices, cells treated with 70% ethanol for 45 min (positive control) showed ruptured and depolarized membranes (100%; Figs. 3 and 4). Similar results were observed to *S. cerevisiae* cells in cashew and guava juices with MPEO, where 99.6 and 99%, respectively, presented ruptured and depolarized membranes (Figs. 3 and 4). *S. cerevisiae* cells in cashew and guava juices without MPEO remained non-permeabilized and largely polarized (90.1 and 90.4%, respectively) as well as in PBS (92.5%).

Although the membrane potential strongly contributes to homeostasis because the balance between the amounts of ions inside and outside cell, reduced membrane potential alone indicates decreased cell activity but not cell death (Léonard et al., 2016). Membrane depolarization is probably an event prior to membrane permeabilization commonly achieved when a sufficient amount of a molecule accumulates into cell membrane, increasing the permeability to ions and dissipation of transmembrane ions gradient (Díaz et al., 2010; Hammer and Heel, 2012). Probably, MPEO affected *S. cerevisiae* cells membrane

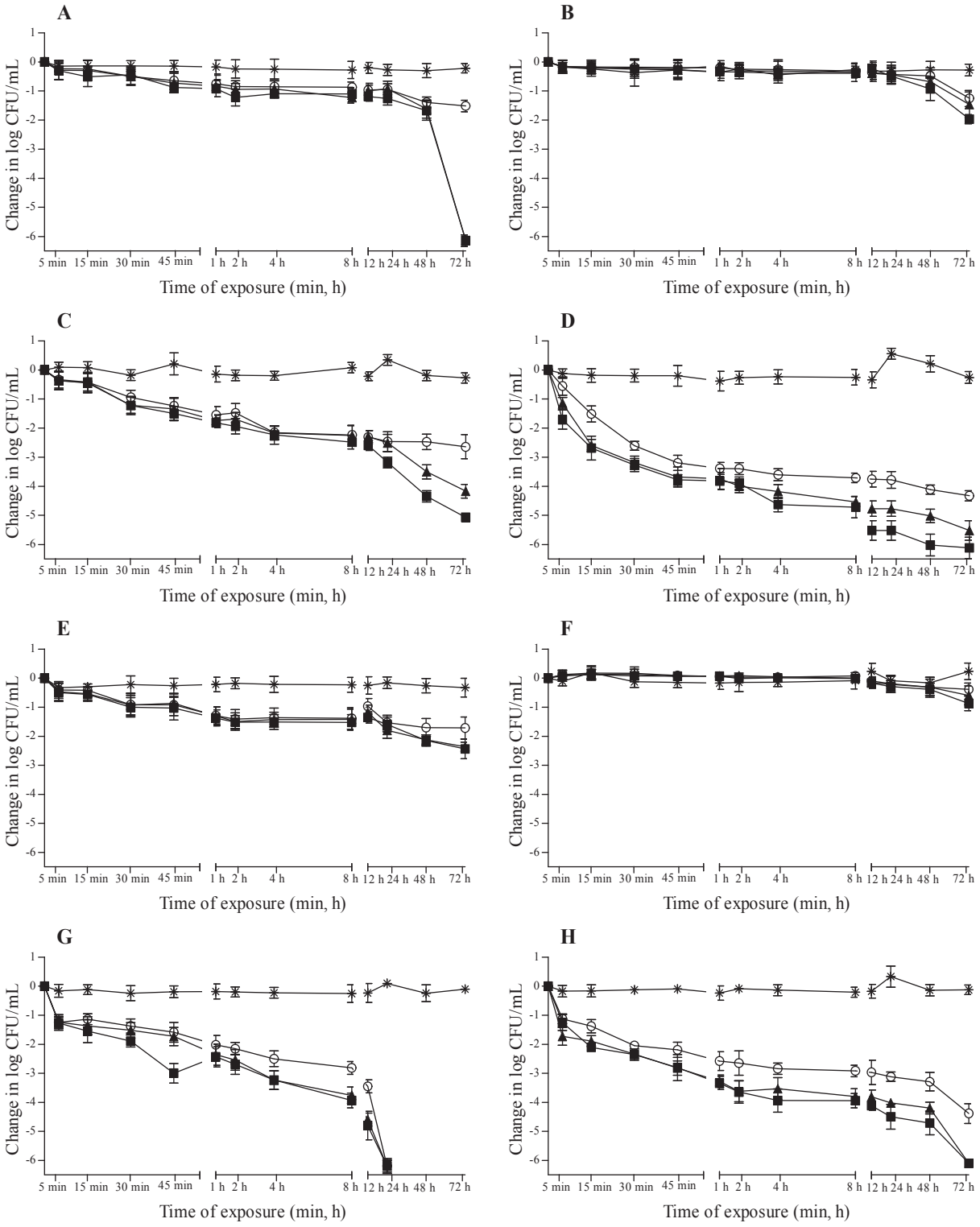

