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ERIKA TAYSE DA CRUZ ALMEIDA

EFICÁCIA DE ÓLEOS ESSENCIAIS DE *Mentha* spp. NO CONTROLE DE LEVEDURAS DETERIORANTES EM SUCOS DE FRUTAS

JOÃO PESSOA – PB

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Tese apresentada ao Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Centro de Tecnologia, Universidade Federal da Paraíba, em cumprimento aos requisitos para obtenção do título de Doutor em Ciência e Tecnologia de Alimentos.

Orientador: Prof. Dr. Evandro Leite de Souza

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A toda minha família,

em especial,

ao meu daddy, José Almeida (in memoriam) e a minha mãe, Edneide da Cruz

Exemplos de resiliência, amor e fé...

Com todo meu amor

Dedico.

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Se vencemos, Alguém esteve conosco. Se nada conseguimos, Ele continua junto de nós. Se persistimos, vemos realmente que Quem nos fez continuar, sorrirá para nós, mesmo que Dele, na felicidade, nos tenhamos esquecido. Agradeço à Deus por estar sempre presente em minha vida, e tornar sonhos em realidade.

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Muito Obrigada!

Uma noite eu tive um sonho
Sonhei que andava na praia
E através do céu
Passavam cenas da minha vida
Em cada cena que passava
Percebi que na areia
Dois pares de pegadas eram deixados
Um era meu e, o outro do Senhor
Mais uma cena me entristeceu
Em algumas horas da minha vida
Só havia um par de pegadas
E aconteceu nos momentos difíceis
Da minha vida
Eu que tanto que confiei no Senhor
Me senti abandonado e perguntei
Senhor por que me abandonaste?
E o Senhor me respondeu
Filha minha, eu te amo!
Jamais te deixaria nas horas
De tua prova, do teu sofrimento
Quando na areia,
Vistes só um par de pegadas
Foi aí que eu te carreguei
(Júnior Dias)

RESUMO

Os sucos de fruta, amplamente consumidos devido às suas características de sabor atraente, aspecto refrescante e riqueza em nutrientes, são susceptíveis à deterioração microbiana, principalmente, por leveduras. Diversas tecnologias de conservação são aplicadas com intuito de extender a vida de prateleira de sucos de frutas, no entanto, há uma crescente demanda dos consumidores para um menor emprego de antimicrobianos nesses produtos. O objetivo deste estudo foi avaliar a eficácia de óleos essenciais de *Mentha piperita* L. (OEMP), *M. spicata* L. (OEMS) e *M. x villosa* Huds. (OEMV) na inativação de leveduras deteriorantes (*Candida albicans*, *C. tropicalis*, *Pichia anomala* e *Saccharomyces cerevisiae*) em caldo Sabouraud dextrose (CSD) e em sucos de caju, goiaba, manga e abacaxi durante 72 h de armazenamento refrigerado. Os efeitos da incorporação de uma dose efetiva do OEMS para inibir as leveduras testadas sobre as características físico-químicas e sensoriais dos sucos de fruta foram avaliados. Os efeitos do OEMP sobre a integridade da membrana, potencial de membrana, atividade enzimática e atividade de bomba de efluxo de *S. cerevisiae* nos sucos de caju e goiaba através do emprego de iodeto de propídio (PI), trimetina de oxonol ($DIBAC_4(3)$), diacetato de fluoresceína (FDA) e brometo de etídio (EB) foram avaliados utilizando citometria de fluxo. O OEMP e OEMS apresentaram concentração inibitória mínima (CIM) de 1,875 µL/mL frente as cepas de leveduras testadas, enquanto o OEMV apresentou CIM de 3,75 µL/mL. Reduções $\geq$ 5 log nas contagens de *C. albicans*, *P. anomala* e *S. cerevisiae* foram observadas em sucos de caju e goiaba contendo 7,5 e 3,75 µL/mL do OEMP; no entanto, essas concentrações não foram eficazes para causar a mesma redução nas contagem de leveduras em sucos de manga e abacaxi ao longo do tempo de armazenamento avaliado. A incorporação de 3,75 µL/mL de OEMS ou 15 µL/mL de OEMV causou redução $\geq$ 5 log nas contagens de *C. albicans*, *P. anomala* e *S. cerevisiae* em CSD; nos sucos de caju e goiaba, 1,875 µL/mL de OEMS ou 15 µL/mL de OEMV causaram reduções $\geq$ 5 log nas contagens de *P. anomala* e *S. cerevisiae*. No suco de abacaxi, 3,75 µL/mL de OEMS causou redução $\geq$ 5 log ufc/mL nas contagens de *P. anomala* e *S. cerevisae*; 15 µL/mL de OEMV causaram reduções $\geq$ 5 log nas contagens de *S. cerevisae* em suco de manga. A incorporação de 1,875 µL/mL do OEMS não afetou os parâmetros físico-químicos (sólidos solúveis, pH e acidez titulável) dos sucos testados, bem como não causou impactos negativos para causar sua rejeição sensorial. Os sucos de fruta com OEMS foram reportados com cor característica de suco, sabor agradável e sabor refrescante; entretanto, também obtiveram elevados scores para aroma não característico de fruta e odor e sabor de menta. A incorporação de 1,875 µL/mL de OEMP em sucos de caju e goiaba comprometeu fortemente a permeabilidade da membrana, o potencial de membrana, a atividade enzimática e de bomba de efluxo em células de *S. cerevisiae*, revelando um mecanismo de ação que envolve a perturbação de diferentes funções na célula alvo. Os resutados obtidos nesse estudo mostram, de forma geral, o potencial de óleos essenciais extraídos de espécies de *Mentha*, particularmente o OEMP e OEMS, como estratégias de controle de leveduras contaminantes de sucos de fruta.

Palavras-chave: Menta, efeito antifúngico, sucos, mecanismos de ação, citometria de fluxo.

ABSTRACT

Fruit juices, widely consumed due to their attractive taste characteristics, refreshing aspect and nutrient richness, are susceptible to microbial spoilage, mainly by yeast. Several conservation technologies are applied in order to extend the shelf life of fruit juices, however, there is a growing consumer demand for less use of synthetic preservatives in these products. The objective of this study was to evaluate the efficacy of essential oils of *Mentha piperita* L. (MPEO), *M. spicata* L. (MSEO) and *M. x villosa* Huds. (MVEO) to inactivate spoilage yeasts (*Candida albicans*, *C. tropicalis*, *Pichia anomala* and *Saccharomyces cerevisiae*) in Sabouraud dextrose broth (SDB) and in cashew, guava, mango and pineapple juice for 72 h refrigerated storage. The effects of incorporating of an effective dose of MSEO (1.875 μL/mL) to inhibit yeasts tested on physicochemical and sensory characteristics of fruit juices were evaluated. The effects of MPEO on membrane integrity, membrane potential, enzyme activity and efflux pump activity of *S. cerevisiae* in cashew and guava juice through the use of propidium iodide (PI), oxonol trimetin ($DIBAC_4$ (3)), fluorescein diacetate (FDA) and ethidium bromide (EB) were evaluated using flow cytometry. The MPEO and MSEO displayed a minimum inhibitory concentration (MIC) of 1.875 μL/mL against the yeast strains tested, while the MVEO displayed a MIC of 3.75 μL/mL. A $\geq$ 5 log reductions in the counts of *C. albicans*, *P. anomala* and *S. cerevisiae* were observed in cashew and guava juices containing 7.5 and 3.75 μL/mL of the MPEO; however, these concentrations were not effective to cause the same reduction in yeast counts in mango and pineapple juices over storage time evaluated. The incorporation of 3.75 μL/mL of MSEO or 15 μL/mL of MVEO caused a $\geq$ 5 log reductions in the counts of *C. albicans*, *P. anomala* and *S. cerevisiae* in SDB; 1.875 μL/mL of MSEO or 15 μL/mL of MVEO caused $\geq$ 5 log reductions in the counts of *P. anomala* and *S. cerevisiae* in cashew and guava juices. In pineapple juice, 3.75 μL/mL of MSEO caused $\geq$ 5 log reductions in the counts of *P. anomala* and *S. cerevisae*; 15 μL/mL of MVEO caused $\geq$ 5 log reductions in *S. cerevisae* counts in mango juice. The incorporation of 1.875 μL/mL of MSEO did not affect the physicochemical parameters (soluble solids, pH and titratable acidity) of the tested juice as well as did not induce negative impacts to cause its possible sensorial rejection. Particularly, fruit juices with MSEO were reported as having characteristic juice color, pleasant taste and refreshing taste; however, they also obtained high scores for non-characteristic fruit aroma and mint odor and taste. The incorporation of 1.875 μL/mL of MPEO into cashew and guava juices strongly compromised membrane permeability, membrane potential, enzymatic activity and efflux pump activity in *S. cerevisiae* cells, revealing a action mechanism involving different functions in the target cell. The results obtained in this study show, in general, the potential of essential oils extracted from *Mentha* species, particularly MPEO and MSEO, as antimicrobials for use in the formulation strategies of contaminant yeast control of fruit juice.

Key-words: Mint, antifungal effects, juices, action mechanisms, flow citometry.

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LISTA DE ABREVIATURAS E SIGLAS

ANOVA – Análise de Variância

ASD – Ágar Sabouraud Dextrose

CATA – *Check all that apply*

CSD – Caldo Sabouraud Dextrose

CIM – Concentração Inibitória Mínima

$DiBAC_4(3)$ - Trimetina oxonol do ácido bis-(1,3-dibutilbarbitúrico)

DO – Densidade óptica

EB - Brometo de etídio

FDA - Diacetato de fluoresceína

GRAS – Generally Recognized as Safe

OEs – Óleos essenciais

OEMP – Óleo essencial de *Mentha piperita*

OEMS – Óleo essencial de *Mentha spicata*

OEMV – Óleo essencial de *Mentha* x *villosa*

PI – Iodeto de propídio

PBS – Tampão fosfato salino

SUMÁRIO

1 INTRODUÇÃO

Nos últimos anos, os consumidores têm demonstrado uma maior conscientização em relação à adoção de hábitos alimentares mais saudáveis em decorrência de mudanças no estilo de vida e vêm buscado por produtos mais naturais, prontos para consumo, porém que se apresentem seguros em relação aos seus aspectos sanitários e que sejam nutricionalmente adequados (CARRILLO et al., 2018; FERRARIO et al., 2015).

Nesse contexto, os sucos de frutas se enquadram, de forma geral, neste perfil de alimentos exigidos, pois são reconhecidos como boas fontes de carboidratos, sais minerais, carotenoides, vitaminas e outros elementos importantes para a manutenção e promoção da saúde dos indivíduos (ABRAMS; DANIELS, 2017; O'NEIL et al., 2012).

No entanto, em consequência de suas características físico-químicas particulares inerentes (elevada acidez e teor considerável de açúcares) e, por vezes, pelas condições higiênico-sanitárias inadequadas de produção, os sucos de frutas são comumente contaminados por micro-organismos que podem causar alterações nas suas características próprias, além de poder representar riscos à saúde dos consumidores (ANEJA et al., 2014).

As leveduras são consideradas os micro-organismos predominantes na deterioração de sucos, pois são tolerantes à elevada acidez. Além disso, uma variedade de espécies possui a capacidade de se multiplicar em ambientes sem oxigênio, a exemplo da condição fornecidas em sucos embalados (ANEJA et al., 2014; ICMSF, 2005; STRATFORD, 2006; TRIBST et al., 2009).

Durante seu metabolismo, as leveduras consomem açúcares e produzem gás carbônico, álcoois e outros compostos orgânicos (ácidos, aldeídos, cetonas, ésters), levando à deterioração dessas matrizes. Além disso, as leveduras podem sintetizar enzimas responsáveis pela degradação das substâncias pécticas, causando sedimentação e formação de turvação indesejável (LAWLOR et al., 2009). *Candida*, *Pichia*, *Rhodotorula* e *Saccharomyces* são exemplos de gêneros de leveduras frequentemente envolvidos na contaminação e deterioração desses produtos (ANEJA et al., 2014; VANTARAKIS et al., 2011).

Com o propósito de prevenir a ocorrência de alterações nutricionais e sensoriais como consequência da presença desses micro-organismos, as indústrias de sucos e polpas de frutas fazem uso da aplicação de processamento térmico e/ou a incorporação de conservantes sintéticos em seus produtos, tais como, a pasteurização e o emprego de benzoato de sódio e sorbato de potássio, respectivamente.

Contudo, a aplicação destes agentes pode provocar perdas significativas na qualidade sensorial e nutricional, bem como formação de substâncias alergênicas e cancerígenas, portanto, nocivas à saúde dos consumidores (AMIRPOUR et al., 2015; SÁNCHEZ et al., 2017; TRIBST et al., 2009; VALLY et al., 2009). Dentre as diversas alternativas aos tratamentos convencionais para a conservação de sucos que atendem o propósito de produzir produtos com menores quantidades ou ausentes de conservantes sintéticos, e com vida de prateleira prolongada, destaca-se o uso de óleos essenciais (OEs) de plantas aromáticas.

Os OEs, classificados como GRAS – *Generally Recognized as Safe* sob as condições de uso pretendido em alimentos, são tradicionalmente utilizados como flavorizantes de alimentos e bebidas e têm sido reconhecidos como alternativas para uso como antimicrobianos em sistemas de conservação empregados nesses produtos. Os diversos constituintes presentes nos OEs geralmente estabelecem seus efeitos antimicrobianos por meio de distintos mecanismos, com consequente dificuldade de adaptação a estes efeitos por parte dos micro-organismos alvo (BARBOSA, 2010; BAKKALI, 2008; de SOUZA GUEDES et al., 2016; USFDA, 2015).

Conhecido por suas propriedades aromáticas e medicinais, o gênero *Mentha* apresenta diversas espécies, das quais hortelã-pimenta (*Mentha piperita* L.), menta verde (*Mentha spicata* L.), menta japonesa (*Mentha arvensis* L.), menta-silvestre (*Mentha longifolia* L.) e bergamota/alevante (*Mentha citrata* Ehrh.) são as estudadas e empregadas por diversos segmentos da indústria, incluindo a indústria de alimentos (MARTÍNEZ, 2016; OLIVEIRA et al., 2011; PARK et al., 2016). Além de possuírem propriedade aromatizante, os OEs de *M. piperita* (OEMP) e *M. spicata* (OEMS) são reconhecidos como agentes antimicrobianos e, portanto, considerados promissores para fins de conservação de alimentos. (BURT, 2004; NGUYEN, MITTAL, 2007). Entretanto, estudos sobre as propriedades biológicas da espécie *M.* x *villosa* Hudson (OEMV) são ainda escassos, embora existam relatos de suas propriedades antimicrobianas (GUERRA et al., 2015).

Considerando os aspectos acima mencionados e o reconhecido potencial antimicrobiano dos OEs de *M. piperita* L., *M. spicata* L. e *M. x villosa* Huds., este estudo teve como objetivo avaliar a eficácia de sua aplicação no controle de leveduras deteriorantes (*C. albicans, C. tropicalis, P. anomala* e *S. cerevisiae*) em sucos de frutas tropicais (abacaxi, caju, goiaba e manga).

2 REVISÃO DE LITERATURA

2.1 ARTIGO DE REVISÃO DE LITERATURA

Comprehensive REVIEWS in Food Science and Food Safety

The Potential of the Incorporation of Essential Oils and Their Individual Constituents to Improve Microbial Safety in Juices: A Review

Evandro Leite de Souza, Erika Tayse da Cruz Almeida, and Jossana Pereira de Sousa Guedes

Abstract: The juice sector is one of the fastest growing sectors in the food industry. Although juices are important because of their nutritional value and convenience, their composition and physicochemical properties affect their microbiological safety and overall quality during their shelf-life. Furthermore, the thermal process classically applied in juices partially reduces the occurring microflora, and the use of chemical additives is perceived negatively by consumers. For these reasons, researchers have proposed the use of nonthermal technologies as antimicrobial preservatives in juices. This paper covers the recent literature on the use of essential oils (EOs) and the individual constituents (ICs) found therein, used alone or in combination with other emerging technologies, for the preservation of juices. From this perspective, this paper discusses the growing importance of the use of EOs and their ICs, either alone or in association with other emerging technologies, in juices and their effects on the safety and physicochemical and sensory quality attributes of these products. The results of papers currently available in the literature reveal that EOs and their ICs are promising alternatives to achieve microbial safety and stability in juices. However, extensive studies considering the effects of each EO or IC on sensory characteristics, primarily taste and aroma, are still needed to establish each of these substances/compounds as feasible preservatives for use in juices. Finally, further studies could focus on the combination of low amounts of EOs or ICs with other nonthermal technologies to achieve a balance between the microbial safety and sensory acceptability of juices.

Keywords: antimicrobial effects, food preservation, juice, plant substances

Introduction

The demand for fresh and natural food in the market has increased in recent years because consumers have modified their eating habits and become aware of the relationship between diet and disease prevention. Hence, the consumption of fruit, vegetables, and juices as natural sources of carbohydrates, vitamins, minerals, and other important components for human health, such as fibers and antioxidants, has risen substantially. However, as a consequence of inappropriate manipulation, storage conditions, and consumption of unpasteurized juices, spoilage and pathogenic microorganisms may contaminate these products, thus increasing the possibilities of changes in their proper characteristics and the risks to consumers. Several salmonellosis and enterohemorrhagic *Escherichia coli* outbreaks associated with the consumption of a variety of unpasteurized juices have been reported in recent years (Parish 2009; Raybaudi-Massilia and others 2009; CDC 2011; EFSA 2013, 2014, 2015).

MS 20160152 Submitted 26/1/2016, Accepted 21/3/2016. *Authors are with Laboratory of Food Microbiology, Dept. of Nutrition, Health Science Center, Federal Univ. of Paraíba, João Pessoa, Brazil. Direct inquiries to author de Souza (E-mail: evandroleitesouza@ccs.ufpb.br)*

Because total common microbial contamination levels in juices often range from 3 to 5 log10 cfu/mL, microbiological spoilage can occur. It is mainly associated with the presence of yeasts and lactic acid bacteria, producing an unpleasant aroma of slight fermentation (Stratford and others 2000; Tournas and others 2006; Tyagi and others 2014b). Nevertheless, even in juices presenting pH values unfavorable for the growth of most pathogenic bacteria, contaminants such as *Salmonella* spp., *E. coli* O157: H7, and *Listeria* spp. may survive and cause disease following the ingestion of these foods (Friedman and others 2004; Kiskó and Roller 2005; Mosqueda-Melgar and others 2007; Parish 2009; Raybaudi-Massilia and others 2009). Consequently, the United States Food and Drug Administration (USFDA) has recommended that juices must be processed to achieve 5 log10 cfu/mL reductions (99.999%) in the population of pathogens of public health concern (USFDA 2001); however, the agency does not specify the method to reach this inactivation level.

Traditionally, the shelf-life stability of juices has been achieved through thermal processing, and the recommended temperatures for pasteurization by high-temperature short-time are in the range of 72 to 82 °C (FDA 2004). In addition to thermal treatment, chemical preservatives such as potassium sorbate and sodium benzoate are widely used to extend the shelf-life of

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juices (Amirpour and others 2015). Sulfur dioxide has been also extensively used to preserve juices because of its antioxidant properties and selective inhibitory effects on enzymatic and microbial activities (Toniolo and others 2010). However, heat treatment commonly reduces product quality and freshness in juices, and chemical preservatives have been often rejected by consumers, which has increased interest in exploiting new and effective natural-origin and safe juice-preservation strategies. Therefore, several nonthermal pasteurization methods have been proposed over the last few decades, including pulsed electric field, high-pressure homogenization, high hydrostatic pressure, and ultrasound, to preserve juices (Rupasinghe and Yu 2012).

These emerging preservation techniques seem to have the potential to provide "fresh-like" juices, and they have been associated with "green" antimicrobials, such as essential oils (EOs) or their individual constituents (ICs), possibly leading to synergistic or additive interactions (Mosqueda-Melgar and others 2012; Patrignani and others 2013; Espina and others 2014b; Tyagi and others 2014b). The synergistic interactions resulting from the combination of these physical techniques and antimicrobial substances/compounds could in practice prolong the shelf-life of juices and become possible strategies for the replacement of traditional pasteurization methods and synthetic antimicrobial preservatives (Rupasinghe and Yu 2012); hurdle technology (the Hurdle principle; Leistner 1978) has offered greater lethality against microorganisms than any single treatment (Duan and Zhao 2009).

EO is a product obtained from natural raw material of plant origin by steam distillation, mechanical processes from the epicarp of citrus fruits or dry distillation, after the separation of the aqueous phase, if there is any, by physical processes (Intl. Organization for Standardization 9235 2013). These substances are complex mixtures that can contain approximately 20 to 80 ICs at different concentrations. The chief group of ICs forming the EOs results from the union of terpenes and terpenoids, and the other group contains aromatic and aliphatic constituents (Burt 2004). Most EOs and their ICs are cited as "generally recognized as safe" (GRAS) by the USFDA and are registered by the European Commission for use as flavoring substances in foods (Anonymous 1999); they have been cited to have no significant or marginal toxic effects regarding the possible amount of use in food (British Pharmaceutical Codex 1979; Burt 2004).

Considering that the outer membrane acts as an impermeable barrier to hydrophobic compounds, the damage in this cell structure might represent an interesting opportunity to design combined or sequential processes that facilitate the action of antimicrobial compounds or procedures (Ait-Ouazzou and others 2011). Hydrophobicity is an important characteristic of EOs and their ICs, which enables them to partition in the lipids of the bacterial cell membrane, disturbing the structures, rendering them more permeable, and causing leakage of ions and other cell components (Burt 2004). In juices, it appears that the application of EOs and their ICs, in combination or in sequential association with other emerging techniques, has been successful due to the decrease in the concentrations of antimicrobials and the temperatures applied in thermal treatment and also to the increase of the effects of nonthermal techniques (Nguyen and Mittal 2007; Mosqueda-Melgar and others 2008a,b,c; Espina and others 2011, 2012, 2013a,b; Ait-Ouazzou and others 2013).

Quality losses in juices may occur as a consequence of microbiological, enzymatic, chemical, or physical alterations. Therefore, interest in the use of nonsynthetic substances or compounds to prevent microbiological spoilage, although assuring safety and maintaining quality characteristics in juices, has significantly increased in the last few years (Raybaudi-Massilia and others 2009). In this context, the aim of this paper is to provide an overview of the use of EOs and/or their ICs, used alone or in association with other emerging technologies, in fruit and vegetable juices to maintain their safety and quality characteristics.

Use of EOs as Antimicrobials in Juices

Although the use of natural antimicrobial preservatives in juices shows an upward trend, some researchers have stated that high concentrations of EOs are necessary to achieve the desired antimicrobial effects when these substances are the single hurdle to controlling microbial growth, implying likely undesirable sensory characteristics (Hyldgaard and others 2012). Apart from these potential negative sensory effects, studies have found interesting antimicrobial effects when EOs and/or ICs are incorporated into a variety of juices, as summarized in Table 1.

The effects of the incorporation of clove EO (4500 and 9000 mg/L) on the mesophilic count in watermelon juice during 7 d of storage at 37 °C was studied. At the end of the incubation period, 9000 mg/mL of clove EO decreased the mesophilic count in juice in 6 to 8 log cycles, whereas this decrease was approximately of 4 log cycles when the EO was incorporated into the juice at 4500 mg/mL (Siddiqua and others 2014). The study of the effects of the incorporation of black pepper EO on the shelf-life of orange juice stored at 4 °C over 28 d detected that the mesophilic and fungi counts increased over time. However, the addition of 0.2 μL/mL of the EO slightly decreased (less than 1 log cycle) these counts in the juice compared to the counts obtained in juice without the EO (Kapoor and others 2014). The incorporation of lemon EO (0.08%, 0.12%, and 0.16%) into lemon juice concentrate provoked the total inhibition of the germination and outgrowth of *Acinetobacter acidoterrestris* spores under refrigerated storage over 11 d (Maldonado and others 2013). The incorporation of 5 μL/100 mL of cinnamon EO in tyndallized carrot broth inhibited the germination of spores of psychrotrophic *Bacillus cereus* (EPSO-35AS and INRA TZ415) over 60 d of storage at either 8 or 12 °C. However, the spores of *B. cereus* were capable to germinate and persist as vegetative cells in juice stored at 16 °C, even when the cinnamon EO was incorporated (Hernández-Herrero and others 2008). These findings suggested an enhanced antimicrobial effect in juices resulting from the use of cinnamon EO and storage under lower temperatures. Few studies have attempted to elucidate the action mechanism of EOs on bacterial spore coats, but ultrastructural studies have shown damage in bacterial spores challenged with EOs, such as shriveling and dehydrated morphology, which may be associated with the possible loss of intracellular contents (Young and Setlow 2003). In addition, studies have suggested that ICs may negatively interfere with the action of nutrient receptors connected to a cascade of alterations that lead spores to commit to germination (Cortezzo and others 2004).

The assessment of the effects of the EO from *Litsea cubeba* on *Lactobacillus plantarum* in orange-milk beverage revealed that 6000 μg/g of the EO provoked a complete inactivation of the *L. plantarum* population and that the inhibitory effects increased nearly 3-fold when the EO concentration doubled (Liu and Yang 2012). A study evaluated the inhibitory effects of the EOs from clary sage, juniper, lemon, and marjoram against *Geotrichum candidum*, *Pichia anomala*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* in cloudy (unfiltered or unclarified juice—containing particles in suspension that not precipitate out) and clear apple juice (Tserennadmid and others 2011). In general, the

Table 1—Studies testing the antimicrobial activity of essential oils in juices.