Fig. 1. Changes (log CFU/mL; n = 6) in the initial counts of *C. albicans* ATCC 90028 (A, E), *C. tropicalis* ATCC 28707 (B, F), *P. anomala* ATCC 40101 (C, G) and *S. cerevisiae* ATCC 2601 (D, H) as a function of the concentration of *Mentha piperita* L. essential oil in cashew juice (A–D) and guava juice (E–H) stored at 4 ± 0.5 °C. (■): 7.5 μL/mL, (▲): 3.75 μL/mL, (○): 1.875 μL/mL, (*) control: 0 μL/mL. Detection limit of the test: 1 log CFU/mL.

by reducing polarity and increasing permeability.

FDA is a lipophilic dye and non-fluorescent precursor that promptly diffuses across membranes, being employed primarily for evaluation of cell enzymatic activity. Into metabolically active cells, FDA undergoes hydrolysis by unspecific esterases into fluorescein, which is a polar membrane-impermeant fluorescent molecule retained in cells with intact membrane (Schenk et al., 2011). Incorporation of MPEO in cashew and guava juices led to a cell shift from the right gate (cells with enzymatic activity) to the left gate (cells with compromised enzymatic activity) compared to negative controls (juices without MPEO and PBS). Percentage of *S. cerevisiae* cells with compromised enzymatic activity was of 8.2% in cashew juice without MPEO and of 99.7% in cashew juice with MPEO (Fig. 3); percentage of *S. cerevisiae* cells with compromised enzymatic activity was of 5.8% in guava juice without MPEO