Juice (pH)	Essential oil	Applied concentrations	Target microorganism (s) (inoculation level)	Main results	References
Apple (3.7)	Apricot, bergamot, cinnamon bark, cinnamon cassia, cinnamon leaf, clove bud, grapefruit, lavender, lemon, lemongrass, lime, Melissa, orange bitter, orange Mandarin, oregano Spanish, orange sweet, and tangerine	0.00065% to 0.67%	*Escherichia coli* O157:H7 and *S. enterica* (≈8log 10 cfu/mL)	Stronger killing effects (BA50; concentration capable of killing 50% of the bacterial population) of EOs were found when the juices were incubated at 37 °C during 60 min.	Friedman and others (2004)
Apple (4.2) Pear (4.0) Melon (5.9)	Cinnamon Lemongrass	2, 3, 5, 6, 8, and 10 μL/mL	*E. coli*, *Listeria innocua* and *Salmonella* Enteritidis (6 log cfu/mL)	In apple and pear juices, 2 μL/mL of the EOs displayed killing effects against all bacteria. In melon juices, 5 and 10 μL/mL of lemongrass and cinnamon EOs, respectively, showed killing effects against all bacteria.	Raybaudi-Massilia and others (2006)
Carrot (6.2)	Cinnamon	5 μL/100 mL	*Bacillus cereus* EPSO-35AS and *B. cereus* INRA TZ415 spores (2 log spores/mL)	Inhibition of the spores germination over 60 d at 8 and 12 °C; however, the spores were capable to germinate and persist as vegetative cells at 16 °C.	Hernández-Herrero and others (2008)
Apple/carrot (4.8)	Thyme	0.5% (v/v)	*Listeria monocytogenes* AUFE 39237 and *Candida albicans* ATCC 10231 (7 log 10 cfu/mL)	Reductions in counts of *L. monocytogenes* and *C. albicans* of 0.71 and 0.63 log cycles, respectively, after 5 d at 4 °C.	Irkin and Korukluoglu (2009)
Cloudy apple juice (4.3) Clear apple juice (3.8)	Lemon	0.0625 to 4 μL/mL	*Pichia anomala* MB-196, *Saccharomyces cerevisiae* MB-21 and *Schizosaccharomyces pombe* MB-89 (≈5 log cfu/mL)	Minimum inhibitory concentrations (MICs) were in a range of 1 to 4 μL/mL; higher MICs were verified in cloudy juice.	Tserennadmid and others (2011)
Orange-milk	*Litsea cubeba*	1500, 3000, and 6000 μg/g	*Lactobacillus plantarum* BCRC 10069 (5 log cfu/mL)	The EO at 6000 μg/g caused complete inactivation of bacterial population.	Liu and Yang (2012)
Lemon	Lemon	0.08%, 0.12%, and 0.16%	*Alicyclobacillus acidoterrestris* (6 log spores/mL)	All EOs tested concentrations caused total inhibition of germination and outgrowth of spores under refrigerated storage over 11 d.	Maldonado and others (2013)
Orange (4.0)	Black pepper	0.2 μL/mL	Mesophilic count Yeast and mold count	The EO reduced the counts at less than 1 log cycle.	Kapoor and others (2014)
Vegetable/fruit (3.9)	Cinnamon bark Clove bark Star anise	0.05% and 1.0%	*E. coli* O157:H7 (5 log cfu/mL)	At 5 °C, the EOs (0.05%) caused reductions in *E coli* counts of until 2.1 log cycles over 7 d; clove and cinnamon EOs (0.1%) reduced the counts to undetected levels after 1 and 7 d, respectively. At 20 °C, all the EOs (0.05%) reduced the counts to undetected levels after 7 d; clove, cinnamon or star anise EOs (0.1%) reduced the counts to undetected levels after 1, 2 and 3 d. At 35 °C, all the EOs (0.05%) reduced the counts to undetected levels after 1 day.	Pan and others (2014)
Watermelon	Clove	4500 and 9000 mg/L	Naturally occurring microbiota	The EO at 9000 mg/mL and 4500 mg/mL caused count reductions of 6 - 8 and 4 log cycles, respectively.	Siddiqua and others (2014)
Pineapple (3.9)	Lemongrass	5, 2.5, 1.25, and 0.6 μL/mL	*E. coli* UFPEDA 224, *L. monocytogenes* ATCC 7644, and *Salmonella* Enteritidis (UFPE 414; 8 log cfu/mL)	The EO at 0.6 μL/mL caused reductions in counts of *E. coli* and *L. monocytogenes* ≥5 log cycles after 1 h and 45 min, respectively; for *Salmonella* Enteritidis the same reduction was verified after 12 h.	Leite and others (2016)

EO, essential oil.

EOs presented the same minimum inhibitory concentration (MIC; 1 to 4 μL/mL) in both clear and cloudy apple juices after 48 h of incubation. However, when minor differences in anti-yeast effects (higher MICs) were observed, this occurred in cloudy apple juice. The researchers stated that particles in cloudy apple juice could decrease the antimicrobial effects of EOs because ICs may adhere to particles and precipitate, thus reducing their antimicrobial efficacy.

The antibacterial effects of different EOs (apricot, bergamot, cinnamon bark, cinnamon, clove, grapefruit, lavender, lemon, lemongrass, lime, melissa, orange, tangerine, and oregano) in a range of concentrations (0.00065% to 0.67%) against *E. coli* O157:H7 and *S. enterica* in cloudy and clear apple juices using different exposure periods (5, 30, and 60 min) and storage temperatures (4, 21, and 37 °C) were assessed. The effects of the tested EOs were expressed as the concentration (%) of each EO capable of killing 50% of the bacterial population (BA50). Overall, the findings of this study revealed that, with some exception, the lowest BA50 values were observed when the juices were maintained at higher incubation temperatures, and the antibacterial effects were lower in the cloudy juice as compared to the clear juice, against both *E. coli* and *S. enterica* (Friedman and others 2004). The researchers proposed that these decreased antimicrobial effects in cloudy juices may be associated with the adsorption of some ICs to the surface of the apple pulp present in cloudy juices. Moreover, in these studies, the antibacterial effects of the EOs increased with the contact time. Exceptionally, thyme EO was capable of killing target pathogens at a refrigeration storage temperature (4 °C).

The effects of the incorporation of 0.5% of thyme EO on the inhibition of *Listeria monocytogenes* and *Candida albicans* in apple–carrot mixed juice during storage at 4 °C for 5 d were analyzed. The thyme EO was effective in inhibiting both *L. monocytogenes* and *C. albicans*, provoking count reductions of 0.71 and 0.63 log cycles, respectively, at the end of the assessed storage period (Irkin and Korukluoglu 2009). The antimicrobial effects of different concentrations (2 to 10 μL/mL) of lemongrass, cinnamon, clove, and palmarose EOs on *Salmonella* Enteritidis, *E. coli*, and *Listeria innocua* in apple, pear, and melon juices were evaluated. In apple and pear juices, 2 μL/mL of cinnamon EO or lemongrass EO displayed killing effects against both target bacteria after 24 h of incubation at 35 °C. In the case of melon juices, lethal effects against the target bacteria were noted when 5 and 10 μL/mL of lemongrass and cinnamon EO, respectively, were added to juices. However, the cidal effects against these bacteria were also observed in juices without EO, and this fact was possibly associated with the influence of the juice pH; apple and pear juices are more acidic (pH 4.2 and 3.9, respectively) than melon juice (pH 5.9). The ultrastructural observations revealed that 5 μL/mL of lemongrass EO in apple juice resulted in the deterioration of membrane permeability, the disruption of the cellular membrane, and the leakage of cell content in *Salmonella* Enteritidis (Raybaudi-Massilia and others 2006).

The efficacy of lemongrass EO was evaluated regarding the capability to induce a ≥5-log reduction of a mixed composite of *E. coli*, *L. monocytogenes*, and *Salmonella* Enteritidis in pineapple juice. The incorporation of EO into juice at all tested concentrations (0.6, 1.2, 2.5, and 5 μL/mL) decreased the counts of all target bacteria. In juice containing 0.6 μL/mL of EO, ≥5-log reductions in *E. coli* and *L. monocytogenes* counts were observed after 1 h and 45 min, respectively whereas 12 h of incubation was needed to achieve a similar reduction in *Salmonella* Enteritidis. The presence of high amounts of oxygenated monoterpenes in lemongrass EO (77.9%), particularly of citral, neral, and geraniol, could be associated with the inhibitory effects against the tested bacterial strains. However, the intrinsic low pH of pineapple juice likely also contributed to these inhibitory effects because of the increased sensitivity of bacteria to EOs at low pH values (Leite and others 2016). Bacterial susceptibility to EOs appears to increase with a decrease in the pH of food, because at a low pH, the hydrophobicity of an EO increases, enabling it to more easily dissolve in the lipids of the cell membrane of the target bacteria (Burt 2004).

Use of ICs as Antimicrobials in Juices

EOs present a diversity of ICs, although in most cases the major constituents found therein are 2 or 3 with concentrations ≥1%, compared with other constituents present in smaller concentrations (0.1% to <1%) and traces (<0.1%; Bakkali and others 2008). The antimicrobial effects of different EOs may depend only on their major ICs; however, evidence has revealed that, in some EOs, these properties may also be influenced by interactions between major and minor ICs through synergism and additive or antagonistic effects (Deba and others 2008; Loeffler and others 2014; Aznar and others 2015). Some investigations have exploited synergies between ICs to overcome drawbacks related to the possible negative sensory effects of high concentrations of whole EOs in foods. This approach has allowed the use of new and more potent antimicrobial mixtures containing ICs, which simultaneously act on distinct targets in microbial cells (Hyldgaard and others 2012; Loeffler and others 2014).

Studies approaching the use of ICs as antimicrobials in juices are presented in Table 2. Overall, cinnamaldehyde, or trans-cinnamaldehyde, followed by carvacrol and *p*-cymene, are the ICs most tested for use as antimicrobial preservatives in juices. Notably, *p*-cymene (the precursor of thymol and carvacrol) presents slight or no antimicrobial effects; however, some studies have combined *p*-cymene with other ICs to assess possible enhancements in antimicrobial effects (Delgado and others 2004; Kiskó and Roller 2005; Rattanachaikunsopon and Phumkhachorn 2010).

Cinnamaldehyde

The interest in studying trans-cinnamaldehyde in juices may be justified by its higher solubility in water when compared to other ICs. This phenylpropanoid is a major component of cinnamon bark EO, and acts in microbial cells by increasing membrane permeability, causing the depletion of intracellular protons and, consequently, the disruption of adenosine triphosphate (ATP) synthesis (Lambert and others 2001; Bakkali and others 2008; Horváth and others 2016). The investigation of the antimicrobial effects of trans-cinnamaldehyde emulsions (0.8%, 2.4%, and 4%, w/v) against *Salmonella* Typhimurium, *E. coli*, and *Staphylococcus aureus* in watermelon juice over 48 h at 37 °C verified that the emulsion containing 0.8% of trans-cinnamaldehyde was capable of inhibiting *Salmonella* Typhimurium and *S. aureus* in juice, as measured by optical density at 600 nm; whereas the emulsions presented no inhibitory effect against *E. coli*. The antimicrobial effects against *Salmonella* Typhimurium increased with the increase in the trans-cinnamaldehyde concentration in emulsions; however, the inhibitory effects against *E. coli* and *S. aureus* were not affected by the increase in trans-cinnamaldehyde concentrations during the assessed incubation period (Jo and others 2015).

The antimicrobial effects of dual combinations among trans-cinnamaldehyde, perillaldehyde, and citral emulsions against

Table 2—Studies testing the antimicrobial activity of individual constituents of essential oils in juices.

Juice (pH)	Individual constituent	Applied concentrations	Target microorganism (s) (inoculation level)	Main results	References
Apple (3.7)	Carvacrol, trans-cinnamaldehyde, citral, eugenol, geraniol, linalool, linalyl acetate, terpinene, and terpinen-4-ol	0.00065% to 0.67%	*E. coli* O157:H7 and *S. enterica* (≈8 log cfu/mL)	At 37 °C by 30 min, 0.044% and 0.056% of trans-cinnamaldehyde caused cidal effects against *E. coli* occurred at 0.044%, and 0.056%; 0.047% and 0.05% caused cidal effects against *S. enterica*. At 60 min at 37 °C, 0.0097%, and 0.02% of carvacrol caused ciddal effects against *E. coli*; 0.019%, and 0.0054% caused cidal effects against *S. enterica*. The stronger antibacterial effects were observed in cloudy than in clear juices.	Friedman and others (2004)
Carrot (6.47)	Thymol *p*-cymene	0.5, 1, 2, and 4 mmol/L	*B. cereus* INRA-AVTZ415 and *B. cereus* INRA-AVZ421 (5 log cfu/mL)	Thymol at 0.5, 1.0, 2.0, and 4.0 mmol/L caused count reductions that varied from 1.0 to 5.5 log cycles; *p*-cymene at 4.0 and 2.0 mmol/L caused reductions of approximately 0.5 log cycles. The combinations of thymol and *p*-cymene reduced the count in a range of 2.0 to 7.0 log cycles.	Delgado and others (2004)
Apple (3.2)	Carvacrol *p* -cymene	0.5 and 1.25 mM 0.25 and 1.25 mM,	*E.* O157;H7 (4 log cfu/mL), total bacterial count and yeast and mold count	At 25 °C, 1.25 mM of carvacrol caused reductions in mesophilic and yeast counts of approximately 2 log cycles after 2 d; the reductions in *E. coli* O157:H7 counts were below 1 log cycle. The *p*-cymene at 1.25 mM delayed the mesophlilic and yeasts growth for 12 d, although reduced the *E. coli* counts to undetected levels. The combination of 0.5 mM of carvacrol and 0.25 mM of *p*-cymene at 4 °C reduced the *E. coli* counts to undetected levels, and reduced the mesophilic and yeast counts in approximately 2 log cycles.	Kiskó and Roller (2005)
Apple (4.2) Pear (4.0) Melon (5.9)	Geraniol	2, 3, 5, 6, 8, and 10 μL/mL	*E. coli*, *L. innocua* and *Salmonella* Enteritidis (6 log cfu/mL)	Geraniol at 2 μL/mL reduced the *Salmonella* Enteritidis counts to near 1 log cycle, whereas 6 μL/mL of geraniol caused complete inactivation of *E. coli* or *L. innocua* population.	Raybaudi-Massilia and others (2006)
Carrot (6.2)	Carvacrol Cinnamaldehyde Eugenol	5 μL/100 mL 2 μL/100 mL 35 μL/100 mL	*B. cereus* EPSO-35AS and *B. cereus* INRA TZ415 spores (2 log spores/mL)	Cinnamaldehyde at 2 μL/100 mL caused complete inactivation of *B. cereus* EPSO-35AS population over 60 d at 8 and 12 °C. Carvacrol at 5 μL/100 mL and eugenol at 35 μL/100 mL were not effective in inhibiting *B. cereus* EPSO-35AS spores at 12 and 16 °C within 60 d, but no growth was observed within 60 d at 8 °C. Eugenol at 35 μL/100 mL were not effective in inhibiting *B. cereus* INRA TZ415 spores germination within 60 d at all temperatures tested.	Hernández-Herrero and others (2008)

(Continued)

Table 2—Continued

Juice (pH)	Individual constituent	Applied concentrations	Target microorganism (s) (inoculation level)	Main results	References
Apple (3.8)	Trans-cinnamaldehyde	0.025%, 0.075%, and 0.125 % (v/v)	*E. coli* O157:H7 (≈ 6 log cfu/mL)	Trans-cinnamaldehyde at 0.025%, 0.075% and 0.125% caused complete inactivation of bacterial population after 5, 3, and 1 d at 23 °C, respectively. Trans-cinnamaldehyde at 0.025%, 0.075%, and 0.125% caused complete inactivation of bacterial population after 14, 5, and 3 d at 4 °C, respectively.	Baskaran and others (2010)
Carrot (6.7)	Carvacrol *p*-cymene	7.5, 10, 12.5, and 15 ppm	*Vibrio cholerae* ATCC 14033, *Vibrio cholerae* VC1 and *Vibrio cholerae* VC7 (≈ 3, 5, and 7 log cfu/mL)	Carvacrol and *p*-cymene at 5 ppm or 7.5 ppm caused total inactivation of bacterial population; the inhibitory effects were stronger in lower storage temperatures.	Rattanachaikunsopon and Phumkhachorn (2010)
Orange	Eugenol	0.3%	Mesophilic count	Reduction in mesophilic counts of approximately 3.5 log cycles over 24 h at 4 °C; at 25 °C, the reduction was of approximately 2.5 log cycles.	Ghosh and others (2014)
Apple (3.4)	Cinnamaldehyde Perillaldehyde Citral	50, 62.5, 100, 125, 200, and 400 μg/mL	*Zygosaccharomyces bailii* ATCC 60484 and *Z. bailii* 906 Pepsi (≈2 log cfu/mL)	Dual combination of trans-cinnamaldehyde-citral, trans-cinnamaldehyde-perillaldehyde or perillaldehyde-citral at 100, 125, and 400 μg/mL, respectively, caused complete inactivation of yeast population in clear and cloudy juices.	Loeffler and others (2014)
Vegetable/fruit (3.9)	Eugenol	0.05 and 1.0%	*E. coli* O157:H7 (5 log cfu/mL)	At 5 °C, 0.05% of eugenol caused count reduction of 1.6 log cfu/mL after 7 d; 0.1% caused complete inactivation of bacterial population after 1 day. At 20 °C, 0.1% and 0.05% of eugenol caused complete inactivation of bacterial population after 1 and 7 d, respectively. At 35 °C, 0.05% of eugenol caused complete inactivation of bacterial population after 1 day.	Pan and others (2014)
Watermelon	Trans-Cinnamaldehyde Clove EO	10000, 5000, 1250, and 625 mg/L	Naturally occurring microorganisms	The combination of 1250 mg/L of both cinnamaldehyde and clove EO was more effective than clove EO alone at 10000 mg/L, with count reductions > 7 log cycles after 5 d.	Siddiqua and others (2014)
Watermelon	*Trans*-cinnamaldehyde	0.8, 2.4, and 4.0 % (w/v)	*Salmonella* Typhimurium KCCM 11862, *Staphylococcus aureus* ATCC 12692 and *E. coli* O157:H7 933 (7 log cfu/mL)	The emulsion containing 0.8% of *trans*-cinnamaldehyde inhibited *Salmonella* Typhimurium and *S. aureus*.	Jo and others (2015)
Tomato (3.8)	Thymol	0.1, 0.3 and 0.5 mmol/L	*Candida lusitaniae* CECT 12006 (≈3 log cfu/mL)	The incorporation of 0.5, 0.3, or 0.1 mmol/L of thymol in juice caused count reductions of approximately 4.6, 2.7, and 0.7 log cycles, respectively.	Aznar and others (2015)

IC, individual constituent; EO, essential oil.

acid-resistant *Zygosaccharomyces bailii* strains (906 Pepsi and ATCC 60484) in clear and cloudy apple juices stored at 20 °C for 27 d was assessed. For both test strains, no growth was observed in clear and cloudy apple juices after the incorporation of the emulsion containing the dual combinations of 100 μg/mL of trans-cinnamaldehyde-citral, 125 μg/mL of trans-cinnamaldehyde-perillaldehyde, or 400 μg/mL of perillaldehyde-citral (Loeffler and others 2014). However, the dual combinations of 50, 62.5, and 200 μg/mL of trans-cinnamaldehyde-citral, trans-cinnamaldehyde-perillaldehyde, and perillaldehyde-citral, respectively, were ineffective in inhibiting yeast growth in both clear and cloudy juices.

Another study investigated the inhibitory effects of 5000 and 10000 mg/L of trans-cinnamaldehyde alone or the combination of 1250 or 625 mg/L of trans-cinnamaldehyde and clove EO on mesophilic counts in watermelon juice over 7 d of storage at 37 °C. The results showed that the combinations of 1250 mg/L of both trans-cinnamaldehyde and clove EO were more effective than 10000 mg/L of clove EO alone, presenting count reductions of >7 log cycles at the end of 5 d of storage (Siddiqua and others 2014). The efficacy of trans-cinnamaldehyde to inactivate *E. coli* O157:H7 in apple juice over 21 d of storage under refrigeration and at room temperature was also verified (Baskaran and others 2010). Trans-cinnamaldehyde at 0.025%, 0.075%, and 0.125% reduced the *E. coli* O157:H7 counts to undetectable levels (<1 cfu/mL) on days 5, 3, and 1 of storage at 23 °C, whereas trans-cinnamaldehyde at 0.025% reduced the counts to undetectable levels only on day 5 of storage. In juices stored at 4 °C, the incorporation of trans-cinnamaldehyde at 0.025%, 0.075%, and 0.125% decreased the counts of *E. coli* O157:H7 to undetectable levels on days 14, 5, and 3 of storage, respectively. The stronger antibacterial effects displayed by trans-cinnamaldehyde at 23 °C were associated with a possible higher metabolic activity, growth, and the bacterial death rate. Moreover, storage at room temperature may increase the solubility of trans-cinnamaldehyde and change the fatty acid profile of the bacterial membrane, facilitating the antimicrobial activities of trans-cinnamaldehyde (McElhaney 1976; Gill and Holley 2006).

The study of the effects of 2 μL/100 mL of trans-cinnamaldehyde on the growth/death kinetics of *B. cereus* (EPSO-35AS) in tyndallized carrot broth stored under different storage temperatures (8, 12, and 16 °C) detected complete inactivation in juice over 60 d of storage at 8 and 12 °C (Hernández-Herrero and others 2008). However, the *B. cereus* cells were capable of surviving in juices that contained trans-cinnamaldehyde and were stored at 16 °C; this capability was associated with the more active metabolism of microbial cells when cultivated at a higher incubation temperature (Prescott and others 2004).

The effect of different trans-cinnamaldehyde concentrations (0.00065% to 0.67%) and time–temperature combinations (21 °C for 5 min and 37 °C for 60 and 120 min) on cidal activities (BA50 values) against *E. coli* O157:H7 and *S. enterica* in cloudy and clear (Mott's) apple juices and in fresh apple (Arkansas black, empire, gala, and Fuji) juices was investigated. In some cases, the apple variety affected the antibacterial effects of trans-cinnamaldehyde under the same experimental conditions as follows: at 37 °C by 30 min, the cidal effects against *E. coli* occurred at 0.044% in Empire apple juice and at 0.056% in Fuji apple juice; for *S. enterica*, the cidal effects occurred at 0.047% in Arkansas Black juice and at 0.05% in both Empire and Gala apple juice (Friedman and others 2004).

Carvacrol

Carvacrol, a monoterpenoid phenol, is frequently referred to as the main constituent of oregano EO (Luz and others 2012; Horváth and others 2016). The antimicrobial mechanisms of carvacrol involve membrane damage and increase in membrane permeability to ions, the depletion of intracellular ATP, and the disruption of the proton-motive force (Helander and others 1998; Ultee and others 1999). The antimicrobial effects of carvacrol and *p*-cymene, alone and in combination, were assessed in carrot juice inoculated with *Vibrio cholerae* (ATCC 14033, VC1, and VC7). After 4 d of storage at 25 °C, the incorporation of carvacrol alone (2.5, 5.0, and 7.5 ppm) into juice presented dose-dependent inhibitory effects, with reductions in *V. cholerae* counts varying from 1.59 to 3.64 log cycles, whereas *p*-cymene presented no inhibitory effects. The combinations of 5 or 7.5 ppm of carvacrol and *p*-cymene were effective in causing the total inactivation of *V. cholerae* strains in carrot juice (Rattanachaikunsopon and Phumkhachorn 2010). The same study investigated the influence of different storage temperatures (4, 15, and 25 °C) on the inhibitory effects of the combination of 7.5 ppm of carvacrol and *p*-cymene against *V. cholerae* in carrot juice and observed that the bacterial sensitivity decreased as the storage temperature decreased. The researchers stated that the low storage temperature might have changed the properties and, consequently, the *V. cholerae* membrane fluidity, in addition to affecting the synthesis of target sites in the cytoplasmic membrane of bacterial cells and thereby influencing the sensitivity to ICs.

The effects of the incorporation of carvacrol and *p*-cymene, alone or in combination, against artificially inoculated *E. coli* O157:H7 and naturally present mesophilics and yeasts in unpasteurized apple juice stored at 4 and 25 °C up to 20 d were assessed. Carvacrol at 1.25 mM provoked reductions in mesophilic and yeast counts of approximately 2 log cycles until the 20th day of storage at 25 °C, followed by a subsequent increase in mesophilic counts until the 20th day of storage. The yeast counts continued to decline, and *E. coli* O157:H7 counts decreased to below the detection limit (1 log cfu/mL). Overall, the antimicrobial effects of carvacrol and *p*-cymene incorporated individually in juices stored at 4 °C were relative because a gradual decline occurred in mesophilic and yeast counts over time, although the survival of *E. coli* O157:H7 extended substantially over the assessed storage period. When the combination of 0.5 mM of carvacrol and 0.25 mM of *p*-cymene was incorporated into juice stored at 4 °C, none *E. coli* O157:H7 cell was detected after day 1 of storage and mesophilic counts were reduced to near 2 log cfu/mL. Therefore, the combination of carvacrol and *p*-cymene at the tested concentrations was considered a potential cidal treatment against naturally present spoilage mesophilics and yeasts and *E. coli* O157:H7 in unpasteurized apple juice, particularly when stored at low temperatures (Kiskó and Roller 2005).

The BA50 of carvacrol in the function of different time/temperature conditions (4, 21, and 37°C by 5, 60, and 120 min) in fresh (Arkansas Black, Empire, Fuji, Gala) apple juices inoculated with *E. coli* and *S. enterica* was studied. At 60 min at 37 °C, the carvacrol BA50 values toward *E. coli* varied from 0.0097% in Gala apple juice to 0.02% in Fuji apple juice; the carvacrol BA50 values toward *S. enterica* varied from 0.019% in Fuji apple juice to 0.0054% in Gala apple juice. Furthermore, carvacrol inactivated the bacteria cells in a contact time as fast as 5 min, and the inactivation rates were higher when the contact time was extended (Friedman and others 2004). Another study

verified that 5 μL/100 mL of carvacrol provoked no growth of *B. cereus* EPSO-35AS spores in tyndallized carrot broth stored at 8 °C within 60 d. Meanwhile, carvacrol appeared to trigger slowness in the growth of *B. cereus* germinated cells in juices under 16 and 12 °C (Hernández-Herrero and others 2008). This behavior was associated with a probable lower active bacterial metabolism at low temperatures (Prescott and others 2004).

Eugenol

Eugenol belongs to the monoterpenes (phenylpropanoid) class, and it is the major component of clove EO. The antimicrobial mechanism action of eugenol is associated with a disruption of the cytoplasmic membrane and a consequent increase in nonspecific permeability that results in a loss of ions and proteins, causing microbial death (Burt 2004; Gill and Holley 2006). The effects of a nanoemulsion (obtained by ultrasound emulsification) containing 3% of eugenol on mesophilic counts in orange juice stored at 4 and 25 °C over 72 h was evaluated. A reduction in mesophilic counts of approximately 3.5 log cycles in juices containing carvacol was achieved over 24 h of storage at 4 °C; however, when the juice was stored at 25 °C, this decrease (approximately 2.5 log cycles) occurred up to only 6 h of storage, with increases in counts in further assessed storage time points (Ghosh and others 2014). In another study, spores of *B. cereus* EPSO-35AS inoculated into tyndallized carrot broth containing 35 μL/100 mL of eugenol were not capable of germinating within 60 d in storage at 8 °C. However, in the higher tested storage temperatures (12 and 16 °C), the incorporation of eugenol into juice was not capable of inhibiting spore germination or cell growth over time. Furthermore, eugenol at 35 μL/100 mL did not inhibit the growth of *B. cereus* INRA TZ415 at all storage temperatures tested (Hernández-Herrero and others 2008).

Thymol

Thymol is a monoterpenoid phenol and one of the major constituents of thyme EO. Although the antimicrobial action modes of thymol is not fully understood, it is believed to include outer- and inner-membrane severance, affecting the structure of membrane proteins and intracellular targets (Hyldgaard and others 2012; Horváth and others 2016). The incorporation of 0.5, 0.3, or 0.1 mmol/L of thymol in tomato juice stored at 25 °C for 48 h provoked reductions of approximately 4.6, 2.7, and 0.7 log cycles, respectively, in counts of *Candida lusitaniae* (Aznar and others 2015). The incorporation of 0.5, 1.0, 2.0, and 4.0 mmol/L of thymol alone in carrot juice stored at 30 °C reduced the counts of *B. cereus* in a range of 1.0 to 5.5 log cycles after 24 h of incubation; this killing effect occurred in a dose-dependent manner. However, 4.0 and 2.0 mmol/L of *p*-cymene tested alone were capable of provoking only approximately 0.5 log-cycle reductions in counts of *B. cereus* in juices after 24 h of incubation. After the 24-h incubation period, the combinations of thymol and *p*-cymene induced a greater killing effect against *B. cereus*, with reductions in counts varying from 2.0 to 7.0 log cycles (Delgado and others 2004).

Geraniol

The inhibitory effects of geraniol on *Salmonella* Enteritidis, *E. coli*, and *L. innocua* in apple, pear, and melon juices were assessed. Geraniol at 2 μL/mL was effective in decreasing the counts of *Salmonella* Enteritidis to near 1 log cycle after 24 h of incubation at 35 °C in melon juice, whereas no count of *E. coli* or *L. innocua* was observed in juice containing 6 μL/mL of geraniol (Raybaudi-Massilia and others 2006). The antibacterial activities of citral, geraniol, linalool, linalyl acetate, terpinene, and terpinen-4-ol against *E. coli* O157:H7 and *Salmonella* Enteritidis in clear and cloudy apple juices was also verified (Friedman and others 2004). The results of this study revealed that for both target bacteria, the inactivation effects induced by geraniol, linalool, and terpinen-4-ol increased with the contact time, and the beginning of bacteria inactivation occurred after as little as 5 min of incubation, suggesting the test compounds were fast-acting antimicrobials. Overall, *Salmonella* Enteritidis was the most sensitive bacteria regardless the type of juice, temperature, and contact time, and stronger antibacterial effects were observed in cloudy than in clear juices.

Use of EOs or ICs in Association with Other Preservation Techniques in Juices

Some studies have approached the combined use of EOs or their ICs and physical emerging technologies such as mild heat treatment, pulsed electric field, high hydrostatic pressure, high pressure homogenization, and ultrasound to maintain the safety and quality of juices, as summarized in Table 3. Thermal processing with a temperature higher than 60 °C is the most widely used technology for the pasteurization of juices, using different time–temperature combinations. Juices are traditionally pasteurized by batch heating using low temperature (63 to 65 °C) for a relatively long time (LTLT), but this method has been progressively replaced by high-temperature short-time (HTST) treatment to avoid undesirable quality changes in the final product. The HTST treatment uses a shorter heat treatment (90 to 95 °C for 15 to 30 s, 77 to 88 °C for 25 to 30 s) and is currently the most widely applied method for the heat treatment of juice; however, this technique also tends to reduce the product quality and freshness (Rupasinghe and Yu 2012). An alternative to these heat treatments is the application of mild temperatures in combination with antimicrobial substances or compounds in juices, forming a new strategy to inhibit or delay microbial growth and to avoid the problems of organoleptic effects on these products.

Mild heat treatment

The combined efficacy of mint, eucalyptus, and lemongrass EOs and thermal treatment on the preservation of a mixed fruit juice (apple and orange) was evaluated. The use of the thermal treatment alone (70 or 80 °C for 30, 60, and 90 s) was ineffective in preventing juice spoilage by *S. cerevisiae*, whereas a complete inhibition was induced when each of the mint (1.13 mg/mL), eucalyptus (4.5 mg/mL), and lemongrass (1.13 mg/mL) EOs was incorporated into juice stored for 8 d at room temperature. The yeast count in juice was inhibited by the EOs in a dose-dependent manner, and the combination of treatments reduced the effective EO dose requirement, showing that it is a highly useful synergy to inhibit *S. cerevisiae* in juice (Tyagi and others 2013, 2014a,b). The use of only one tested treatment (EO or thermal treatment) was not capable of guaranteeing the microbial stability of juices without affecting the final sensory properties (Belletti and others 2010). Thermal treatment may enhance the antimicrobial efficacy of EOs by influencing the vapor phase of the volatile molecules forming the EOs, which in turn improves the possibility of solubilizing the yeast–cell membrane (Lanciotti and others 2004; Belletti and others 2007).

The incorporation of 0.1% or 1.2% of mint EO in pasteurized tomato juice that was treated with mild heat (50 °C for 30 min) caused 4.77 and 8.34 log-cycle reductions, respectively, in naturally occurring microorganisms (Nguyen and Mittal 2007).

Table 3—Studies testing the effect of essential oils and/or their individual constituents combined with other preservation methods in juices.

Juice (pH)	Essential oil/ individual constituent	Applied concentrations	Combined treatment	Target microorganism (s) (inoculation level)	Main results	References
Tomato	Oregano Thyme	0.1% and 0.5%	HHP (200 or 400 MPa for 5 to 20 min)	Naturally occurring microorganisms (lactic acid bacteria)	Increase in the microbiological shelf-life in 3 weeks when 200 MPa 10 min and 0.1% of thyme EO were used in combination.	Mohácsi-Farkas and others (2002)
Apple (3.7)	Cinnamon	0.1%, 0.2%, and 0.3 % (w/v)	Sodium benzoate (0.1%) Potassium sorbate (0.1%)	*E. coli* O157:H7 7927 (5 log cfu/mL)	Reduction of 5.2 log cycles in 11 or 14 d when 0.3% of EO and 0.1% of sodium benzoate or potassium sorbate, respectively, were used in combination at 8 °C.	Ceylan and others (2004)
Tomato (4.2)	Clove Mint	0.1 % (w/w) 0.1, 0.5, and 1.2 % (w/w)	Mild heat treatment (44, 50, 52, or 54 °C)	Naturally occurring microorganisms	Reductions of 4.0 and 5.55 log cycles when 0.5 and 1.0% of mint EO and 44 °C were used in combination. Reductions of 4.77 and 8.34 log cycles when 0.1 or 1.2% of mint EO and 50 °C were used in combination. Reduction of 3.9 log cycles when 0.1% of clove EO and 50 °C were used in combination.	Nguyen and Mittal (2007)
Orange	Vanillin Citral	1000, 1500, and 2000 ppm 75, and 100 ppm	US (600 W, 20 kHz, 95.2-μm wave amplitude)	*L. monocytogenes*	Total inactivation of bacterial population between 1.6 and 2.6 min when vanillin or citral and US were used in combination.	Ferrante and others (2007)
Apple (4.5) Pear (4.40 Orange (3.4) Strawberry (3.2)	Cinnamon bark	0.05%, 0.1%, 0.2%, and 0.3 % (v/v)	HIPEF (35 kV/cm, for 1575 to 1700 μs at 100 to 235 Hz, 4 μs pulse length)	*Salmonella* Enteritidis and *E. coli* O157:H7 (7 to 8 log cfu/mL)	Reduction of >5 log cycles when HIPEF and 0.1% of EO were used in combination in apple and pear juices, and with 0.05% of EO in strawberry juice.	Mosqueda-Melgar and others (2008a)
Melon (6.1) Watermelon (5.7)	Cinnamon bark	0.05%, 0.1%, 0.2%, and 0.3% (v/v)	HIPEF (35 kV/cm for 1682 to 1709 μs at 193 Hz, 4 μs pulse length)	*E. coli* O157:H7, *Salmonella* Enteritidis 1.82 and *L. monocytogenes* 1.131 (7 to 8 log cfu/mL)	Reduction of >5 log cycles when HIPEF and 0.20% of EO were used in combination in juices.	Mosqueda-Melgar and others (2008b)
Tomato (4.3)	Cinnamon bark	0.05%, 0.1%, 0.2%, and 0.3% (v/v)	HIPEF (35 kV/cm for 200 to 1000 μs at 100 to 200 Hz, 4 μs pulse length)	*Salmonella* Enteritidis (7 log cfu/mL)	Reduction of 6.04 log cycles when HIPEF at 35 kv/cm for 1000 μs at 100 Hz, 4-μs pulse length and 0.1% of EO were used in combination.	Mosqueda-Melgar and others (2008c)
Strawberry	Lemongrass Cinnamon leaf	0.1, 0.3, and 0.5 μL/mL	Freeze–thaw treatment (Freezing −23 °C/24 or 48 h; thawing at 7 °C for 4 h)	*E. coli* O157:H7 ATCC 43894 and *Salmonella* Enteritidis ATCC 13076 (7 to 8 log cfu/mL)	Reduction of ≥5 log cycles when lemongrass or cinnamon EOs were added in juice either before or after freeze–thaw treatment.	Duan and Zhao (2009)
Citrus-based concentrated beverages	Citral Linalool and β-pinene	30, 60, 90, and 120 ppm 15, 30, 45, and 60 ppm	Mild heat treatment (55 °C for 15 min)	*Saccharomyces cerevisiae* (50 cfu/mL)	No occurrence of spoilage in juices after 60 d of storage when the thermal treatment and citral at their highest concentrations/levels were used in combination.	Belletti and others (2010)
Orange (3.5)	Citral Vanillin	25 ppm 900 or 1100 ppm	Mild thermal treatment (52, 57, 59, and 61 °C for 10 min)	*L. innocua* (≈8 log cfu/mL)	Reduction of 5 log cycles in 2.4, 1.0, and 1.3 min when the thermal treatment at 57, 59, and 61 °C, respectively, and 25 ppm of citral were used in combination. Reduction of 5 log cycles in 1 and 0.5 min when the thermal treatment at 52 and 57 °C and 1000 ppm of vanillin were used in combination. Reduction of 5 log cycles in 5.1 and 4.5 min when the thermal treatment at 52 and 57 °C and 25 ppm of citral and 900 ppm of vanillin were used in combination.	Char and others (2010)

(Continued)

Table 3–Continued.

Juice (pH)	Essential oil/ individual constituent	Applied concentrations	Combined treatment	Target microorganism (s) (inoculation level)	Main results	References
Apple (3.5) Orange (3.7)	α-Pinene, β-pinene, p-cymene, thymol, carvacrol, borneol, linalool, terpineol-4-ol, 1,8-cineole, α-terpinyl acetate, camphor	0.2 μL/mL	Mild heat treatment (54 °C for 10 min) Pulsed electric fields (30 kV/cm/25 pulses)	*E. coli* O157:H7 VTEC (Phage type 34; ≈7 log cfu/mL)	Reduction of 0.5 log cycles when the thermal treatment at 54 °C and ICs were used in combination. Reduction of 2 log cycles in orange juice and nearly 5 log cycles in apple juice when HIPEF and ICs were used in combination.	Ait-Ouazzou and others (2011)
Apple (4.2) Pear (4.8) Tomato (4.3) Strawberry (3.3) Orange (3.3)	Cinnamon bark	0.05% and 0.1%	HIPEF (35 kV/cm for 1000 to 1700 μs at 100 to 235 Hz, 4 μs pulse length)	Mesophilic, molds and yeasts, and psychrophilic microorganisms	Total inhibition of microflora for more than 91 d at 5 °C when 0.1% of EO and HIPEF were used in combination.	Mosqueda-Melgar and others (2012)
Apple (3.7)	*Citrus lemon* L.	200 μL/mL	Mild heat treatment (54 °C for 10 min)	*E. coli* O157:H7 VTEC (Phage type 34; 3 × 104; 3 × 107 cfu/mL)	Reduction of 5 log cycles in 5 min when the EO and thermal treatment were used in combination.	Espina and others (2012)
Apple (3.5)	Mentha pulegium L. Thymus algeriensis L.	0.2 μL/mL	Mild heat treatment (54 °C for 10 min)	*E. coli* O157:H7 VTEC (Phage type 34; 2 × 107 or 2 × 104 cfu/mL)	Reduction of 5 log cycles in 8.2 and 5.0 min in apple juice when the thermal treatment at 54 °C and *M. pulegium* and *T. algeriensis*, respectively, were used in combination.	Ait-Ouazzou and others (2012)
Apricot (3.3)	Citral	50 mg/L	HPH (100 MPa 1, 3, 5, and 8 passes)	*S. cerevisiae* SPA (4.5 log cfu/mL)	Increase in 6 d of the time necessary to attain 6.0 log cfu/mL at 10 °C when HHP (8 passes at 100 MPa) and citral were used in combination.	Patrignani and others (2013)
Apple (3.6) Orange (3.8)	(+)-Limonene	200 μL/mL	Mild heat treatment (54 °C for 10 min) HIPEF (30 kV/cm/25 pulses)	*E. coli* O157:H7 VTEC (Phage type 34; 3 × 107 cfu/mL)	Reduction of 5 log 10 cycles in 5 min in both juices when the thermal treatment and (+)-limonene were used in combination.	Espina and others (2013a)
Apple (3.6) Orange (3.8)	Citrus sinensis L. Citrus reticulata L. (+)-limonene	200 μL/mL	HPH (175 to 400 MPa for 5 to 20 min)	*E. coli* O157:H7 VTEC (Phage type 34; 3 × 104 or 3 × 107 cfu/mL)	Reduction of 3 and 1.5 to 2 log cycles in orange and apple juices, respectively, when the EOs and HHP were used in combination. Reduction of ≥5 log cycles in apple or orange juice, respectively, when (+)-limonene and HPP were used in combination.	Espina and others (2013b)
Apple + orange	Mentha	1.13, 0.56, and 0.28 mg/mL	Thermal treatment (80 °C for 30, 60, and 90 s)	*S. cerevisiae* SPA (3 log10 cfu/mL)	Total inhibition for 8 d at room temperature when 1.13 and 0.56 mg/mL of EO and thermal treatment were used in combination. Reduction of 1.03 log cycles when 0.28 mg/mL of EO and thermal treatment were used in combination.	Tyagi and others (2013)
Apple + orange	Eucalyptus	4.5, 2.25, and 1.125 mg/mL	Thermal treatment (70 °C for 30, 60, and 90 s)	*S. cerevisiae* SPA (3 log cfu/mL)	Total inhibition for 8 d at room temperature when 4.5 mg/mL of EO and thermal treatment were used in combination. Reduction of 3.2 and 6.2 log cycles when 2.25 and 1.125 mg/mL of EO and thermal treatment were used in combination.	Tyagi and others (2014a)
Apple + orange	Lemongrass	1.13, 0.56, and 0.28 mg/mL	Thermal treatment (80 °C for 30, 60, and 90 s)	*S. cerevisiae* SPA (3 log cfu/mL)	Total inhibition for 8 d when 1.13 mg/mL of EO and thermal treatment were used in combination.	Tyagi and others (2014b)

(Continued)

Table 3–Continued.

Juice (pH)	Essential oil/ individual constituent	Applied concentrations	Combined treatment	Target microorganism (s) (inoculation level)	Main results	References
Orange (3.7)	(+)-Limonene *Citrus sinensis* L.	50, 100, and 200 ppm	Mild thermal treatment (54 °C for 10 min)	*E. coli* O157:H7 VTEC (Phage type 34; 3 × 107 cfu/mL)	Reduction of 5 log cycles in less than 20 and 12 min when the thermal treatment and 200 ppm of EO or (+)-limonene, respectively, were used in combination.	Espina and others (2014b)
Pineapple	*Eryngium foetidum*	15 ppm	Mild thermal treatment (60 °C)	*L. monocytogenes*	Reduction in 6.5 min the time necessary to achieved 4 log cycle reduction when the EO and thermal treatment were used in combination.	Ngang and others (2014)
Orange nectar (4.2)	Trans-cinnamaldehyde	0.26, 0.39, and 0.52 µL/mL	Nisin (46.8 IU/ml)	*Alicyclobacillus acidoterrestris* ATCC 49025 spores (2 × 108 spores/mL)	Extension of total inactivation in 45 d at 25 °C and total inactivation of the spore germination for 33 d when trans-cinnamaldehyde and nisin were used in combination.	Khallaf-Allah and others (2015)
Apple (≈ 3.4)	*Mentha piperita* L.	0 to 1000 ppm	Sodium benzoate (0% to 0.1%)	*Z. rouxii* DSM 70540 and *Z. bailii* DSM 70492 (6 to 7 log cfu/mL)	Increase in yeast counts when the EO and sodium benzoate were used in combination. The lowest yeast counts (0.017 or 0.271 log cfu/mL) were verified when 100 ppm of the EO was used alone.	Karaman and others (2016)
Orange (3.8)	*Citrus sinensis* L.	250 µL/mL	US (20 kHz/100 w/0.4 W/mL/15 min/< 30 °C)	Total bacteria and yeast counts	Total inhibition of microflora over 8 weeks of storage at 4 °C when EO and US were used in combination.	Khandpur and Gogate (2016)

EO, essential oil; IC, individual constituent; HHP, high hydrostatic pressure; US, high-intensity ultrasound; HIPEF, high-intensity pulsed electric fields; HPH, high pressure homogenization.

A 0.74 log-cycle reduction in the native microbiota of tomato juice submitted only to heat treatment was observed. At 44 °C, 0.5% of mint EO reduced the native microbiota by 4.0 log cycles. In the same study, 0.1% of clove EO and heat treatment reduced the microbial counts to approximately 3.9 log cfu/mL in tomato juice, whereas untreated juice presented an increase in microbial counts over time (7.34 log after 7 d at 4 °C). Overall, clove and mint EOs at low concentrations applied in combination with mild heat provided strong microbial inhibition in juices.

In addition to studying EOs as a whole, research has tested ICs in combination with mild heat treatments in juices. The effect of the incorporation of citral, linalool, and β-pinene combined with a mild heat treatment (55 °C for 15 min) on the survival of a wild strain of *S. cerevisiae* in orange-based soft drink after 60 d of storage at 28 °C was assessed. Neither the thermal treatment alone nor the presence of the ICs alone at their higher tested concentrations (60 and 120 µL/L) were capable of guaranteeing the microbial stability of the beverages, but the antimicrobial activity of all 3 ICs was potentiated by the applied thermal treatment. In this case, the inhibition of the yeast growth may have been the result of the cumulative damages caused by the sublethal thermal treatment and the presence of ICs in the beverage (Belletti and others 2010).

The growth of *L. innocua* (a surrogate of *L. monocytogenes*) was affected by combinations of vanillin, citral, and mild heat treatments in orange juice. The addition of 25 ppm of citral reduced the inactivation time to reach a 5 log-cycle reduction of *L. innocua* in 2.4, 1.0, and 1.3 min at 57, 59, and 61 °C, respectively. The combination of the applied heat treatment with 900 ppm of vanillin produced a 5 log-cycle inactivation of *L. innocua* in 5.1 and 4.5 min at 52 and 57 °C, respectively. The inhibitory effects of the ICs in orange juice were enhanced with the increase in vanillin concentration (1000 ppm) and/or the heating temperature (57 °C; Char and others 2010).

The effects of the use of 0.2 µL/mL of the EOs from *Mentha pulegium* L. and *Thymus algeriensis* L. in combination with mild heat treatment (54 °C for 10 min) was also evaluated against *E. coli* O157:H7 VTEC (Phage type 34) in apple and orange juices (Ait-Ouazzou and others 2011, 2012). The incorporation of the EOs into apple juice at 54 °C for 10 min caused the 5 log-cycle reduction of *E. coli* O157:H7 (8.2 and 5.0 min, respectively) to be faster than the heat treatment acting alone (27 min). Moreover, the *M. pulegium* L. and *T. algeriensis* L. EOs caused sublethal injury in approximately 3 and 4 extra log cycles of *E. coli* O157:H7 survivors, respectively. The occurrence of sublethal injuries caused by the tested treatments seemed to drive cells to subsequent inactivation in juices. Similar results were obtained when the same experiments were performed at 57 and 60 °C. Furthermore, no differences in the inactivation rates and injury effects were observed when the target strain was assessed with different inoculum concentrations (approximately 4 log cfu/mL vs. approximately 7 log 10 cfu/mL; Ait-Ouazzou and others 2012).

In another study, the exposure of *E. coli* O157:H7 to mild thermal treatment (54 °C for 10 min) alone, either in apple or orange juice, caused the inactivation of approximately 0.5 log cycles of cells and injured approximately 3 and 4 extra log cycles of survivors, whereas the exposure of this bacterium to each α-pinene, β-pinene, camphor, *p*-cymene, thymol, carvacrol, borneol, linalool, terpineol-4-ol, α-terpinyl acetate, and 1,8-cineole alone caused a <0.5 log-cycle inactivation and caused only slight sublethal injury in cells. The application of combined treatments including heat treatment and each of the ICs resulted in greater inactivation (5 log cycles) to cells than did the sum of both

methods acting separately, and all ICs were able to interact with cell envelopes; sublethal injuries were inflicted to most cells forming the target bacterial population (Ait-Ouazzou and others 2011).

The occurrence of sublethal injury in bacteria after a challenge with different food-preservation methods, or as a consequence of other stressing conditions, is an already well-known phenomenon (Mañas and Pagán 2005), although the underlying mechanism is still not fully understood. However, the inflicted damages in sublethally injured cells demonstrate the efficacy of combining mild heat treatments with EOs or ICs, as hydrophobic antimicrobials (Somolinos and others 2010). Damage induced by heat might facilitate the access of hydrophobic compounds to the cytoplasmic membrane, which is the primary site of toxic action of terpenes, or might enable ICs to be more easily transported into the cell (Burt 2004).

Other studies also evaluated the effects of EOs from citrus fruit peel, such as orange and lemon, or their ICs in a sequential application with mild heat treatment (54 °C for 10 min) on the survival of *E. coli* O157:H7 VTEC (Phage type 34) inoculated in apple and orange juices. The pretreatment of *E. coli* O157:H7 with 200 μL/L of lemon EO in apple juice (Espina and others 2012) and with orange EO or (+)-limonene in orange juice (Espina and others 2013a, 2014b) increased the bacterial inactivation effects of mild heating (54 °C). In apple juice containing each of the EOs or (+)-limonene, only 5 min was necessary to inactivate 5 log cycles of *E. coli* O157:H7; in orange juice, this reduction was reached in less than 20 min, with the exception of juice supplemented with (+)-limonene, where the same inactivation level was achieved in 12 min. The decreased heat tolerance imposed by the tested EOs or ICs on *E. coli* 0157:H7 was similar with the application of increasing temperatures (up to 60 °C). Furthermore, approximately 3.0, 2.0, and 4.0 log cycles of the *E. coli* O157:H7 survivor population presented sublethal injuries in their outer membrane when cultivated in juice containing lemon EO, orange EO, and (+)-limonene alone, respectively (Espina and others 2012, 2013a, 2014b). Overall, the addition of the tested citrus EOs or (+)-limonene before heating provoked more than 4 extra log cycles of inactivation than the antimicrobials or mild heat treatment acting alone, revealing an enhancement (such as synergism action) of their anti-*E. coli* effects as a consequence of their sequential application. The researchers stated that, because the inactivation kinetics of target bacteria in juices containing orange EO or in juices treated with the mild temperature were highly similar, it could be proposed that the enhanced antibacterial effects were likely associated with the inactivation of heat-injured cells, primarily of those damaged in their outer membranes because of the action of heat treatment. This structural damage in cells may facilitate the access of ICs (such as (+)-limonene) to the periplasmic space and cytoplasmic membrane, resulting in the further inactivation of *E. coli* O157:H7 cells in juice (Espina and others 2013a, 2014b).