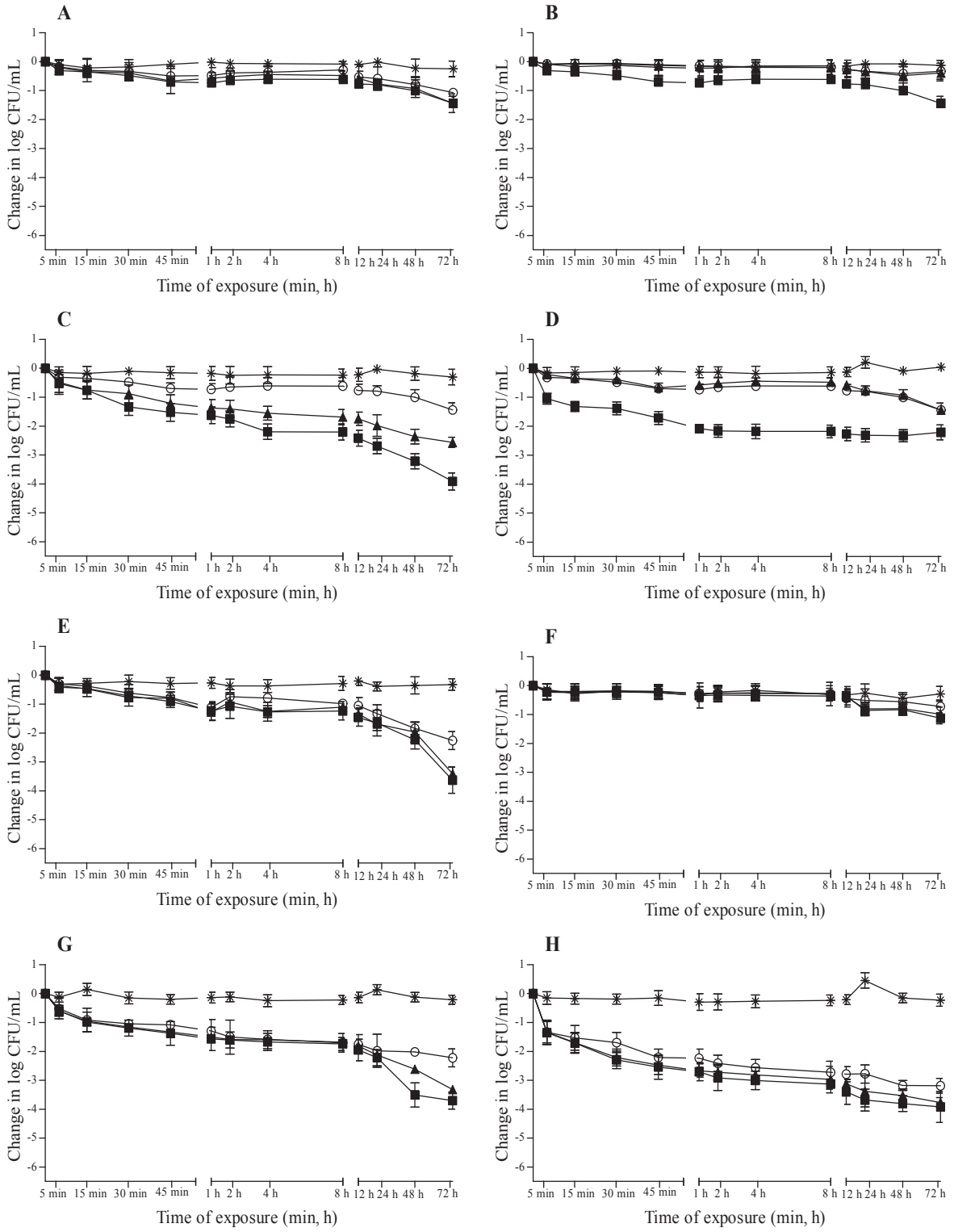

Fig. 2. Changes (log CFU/mL; n = 6) in the initial counts of *C. albicans* ATCC 90028 (A, E), *C. tropicalis* ATCC 28707 (B, F), *P. anomala* ATCC 40101 (C, G) and *S. cerevisiae* ATCC 2601 (D, H) as a function of the concentration of *Mentha piperita* L. essential oil in mango juice (A–D) and pineapple juice (E–H) stored at 4 ± 0.5 °C. (■): 7.5 μL/mL, (▲): 3.75 μL/mL, (○): 1.875 μL/mL, (*) control: 0 μL/mL. Detection limit of the test: 1 log CFU/mL.

and of 95.6% in guava juice with MPEO (Fig. 4). These findings indicate that enzymatic activity in *S. cerevisiae* cells was strongly compromised in cashew and guava juice with MPEO.

Efflux pumps are integral membrane proteins that exert important role in yeast tolerance to antimicrobials, since they cause active extrusion of antimicrobials avoiding their accumulation to lethal levels inside cells (Ling et al., 2013). EB is a membrane-permeant that enters intact cell membranes but is actively pumped outside through the action of non-specific proton anti-port transport system. In cells with compromised membrane and altered efflux pump activity, EB is not extruded from cells and can, therefore, stain intracellular DNA (Díaz et al., 2010; Kim et al., 2017). MPEO in cashew and guava juices led to a cell shift from the left gate (cells with efflux pump activity) to the right gate (cells with compromised efflux pump activity) compared to

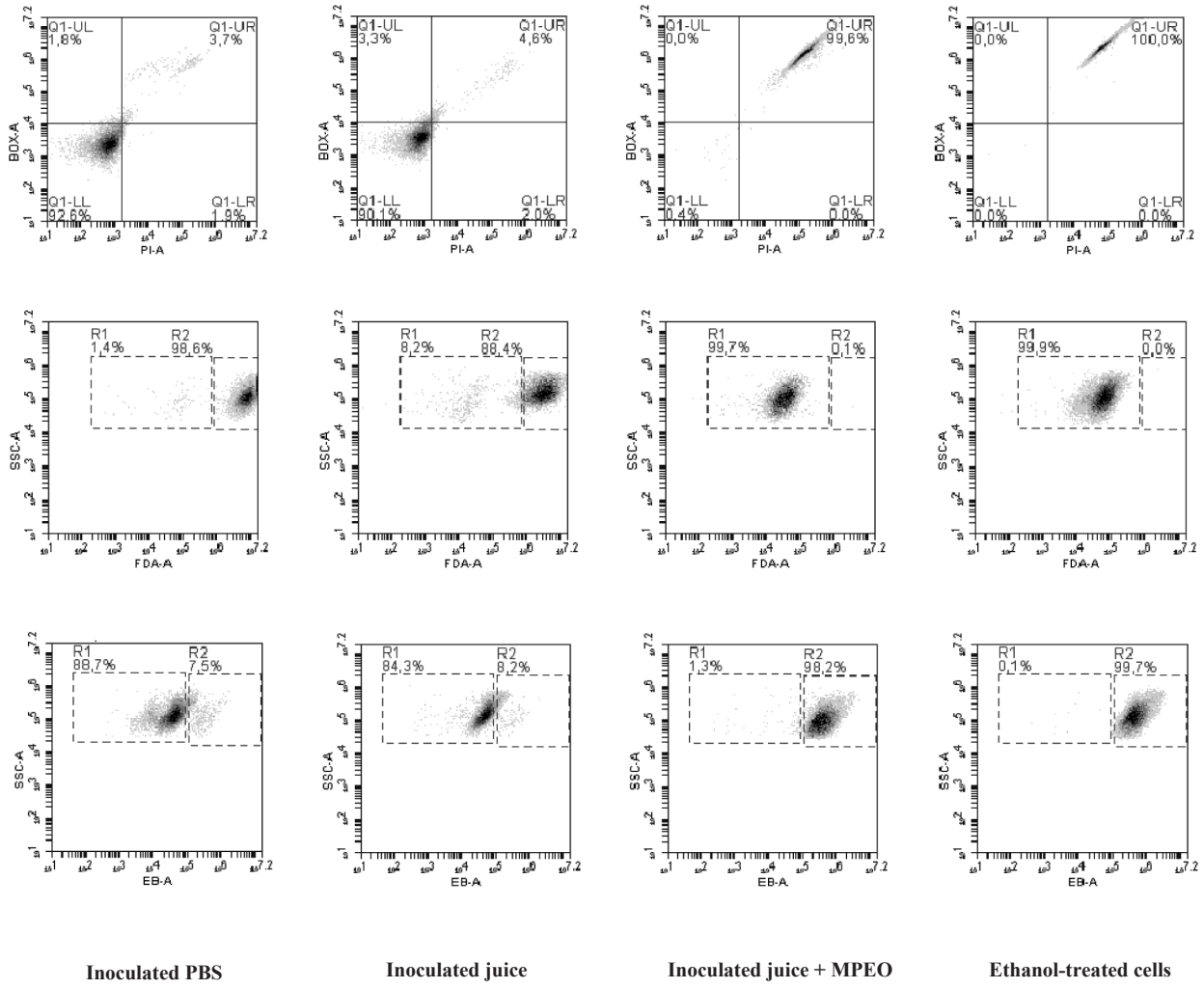

Fig. 3. Fluorescence density plots of *S. cerevisiae* in response to staining with PI and $DiBAC_4(3)$, FDA and EB after a 45-min exposure to 1.875 μL/mL *Mentha piperita* L. essential oil (MPEO) in cashew juice stored at 4 ± 0.5 °C. The vertical axis indicates the fluorescence intensity of $DiBAC_4(3)$ and side-light scatter intensity; the horizontal axis indicates the fluorescence intensity of PI, FDA and EB. The negative stain subpopulation was gated in the left rectangles; the positive stain subpopulation was gated in the right rectangles. Percentages of cell populations that fell in each gate are shown in each plot.

negative controls (juices without MPEO and PBS). Percentage of cells with compromised efflux pump activity was of 8.2% in cashew juice without MPEO and of 98.2% in cashew juice with MPEO; percentage of cells with compromised efflux pump activity was of 1.4% in guava juice without MPEO and of 97.6% in guava juice with MPEO. These data indicate that efflux pump activity in *S. cerevisiae* cells was sharply affected by MPEO in cashew and guava juice (Figs. 3 and 4).