The inactivation kinetics of *L. monocytogenes* in pineapple juice containing *Eryngium foetidum* EO and/or treated with low pasteurization temperatures was also assessed. The use of only 15 ppm of the EO during pasteurization (60 °C) of pineapple juice reduced the time required to achieve a 4-log-cycle reduction in the *L. monocytogenes* population (from 8.5 to 2.1 min) compared with heat treatment without EO. These studies also detected that the inhibitory effects of *E. foetidum* EO on *L. monocytogenes* increased with the reduction of pH and supported its possible use at sublethal concentrations as possible strategy for an EO-assisted pasteurization of juices (Ngang and others 2014).

High-intensity pulsed electric field

Because thermal treatment causes undesirable effects, nonthermal pasteurization methods have been proposed over the last few decades for use in juices, including the pulsed electric field process, also known as the high-intensity pulsed electric field (HIPEF) process. HIPEF treatment has been shown to be able to inactivate microorganisms, to decrease the activity of enzymes and to extend the shelf-life of foods without a significant loss of flavor, color, or nutrients (Cserhalmi and others 2006; Elez-Martínez and others 2006; Mosqueda-Melgar and others 2008a,b,c). This technology involves the application of short pulses (1 to 10 μs) of a high-intensity electric field (typically 20 to 80 kV/cm) to fluid foods placed between 2 electrodes in a batch or a continuous flow system. This treatment induces structural changes in the membranes of microbial cells because of the formation of pores, consequently leading to microbial destruction and inactivation (Tsong 1991). The HIPEF treatment application has received USFDA approval, and it is currently used in food processing on a commercial scale.

Combinations of HIPEF (35 kV/cm, 4-μs pulse length, without exceeding 40 °C) with cinnamon bark EO against *Salmonella* Enteritidis and *E. coli* O157:H7 populations in apple, pear, orange, and strawberry juices were evaluated, and enhanced (like additive) inhibitory effects were detected when the EOs were assayed in concentrations varying from 0.05% to 0.1%. Nonetheless, to achieve a >5 log-cycle reduction in bacteria populations, the association of HIPEF treatment with 0.1% of cinnamon bark EO in apple and pear juices and with 0.05% in strawberry juices was needed (Mosqueda-Melgar and others 2008a). In tomato juice, a synergistic effect was observed using HIPEF treatment (35 kv/cm for 1000 μs at 100 Hz, 4-μs pulse length) and 0.1% of cinnamon bark EO. This combined treatment was also enough to provoke a ≥5 log-cycle reduction in the *Salmonella* Enteritidis population, thus achieving the pasteurization standard required by the USFDA (Mosqueda-Melgar and others 2008c). The authors proposed that the mechanisms for the enhanced antimicrobial effect of the combined application of HIPEF and cinnamon bark EO were likely related to the formation of pores on the cell membrane when HIPEF was applied, favoring the diffusion of the ICs to cells inside and causing damage to vital cell functions.

Indeed, the consumption of melon and watermelon juices can provide potential health benefits. However, without a minimal processing, these products could potentially be a source of microbiological diseases because of their mild acidity (pH 5.2 to 6.7) and high water activity (0.97 to 0.99), which both favor the growth of pathogenic microorganisms (USFDA 2001); this concern may be expanded to other low-acidity fruit. Different concentrations of cinnamon bark EO (0.05% to 0.3%) applied alone were effective in reducing *E. coli* O157:H7, *Salmonella* Enteritidis, and *L. monocytogenes* counts in melon and watermelon juices. Contrary to other findings (Mosqueda-Melgar and others 2008a), synergistic effects of the combined treatment using 0.05% and 0.10% of cinnamon bark EO and HIPEF (35 kV/cm for 1682 to 1709 ms at 193 Hz and 4-ms pulse duration) were detected against *Salmonella* Enteritidis and *L. monocytogenes* in both melon and watermelon juice. An additive effect was observed only against *E. coli* O157:H7 in melon and watermelon juice when 0.05% of cinnamon bark EO was combined with HIPEF. Nevertheless, in order to inactivate the bacterial populations in both juices by more than 5 log cycles, combinations of 0.20% of cinnamon bark EO and HIPEF treatment were needed (Mosqueda-Melgar and others 2008b).

Changes in the microbiological population of apple, pear, tomato, strawberry, and orange juices treated with cinnamon bark EO and HIPEF and stored at 5 °C were investigated (Mosqueda-Melgar and others 2012). The shelf-life of apple, pear, and tomato juices treated by HIPEF alone was extended approximately 27, 37, and 44 d at 5 °C more than untreated juices, respectively. In turn, the juices treated by cinnamon bark EO (0.1%) and HIPEF presented a total inactivation of background microflora (mesophilic, molds, and yeasts and also psychrophilic) for more than 91 d at 5 °C. The microbial populations were completely inactivated when the strawberry and orange juices were treated by HIPEF, alone or combined with the EO, and the shelf-life for all was extended by more than 91 d.

The possible enhanced lethal effects of the combined (sequential) use of HIPEF (25 pulses at 30 kV/cm) and each of the ICs, α-pinene, β-pinene, *p*-cymene, thymol, carvacrol, borneol, linalool, terpineol-4-ol, 1,8-cineole, α-terpinyl acetate, and camphor, at 0.2 μL/mL each, against *E. coli* O157:H7 VTEC (Phage type 34) in apple and orange juice, was also evaluated. Only the combination of HIPEF and carvacrol caused the inactivation of 2 log cycles of bacterial cells in orange juice and nearly 5 log cycles in apple juice. To understand the outstanding synergistic effect of PEF and carvacrol, instead of applying both barriers simultaneously, carvacrol was added to the apple juice 3 min immediately after the HIPEF treatment. As a result, again, nearly 5 log cycles of cells were inactivated, suggesting that changes caused by HIPEF were not instantaneously reversible, and most survivors were sensitive to the subsequent challenge with carvacrol. This detected synergistic effect appeared to be promising for the improvement of the antibacterial efficacy of HIPEF treatments, allowing higher levels of inactivation at lower intensities of pulses (Ait-Ouazzou and others 2011). In another study, the level of inactivation of *E. coli* O157:H7 resulting from the associated application of (+)-limonene (200 mL/L) and HIPEF (25 pulses at 30 kV/cm) was additive, that is, it was equal to the sum of the levels of inactivation of both treatments applied separately; and no extra inactivation because of the followed application of the methods was observed. When applied separately, the treatments inactivated fewer than 0.5 log cycles of the initial *E. coli* O157:H7 population (Espina and others 2013a).

High-pressure homogenization and high hydrostatic pressure

Other nonthermal pasteurization methods for use in juices are high-pressure homogenization (HPH) and high hydrostatic pressure (HHP), these are processes that use pressures up to 1000 MPa, with or without heat, to inactivate microorganisms in food products (Ramaswamy and others 2005). Some studies have shown the efficacy of HPH treatments to inactivate both spoilage and pathogenic microorganisms and to extend the shelf-life of juices (Briñez and others 2006a,b, 2007; Patrignani and others 2009, 2010; Tribst and others 2011). HHP has been shown to meet the FDA requirement of a 5 log-cycle reduction of microorganisms in juices without negatively affecting their sensory and nutritional attributes (San Martín and others 2002). The application of a multipass HPH treatment (100 MPa/1 to 8 passes) was capable of potentiating the antimicrobial activity of 50 mg/mL (sublethal concentrations) of citral against *S. cerevisiae*, resulting in an increased shelf-life of apricot juice. The yeast counts decreased with the applied number of passes at 100 MPa, regardless of the added citral concentration. The relationship between the decreases in yeast counts and the number of passes followed a linear trend; hence, after 8 passes, cell counts of 1.2 and 0.3 log10 cfu/mL were detected in juice samples containing and not containing citral, respectively (Patrignani and others 2013). The observed yeast-count decreases were in agreement with the hypothesis that in the multipass treatment, the effect of each pass is additive, and, therefore, each homogenization pass causes the same reduction of the counts of the microbial population (Diels and Michiels 2006; Patrignani and others 2010). The yeast cells' latency time in juices without citral increased (approximately 10-fold) with 8 passes at 100 MPa, from 0.56 to 5.89 d, and the presence of citral increased to 6 to 8 d the time necessary to attain counts of 6.0 log10 cfu/mL at 10 °C, compared to juices not containing citral. The unpressurized and non-citral-juice samples reached the critical spoilage value after 3.8 d, whereas for samples containing citral, this occurred only after 9.5 d of storage. It has been suggested that the cumulative damages caused by HPH treatment and the presence of citral-injured cells leads to a major inhibition of *S. cerevisiae* growth in samples subjected to the combined strategy adopted (Patrignani and others 2013).

The effects of the combined application of the EOs from *Citrus sinensis* L. and *Citrus reticulata* L. or (+)-limonene with HHP on *E. coli* O157:H7 VTEC (Phage type 34) in apple and orange juices were assessed (Espina and others 2013b). HHP treatments caused a low level of inactivation, but a high level of sublethal injury, to treated cells, and lower pH values of juices contributed to establishing higher cell-inactivation rates via HHP treatment. In both apple and orange juices, HHP treatment at 300 MPa for 20 min inactivated fewer than 0.5 log cycles of the initial population (approximately 7 log10 cfu/mL). The addition of 200 μL/L of *C. sinensis* L. or *C. reticulata* L. EO resulted in inactivation levels of approximately 3 and 1.5 to 2 extra log cycles in orange and apple juices, respectively. The addition of (+)-limonene inactivated more than 5 log cycles of the initial cell population in apple and orange juices, increasing the pressure intensity maintained or increased this effect. Storage of the treated samples under refrigeration resulted in the inactivation of up to 3 extra log cycles, compared with the inactivation achieved right after the combined treatments (Espina and others 2013b).

Tomato juices were treated in several experimental trials using HHP alone or in combination with 0.1% and 0.5% of oregano or thyme EOs. Lactic acid bacteria formed the dominating component of the spoilage microbiota during postprocessing storage at 15 °C of juices, causing spoilage of the untreated samples within 4 d. A 0.1% concentration of oregano or thyme EO at least doubled the microbiological shelf-life of tomato juice, and their respective concentrations of 0.5% alone or 400 MPa 5 to 20 min HHP treatment alone resulted in microbial stability for at least 2 weeks. Two-hundred MPa for 10 min resulted in a spoilage delay of only approximately 3 d, whereas 0.1% of thyme EO increased the efficacy of this moderate HHP treatment, resulting in a stable product for at least 3 weeks at the applied storage temperature (Mohácsi-Farkas and others 2002).

Ultrasound

High-intensity ultrasound (US) combined with EOs or ICs may also be an alternative technology for juice preservation. The response of *L. monocytogenes* in orange juice to combined treatments involving a moderate temperature (45 °C), US (600 W, 20 kHz, 95.2-μm wave amplitude), and the addition of different concentrations of vanillin (1000, 1500, and 2000 ppm), citral (75 and 100 ppm), or both was investigated to determine the most effective inactivation treatment. The presence of vanillin or citral

greatly increased the cidal effect, and when both compounds were added together to juice and US was applied, the average bacterial death times were between 1.6 and 2.6 min (Ferrante and others 2007). A study investigated the efficacy of the use of US (20 kHz/100 w/0.4 W/mL/15 min/<30 °C) in combination with orange EO (250 μL/mL) to enhance the shelf-life of orange juice (Khandpur and Gogate 2016). The combined application of US and orange EO caused higher reductions in mesophilic counts in juice, as compared to US or orange EO acting alone.

Chemical preservatives

Chemical preservatives are also widely used for the extension of the shelf-life of juices. Two of the most commonly used preservatives are potassium sorbate and sodium benzoate; however, these substances demonstrate a slight killing effect toward pathogens (such as *E. coli* O157:H7; Rupasinghe and Yu 2012). The antimicrobial synergistic effect of the combinations of cinnamon EO (0.1%, 0.2%, and 0.3% [w/v]) with sodium benzoate (0.1%) or potassium sorbate (0.1%) against *E. coli* O157:H7 in apple juice at different temperatures and storage periods (8 °C for 14 d and 25 °C for 3 d) was investigated (Ceylan and others 2004). Combinations of the antimicrobials exhibited a greater inhibition of target bacteria than sodium benzoate or potassium sorbate alone. The antimicrobial effects increased with the increase in the concentration of cinnamon EO and a decrease in the storage temperature, although a ≥5 log-cycle of bacterial inactivation was reached under both tested storage conditions. The effect of nisin and cinnamaldehyde alone or combined to extend shelf-life of pasteurized (90 °C for 15 s) orange nectar during storage at 25 and 45 °C was also studied. The combination of the compounds extended the total inhibition of *Alicyclobacillus acidoterrestris* growth in 45 d at 25 °C and demonstrated an increased antimicrobial effect compared with nisin (46.8 IU/mL) or trans-cinnamaldehyde (0.39 μL/mL) acting alone, which extended the shelf-life by 18 and 39 d, respectively. The combination of nisin and trans-cinnamaldehyde also induced the complete inhibition of the spore germination of *A. acidoterrestris* for a longer time (33 d) compared with nisin (9 d) and trans-cinnamaldehyde alone (21 d; Khallaf-Allah and others 2015). However, an antagonistic effect was observed when mint EO (1000 ppm) and sodium benzoate (0.1%) were used in combination against *Zygosaccharomyces rouxii* and *Z. bailii* in apple juice (Karaman and others 2016).

Freeze–thaw treatment

In addition to the investigations of heat treatment, PEF, HHP, and chemical substances in combination with EOs and their ICs, only one available study was found that reported the combined use of lemongrass (0.1, 0.3, 0.5, and 1 μL/mL), cinnamon leaf (2 μL/mL), and basil (2 μL/mL) EOs with a freeze–thaw treatment (FTT; freezing −23 °C/24 or 48 h; thawing at 7 °C for 4 h) to reduce the counts of *E. coli* O157:H7 and *Salmonella* Enteritidis in strawberry juice stored at 7 °C (Duan and Zhao 2009). FTT is a common method for extending the shelf-life of apple cider, and its antibacterial effects are associated with the induction of irreversible damages to bacterial cells by the formation of intra- and extracellular ice crystals (Uljas and Ingham 1999). The combination of each of the tested EOs and FTT resulted in an enhanced antimicrobial effect, and the addition of EO before the FTT resulted in a shorter inactivation time of the *E. coli* O157:H7 population. A possible reason for this behavior is that the membrane damage and cell leakage caused by EO may increase the cell injury resulting from freezing stresses (Duan and Zhao 2009).

Effects of EOs and Their ICs on Quality Parameters of Juices

During the storage period, fruit and vegetable juices experience changes in their physicochemical parameters that negatively affect nutritional composition, sensory characteristics, and market value. However, the incorporation of EOs or their ICs in juices commonly influence the alterations in a variety of physicochemical and sensory parameters over time, as summarized in the studies presented in Table 4.

The addition of 0.2 μL/mL of black pepper EO in orange juice induced a decrease in acidity, ascorbic acid, and total sugar content, in addition to slow nonenzymatic browning over 28 d of refrigerated storage (Kapoor and others 2014). Under the same storage conditions and period, similar effects on acidity, total sugar, and ascorbic acid were induced by the incorporation of 0.1% of cardamom EO into sweet orange juice (Kapoor and others 2011). The incorporation of 0.05% of tejpat EO was evaluated for its effects on the pH value, total acidity, reducing sugars, ascorbic acid contents, and peroxide values in pineapple juice over 7 d of storage at 10 °C. The tejpat EO did not protect, caused lower reductions, and caused no changes in the peroxide value, sugar content, ascorbic acid, and titrable acidity or pH of pineapple juice during the monitored storage-time interval (Kapoor and others 2008).

The incorporation of (+)-limonene or orange EO (50, 100, and 200 ppm) was assessed for changes in the sensory parameters of orange juice submitted to different thermal treatments (60 °C for 2.1, 2.4, 2.9, 3.4, and 3.9 min) and further stored at 4 °C for 12 h. The incorporation of (+)-limonene, in concentrations enough to reduce the applied thermal processing time, resulted in a lower sensory acceptance of juices, with worse results for juice supplemented with 200 ppm of (+)-limonene. The orange juices that contained up to 100 ppm of (+)-limonene or 200 ppm of orange EO and were further submitted to heat treatment presented a similar sensory acceptance as the orange juice without the antimicrobial tested substances (Espina and others 2014b).

The incorporation of 0.25 μL/mL of lemon EO positively affected the taste of clear and cloudy apple juice, and panelists reported that juices containing lemon EO were refreshing and harmonic. However, the odor of juices containing lemon EO was perceived as unpleasant in clear apple juice; this parameter was better judged in cloudy apple juice (Tserennadmid and others 2011). A study assessed the effects of 4 different EOs (lemon, pennyroyal mint, thyme, and rosemary) and 2 ICs (carvacrol and *p*-cymene) at varying concentrations (20, 100, and 200 μL/L) on the taste acceptance of tomato juice. The results showed that the lowest tested concentration of pennyroyal mint and lemon EO did not change the taste acceptance of tomato juice. On the contrary, the pennyroyal mint EO increased the taste acceptance of tomato juice, and a remarkable proportion of panelists responded positively to the incorporation of the other concentrations. The other assessed EOs and ICs at all tested concentrations negatively affected the taste acceptance of juices (Espina and others 2014a).

The effect of the incorporation of mint EO on the sensory acceptance of an apple-orange mixed beverage was investigated. The incorporation of 1.13 mg/mL of mint EO did not affect the juice sensory acceptance over 8 d of refrigerated storage in comparison to the incorporation of 0.56 and 0.25 mg/mL of the EO. Nonetheless, the incorporation of mint EO did not undesirably alter the odor or color of the evaluated mixed juice

Table 4–Studies testing the effect of essential oils and/or their individual constituents on quality parameters of juices.

Juice (pH)	Essential oil/ Individual constituent	Applied concentrations	Combined treatment	Assays	Main results	References
Orange	Vanillin citral	1000, 1500, and 2000 ppm 75, and 100 ppm	US (600 W, 20 kHz, 95.2-μm wave amplitude)	Sensory evaluation	Treatments not affected juices and consumers considered juice pleasant when US and ICs were used in combination.	Ferrante and others (2007)
Pineapple (3.4)	Tejpat	0.05%	NA	Peroxide value, total and reducing sugars, titrable acidity, pH, and ascorbic acid	The EO caused no changes in peroxide value, total and reducing sugars, titrable acidity, pH, and ascorbic acid of juice.	Kapoor and others (2008)
Melon (6.1) Watermelon (5.7)	Cinnamon bark	0.05%, 0.1%, 0.2%, and 0.3 % (v/v)	HIPEF (35 kV/cm for 1682 to 1709 μs at 193 Hz, 4-μs pulse length)	Sensory evaluation	Treatments affected negatively odor, taste, sourness, and overall acceptability of melon juice; and taste, sourness, and overall acceptability of watermelon juice.	Mosqueda-Melgar and others (2008b)
Orange (3.5)	Citral vanillin	25 ppm 900 or 1100 ppm	Mild thermal treatment (52 or 57 °C for 10 min)	Sensory evaluation	Treatments affected negatively sensory properties of juice; and the panelists reported that the addition of ICs imparted pleasant but unfamiliar flavor to the juice.	Char and others (2010)
Cloudy apple juice (4.3) Clear apple juice (3.8)	Lemon	0.25 μL/mL	NA	Sensory evaluation	The EO affected positively the taste of juice; and panelists reported that juices were refreshing and harmonic, but the odor was perceived as unpleasant in clear apple juice.	Tserennadmid and others (2011)
Orange (≈4.2)	Cardamom	0.1%	NA	pH, total and reducing sugars, ascorbic acid, titrable acidity, and percent weight loss	The EO induced a decrease in acidity, ascorbic acid, and total sugar content in juice over 28 d of refrigerated storage.	Kapoor and others (2011)
Pear Orange	D-limonene	1.0, 5.0 and 10 g/L	NA	Soluble solids, pH, and color	D-Limonene did not modify the °Brix, pH, and color of juices during storage.	Donsi and others (2011)
Apple (3.7)	D-limonene	900 ppm	HPH (20 MPa)	Consumer test	D-Limonene affected negatively the sensory properties of juice, whereas HPP caused a reduction of juice acceptability. The combination of D-limonene and HPP did not change the acceptability of juice.	Bevilacqua and others (2012)
Apple (4.2) Pear (4.8) Tomato (4.3) Strawberry (3.3) Orange (3.3)	Cinnamon bark	0.05% and 0.1%	HIPEF (35 kV/cm for 1000 to 1700 μs at 100 to 235 Hz, 4-μs pulse length)	Sensory evaluation	Treatments alone did not induce changes in the color of juices. The juices treated with the combination of EO and HIPEF presented the lowest scores for aroma, taste, sourness, and overall acceptability.	Mosqueda-Melgar and others (2012)

(Continued)

Table 4—Continued.

Juice (pH)	Essential oil/ Individual constituent	Applied concentrations	Combined treatment	Assays	Main results	References
Orange-milk	*Litsea cubeba*	1500, 3000, and 6000 μg/g	NA	Sensory evaluation	The EO did not affect the sensory properties of juice.	Liu and Yang (2012)
Apple (3.7)	*Citrus lemon* L.	200 μL/mL	Mild heat treatment (54 °C for 10 min)	Sensory evaluation	Treatments did not affect sensory properties of juice.	Espina and others (2012)
Apricot (3.3)	Citral	50 mg/L	HPH (100 MPa 1, 3, 5, and 8 passes)	pH, water activity, viscosity, and aroma profile	The HPH decrease and increase the pH values and viscosity of juices, respectively. When the citral was incorporated into juice before HPH treatment, no modification was noted in the pH and viscosity.	Patrignani and others (2013)
Apple + orange	Mentha	0.28, 0.56, and 1.13 mg/mL	Thermal treatment (80 °C for 30, 60, and 90 s)	Sensory evaluation	The EO at 1.13 mg/mL did not affect the juice sensory acceptance over 8 d of refrigerated storage.	Tyagi and others (2013)
Tomato	Lemon Pennyroyal mint Thyme Rosemary Carvacrol *p*-cymene	20, 100, and 200 μL/L	NA	Sensory evaluation	Pennyroyal mint or lemon EO at 20 μL/L did not affect the taste of tomato juice; however, higher concentrations of these compounds or any concentration of the other 4 ICs did.	Espina and others (2014a)
Orange (3.7)	(+)-Limonene *Citrus sinensis* L.	50, 100, and 200 ppm	Mild thermal treatment (54 °C for 10 min)	Sensory evaluation	The (+)-limonene at 200 ppm caused a lower sensory acceptance of juices. The (+)-limonene at 100 ppm and orange EO at 200 ppm combined with heat treatment did not affect sensory acceptance of juice.	Espina and others (2014b)
Orange (4.0)	Black pepper	0.2 μL/mL	NA	pH, ascorbic acid, total and reducing sugar, titratable acidity, no enzymatic browning, and percent weight loss over	The EO induced a decrease in acidity, ascorbic acid, and total sugar content in juice, in addition to slow nonenzymatic browning over 28 d of refrigerated storage.	Kapoor and others (2014)
Pineapple (3.9)	Lemongrass	5, 2.5, 1.25, and 0.6 μL/mL	NA	pH, soluble solids, and titratable acidity	The EO at 2.5 and 1.25 μL/mL did not induce changes in pH, titratable acidity, and °Brix in juice. The EO did not affect the appearance, odor, and viscosity, although noticeable unsatisfactory changes were found in the taste, aftertaste, and overall acceptability.	Leite and others (2016)
Apple (≈3.4)	*Mentha piperita* L.	0 to 1000 ppm	Sodium benzoate (0% to 0.1%)	Sensory evaluation Total soluble solids, pH, and titratable acidity.	The EO and sodium benzoate in combination did not affect °Brix, pH, and acidity in juice.	Karaman and others (2016)

EO, essential oil; IC, individual constituent; US, high-intensity ultrasound; HIPEF, high-intensity pulsed electric fields; HPH: high-pressure homogenization; NA, not applied.

(Tyagi and others 2013). The effects of 2.5 and 1.25 μL/mL of lemongrass EO on the physicochemical parameters of pineapple juice over 72 h of storage under refrigeration (4 ± 1 °C) were assessed. Overall, the incorporation of lemongrass EO preserved the physicochemical properties of pineapple juice, as measured by pH values, titratable acidity, and °Brix. The evaluation of the sensory quality of pineapple juices containing lemongrass EO after refrigeration for 24 h demonstrated they were acceptable in terms of appearance, odor, and viscosity, although noticeable unsatisfactory changes were found in the taste, aftertaste, and overall acceptability. Nonetheless, the researchers stated that the overall acceptability of pineapple juice samples containing lemongrass EO was likely affected by the taste and aftertaste perceptions of the panelists (Leite and others 2016). Otherwise, the study of effects of the incorporation of *L. cubeba* EO (375 to 6000 μg/g) on the aroma and taste of orange-milk beverages revealed no negative effect in terms of product characteristics (Liu and Yang 2012).