The 45-min exposure to 1.875 μL/mL MPEO in cashew and guava juices caused reductions in counts of *S. cerevisiae* of 3.19 log and 2.12 log, respectively. Despite the small difference in count reductions, the results of FC analysis showed consistent data that revealed higher total percentage of *S. cerevisiae* cells presenting abnormal physiological functions in cashew juice than in guava juice with MPEO. Available literature has mostly attributed the antimicrobial properties of MPEO to menthol, which was the majority constituent in MPEO used in this study. Menthol has been shown to exert antifungal effects through the partition into cell membranes, causing inhibition of plasma membrane H + -ATPase and intracellular acidification, as well as trough the inhibition of ergosterol biosynthesis pathway, reducing the ergosterol amounts in cell membranes with disturbance of membranes fluidity and cell integrity (Samber et al., 2015). Effects of menthol disturbing yeast cell structures could be primarily implicated with the altered physiological functions observed in *S. cerevisiae* cells in cashew and guava juice with MPEO. However, other components detected in lower amounts in MPEO (e.g., isomenthone, eucalyptol and limonene) could also potentiate these effects in yeast cells (de Sousa Guedes et al., 2017). In addition to the disturbance of the physiological functions in yeast cells measured in this study, a previous investigation observed that MPEO can induce apoptotic cell death in *S. cereviseae* cells in laboratory media, having the ability to exert pro-oxidant effects at the cytosol and mitochondria, with yeast cell inactivation relating with both of these effects (Ferreira et al., 2014).

3.4. Sensory evaluation of fruit juices

The sensory characteristics of cashew, guava, mango and pineapple juices with 1.85 μL/mL MPEO were evaluated using an acceptance test (Table 2). The attributes appearance, odor and viscosity were not affected ($p > 0.05$) in juices with MPEO. All juices with or without MPEO received hedonic scores varying between “like slightly” and “really enjoyed” for appearance, odor and viscosity. In turn, taste and aftertaste of juice with MPEO received lower scores ($p \leq 0.05$) than