Nanoemulsions containing terpenes (prepared by HPH) were incorporated into orange and pear juices inoculated with *Lactobacillus delbrueckii*, which were further evaluated for their physicochemical characteristics during storage at 32 °C. The °Brix, pH, and color of both orange and pear juices were not modified by the incorporation of the tested nanoemulsions during storage. The main color deviations were ascertained when 10 g/L of each of the nanoemulsions was incorporated into juices, and the combined incorporation of both tested nanoemulsions at lower concentrations (5 and 1 g/L) was considered acceptable because it induced minor color deviations (Donsì and others 2011).

The effects of the combined application of 50 mg/L of citral and HPH on the quality parameters of apricot juice stored at 4 °C for 20 d were investigated, and the juice treated with HPH presented a decrease and increase in pH values and viscosity, respectively, after the 1st pass at 100 MPa, with a slight decrease in these parameters in further passes. When the citral was incorporated into apricot juice before HPH treatment, no modification was noted in the pH values or viscosity (Patrignani and others 2013). The influence of the incorporation of 0.1% of cinnamon bark EO and further HIPEF treatment on the aroma, color, taste, sourness, and overall acceptability of strawberry, orange, apple, pear, and tomato juices after maximum 12 h storage at 4 °C was also evaluated. The dual treatment (HIPEF and cinnamon bark EO) did not induce changes in the color of any treated juice, whereas the juices submitted to this combination presented the lowest scores for aroma, taste, sourness, and overall acceptability compared to HIPEF alone (Mosqueda-Melgar and others 2012). Sensory evaluations of melon and watermelon juices treated with the combination of HIPEF and 0.2% of cinnamon EO immediately after processing revealed that incorporation of the EO affected all organoleptic properties, although it varied according to the type of juice. Lower scores in odor, taste, sourness, and overall acceptability were found for melon juice, whereas lower scores for taste, sourness, and overall acceptability were found for watermelon juice (Mosqueda-Melgar and others 2008b).

The investigation of the hedonic acceptability of thermally treated (60 °C for 0.58 min and 60 °C for 3.12 min) apple juice containing 75 μL/L of lemon EO revealed no alteration in the sensory properties in comparison to the product treated only by heat. Moreover, in a simple preference test, juice that contained lemon EO and was submitted to short thermal treatment (75 μL/L; 60 C for 0.58 min) was preferred to juice without EO and submitted to longer thermal treatment (60 °C for 3.12 min; Espina and others 2012). Otherwise, the use of 900 ppm of limonene and HPH (20 MPa) negatively affected the purchase intention of pasteurized apple juice, and this effect was primarily associated with a strong lemon odor in juice (Bevilacqua and others 2012). The incorporation of 900 ppm of vanillin and 25 ppm of citral into thermally treated (52 °C for 4.7 min) orange juice induced slight sensory changes in the product. The panelists reported that the addition of these constituents imparted pleasant but unfamiliar flavor to the orange juice (Char and others 2010). Although there has been some skepticism about the practical use of EOs or ICs as antimicrobials in foods, mainly because their possible negative effects on sensory characteristics of foods, the findings of these studies cited above show that these desirable or undesirable sensory impacts have varied with the kind of juice and the incorporated EO/IC as well their final concentration in juice.

Conclusions

The information compiled in this review demonstrates that different EOs or their ICs incorporated into fruit and vegetable juices can effectively reduce or inhibit pathogenic and spoilage microorganisms. From the reported studies, it can be inferred that the use of EOs or ICs in association with other nonthermal emerging food-preservation technologies to preserve fruit and vegetable juices are innovative and potentially useful alternatives to replace the use of chemical additives and intense heat treatments. However, the conditions that are capable of provoking synergistic effects when the EOs or ICs and other nonthermal technologies are applied in juices remain focus areas for further research. Adding EOs or ICs as antimicrobial preservatives into juices without adversely affecting the sensory characteristics remains a challenge because the concentrations that are necessary to ensure microbial safety in some of these products are higher than those normally accepted by consumers. Therefore, the study of the synergistic effects among EOs or ICs and emerging technologies may be utilized to make the best use of their antimicrobial properties, to reduce the concentrations required to achieve a safe antibacterial effect, and to guarantee sensory acceptance during the shelf-life.

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Authors' Contributions

E.T.C. Almeida and J.P. de Sousa Guedes researched prior studies. E.T.C. de Almeida, J.P. de Sousa Guedes, and E.L. de Souza interpreted the results, compiled the data, and drafted the manuscript.

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2.2 CONTAMINAÇÃO DE SUCOS DE FRUTAS

Suco de fruta é definido como bebida não fermentada, não concentrada e não diluída, obtida da fruta madura e sadia, ou parte do vegetal de origem, obtido através de processamento tecnológico adequado e submetido a tratamento que assegure sua conservação e manutenção de suas características nutricionais, sensoriais e microbiológicas, até o momento de seu consumo (BRASIL, 2009). Apesar da crescente demanda de consumo, a produção de sucos de fruta é dificultada em decorrência do ritmo de vida mais acelerado da sociedade. Isso despertou o interesse das indústrias de sucos de frutas para a produção de produtos prontos para consumo com as características mais próximas possível daquelas apresentadas pelos sucos de fruta frescas (CARRILLO et al., 2018).

Como cada espécie de fruta abriga uma microbiota dominante, a qual pode colonizar este substrato durante o seu desenvolvimento, ou ainda ser agregada ao produto devido à contaminação durante práticas inadequadas de colheita, transporte, beneficiamento e industrialização, os produtos de frutas, tais como os sucos, podem ser fontes de micro-organismos deteriorantes e patogênicos, podendo proporcionar problemas de qualidade, fator preocupante para as indústrias processadoras de sucos de fruta, visto que podem ocasionar desperdício, insatisfação do consumidor e ameaças à proteção da marca, além de maiores probabilidades de ocorrência de surtos de doenças (ANEJA et al., 2014; SNYDER; WOROBO, 2018).

Os surtos de doenças transmitidas por alimentos resultantes do consumo de sucos de frutas frescos contaminados continuam a ocorrer em todo o mundo. Entre 1974 e 2014, estes produtos estiveram envolvidos em pelo menos 48 surtos de doenças transmitidas por alimentos envolvendo bactérias (*Salmonella*, *Shigella flexneri*, *Escherichia coli*, produtora de toxina Shiga, incluindo grupos O:157 e O:111, e *E. coli* enterotoxigênica), vírus (norovírus genótipo II , vírus da hepatite A) ou protozoários (*Cryptosporidium* e *Trypanosoma cruzi*) como agentes etiológicos (MARTÍNEZ-GONZÁLES; CASTILLO, 2016). No entanto, vale ressaltar que, os sucos de frutas por apresentar características únicas (acidez eleveda e alto teor de açúcares) são considerados substratos/ambientes adequados para o desenvolvimento de agentes específicos e potencialmente deteriorantes, tais como bactérias heterotróficas, bolores e, particularmente, as leveduras (BATRA et al., 2018).

As leveduras são reconhecidas como as causas mais frequentes de deterioração de sucos de frutas em virtude da sua capacidade de sobreviver à elevada acidez, às baixas temperaturas e, no caso de algumas espécies, em ambientes sem oxigênio.

2.1.1 Aspectos gerais de leveduras

Tratam-se de células eucarióticas, unicelulares que se reproduzem na maioria das vezes de maneira assexuada por meio de brotamento (único ou múltiplo) e, outras, por divisão binária ou cissiparidade. Algumas leveduras se multiplicam sexuadamente por meio de ascopóros (esporos sexuados endógenos formados no interios das hifas em forma de sacos, denominados ascos). Possuem temperatura ótima de crescimento na faixa de 20 a 30 °C, embora algumas espécies possam sobrevier à exposição a altas temperaturas (65 a 70 °C). Seu desenvolvimento pode promover a produção de CO_2 e etanol, mas também pode causar a formação de películas e floculação, o que, consequentemente, aumenta a turvação dos sucos. Além disso, o aumento da população de certas leveduras em sucos de fruta pode produzir acetaldeído e formação de gases, o que contribui para promover odor fermentado e formação de sabores e odores desagradáveis, respectivamente, no produto (PAULA et al., 2011).

Candida, Pichia e *Saccharomyces* são gêneros citados na literatura como sendo responsáveis pela deterioração de sucos (ANEJA et al., 2014).

2.2.1.1 Leveduras do gênero *Candida*

O gênero *Candida*, importantes agente causador de infecções, principalmente, em indivíduos imunodeficientes, possui distribuição universal, podendo ser encontradas nos homens ou nos animais, como comensais ou patogênicas, no solo, na água, nos vegetais e, inclusive, podem ser isoladas de alimentos (CALDERONE; FONZI, 2001).

C. albicans é polimórfica, ou seja, capaz de crescer isotropicamente (leveduras) ou apicalmente (pseudo-hifas e hifas), bem como, são fermentativas, utilizando açúcares como fontes de carbono, com a produção de etanol e CO_2 como produtos finais desse metabolismo (TORTORA et al., 2005).

C. tropicalis é uma levedura osmotolerante, dimórfica e esporogênica, que possui aplicações industriais importantes devido a sua capacidade de assimilação de n-alcanos para a produção de ácidos carboxílicos de cadeia longa, com consequente produção de poliamida e poliéster, além de sua habilidade em fermentar xilose, com formação de xilitol. No entanto, *C. tropicalis* pode ser um importante micro-organismo deteriorante de sucos de frutas, com capacidade de produção de etanol e CO_2 (WANG et al., 2015).

2.2.1.2 Pichia anomala

P. anomala, recentemente classificada como *Wickerhamomyces anomalus*, pode crescer sob condições extremas de estresse ambiental, como baixo e alto pH, baixa atividade de água, alta pressão osmótica e condições anaeróbicas, sendo considerada um micro-organismo deteriorante de produtos com alto teor de açúcar, a exemplo de sucos de frutas (PASSOTH et al., 2005). Além disso, tem habilidade em produzir quantidades significantes de acetato de etila, um agente causador de *off-odour* (KURITA, 2008). Ainda podem descarboxilar o ácido ferúlico à 4-vinilguaiacol (4-hidroxi-3-metoxiestireno), composto responsável por *off-flavor* em sucos (LATEEF, et al., 2004)

2.2.1.3 Saccharomyces cerevisiae

S. cerevisiae caracteriza-se como levedura altamente fermentativa, capaz de produzir acetaldeído, etanol e uma variedade de álcoois superiores, incluindo n-propanol, n-butanol, 2-metil-1-propanol, 2-metil-1-butanol, 3-metil-1- butanol e n-pentanol (SAXBY, 1996). Algumas cepas de *S. cerevisiae* possuem atividade pectinolítica, o que provoca diminuição da turvação de sucos (RIIKKA et al., 2011). Sulfeto de hidrogênio e compostos de enxofre voláteis, produzidos por *S. cerevisiae* durante a fermentação por meio da redução de sulfato, são responsáveis por causar sabores anormais descritos como similares àqueles observados em ovos podres (LUCION, 2015). Além disso, também podem metabolizar ácido ferúlico e produzir 4-vinilguaiacol (LATEEF et al., 2004).

A inativação de micro-organismos e a garantia da segurança alimentar são considerados as tarefas mais importantes do processamento moderno de alimentos, porque qualquer deficiência na segurança alimentar significa que nenhum outro atributo de qualidade será valioso. A inativação de microrganismos durante o processamento de sucos de frutas é crucial para uma melhor segurança e vida útil prolongada, pois, como mencionado, os sucos são uma fonte rica em nutrients que apoiam o crescimento e atividades microbianas (ROOBAB et al., 2018).

Tratamentos tradicionais, tais como pasteurização e/ou adição de conservantes sintéticos têm sido utilizados no processamento de sucos para reduzir as contagens microbianas a níveis seguros que eliminam os riscos à saúde e garantam ao consumidor segurança alimentar, no entanto, este nível de segurança é comumente alcançado em detrimento da qualidade nutricional de um produto, isto é, formação de sabor indesejável, degradação oxidativa e perdas de pigmentos e vitaminas, além de possível formação de substâncias nocivas a saúde (ROOBAB et al., 2018). E hoje em dia, em virtude da preocupação dos consumidores em relação aos riscos inerentes ao consumo desses alimentos, resultou em atenção especial aos métodos de conservação alternativos, dentre os quais, merece destaque, os óleos essenciais (PANDEY et al., 2017).

2.3 ÓLEOS ESSENCIAIS

Os óleos essenciais (OEs), também conhecidos como óleos voláteis, óleos etéreos ou essências, são produzidos, geralmente, por estruturas secretoras especializadas, sejam elas localizadas em toda planta ou em partes específicas. São caracterizados como misturas complexas de substâncias de baixo peso molecular, geralmente, odoríferas, líquidas e de aparência oleosa à temperatura ambiente, com coloração amarelada e pouco estáveis na presença de ar, luz e altas temperaturas, (RAD et al., 2017; SIMÕES et al., 2007).

A composição química dos OEs varia em relação à presença e quantidade de hidrocarbonetos terpênicos, álcoois simples e terpênicos, aldeídos, cetonas, fenóis, ésteres, éteres, óxidos, peróxidos, furanos, ácidos orgânicos, lactonas, cumarinas e compostos com enxofre. No entanto, os OEs são constituídos, principalmente terpenos/terpenóides e, eventualmente, por fenilpropanóides que se originam a partir do ácido chiquímico, o qual forma as unidades básicas dos ácidos mevalônico e cinâmico, respectivamente, e, por sua vez,

formam as unidades de isopreno (BAKKALI et al., 2008; BURT, 2004; SIMÕES et al., 2007). Em virtude dessa complexidade, os efeitos biológicos dos OEs podem ser em consequência da atividade do seu constituinte majoritário ou mesmo ser resultante do sinergismo entre os seus diferentes constituintes (BURT, 2004).

As principais vantagens do uso de OEs como agentes antimicrobianos são o seu amplo espectro de atividade e sua origem natural, o que pode proporcionar uma maior segurança para os consumidores e para o meio ambiente. Destaca-se, ainda, o baixo risco de desenvolvimento de resistência microbiana a estas substâncias, o que é atribuído, principalmente, a composição complexa dos OEs, proporcionando diferentes mecanismos de ação por parte de seus constituintes. Isso torna mais difícil uma possível adaptação por parte dos micro-organismos frente a sua ação (GOMES NETO et al., 2012; SOUZA et al., 2016).

Até o momento, mais de 3000 diferentes OEs já forma descritos, dos quais, aproximadamente, um décimo é considerado relevante para as indústrias farmacêutica, alimentícia ou cosmética (RAD et al., 2017), estando os OEs extraídos de espécies *Mentha* entre os mais comercializados no mercado mundial (BIZZO et al., 2009).

2.3.1 Aspectos gerais de *Mentha*

Mentha é um dos gêneros botânicos mais antigo, do qual a origem de seu nome provém da palavra grega "Menthe" (TESKE; TRENTINI, 1997). O gênero *Mentha,* originário da Europa e amplamente cultivado em todo o mundo, foi introduzido no Brasil ainda no período de colonização (GONÇALVES, 2017). As espécies deste gênero podem facilmente produzir formas intermediárias, seja por hibridização ou poliploidia, proporcionando papel considerável na especiação e, consequentemente, tornando o número de espécies taxonomicamente válidas um assunto de controvérsia (KUNDALIC et al., 2009). As diferenças na sua composição química oferecem uma variedade de espécies com alto teor de mentol, mentona, carvona, linalol ou outros terpenóides, dos quais são sintetizados pela via do ácido mevalônico (DESCHAMPS et al., 2008).

Mentha piperita L., *M. arvensis* L. e *M. spicata* L. popularmente conhecidas como hortelã-pimenta, menta-japonesa e menta, respectivamente, são as espécies mais importantes, sendo amplamente utilizadas nas indústrias de alimentos, bebidas e cosméticos, principalmente, em virtude de suas propriedades aromatizantes (KUMAR et al., 2011;

VERMA et al., 2010). Além disso, existem dados de vários estudos pré-clínicos que mostram diversos efeitos farmacológicos destas espécies (AMARAL, 2015; LIMA et al., 2014; MATOS ROCHA et al., 2016SEVINDIK, 2018; OGALY et al., 2018; WANGJIT et al., 2016).

2.3.1.1 Mentha piperita L.

M. piperita L. caracteriza-se por ser uma espécie herbácea, perene e quase rasteira, com folhas e ramos de cor variando de verde escuro à roxo purpúrea (Figura 1). Esta espécie é um híbrido natural oriundo do cruzamento entre *Mentha aquatica* L. e *M. spicata* L. (*Mentha longifolia* Huds. x *Mentha suaveolens*) (TIWARI, 2016). *M. piperita* é conhecida popularmente como hortelã, hortelã-pimenta, menta, hortelã-apimentada, hortelã-das-cozinhas, menta-inglesa e sândalo, e apresenta um odor característico de mentol fresco e um sabor picante, seguido por uma sensação refrescante (HERRO; JACOB et al., 2010).

Figura 1: Planta de *Mentha piperita*

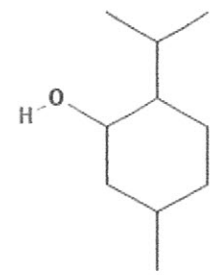
FONTE: Herbario Virtual de Banyeres de Mariola y Alicante

O Brasil já foi considerado o maior produtor mundial de OEMP, no entanto, devido ao baixo nível tecnológico de produção empregado na região sul (cultivo em maior escala) passou a importá-lo (BIZZO et al., 2009).

Adicionalmente ao uso na culinária, o OEMP é reconhecido por suas atividades anticancerígena, antifibrogênica, anti-inflamatória, antioxidante, antibacteriana, antifúngica e antiviral (LOOLAIE et al., 2017; OGALY et al., 2018; OLIVEIRA, K. et al., 2017; SOUSA GUEDES et al., 2016; SUN et al., 2014). Mentol (30 - 55%; Figura 2) é o constituinte volátil

mais importante da espécie e considerado como sendo responsável pelo seu aroma e sabor típicos (BARROS et al., 2015; KAMATOU et al., 2013). O mentol é capaz de causar perturbação na membrana plasmática de micro-organismos, induzindo alterações de permeabilidade e liberação de materiais intracelulares, que ocorre, em virtude de apresentar capacidade de migrar em ambientes aquosos e interagir com os fosfolipídios de membrana das células alvo (SCHELZ et al., 2006; TROMBETTA et al., 2005).

Figura 2: Estrututa química do mentol



FONTE: PubChem

A qualidade dos OEs extraídos de *M. piperita* depende da concentração de mentol, mentona, mentofurano e pulegona, ou seja, melhora com o aumento no conteúdo de mentol e diminuição do conteúdo de mentofurano e pulegona. Um dos fatores desencadeantes dessa variação é a temperatura do ambiente, visto que a síntese de monoterpenos em *M. piperita* é aumentada em temperaturas mais elevadas (BARROS et al., 2015).

2.3.1.2 Mentha spicata L.

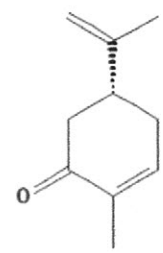
A espécie *M. spicata* L. é uma planta rizomatosa rastejante, perene glabra, com folhas mais estreitas verde-escuras (Figura 3), oriunda de um híbrido resultante do cruzamento entre as espécies *M. suaveolens* e *M. longifolia* (HARLEY; BRIGHTON, 1977). *M. spicata* L. é popularmente conhecida como menta de jardim, hortelã, hortelã-comum, hortelã das hortas, hortelã dos temperos, hortelã-verde, hortelã rasteira, menta verde, menta romana e menta enrugada. A planta na sua forma fresca ou desidratada, bem como o seu óleo essencial, são amplamente utilizados em alimentos, cosméticos, confeitaria, na formulação de gomas de mascar e dentifrício e indústria farmacêutica (LAWRENCE, 2006).

Figura 3: Planta de *Mentha spicata*

FONTE: Herbario Virtual da Universidade de Valência

Os principais componentes do OEMS são os derivados do terpeno, incluindo carvona (Figura 4) e limoneno, os quais são fortemente relacionados ao seu odor característico. Carvona e limoneno demonstram várias propriedades farmacológicas, a citar: anti-inflamatória, antiespasmódica, antioxidante, antibacteriana, antifúngica e antitumoral (KOKKINI et al., 1995; ULBRICHT et al., 2010; WANGJIT et al., 2016).

Figura 4: Estrututa química da carvona



FONTE: PubChem

O mecanismo de ação antimicrobiana da carvona não é ainda totalmente esclarecido, no entanto, tem sido demonstrado que este constituinte causa desestabilização da estrutura da bicamada fosfolipídica e interage com enzimas e proteínas da membrana de micro-organismos (SHABAZI, 2015).

2.3.1.3 Mentha x villosa Huds.

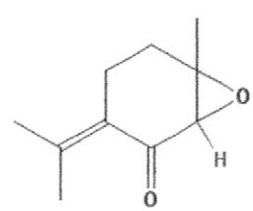
A espécie *M. x villosa* Hudson, cultivada no Nordeste brasileiro, é uma erva perene com folhas ovais e verde-escuras (Figura 5), sendo reportada na literatura também como *M. crispa* e popularmente conhecida como hortelã-da-folha-miúda. *M. villosa* é originária do retrocruzamento espontâneo de *M. suaveolens* e *M. spicata* (*Mentha longifolia* Huds. x *Mentha suaveolens*) (LORENZI; MATOS, 2002).

Figura 5: Planta de *Mentha x villosa*

FONTE: Herbário da Universidade de Michigan

M. villosa apresenta uma grande variedade de constituintes na sua composição, sendo a rotundifulona ou óxido de piperitenona (Figura 6), o composto majoritário mais comumente dectectado, seguido por limoneno, germacreno-D e mirceno, porém, geralmente, em quantidade bastante reduzidas em relação à rotundifolona (LIMA et al., 2014).

Figura 6: Estrututa química da rotundifolona



FONTE: PubChem

Estudos têm mostrado diversas propriedades biológicas do OEMV, a citar: antimicrobiana (ARRUDA, 2007; 2006; FREITAS et al., 2014; GUERRA et al., 2015), antioxidante (FREITAS et al., 2014), larvicida (LIMA et al., 2014), anti-parasitária (MATOS ROCHA et al., 2016), anti-câncer (AMARAL, 2015), cardiovascular (GUEDES et al., 2004; LAHLOU et al., 2002) e antinoceptiva (SOUSA CUNHA et al., 2009).

De modo geral, o mecanismo de ação antimicrobiana do OEMV está associado com alteração na membrana citoplasmática, interferência nas bombas de prótons e efluxo de eletrólitos e coagulação do conteúdo celular (ARRUDA, 2007).

2.3.1.4 Toxicidade dos OEs de Mentha spp.

Apesar de suas características biológicas já reconhecidas, a utilização de produtos naturais, tais como os OEs extraídos do gênero *Mentha,* não está isenta de riscos para a saúde pública, pois há a possibilidade de uma má utilização destas misturas, principalmente em decorrência da adição de teores excessivos. Sendo assim, a avaliação da segurança de seu uso, como aditivos alimentares, está atrelada à informações quanto ao processo de fabrico, destino no alimento e respetivos dados toxicológicos (ISMAILI et al., 2017).

Ainda são escassos os estudos de toxicidade dos OEs de *Mentha*; não foram encontrados dados na literatura sobre os efeitos tóxicos de OEMS, a maioria dos estudos está relacionado à extratos aquosos do mesmo.

Estudo de toxicidade aguda OEMP demonstrou que doses de de 100 a 2000 mg/kg por via oral (dose única) apenas um efeito tóxico mínimo nos ratos (SOKOVIĆ et al., 2006).

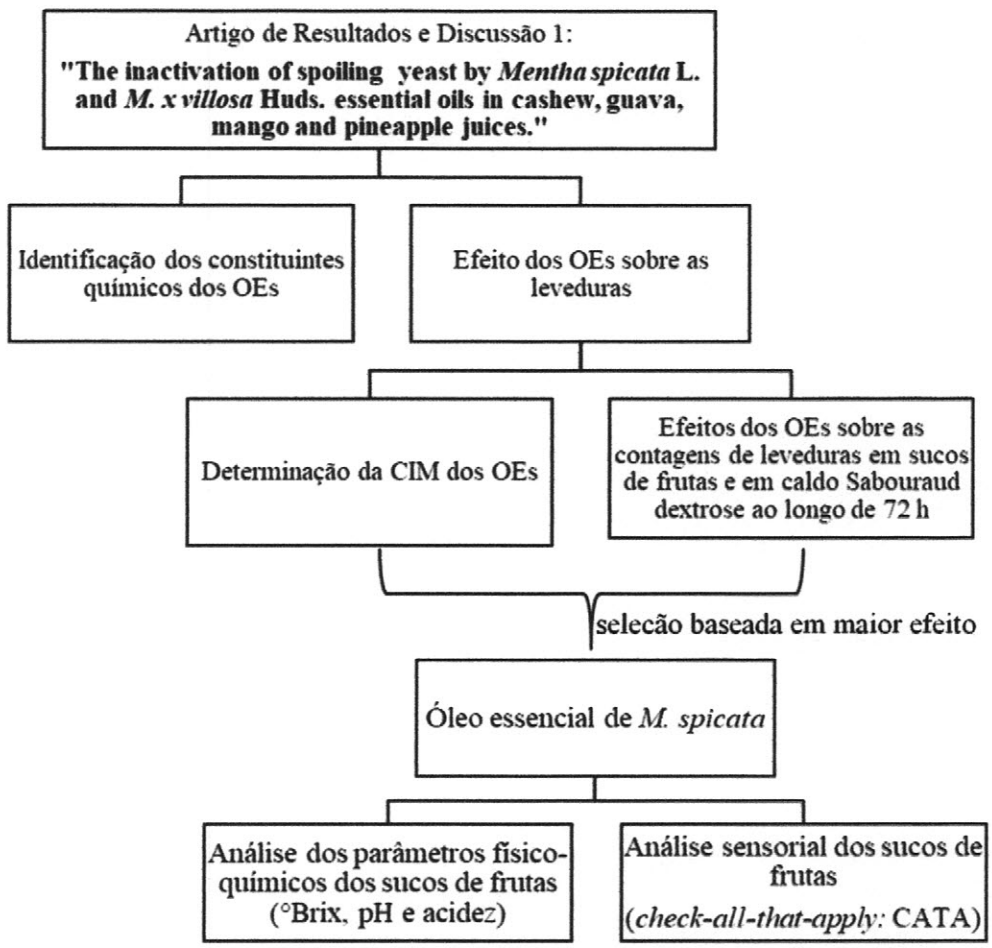
Em estudo *in vivo,* OEMV não alterou significativamente os parâmetros hematológicos e bioquímicos em ratos e camundongos tratados por via oral durante 30 dias com doses equivalentes a 10 e 20% da DL50 (OLIVEIRA, L. et al., 1998). Além disso, em outro estudo, foi observado ausência de toxicidade para ratas grávidas ao ser administrado óleo essencial de *Mentha x villosa* à 10, 25 e 50 µg/kg por gavagem, no entanto, mesmo com a ausência de malformação esquelética e visceral observada nos fetos expostos, os resultados revelaram o óleo essencial apresenta efeito tóxico fetal, devido aos pontos hemorrágicos visualizados no encéfalo, mas mais frequentemente, nos rins, fígado e vasos próximo ao coração, de alguns fetos expostos às três doses (GUERRA, K. et al., 2012).

Apesar da escassez de dados sobre toxicidade, vale ressaltar que, os OEs são geralmente seguros e que as concentrações empregadas com função antimicrobiana em alimentos, são mínimas e, portanto, acredita-se que seu uso não implique em prejuízos à saúde dos consumidores.

3 MATERIAL E MÉTODOS

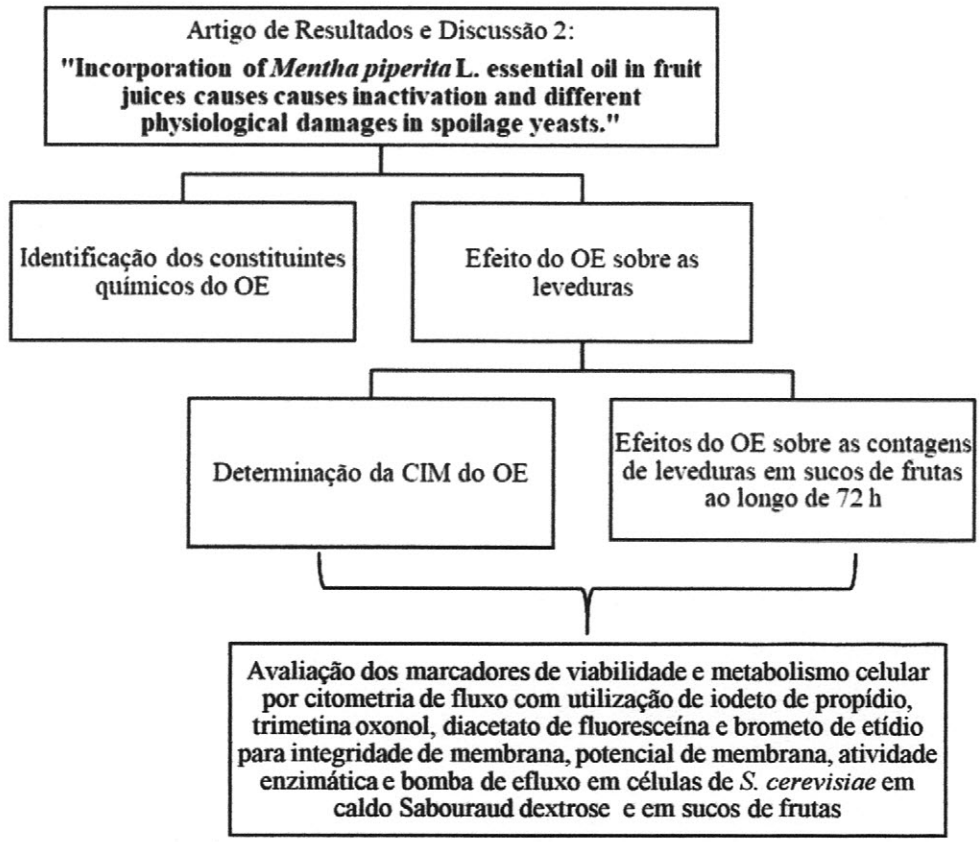
As análises foram realizadas no Laboratório de Microbiologia de Alimentos/Departamento de Nutrição/Centro de Ciências da Saúde, bem como no ~~IPeFArM – Instituto de Pesquisas em Fármacos e Medicamentos/UNICAL – Unidade de~~ Caracterização e Análises, ambos da Universidade Federal da Paraíba e situados no ~~Campus I (João Pessoa – PB). Abaixo estão apresentados os esquemas (Fluxograma 1 e 2)~~ dos desenhos experimentais referentes aos dois artigos elaborados com os resultados obtidos ao longo da execução dos experimentos.

Fluxograma 1: Etapas experimentais contidas no artigo 1



FONTE: Próprio autor, 2018.

Fluxograma 2: Etapas experimentais contidas no artigo 2



FONTE: Próprio autor, 2018.

3.1 MATERIAL

3.1.1 Obtenção dos óleos essenciais

Os OEs de *M. piperita* (lote 185; densidade a 20 °C, 0,900; índice de refração a 20 °C, 1,460; pH 5,17) e *M. spicata* (lote 190; densidade a 20 °C, 0,930; índice de refração a 20 °C, 1,490; e pH 5,17) foram adquiridos na Ferquima Ind. Com. Ltda. (São Paulo, Brasil), enquanto que o OE de *M. x villosa* foi cedido pelo Laboratório Hebrom® por intermédio do Prof. Dr. José Maria Barbosa Filho (Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Federal da Paraíba – Campus I, João Pessoa). Todos os OEs foram extraídos por meio de destilação à vapor de água das folhas das respectivas espécies vegetais.

3.1.2 Obtenção das leveduras e preparação do inóculo

Diferentes espécies de leveduras citadas como potenciais agentes deteriorantes de sucos de fruta (ANEJA et al., 2014; TOURNAS et al., 2006; VANTARAKIS et al., 2011) foram utilizadas como micro-organismos teste, a citar: *Candida albicans* (ATCC 90028), *C. tropicalis* (ATCC 28707), *Pichia anomala* (ATCC 40101) e *Saccharomyces cerevisiae* (ATCC 2601). As cepas foram fornecidas na forma liofilizada da Coleção de Micro-organismos de Referência, do Instituto Nacional de Controle de Qualidade em Saúde, Fiocruz (Fundação Oswaldo Cruz; Rio de Janeiro, Brasil), as quais foram ativadas utilizando duas passagens consecutivas com duração de 48 h cada em caldo Sabouraud dextrose (CSD; pH 5,6; Acumedia Manufacturers Inc., Lansing, Michigan, EUA) a 30 °C. As cepas estoque foram mantidas em CSD contendo glicerol (15 g/100mL) a -20 °C, enquanto que as cepas de trabalho foram mantidas em ágar Sabouraud dextrose (ASD; pH 5,6; Acumedia Manufacturers Inc., Lansing, Michigan, EUA) a 4 °C e transferidas mensalmente para um novo meio de cultura. Para utilização nos ensaios, as cepas foram, primeiramente, cultivadas em CSD a 30 °C durante 24 h; posteriormente, as culturas foram colhidas por centrifugação (4500 *x g*, 15 min, 4 °C), lavadas duas vezes e resuspensas em solução salina esterilizada (NaCl 0,85 g/100 mL) para obter suspensões de células apresentando leitura de densidade óptica a 625 nm (DO_{625}) de 0,75 para *C. albicans*, *C. tropicalis* e *P. anomala*, e 0,95 para *S. cerevisiae*. Estas suspensões proporcionaram contagens de células viáveis de, aproximadamente, 7 log de unidades formadoras de colônias por mL (log ufc/mL).

3.1.3 Obtenção dos sucos de frutas

As frutas utilizadas para a preparação dos sucos foram abacaxi (*Ananas comosus* L. Merril.), caju (*Anacardium occidentale* L.), goiaba (*Psidium guajava* L.) e manga (*Mangifera indica* L.), as quais foram adquiridas de um distribuidor atacadista local na cidade de João Pessoa – PB e selecionadas em estádio de maturação comercial, considerando também a ausência de danos mecânicos e sinais visíveis de deterioração. Inicialmente, as frutas foram lavadas e sanitizadas por imersão em solução de hipoclorito de sódio (150 ppm, pH 7,2 ajustado com NaOH 1 M) durante 5 min, lavadas com água destilada esterilizada e secas

durante 30 min em cabine de segurança. Em seguida, as frutas foram descascadas assepticamente, picadas e misturadas com água destilada (50 g de frutas em 100 mL de água destilada estéril para suco de goiaba e 60 g de fruta em 100 mL de água destilada esterilizada para os sucos de caju, manga e abacaxi; BRASIL, 2003), utilizando um liquidificador doméstico na velocidade máxima durante 3 min. Os sucos foram filtrados, utilizando uma camada de gaze tripla e esterilizados por meio de membrana filtrante de nylon Wattman® com poros de dimensão de 0,22 μm (Sigma Aldrich, St. Louis, EUA). As amostras de sucos foram armazenadas em alíquotas de 25 mL a -20 °C e, quando necessário, as alíquotas foram descongeladas sob refrigeração (4 ± 0,5 °C) e utilizadas nos ensaios (LEITE et al., 2016; de SOUSA GUEDES et al., 2016).

3.2 MÉTODOS

3.2.1 Identificação dos constituintes dos óleos essenciais

Os constituintes do OEMP, OEMS e OEMV foram identificados por meio de um cromatógrafo gasoso acoplado a um espectrômetro de massas (CGMS-QP2010 Ultra Shimadzu, Kyoto, Japão), utilizando as seguintes condições analíticas: coluna capilar RTX-5MS (proporções de 30 m x 0,25 mm x 0,25 μm); temperatura do programa: 60 a 240 °C (3 °C/min); temperatura do injetor: 250 °C; temperatura do detector: 220 °C; gás de transporte: hélio ajustado com taxa de fluxo de 0,99 mL/min; impacto de elétrons: 70 eV; e faixa de massa (*m/z*): 40 a 500. A identificação de cada constituinte foi realizada comparando seus espectros de massa com as bibliotecas NIST/EPA/NIH Mass Spectral Database (Instituto Nacional de Padrões Tecnológicos, Norwalk, CT) e FFNSC1.3 (Sabor e Fragrância Natural e Compostos Sintéticos), bem como com o índice de retenção de Kovats (ADAMS, 2001). Por sua vez, a quantificação dos constituintes dos OEs foi obtida após a normalização das áreas de cada constituinte detectado, sendo expressos como porcentagem da área (%) (de SOUSA GUEDES et al., 2016).

3.2.2 Ensaios de atividade antimicrobiana

3.2.2.1 Determinação da Concentração Inibitória Mínima (CIM) dos óleos essenciais em Caldo Sabouraud Dextrose

A CIM dos OEs foi determinada por meio de ensaio de microdiluição em caldo utilizando microplacas de 96 poços com fundo na forma de "U" (CLSI, 2008). Emulsões estoque de cada OE na concentração de 240 µL/mL foram preparadas em CSD contendo Tween 80 (1%, v/v; Sigma Aldrich, Saint Louis, USA). Alíquotas de 50 µL das soluções estoque do OEMP, OEMS e OEMV foram distribuídas e homogeneizadas nos poços (primeira linha) da microplaca contendo 50 µL de CSD duplamente concentrado. Subsequentemente, alíquotas de 50 µL contidas nos poços da primeira linha foram transferidas para os poços seguintes (segunda linha) e, por meio de diluições geométricas de razão dois, as concentrações dos EOs variaram de 120 - 0,469 µL/mL. Em seguida, alíquotas de 50 µL da suspensão da levedura testada foram adicionadas a cada poço (contagens finais de células viáveis de, aproximadamente, 7 log ufc/mL), de modo a obter 8 concentrações finais diferentes para cada OE, a saber: 60; 30; 15; 7,5, 3,75, 1,875, 0.937 e 0,496 µL/mL. Em cada microplaca foi utilizado um controle positivo (CSD inoculado) e negativo (CSD não inoculado) para cada cepa de levedura testada. A microplaca foi revestida com filme plástico com o intuito de evitar a volatilização do óleo essencial e incubada a 30 °C durante 48 h. A resazurina, um indicador de oxi-redução, foi empregada para a realização da leitura dos resultados. Para tanto, 40 µL de solução de resazurina (100 µg/mL) foram adicionados aos poços, seguindo-se por incubação a 30 °C por até 2 h; a presença da cor azul e cor rosa foram consideradas indicativas de ausência e presença de crescimento microbiano, respectivamente. A CIM foi considerada como a menor concentração de cada OE necessária para inibir o crescimento visível da levedura testada ao final do período de incubação.

3.2.2.2 Contagem de células viáveis de leveduras expostas aos óleos essenciais em caldo Sabouraud dextrose e em sucos de frutas

Os efeitos das diferentes concentrações do OEMP, OEMS e OEMV sobre o crescimento/sobrevivência das leveduras testadas foram avaliados em CSD e em sucos de abacaxi, caju, goiaba e manga durante 72 h de armazenamento refrigerado (4 ± 0,5 °C) por meio da contagem células viáveis (SOUZA et al., 2007). Soluções estoques dos OEs na concentração de 15 µL/mL (OEMS), 30 µL/mL (OEMP) e 60 µL/mL (OEMV) foram preparadas em CSD contendo Tween 80 (1%, v/v) e em sucos de frutas. Em seguida, alíquotas de 2 mL da solução estoque (CSD e sucos de frutas) foram transferidas para tubos de ensaios contendo 2 mL de CSD e sucos de frutas, resultando concentrações de 7,5, 15 e 30 µL/mL para OEMS, OEMP e OEMV, respectivamente. Após homegeneização, 2 mL dessa mistura foram transferidos para outro tubo de ensaio contendo 2 mL de CSD e sucos de frutas, obtendo concentrações de 3,75, 7,5 e 15 µL/mL para OEMS, OEMP e OEMV, respectivamente. Por fim, mais uma vez, 2 mL dessa última mistura foram transferidos e homogeneizados em CSD e em sucos de frutas contidos em outro tubo de ensaio, originando concentrações de 1,875, 3,75 e 7,5 µL/mL para OEMS, OEMP e OEMV, respectivamente. Sendo assim, três concentrações diferentes para cada OE foram obtidas e ao qual foram adicionados 2 mL de suspensão de levedura (contagem final de células viáveis de, aproximadamente, 7 log ufc/mL) que, por fim, resultaram em misturas de CSD e sucos de frutas contendo OEMS (3,75, 1,875 e 0,937 µL/mL correspondente à CIM x 2, CIM e CIM/2, respectivamente), OEMP (7,5, 3,75 e 1,875 µL/mL correspondente à CIM x 4, CIM x 2 e CIM, respectivamente) e OEMV (15, 7,5 e 3,75 µL/mL correspondente à CIM x 2, CIM e CIM/2, respectivamente). As misturas foram agitadas em vórtex por 30 segundos, em seguida, mantidas a 4 ± 0,5 °C, e em diferentes intervalos de tempo de armazenamento (0 - logo após a homogeneização, 5, 15, 30, 45 min e 1, 2, 4, 8, 12, 24, 48 e 72 h), uma alíquota de 100 µL de cada mistura foi diluída seriadamente em solução salina esterilizada (NaCl 0,85 g/100 mL, p/v). Alíquotas de 10 µL de cada diluição foram inoculadas em ASD utilizando a técnica da microgota (HERIGSTAD et al., 2001). Os ensaios controle com amostras de CSD e dos sucos sem adição do OEMP, OEMS ou OEMV foram realizados de forma semelhante. Após um período de incubação de 48-72 h a 30 °C, as colônias formadas sobre o ágar foram contadas e os resultados expressos como log ufc/mL. As placas inoculadas com alíquotas recolhidas de amostras de CSD ou suco de fruta contendo OEMP, OEMS ou OEMV foram incubadas por

24 h adicionais à temperatura adequada em relação às amostras recolhidas a partir das amostras controle (sem adição de OE).

Estes estudos foram realizados para investigar se o OEMP, OEMS e OEMV testados são capazes de causar uma redução ≥ 5-log na contagem das leveduras testadas, bem como para determinar o tempo necessário para atingir este nível de redução (log N_0 - N, onde N_0 e N se referem à contagem inicial e contagem após incubação, respectivamente, para o intervalo de tempo de armazenamento indicado).

3.2.2.3 Avaliação dos marcadores de viabilidade e metabolismo celular por citometria de fluxo

Para monitorar as respostas fisiológicas de células de *S. cerevisiae* (aproximadamente 7 log cfu/mL) quando exposta ao OEMP (1,875 µL/mL, correspondente a CIM previamente determinada) foi utilizado o método de citometria de fluxo. Quatro sistemas distintos para cada suco testado (caju e goiaba) foram utilizados, a citar: tampão fosfato salino inoculado (controle negativo; PBS; NaCl 8,0 g/L, KCl 0,20 g/L, Na_2HPO_4 1,42 g/L, KH_2PO_4 0,24 g/L, pH 7,4), suco inoculado sem adição do OEMP, suco adicionado de OEMP (1,875 µL/mL) e inoculado, e células tratadas com etanol à 70% (v/v) (controle de morte celular). Após estocagem refrigerada (4 °C) por 45 min, os materiais dos diferentes sistemas foram centrifugados (4500 *g x* 10 min, 4 °C) e os *pellets* obtidos foram, então, lavados duas vezes e ressuspensos em PBS e imediatamente marcados com iodeto de propídio (PI, Sigma-Aldrich, St. Louis, MO, EUA) para avaliar integridade da membrana; trimetina oxonol do ácido bis-(1,3-dibutilbarbitúrico) ($DiBAC_4(3)$, Molecular Probes, Invitrogen, OR, EUA) para avaliar alterações no potencial de membrana; diacetato de fluoresceína (FDA; ThermoFisher Scientific, Molecular Probes ™, F1303) para avaliar alterações na atividade enzimática e brometo de etídio (EB; Sigma-Aldrich, St. Louis, MO, EUA) para avaliar alterações na atividade de bomba de efluxo das leveduras teste (CARRILLO et al., 2018; KIM et al., 2017; SILVA et al., 2011).

Para avaliar a integridade e o potencial de membrana foi utilizado uma dupla marcação. Para isso, os *pellets* ressuspensos em PBS (100 µL) foram incubados por 30 min na presença de 0,1 µL de PI (1 mg/mL) e 0,1 µL de $DiBAC_4(3)$ (1 mg/mL), resultando em concentrações finais dos marcadores de 1 µg/mL (KIM et al., 2017). Para a atividade enzimática, os *pellets*

ressuspensos em PBS (200 µL) foram incubados por 30 min na presença de 0,1 µL de FDA (5 mg/mL), proporcionando concentração final do marcador de 2,5 µg/mL (CARRILLO et al., 2018). Para avaliar a atividade de bomba de efluxo, os *pellets* ressuspensos em PBS com glicose 1% (p/v) em proporção 1:1 (200 µL) foram incubados por 5 min na presença de 0,1 µL de EB (5 mg/mL), proporcionando uma concentração final do marcador de 2,5 µg/mL (SILVA et al., 2011). Após o período de incubação a 37 °C sob abrigo da luz, todas as amostras foram centrifugadas (4500 *g* x 10 min, 4 °C) e lavadas em PBS, e os *pellets* resultantes ressuspensos com mesmo volume inicial de PBS (100, 200 e 200 µL, respectivamente), seguindo-se por análise em citômetro de fluxo.

As análises de citometria de fluxo foram realizadas com uso de citômetro de fluxo equipado com *lasers* azul (488 nm) e vermelho (640 nm), dois detectores de dispersão de luz (FSC e SSC) e quatro detectores de fluorescência (BD Accuri C6, New Jersey, EUA).

O sinal de fluorescência (medições da área de pulso) referentes aos fluorocromos foi coletado por filtros óticos nos canais FL1 ($DiBAC_4$ (3) e FDA) e FL3 (PI e EB). O nível dos limiares (*threshold*) para a aquisição de dados foi definido para FSC igual a 30 000, com o intuito de eliminar ruídos ou partículas considerados muito menores que a levedura intacta. As células de levedura foram identificadas por parâmetros FSC/SSC. Cada aquisição de amostra foi operada em baixa taxa de fluxo (14 µL/min) e um total de 10 000 eventos foram analisados. Todos os citogramas de emissões de fluorescência foram registrados usando o Software BD Accuri C6 (BD®, Becton Dickinson e Company, Franklin Lakes, NJ, EUA). A análise de FL1 *versus* FL3 foi utilizada para estabelecer propriedades de fluorescência da população com dupla marcação. As células $DiBAC_4(3)^+PI^-$ (porta UL) correspondem a células despolarizadas e não permeabilizadas; as células $DiBAC_4(3)^+PI^+$ e $DiBAC_4(3)^-PI^+$ (porta UR e LR) correspondem à população de células despolarizadas e permeabilizadas com diferentes graus de dano; e as células $DiBAC_4(3)^-PI^-$ (gate LL) correspondem a populações não coradas de células intactas, polarizadas e não permeabilizadas (HAMMER; HEEL, 2012). A análise do gráfico de densidade de SSC *versus* FL1 ou FL3 foi aplicada para determinar as propriedades de fluorescência das populações FDA^+ e EB^+, respectivamente, indicando células com atividades enzimáticas e de bomba de efluxo alteradas, respectivamente, as quais tiveram as suas populações identificadas por meio de retângulos localizados no lado direito dos gráficos.

3.2.3 Avaliação de aspectos de qualidade dos sucos de frutas

3.2.3.1 Análises físico-químicas

Os valores de sólidos solúveis totais, pH e acidez titulável foram determinados nos sucos de abacaxi, caju, goiaba e manga adicionados ou não de OEMS (1,875 µL/mL, correspondente à CIM, visto que foi o OE mais eficaz entre os demais) no tempo zero (logo após a incorporação e homogeneização do OE) e após 72 h de armazenamento refrigerado (4 ± 0,5 °C). Estes parâmetros foram selecionados conforme legislação brasileira para monitoramento da qualidade de sucos de frutas não adoçados (BRASIL, 2003). O teor de sólidos solúveis (°Brix) foi analisado utilizando um refractômetro digital (modelo HI 96801, Hanna Instruments, São Paulo, Brasil) (AOAC, 2016). Os valores de pH foram obtidos por meio do uso de potenciômetro digital (modelo Q400AS, Quimis, São Paulo, Brasil) (AOAC, 2016). A acidez titulável foi determinada por meio de titulometria com resultados expressos em gramas por 100 mL de equivalentes de ácido cítrico (AOAC, 2016).

3.2.3.2 Análise sensorial

A avaliação sensorial foi realizada após aprovação no Comitê de Ética em Pesquisa (protocolo 1.125.993/2015) por meio de perguntas *check-all-that-apply* (CATA) para os sucos abacaxi, caju, goiaba e manga contendo OEMS na concentração de 1,875 µL/mL, em consideração à melhor eficácia entre os demais OEs. Os sucos foram produzidos no mesmo dia dos testes sensoriais e mantidos sob refrigeração (4 ± 0,5 °C). Vale ressaltar que os termos dos atributos de aparência (cor caracterísitica ou não característica dos respectivos sucos), sabor (doce, sem doce, sabor forte, sensação refrescante, sabor da fruta em questão, sabor de menta, sabor agradável ou desagradável) e odor (odor caracterísitico ou não característico dos respectivos e odor de menta) de todos os sucos foram estabelecidos por um grupo não treinado para compor as questões da CATA. Cem membros do painel não treinados (17 a 50 anos), para cada suco analisado, foram recrutados e selecionados considerando seus hábitos de consumir sucos de frutas duas ou mais vezes por semana. As análises sensoriais foram

realizadas em quatro sessões de testes, uma para cada suco, conduzidas em cabines individuais com luz branca. Após assinar Termo de Consentimento Livre e Esclarecido (TCLE; Apêndice A), os membros presentes no painel avaliaram as amostras de sucos, imediatamente após a remoção do armazenamento refrigerado, servidas em alíquotas de 30 mL em copos brancos descartáveis, aos quais foi solicitado que marcassem todos os termos de cada atributo (conforme definido anteriormente) apropriados para descrever cada suco avaliado (Apêndice B) (ARES et al., 2010).

Tabela 1: Termos utilizados para compor questões CATA para cada atributo sensorial de sucos de frutas avaliados.