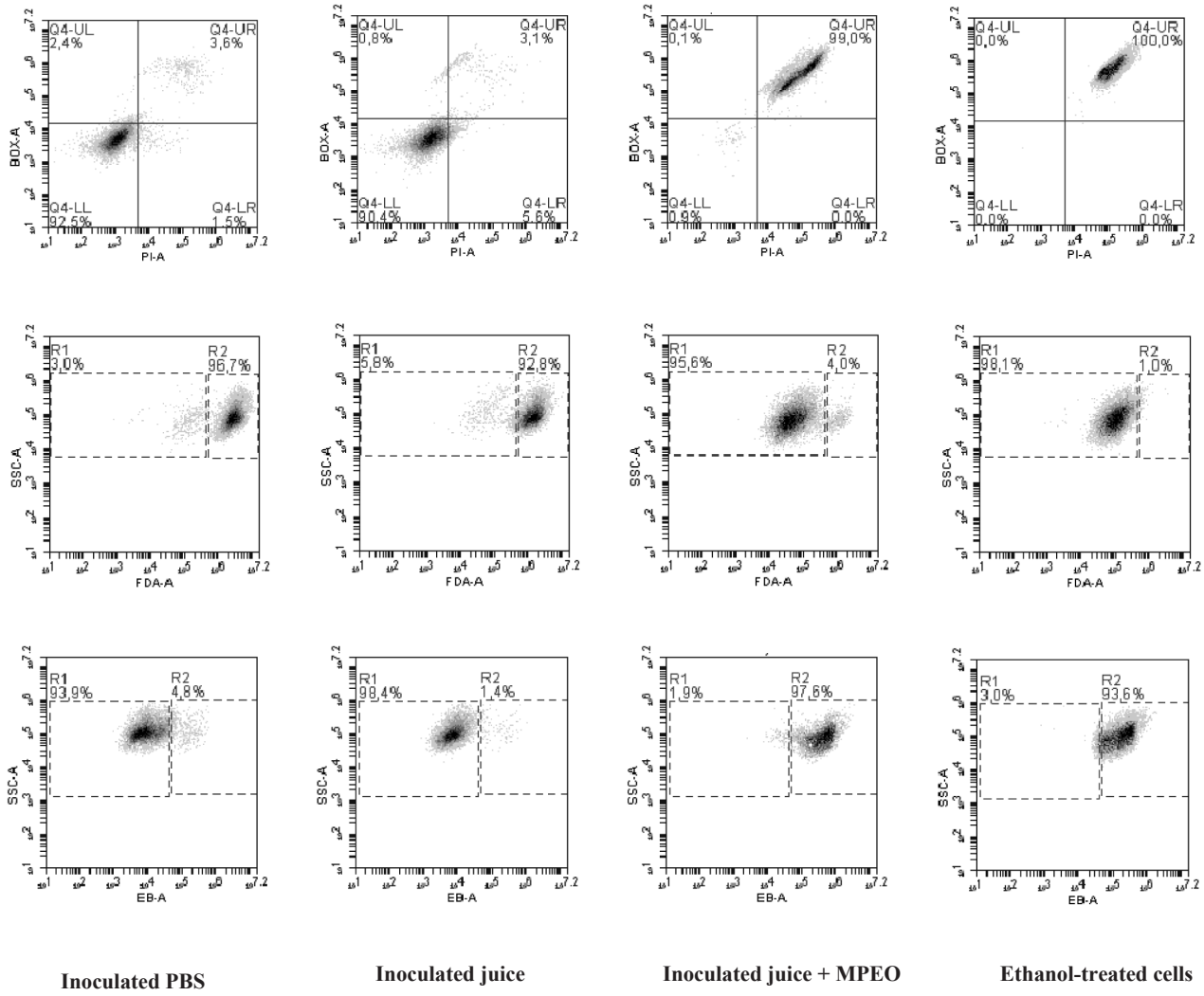

Fig. 4. Fluorescence density plots of *S. cerevisiae* in response to staining with PI and $DiBAC_4(3)$, FDA and EB after a 45-min exposure to 1.875 μL/mL *Mentha piperita* L. essential oil (MPEO) in guava juice stored at 4 ± 0.5 °C. The vertical axis indicates the fluorescence intensity of $DiBAC_4(3)$ and side-light scatter intensity; the horizontal axis indicates the fluorescence intensity of PI, FDA and EB. Negative stain subpopulation was gated in the left rectangles; positive stain subpopulation was gated in the right rectangles.

juices without MPEO. For these parameters, juices without MPEO received hedonic scores varying between "liked moderately" and "really enjoyed", while juices with MPEO received scores varying between "disliked moderately" to "neither liked nor disliked".