Aparência	**Sabor**	**Aroma**
Cor característica do suco	Adoçado	Aroma característico do suco
Cor não característica do suco	Não adoçado	Aroma não característico do suco
	Sabor forte	Aroma de menta
	Sensação refrescante	
	Sabor da fruta	
	Sabor de menta	
	Sabor amargo	
	Sabor agradável	
	Sabor desagradável	

3.2.4 Análise estatística

Os ensaios foram realizados em dois experimentos independentes em triplicata. Diferentes lotes de sucos de fruta (preparados utilizando um conjunto de três frutas diferentes) e do inóculo padronizado de uma única suspensão de levedura preparada a partir de duas culturas independentes de levedura teste foram utilizados em cada experimento independente. As contagens de células viáveis em cada ponto de tempo monitorado foram obtidas a partir de uma única mistura compreendendo cada um dos sucos de fruta testado, a concentração de OEMP ou OEMS ou OEMV e o inóculo da levedura teste. As amostras foram plaqueadas em duplicata e as médias calculadas em cada tempo avaliado. Para os ensaios de determinação da

CIM, os resultados foram expressos como valores modais pois os valores de CIM foram os mesmos em todas as repetições. Para os ensaios de contagem de células viáveis das leveduras e determinação dos parâmetros físico-químicos, a análise estatística foi realizada para determinar diferenças significativas ($p \leq 0,05$) com base no teste *t* de Student e ANOVA, respectivamente. O software Sigma Stat 3.5 (Jandel Scientific Software, San José, Califórnia) foi utilizado para a realização das análises estatísticas. Os dados das perguntas da CATA foram analisados determinando a frequência de uso de cada termo para descrever cada atributo dos sucos de frutas e, as diferenças significativas ($p \leq 0,05$) entre as amostras foram avaliadas usando o Teste Q de Cochran (Manoukian, 1986), através do MedCalc Statistical Software versão 18 (MedCalc Software bvba, Ostend, Bélgica).

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4 RESULTADOS E DISCUSSÃO

Segundo normas estipuladas pelo Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos em nível de Doutorado, a seção de "Resultados e Discussão" da Tese deve ser apresentado em forma de artigos, dos quais, ao menos dois, devem ser elaborados e submetidos para publicação em revistas indexadas em Qualis com conceito mínimo B2 na a área de Ciência dos Alimentos da CAPES. Sendo assim, o formato de apresentação dos artigos segue as normas de "instruções aos autores" das revistas as quais foram submetidos.

4.1 ARTIGO DE RESULTADOS E DISCUSSÃO 1:

O primeiro artigo intitula-se **"The inactivation of spoiling yeasts by *Mentha spicata* L. and *M. x villosa* Huds. essential oils in cashew, guava, mango and pineapple juices** foi publicado na **Frontiers in Microbiology** (ISSN: 1664-302X; fator de impacto: 4,076; periódicos Qualis com classificação A1).

4.2 ARTIGO DE RESULTADOS E DISCUSSÃO 2:

O segundo artigo intitula-se **"Incorporation of *Mentha piperita* L. essential oil in fruit juices causes inactivation and different physiological damages in spoilage yeasts"**, foi elaborado e submetido à International Journal of Food Microbiology (ISSN: 0168-1605; fator de impacto: 3,339; Qualis Periódicos com classificação A1).

4.1 ARTIGO DE RESULTADO E DISCUSSÃO 1

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Inactivation of Spoilage Yeasts by *Mentha spicata* L. and *M.* × *villosa* Huds. Essential Oils in Cashew, Guava, Mango, and Pineapple Juices

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This study evaluated the efficacy of the essential oil from *Mentha spicata* L. (MSEO) and *M.* × *villosa* Huds. (MVEO) to inactivate *Candida albicans*, *C. tropicalis*, *Pichia anomala* and *Saccharomyces cerevisiae* in Sabouraud dextrose broth and cashew, guava, mango, and pineapple juices during 72 h of refrigerated storage. The effects of the incorporation of an anti-yeast effective dose of MSEO on some physicochemical and sensory characteristics of juices were evaluated. The incorporation of 3.75 μL/mL MSEO or 15 μL/mL MVEO caused a ≥5-log reductions in counts of *C. albicans*, *P. anomala*, and *S. cerevisiae* in Sabouraud dextrose broth. In cashew and guava juices, 1.875 μL/mL MSEO or 15 μL/mL MVEO caused ≥5-log reductions in counts of *P. anomala* and *S. cerevisiae*. In pineapple juice, 3.75 μL/mL MSEO caused ≥5-log reductions in counts of *P. anomala* and *S. cerevisiae*; 15 μL/mL MVEO caused ≥5-log reductions in counts of *S. cerevisiae* in mango juice. The incorporation of 1.875 μL/mL MSEO did not affect the physicochemical parameters of juices and did not induce negative impacts to cause their possible sensory rejection. These results show the potential of MSEO and MVEO, primarily MSEO, to comprise strategies to control spoilage yeasts in fruit juices.

Keywords: *Mentha* spp., essential oil, anti-yeast effects, fruit juice, preservation

INTRODUCTION

In last years, there is an increasing search by consumers for foods possessing particular characteristics in their composition (e.g., low contents of simple sugars, sodium, fat, and cholesterol) and the presence of constituents with health promoting effects (e.g., polyphenols and other antioxidants) (Corbo et al., 2014). Consumers have also demanded for fresher foods submitted to minimal processing having low or no amounts of synthetic preservatives (Román et al., 2017). In this context, the consumption and variety of unpasteurized and synthetic additive-free fruit juices in market has been increased. Nonetheless, the non-use of heating treatments and synthetic preservatives facilitate the survival of microbial contaminants in fruit juices resulting in decreased stability and higher risk to consumers (Leite et al., 2016).

In contrast to the harmful effects on some potentially pathogenic bacteria, the low pH and high sugar content in fruit juices provide a favorable substrate to yeast growth (Tribst et al., 2009). Most of the yeast species are highly fermentative and metabolize sugars with the production of alcohols (mostly ethanol), carbon dioxide and other organic compounds causing packing blown and unpleasing taste and flavor in fruit juices. Additionally, yeast growth in fruit juice may result in increased turbidity, flocculation, pellicle formation, and clumping (Lawlor et al., 2009). Reports have cited *Candida*, *Pichia*, *Rhodotorula*, and *Saccharomyces* as yeast genera frequently involved in fruit juice contamination and spoilage (Tournas et al., 2006; Vantarakis et al., 2011; Aneja et al., 2014). Although the fruit juice yeast contamination has not been associated with risks of foodborne diseases, the yeast growth in these products may result in alterations that cause consumption rejection, decreased market value and economic loss to processors (Tournas et al., 2006; Lawlor et al., 2009).

Control of contaminant yeasts in fruit juices has been classically achieved by the application of heat and/or incorporation of synthetics preservatives (Amirpour et al., 2015). Pasteurization is the method more commonly used to control microorganism in fruit juices, but the heating temperature used in this procedure may impact negatively on their physicochemical and sensory characteristics (Sánchez et al., 2017). Additionally, synthetic preservatives more commonly used in fruit juices (e.g., benzoic acid, sorbic acid and sulfur dioxide) may have harmful effects to consumers such as allergies, asthma, and skin rashes (Vally et al., 2009). Efforts have been addressed to the study of non-synthetic antimicrobials to inactivate contaminant microorganisms in fruit juices (Chanukya and Rastogi, 2017).

Interest in using non-synthetic antimicrobials has increased the attention to essential oils as potential inhibitors of microorganisms to preserve fruit juices. Essential oils are categorized as "generally recognized as safe" (GRAS) for use as flavoring ingredients in beverages (U.S. Food and Drug Administration [USFDA], 2015). *Mentha* genus, Lamiaceae family, includes more than 25 plant species that are recognized for their aromatic properties, being exploited in various sectors including pharmacy, cosmetic, agronomy, and foods (Park et al., 2016; Mamadalieva et al., 2017). *Mentha spicata* L., commonly known as spearmint, is one of the most common, popular and extensively studied *Mentha* species. *Mentha* × *villosa* Huds., also reported in literature as *M. crispa* and commonly known as creeping mint, is a hybrid of *M. spicata* and *M. suaveolens*. *M.* × *villosa* is still few explored concerning its biological activities, but has been considered a potential alternative for use as flavoring agent in foods and beverages (Lahlou et al., 2002; Kumar et al., 2011).

Essential oils from *Mentha* species have shown effective to inhibit a variety of pathogenic bacteria and molds (Guerra et al., 2015; Leite et al., 2016; Park et al., 2016; de Sousa Guedes et al., 2016) but few studies have focused on their anti-yeast effects. Previous studies have reported the anti-yeast effects of *M. piperita* essential oil (Tyagi et al., 2013; Ferreira et al., 2014; Karaman et al., 2016); however, there is a lack of studies about the efficacy of the essential oil from *M. spicata* (MSEO) and *M. villosa* (MVEO) to inactivate spoilage yeasts in juices.

Cashew, guava, mango, and pineapple juice are some of the main fruit species cultivated in Brazil (Food and Drug Administration [FAO], 2015), with much of their production destinated for juice processing. A variety of well-accepted fruit products (such as frozen pulps, unpasteurized juices, ready-to-eat fruit-mix salads and minimally processed slices) containing grinded mint (*Mentha* spp.) leaves are available in the market, reinforcing the idea that fruit juices could represent potential matrices to exploit the antimicrobial properties of MSEO and MVEO. This study evaluated the efficacy of MSEO and MVEO to inactive the potentially spoilage yeasts *C. albicans*, *C. tropicalis*, *P. anomala*, and *S. cerevisiae* in pineapple, cashew, guava, and mango juice stored under refrigeration. The effects of the incorporation of an effective anti-yeast dose of MSEO on some physicochemical parameters and sensory characteristics of theses juices were assessed.

MATERIALS AND METHODS

Test Strains and Growth Conditions

Different yeasts species commonly cited as potential spoilage agents of fruit juices (Tournas et al., 2006; Vantarakis et al., 2011; Aneja et al., 2014) were used as test organisms, to cite: *C. albicans* (type-strain ATCC 90028), *C. tropicalis* (type-strain ATCC 28707), *P. anomala* (type-strain ATCC 40101) and *S. cerevisiae* (type-strain ATCC 2601). These type-strains were supplied as freeze-dried isolates by the Collection of Reference Microorganisms, National Institute of Quality Control in Health, Oswaldo Cruz Foundation (Rio de Janeiro, Brazil), and they were activated by two consecutive 48 h-passages in Sabouraud dextrose broth (Acumedia Manufacturers Inc., Lansing, MI, United States) at 30°C. Stocks were kept in Sabouraud dextrose broth containing glycerol (15 g/100 mL) at −20°C; working cultures were maintained in Sabouraud dextrose agar (Acumedia Manufacturers Inc., Lansing, MI, United States) at 4°C, and transferred for fresh Sabouraud dextrose agar monthly. For use in assays, the strains were first cultivated in Sabouraud dextrose agar at 30°C during 48 h, harvested by centrifugation (4500 × *g* × 15 min, 4°C), washed twice in sterile saline solution (0.85 g/100 mL) and re-suspended in sterile saline solution to obtain cell suspensions presenting optical density reading at 625 nm of 0.75 for *C. albicans*, *C. tropicalis*, and *P. anomala*, and of 0.95 for *S. cerevisiae*. These suspensions provided viable counts of approximately 7 log cfu/mL when pour plated onto Sabouraud dextrose agar.

Preparation of Fruit Juices

Cashew (*Anacardium occidentale* L.), guava (*Psidium guajava* L.), mango (*Mangifera indica* L.), and pineapple (*Ananas comosus* L. Merrill) fruit were acquired from a local wholesale distributor (João Pessoa, Brazil) and selected in commercial maturation stage, with absence of mechanical damages and visible signs of infection. Fruit were surface disinfected through immersion in a sodium hypochlorite solution (150 ppm, pH 7.2 adjusted

using 1 M NaOH) for 5 min, washed with distilled water and dried for 30 min in a biosafety cabinet. Fruit were aseptically peeled, chopped and mixed with sterile distilled water (50 g of fruit in 100 mL of sterile distilled water for guava juice and 60 g of fruit in 100 mL of sterile distilled water for cashew, mango, and pineapple; Anonymous, 2003) using a domestic blender (for 3 min). The fruit juices were double filtered using a triple-cheesecloth layer and sterilized using Wattman membrane filters nylon por size 0.22 μm (Sigma-Aldrich, St. Louis, MO, United States). 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.

MSEO and MVEO

MSEO and MVEO extracted through steam distillation were obtained from Ferquima Ind. Com. Ltd. (São Paulo, Brazil) and Hebron® company (Recife, Brazil), respectively. A stock emulsion of each essential oil at a concentration of 240 μL/mL was prepared in sterilized Sabouraud dextrose broth containing Tween 80 (1%, v/v; Sigma-Aldrich, St. Louis, MO, United States) as an emulsifier (de Sousa Guedes et al., 2016). At the highest assayed concentration (1%, v/v), Tween 80 presented no inhibitory effect against the tested yeast strains. Stock emulsions of each essential oil were maintained in an amber screw-capped tube under 4 ± 0.5°C and used in anti-yeast assays after a storage time period no longer than 48 h.

Identification of Essential Oils Constituents

MSEO and MVEO constituents were identified using a gas chromatograph coupled to mass spectrometer (CGMS-QP2010 Ultra Shimadzu, Kyoto, Japan). Analytical conditions were: a RTX-5MS capillary column (30 m × 0.25 mm × 0.25 μm); 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. Identification of each component was performed by comparing their mass spectra with the NIST/EPA/NIH Mass Spectral Database (National Institute of Standards Technology, Norwalk, CT, United States) and FFNSC1.3 (Flavor and Fragrance Natural and Synthetic Compounds) libraries as well as the Kovats retention index (Adams, 2001). Quantification of the essential oils constituents was obtained after normalizing the areas of each detected constituent and expressed as a percentage of area (%) (de Sousa Guedes et al., 2016).

Determination of the Minimum Inhibitory Concentration of Essential Oils

MIC of MSEO and MVEO was determined using a microdilution in broth assay (Clinical and Laboratory Standards Institute [CLSI], 2008). For this, 50 μL-aliquots of the stock emulsion of MSEO or MVEO (240 μL/mL) were dispensed into wells of a 96-well microplate containing 50 μL of double concentrate Sabouraud dextrose broth. Subsequently, 50 μL-aliquots were transferred the following wells, and through geometric dilutions the essential oils concentrations varied from 120 to 0.469 μL/mL. Afterward, 50 μL-aliquots of the yeast suspensions were added to each well (final viable counts were approximately 7 log cfu/mL), and the final essential oils concentrations varied from 60 to 0.234 μL/mL. Each microplate contained a positive (Sabouraud dextrose broth inoculated) and a negative (Sabouraud dextrose broth non-inoculated) control for each yeast strain tested. The microplate was loosely wrapped with cling wrap to avoid essential oil volatilization, and incubated at 30°C for 48 h. MIC was determined as the lowest concentration of each essential oil required to prevent visible yeast growth.

Effects of the Essential Oils on Yeasts Counts in Sabouraud Dextrose Broth and Fruit Juices Over 72 h

The effects of different concentrations of MSEO and MVEO on the yeasts counts were evaluated in Sabouraud dextrose broth and in cashew, guava, mango, and pineapple juice during 72 h of refrigerated storage (4 ± 0.5°C) using a viable cell count method (de Souza et al., 2007). Initially, 2 mL-aliquots of Sabouraud dextrose broth or fruit juice samples containing MSEO or MVEO in an amount enough to provide the required final concentrations of 3.75, 1.875, and 0.937 μL/mL or 15, 7.5 and 3.75 μL/mL, respectively, were inoculated with 2 mL of the yeast suspension and vortexed for 30 s. The mixtures were maintained at 4 ± 0.5°C, and 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 mixture was serially diluted in sterilized saline solution (0.85 g/100 mL, w/v), and 10 μL-aliquots of each dilution were inoculated onto Sabouraud dextrose agar using a microdrop technique (Herigstad et al., 2001). Additionally, 100 μL-aliquot of each mixture was directly inoculated onto Sabouraud dextrose agar. Inoculated control juices not-containing MSEO or MVEO were assayed similarly. After an incubation period of 48–72 h at 30°C, the visible colonies were counted, and the results were expressed as log cfu/mL. Plates inoculated with aliquots collected from juice samples containing MSEO or MVEO were incubated for an additional 24 h at adequate temperature compared with the samples collected from control juice.

Reduction in yeast counts were calculated using the formula: $\log N_0 - \log N$; where $\log N_0$ and $\log N$ were the initial count and count after incubation, respectively, for indicated storage time interval. Results were expressed as log cycle reduction. The detection limit of the test was 1 log cfu/mL.

Analysis of Physicochemical Parameters of Fruit Juices

Total soluble solids, pH and titratable acidity values were determined in cashew, guava, mango, and pineapple juice with and without MSEO (1.875 μL/mL) on time zero (baseline, just after the essential oil incorporation and homogenization) and after 72 h of refrigerated storage (4 ± 0.5°C). These parameters were selected because they comprise the current Brazilian physicochemical standards to unsweetened fruit juices (Anonymous, 2003). Soluble solids content (°Brix) was

analyzed using a digital refractometer (model HI 96801, Hanna Instruments, São Paulo, Brazil) (Association of Official Analytical Chemists International [AOAC], 2016). pH values were determined using a digital potentiometer (model Q400AS, Quimis, São Paulo, Brazil) (Association of Official Analytical Chemists International [AOAC], 2016). Titratable acidity was determined employing phenolphthalein as indicator with NaOH to the 0.1 N, and the results were expressed in grams per 100 mL of citric acid equivalents (Association of Official Analytical Chemists International [AOAC], 2016).

Sensory Analysis of Fruit Juices

Sensory analyses were performed after the approval from an Ethics Research Committee (Federal University of Paraía – Brazil) under a protocol number 1.125.993/2015. Sensory evaluation was performed using check-all-that-apply (CATA) questions for cashew, guava, mango, and pineapple juices with 1.875 μL/mL of MSEO. Juices were produced in the same day of the sensory tests and maintained under refrigeration storage (4 ± 0.5°C). Firstly, most mentioned appropriate terms for each attribute (color, appearance, flavor, odor, and texture) of the all fruit juices were established by an untrained group to compose the CATA questions (**Table 1**). One hundred untrained panelists (17–50 years old), for each juice, were recruited and selected considering their habits of consuming fruit juices two or more times per week. Sensory analyses were performed in four testing sessions, one for each juice, conducted in individual cabins with white light. Panelists evaluated the juice samples, immediately after removal from refrigerated storage, served in 30-mL aliquots in white disposable cups in individual booths with controlled temperature and lighting. Panelists were asked to check all the terms of CATA questions (as previously defined) appropriate to describe each attribute (Ares et al., 2010).

Statistical Analysis

The assays were performed in three 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. Results of MIC determination assays are expressed as modal values because the MIC values were the same in all repetitions. For the yeast count assays and physicochemical parameters, the statistical analyses were performed to determine significant differences ($p \leq 0.05$) based on Student's *t*-test or ANOVA followed by *post hoc* Tukey test. The computational software Sigma Stat 3.5 software (Jandel Scientific Software, San Jose, CA, United States) was used for these statistical analyses.

Data from CATA questions were analyzed by determining the frequency of use of each term for describing each attribute of the fruit juices. Significant differences ($p \leq 0.05$) among samples were evaluated using Cochran's Q Test (Manoukian, 1986). MedCalc Statistical Software version 18 (MedCalc Software bvba, Ostend, Belgium) was used for this analysis.

TABLE 1 | Terms surveyed for check-all-that-apply (CATA) questions of each sensory attribute of the fruit juices evaluated.

Appearance	Flavor	Odor
Characteristic color of the juice	Sweet	Characteristic odor of fruit
Uncharacteristic color of the juice	Not sweet	Uncharacteristic odor of fruit
	Strange flavor	Mint odor
	Refreshing sensation	
	Fruit flavor	
	Mint flavor	
	Bitter flavor	
	Pleasant flavor	
	Unpleasant flavor	

RESULTS AND DISCUSSION

Identification of MSEO and MVEO Constituents

Constituents identified in MSEO and MVEO are shown in **Table 2**. Majority constituents in MSEO were carvone (72.69%) and limonene (14.25%), other constituents were identified in lower amounts, e.g., menthol (2.29%) and menthone (1.07%). Piperitenone oxide (62.39%), eucalyptol (4.46%), and limonene (4.40%) were the majority constituents in MVEO, followed by other constituents in lower amounts, such as isopentyloxyethyl acetate (2.19%), β-pinene (2.14%), myrcene (2.01%), α-pinene (1.52%), sabinene (1.41%), β-caryophyllene (1.34%), 6-methyl-5-hepten-2-ol (1.31%), piperitone oxide (1.25%), germacrene D (1.23%) and *p*-cymene (1.04%). A wide variety of other constituents were identified in amounts < 1% in both MSEO and MVEO.

Existence of different chemotypes based on qualitative and quantitative differences within essential oils from the same plant species is a common characteristic in *Mentha* genus (Edris et al., 2003). Nine different chemotypes has been already reported to *Mentha* species (Kokkini and Vokou, 1989; Mimica-Dukiæ, 2008). Considering the detected majority constituents, MSEO and MVEO evaluated in this study could be classified as belonging to the chemotypes carvone-dihydrocarvone and piperitenone oxide, respectively.

Anti-yeast Effects of MSEO and MVEO

MSEO and MVEO displayed MIC values of 1.875 and 7.5 μL/mL, respectively, against *C. albicans*, *C. tropicalis*, *P. anomala*, and *S. cerevisiae*. No previous studies evaluating the inhibitory effects of MSEO against *C. tropicalis* and *P. anomala* were found in literature, and the available studies reported MIC of 0.78 μL/mL against *S. cerevisiae* (Liu et al., 2012) and MIC varying from 20 to 40 μL/mL against *C. albicans* (Martins et al., 2012; Bona et al., 2016). Regarding the MVEO, most of the available studies has focused on its antiparasitic activities (de Sousa Guedes et al., 2016; Matos-Rocha et al., 2016). Only a previous study reported the efficacy of neat MVEO to inhibit *C. albicans* with the measure of growth inhibition zones using an agar diffusion assay (Arruda et al., 2006).

TABLE 2 | Constituents identified in the essential oil from *Mentha spicata* L. (MSEO) and *M.* × *villosa* Huds. (MVEO) in amounts > 1%.

MSEO				MVEO			
Retention time	**Kovats index**	**%**	**Constituent**	**Retention time**	**Kovats index**	**%**	**Constituent**
8.824	1027	14.25	Limonene	5.893	932	1.52	α-Pinene
13.805	1152	1.07	Menthone	7.002	972	1.41	Sabinene
14.638	1171	2.29	Menthol	7.129	976	2.14	β-Pinene
18.019	1248	72.69	Carvone	7.478	988	2.01	Myrcene
				7.605	993	1.31	6-Methyl-5-hepten-2-ol
				8.661	1023	1.04	*p*-Cymene
				8.814	1027	4.40	Limonene
				8.914	1029	4.46	Eucalyptol
				14.857	1177	2.49	Isopentyloxyethyl acetate
				18.295	1245	1.25	Piperitone oxide
				23.468	1371	62.39	Piperitenone-oxide
				25.564	1420	1.34	β-Caryophyllene
				28.183	1482	1.23	D-Germacrene

Inhibitory effects of MSEO and MVEO toward the test yeast strains could be related to the already reported antifungal properties of their majority compounds. Antifungal effects of carvone involve primarily two different action modes, to cite: (i) partition into the cell membrane, causing inhibition of plasma membrane H+-ATPase and intracellular acidification; and (ii) inhibition of ergosterol biosynthesis pathway, reducing its amounts in cell membranes with disturbance of membrane fluidity and cell integrity (Samber et al., 2015). Piperitone oxide, as an oxygenated monoterpene, may induce disorganization of cell membrane structures, resulting in depolarization and physical or chemical alterations, thereby disrupting fungal metabolic activities (Ait-Ouazzou et al., 2012; Guerra et al., 2015). In addition to the majority constituents, other compounds detected in lower amounts in MSEO and MVEO such as caryophyllene, eucalyptol, myrcene, and pulegone have demonstrated inhibitory effects against a variety of microorganisms (Zengin and Baysal, 2014; Dahham et al., 2015; Guerra et al., 2015).

Effects of 0.937, 1.875, and 3.75 μL/mL MSEO and 15, 7.5, and 3.75 μL/mL MVEO on the counts of *C. albicans*, *C. tropicalis*, *P. anomala*, and *S. cerevisiae* were studied in Sabouraud dextrose broth (Supplementary Figure S1) and in cashew (**Figure 1**), guava (**Figure 2**), mango (**Figure 3**), and pineapple (**Figure 4**) juices during 72 h of refrigerated storage. Incorporation of MSEO and MVEO at all assayed concentrations decreased ($p \leq 0.05$) the counts of either of the tested yeast strains in Sabouraud dextrose broth as well as in fruit juices over time. Tested yeast strains presented a linear growth in Sabouraud dextrose broth and in fruit juices without MSEO and MVEO over the measured storage time period, with counts varying from 7 to 8 log cfu/mL.