The possible negative impacts on taste and aftertaste of beverages caused by MPEO have been tentatively associated with the large amounts of menthol commonly detected in this essential oil (de Sousa et al., 2016). Menthol can induce a "like-mint flavor" in these products that may be not positively perceived by some consumers (Davis et al., 2005; Green, 2005). In a practical point of view, the observed negative impacts on taste and aftertaste of cashew, guava, mango and pineapple juices caused by 1.85 μL/mL MPEO could limit its use to control

Table 2
Scores (average ± standard deviation) for sensory attributes of cashew, guava, mango and pineapple juices with (1.25 μL/mL) or without *Mentha piperita* L. essential oil (MPEO).

Juices	Treatments	Attributes				
		Appearance	Odor	Viscosity	Taste	Aftertaste
Cashew	MPEO	7.95 (± 2.01)[a]	7.89 (± 1.78)[a]	7.55 (± 1.85)[a]	4.62 (± 1.26)[a]	4.77 (± 1.33)[a]
	Control	8.46 (± 1.99)[a]	8.17 (± 1.46)[a]	7.84 (± 1.73)[a]	7.66 (± 1.32)[b]	7.61 (± 1.28)[b]
Guava	MPEO	7.82 (± 1.68)[a]	7.65 (± 1.87)[a]	7.32 (± 1.48)[a]	4.83 (± 1.19)[a]	4.81 (± 1.55)[a]
	Control	8.32 (± 1.54)[a]	8.01 (± 1.48)[a]	7.44 (± 1.39)[a]	7.38 (± 1.45)[b]	7.68 (± 1.09)[b]
Mango	MPEO	8.22 (± 1.95)[a]	7.46 (± 1.45)[a]	7.61 (± 1.85)[a]	5.05 (± 1.22)[a]	5.36 (± 1.05)[a]
	Control	7.98 (± 1.82)[a]	7.99 (± 1.31)[a]	7.83 (± 1.97)[a]	8.01 (± 1.03)[b]	7.48 (± 1.62)[b]
Pineapple	MPEO	8.09 (± 2.02)[a]	7.44 (± 1.73)[a]	7.61 (± 1.44)[a]	5.33 (± 1.18)[a]	5.51 (± 1.24)[a]
	Control	7.79 (± 1.86)[a]	7.86 (± 1.68)[a]	7.72 (± 1.58)[a]	7.75 (± 1.54)[b]	7.78 (± 1.05)[b]

Control: juice without MPEO; Different superscript letters in the same column indicate significant difference ($p \leq 0.05$) among treatments, based on Student *t*-test.

spoilage yeasts in fruit juices. Therefore, the combined application of MPEO with other preservation methods could be a further research focus as an alternative to decrease the effective MPEO dose and, consequently, the occurrence of unsatisfactory changes in taste and aftertaste of these products.

4. Conclusion

The results of this study showed that 1.875, 3.75 and 7.5 μL/mL MPEO were effective to inactivate *C. albicans*, *C. tropicalis*, *P. anomala* and *S. cerevisiae* in cashew, guava, mango and pineapple juices over a 72 h-refrigerated storage, although variations in inactivation rates were evident. An effective anti-yeast dose of MPEO (i.e., 1.875 μL/mL) in cashew and guava juices sharply compromised membrane permeability, membrane potential, enzymatic activity and efflux pump in *S. cerevisiae* cells. However, the same MPEO dose caused negative impacts on taste and aftertaste of cashew, guava, mango and pineapple juices. These results show that MPEO exerts inhibitory effects against spoilage yeasts in fruit juices through a multi-target mechanism that affects simultaneously different physiological functions in yeast cells. Additional studies to exploit the anti-yeast properties of MPEO when used in combination with other technologies for preservation of fruit juices should be necessary to decrease the effective MPEO anti-yeast dose and possible unsatisfactory alterations in particular sensory properties of these products.

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