Incorporation of 3.75 and 1.875 μL/mL MSEO in Sabouraud dextrose broth caused reductions in counts of *C. albicans*≥5-log and of 3.85-log cycles, respectively, after 72 h of exposure; the incorporation of 1.875 μL/mL MSEO in Sabouraud dextrose broth caused a ≥5-log reduction in counts of *P. anomala* after 72 h of exposure, while a higher concentration of 3.75 μL/mL was necessary to reduce ≥5-log in counts of *S. cerevisiae* after 24 h. In relation to MVEO, only the concentration of 15 μL/mL in Sabouraud dextrose broth reduced ≥5-log in counts of *C. albicans*, *P. anomala*, and *S. cerevisiae* after 72, 12, and 72 h of exposure, respectively (Supplementary Figures S1A–H).

In cashew juice, MSEO and MVEO were effective to cause ≥ 5-log reductions in counts of the tested yeast strains over the measured 72 h-storage period, with the exception of *C. tropicalis*. Incorporation of 3.75 and 1.875 μL/mL MSEO in cashew juice caused ≥5-log reductions in counts of *P. anomala* after 48 and 72 h of exposure, respectively (**Figure 1C**); the same reduction was observed in the counts of *S. cerevisiae* after an exposure time interval of 1 and 72 h, respectively (**Figure 1D**). Incorporation of 3.75 μL/mL MSEO or 15 μL/mL MVEO caused ≥5-log reductions in counts of *C. albicans* after 72 h of exposure (**Figures 1A,E**). Cashew juice containing 15 μL/mL MVEO presented ≥5-log reductions in counts of *P. anomala* and *S. cerevisiae* after 72 and 24 h of exposure, respectively (**Figures 1G,H**).

In guava juice, MSEO and MVEO were effective to cause ≥5-log reductions in counts of *C. albicans*, *P. anomala*, and *S. cerevisiae* over the monitored 72 h-storage period. Incorporation of 3.75 and 1.875 μL/mL MSEO in guava juice caused ≥5-log reduction in counts of *P. anomala* in an exposure time interval of 4 h (**Figure 2C**); 3.75 and 1.875 μL/mL MSEO caused ≥5-log reductions in counts of *S. cerevisiae* after 45 min and 8 h of exposure, respectively (**Figure 2D**); incorporation of 3.75 μL/mL MSEO or 15 μL/mL MVEO showed ≥5-log reductions in counts of *C. albicans* after 72 and 48 h of exposure, respectively (**Figures 2A,E**). Incorporation of 15 μL/mL MVEO in guava juice induced ≥5-log reductions in counts of *P. anomala* after 72 h of exposure (**Figure 2G**); this same reduction level was caused by 15 and 7.5 μL/mL MVEO against *S. cerevisiae* after 12 and 48 h of exposure, respectively (**Figure 2H**).

Incorporation of MSEO and MVEO at all tested concentrations was not effective to cause ≥5-log reductions

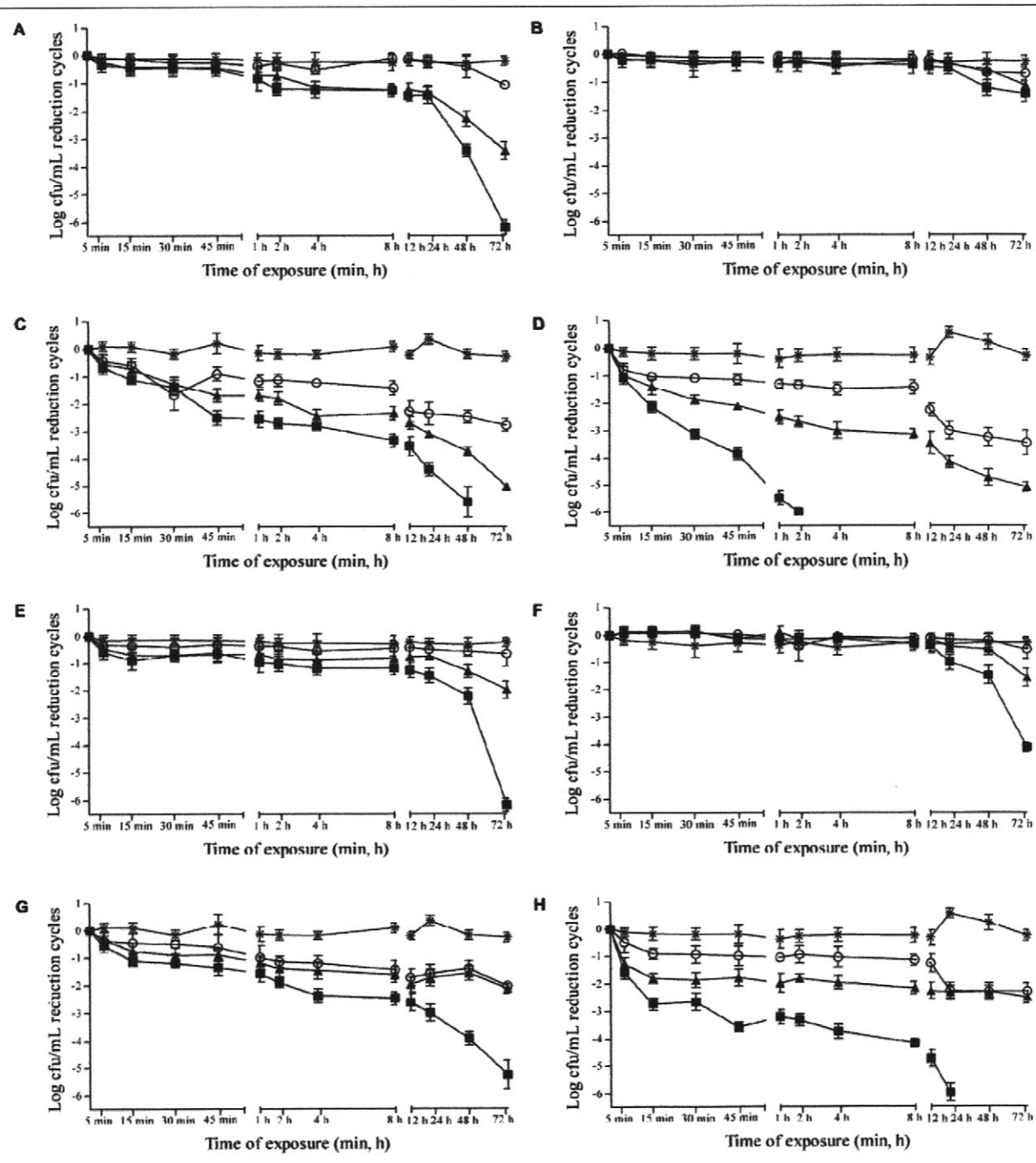

FIGURE 1 | Reduction cycles (log cfu/mL) of the counts of *C. albicans* ATCC 90028 **(A,E)**, *C. tropicalis* ATCC 28707 **(B,F)**, *P. anomala* ATCC 40101 **(C,G)** and *S. corovisiae* ATCC 2601 **(D,H)** in cashew juice at 4 ⊥ 0.5°C as a function of the concentration of *M. spicata* L. essential oil **(A–D)** at (■): 3.75 μL/mL, (▲): 1.875 μL/mL, (○): 0.9375 μL/mL or *M. villosa* Huds. essential oil **(E–H)**. (■): 15.0 μL/mL, (▲): 7.5 μL/mL, (○): 3.75 μL/mL, (*) control: 0 μL/mL. Detection limit of the test: 1 log cfu/mL.

in counts of *C. albicans*, *C. tropicalis*, and *P. anomala* in mango juice over the measured 72 h-storage period. Highest reductions in counts of *C. albicans* (1.33-log and 1.13-log), *C. tropicalis* (1.06-log and 1.08-log), and *P. anomala* (3.79-log and 3.92-log) at the end of the measured storage time period were observed in mango juice containing 3.75 μL/mL MSEO (**Figures 3A–C**) and 15 μL/mL MVEO (**Figures 3E–G**). Incorporation of 3.75 μL/mL MSEO or 15 μL/mL MVEO in mango juice caused

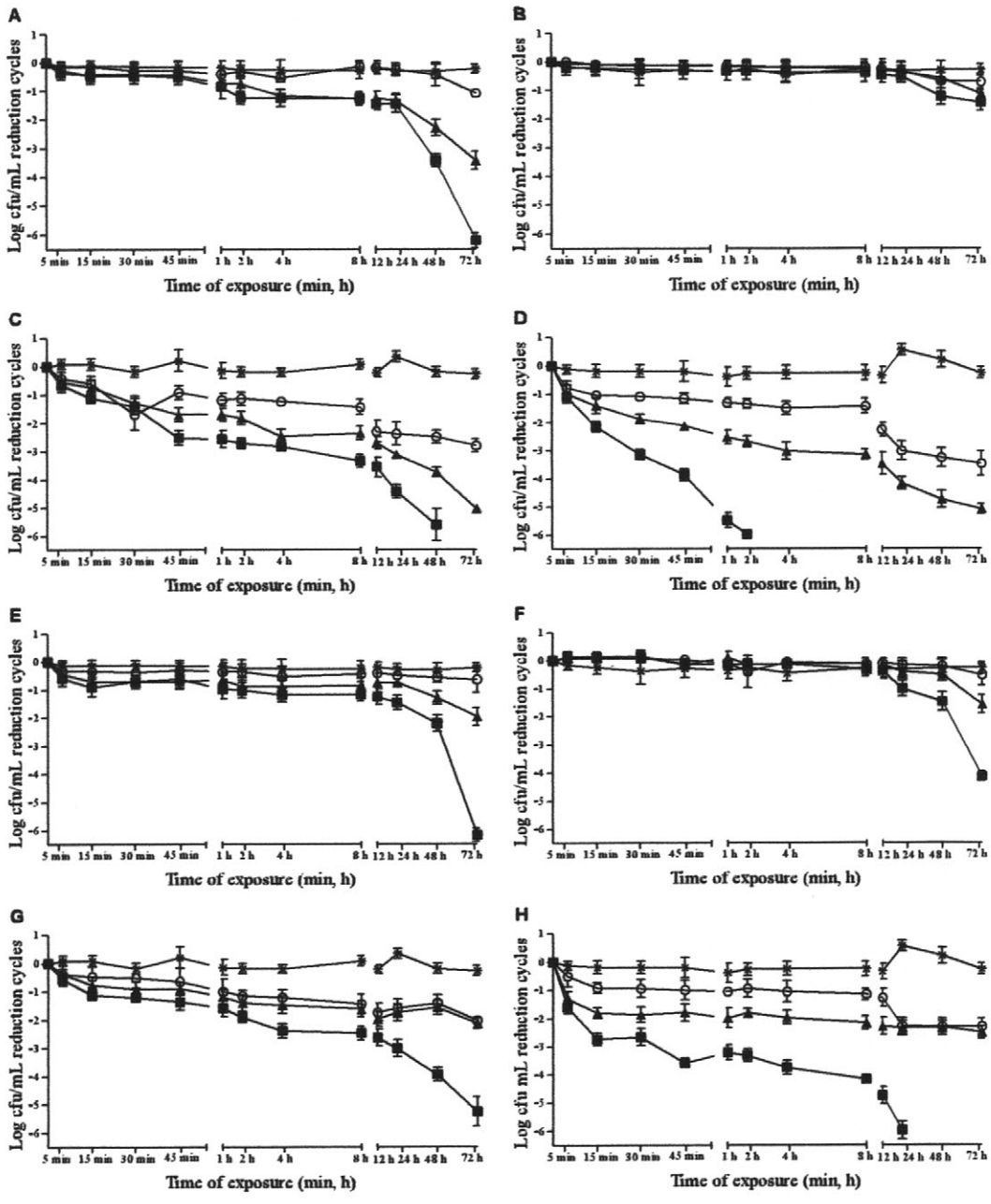

FIGURE 2 | Reduction cycles (log cfu/mL; $n = 6$) of the counts of *C. albicans* ATCC 90028 **(A,E)**, *C. tropicalis* ATCC 28707 **(B,F)**, *P. anomala* ATCC 40101 **(C,G)** and *S. cerevisiae* ATCC 2601 **(D,H)** in guava juice at 4 ± 0.5°C as a function of the concentration of *M. spicata* L. essential oil **(A–D)** at (■): 3.75 μL/mL, (▲): 1.875 μL/mL, (○): 0.9375 μL/mL or *M. villosa* Huds. essential oil **(E–H)**. (■): 15.0 μL/mL, (▲): 7.5 μL/mL, (○): 3.75 μL/mL, (*) control: 0 μL/mL. Detection limit of the test: 1 log cfu/mL.

≥5-log reductions in counts of *S. cerevisiae* after 8 h of exposure (**Figures 3D,H**).

In pineapple juice with 3.75 μL/mL MSEO, ≥5-log reductions in counts of *P. anomala* and *S. cerevisiae* were observed after 48 h of exposure (**Figures 4C,D**). Only in pineapple juice containing 15 μL/mL MVEO a ≥5-log reduction in counts of *S. cerevisiae* was observed after a 4 h-exposure (**Figure 4H**). *C. albicans* and *C. tropicalis* showed count reductions of 1.63-log and 2.45-log cycles, respectively, in pineapple juice with 15 μL/mL MVEO after 72 h of exposure (**Figures 4E,F**).

Considering the data obtained in time-kill studies, the general ranking of sensitivity to MSEO and MVEO was

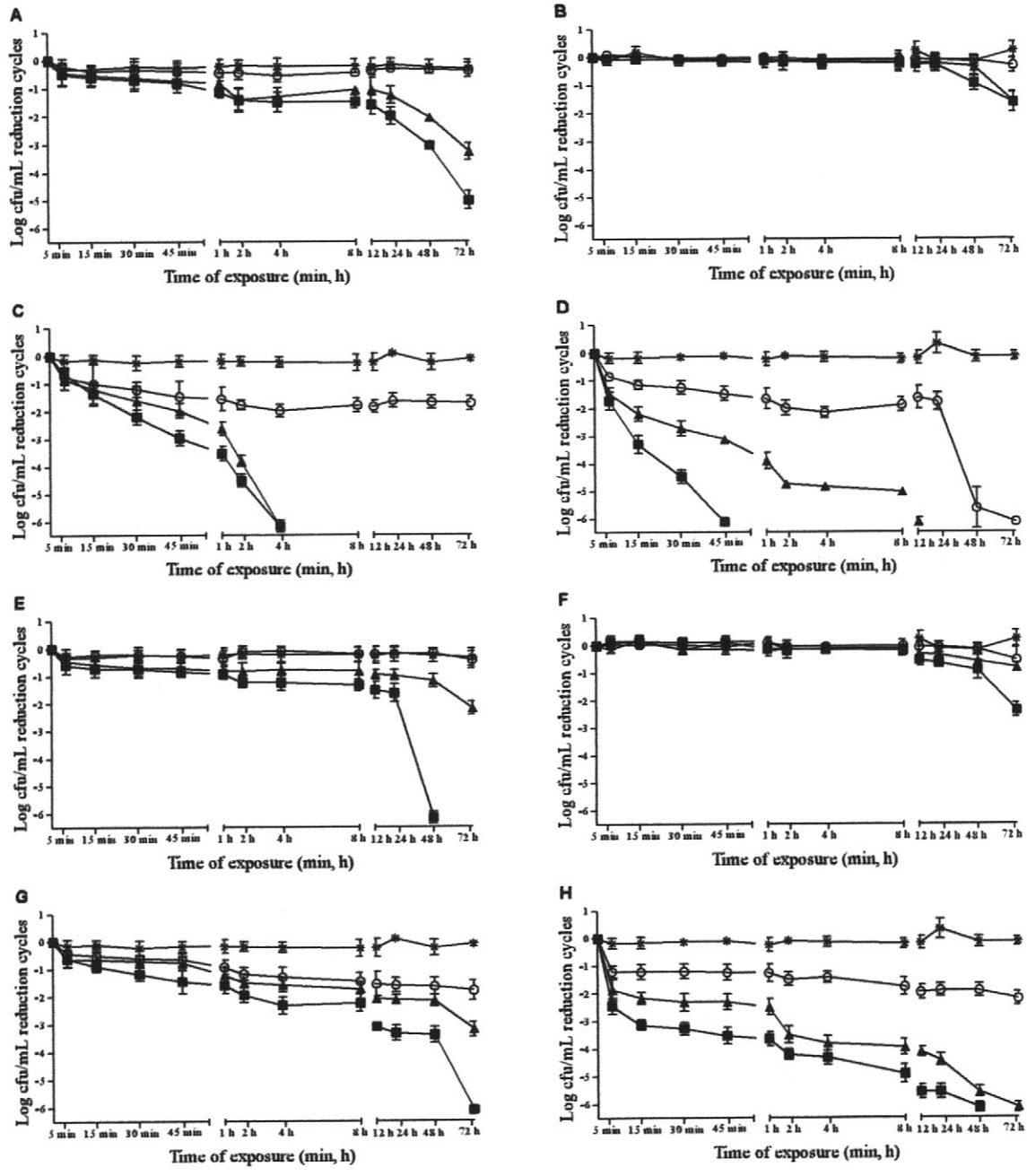

FIGURE 3 | Reduction cycles (log cfu/mL; $n = 6$) of the counts of *C. albicans* ATCC 90028 **(A,E)**, *C. tropicalis* ATCC 28707 **(B,F)**, *P. anomala* ATCC 40101 **(C,G)** and *S. cerevisiae* ATCC 2601 **(D,H)** in mango juice at 4 ± 0.5°C as a function of the concentration of *M. spicata* L. essential oil **(A–D)** at (■): 3.75 µL/mL, (▲): 1.875 µl /ml , (○): 0.9375 µl /ml or *M. villosa* Huds. essential oil **(E–H)**. (■): 15.0 µl /ml , (▲): 7.5 µl /ml , (○): 3.75 µl /ml , (*) control: 0 µl /ml . Detection limit of the test: 1 log cfu/mL.

S. cerevisiae > *P. anomala* > *C. albicans* > *C. tropicalis*; and the MSEO was more effective to cause ≥5-log reductions in yeasts counts than MVEO. Anti-yeast activity of MSEO and MVEO was dose-dependent, i.e., the reductions in yeasts counts were higher and occurred in a shorter exposure time when the concentrations of MSEO or MVEO increased. This dose-dependent effect was reported in other studies on the antimicrobial effects of *Mentha* essential oils (Tyagi et al., 2013; Guerra et al., 2015).

Results showing the ability of MSEO and MVEO to cause reductions in counts of tested yeasts in laboratorial media as

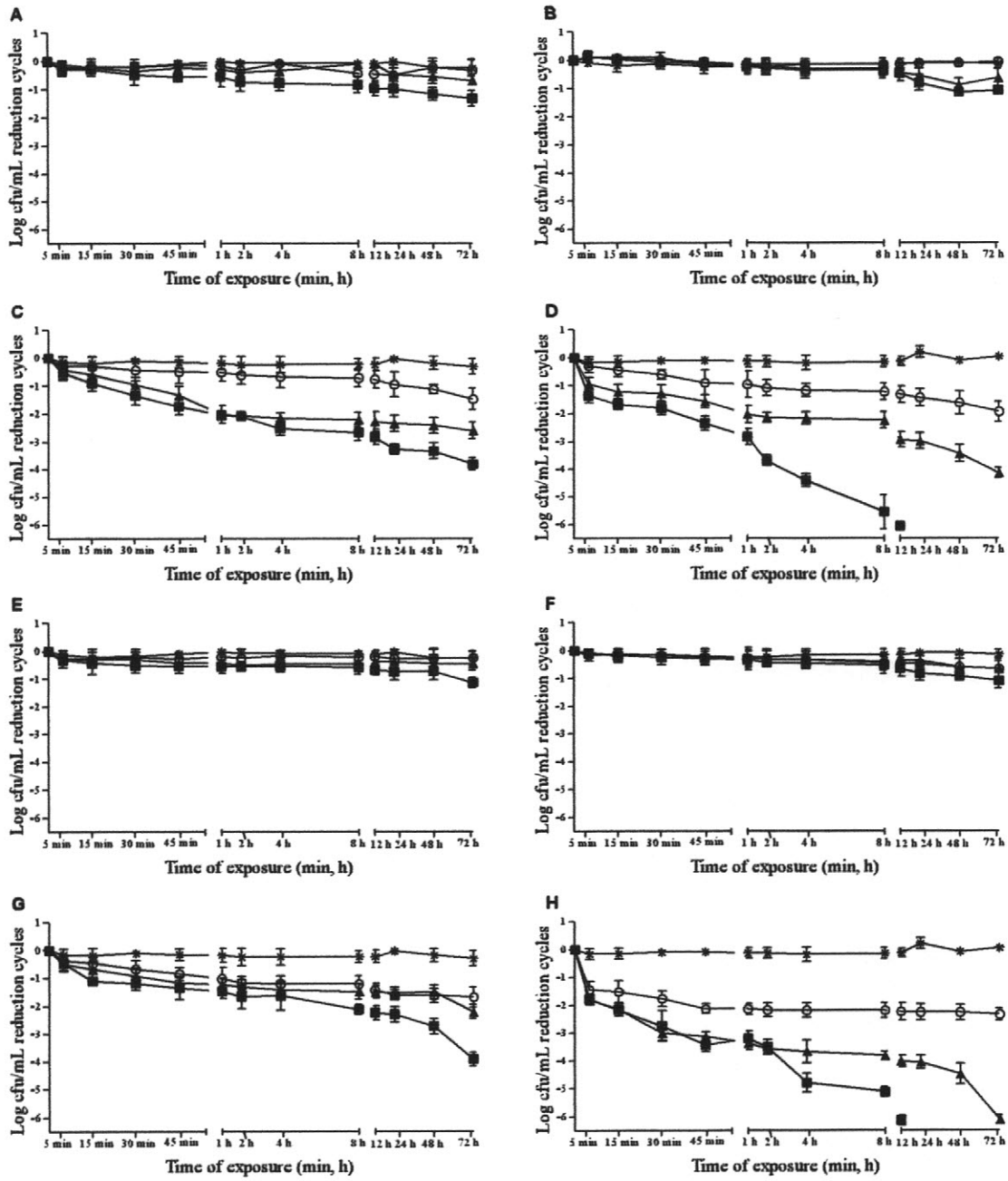

FIGURE 4 | Reduction cycles (log cfu/mL; $n = 6$) of the counts of *C. albicans* ATCC 90028 **(A,E)**, *C. tropicalis* ATCC 28707 **(B,F)**, *P. anomala* ATCC 40101 **(C,G)** and *S. cerevisiae* ATCC 2601 **(D,H)** in pineapple juice at 4 ⊥ 0.5°C as a function of the concentration of *M. spicata* L. essential oil **(A–D)** at (■): 3.75 μL/mL, (▲): 1.875 μL/mL, (○): 0.9375 μL/mL or *M. villosa* Huds. essential oil **(E–H)**. (■): 15.0 μL/mL, (▲): 7.5 μL/mL, (○): 3.75 μL/mL, (*) control: 0 μL/mL. Detection limit of the test: 1 log cfu/mL.

well as in fruit juices are noteworthy because some previous studies have found that, in most cases, the antimicrobial efficacy of essential oils decreases when these substances are incorporated in food models (Gutierrez et al., 2009). Generally, MSEO and MVEO caused the lowest decreases in yeasts counts in mango juice that presents higher total soluble solids content than guava, pineapple, and cashew juices (de Sousa Guedes et al., 2016), reinforcing that carbohydrates may reduce the antimicrobial effects of essential oils (Gutierrez et al., 2009). Highest reductions in yeast counts caused by MSEO in Sabouraud dextrose broth

as well as in fruit juices when compared with MVEO may be associated with the majority constituents found in each essential oil. Carvone (the majority constituent found in MSEO) typically presents stronger antifungal effects than piperitone oxide (the majority constituent found in MVEO). Higher solubility of carvone may also result in better capacity of the former to migrate across the characteristic aqueous medium of fruit juices and, consequently, to interact with yeast target cells to cause damage in cell structure and function (Trombetta et al., 2005; Soković et al., 2009).

There are no previous reports on the anti-yeast effects of MSEO or MVEO in fruit juices; however, previous studies with essential oils from other *Mentha* species have been reported. The detection of 2-log reductions in counts of *S. cerevisiae* in a mixed fruit juice (orange and apple) containing 1.13 mg/mL *M. piperita* essential oil was observed after 8 days of room temperature storage (Tyagi et al., 2013). Another study detected that the incorporation of 1 μL/mL *M. piperita* EO in apple juice caused ≥5-log reductions in counts of *Zygosaccharomyces rouxii* and *Z. bailii* after 15 days of refrigerated storage (Karaman et al., 2016).

The essential oil concentrations and the exposure time needed to achieve ≥5-log reductions in yeasts counts in cashew, guava, mango, or pineapple juices containing MSEO or MVEO observed in the present study were more than those previously observed for *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis in the same fruit juices containing *M. piperita* or *M. arvensis* essential oil (5, 2.5, and 1.25 μL/mL) (de Sousa Guedes et al., 2016). The needed essential oil concentration and the shorter time to achieve ≥5-log reductions in bacterial counts in juices containing *Mentha* essential oils could be associated with the higher sensitivity of bacterial cells to the acidic pH of fruit juices than yeast cells. Acidic pH in fruit juice may injury cells of low-pH sensitive microorganisms resulting in increased sensitivity to the action of essential oils constituents (Gutierrez et al., 2009; Tserennadmid et al., 2011).

Considering the results of assays that measured the anti-yeast effects of the tested essential oils, which demonstrated that MSEO even in lower doses than MVEO caused the highest reductions in yeasts counts in laboratorial media and in fruit juices, as well as the fact that high doses of *Mentha* essential oils typically impact negatively on the sensory attributes of fruit juices (de Sousa Guedes et al., 2016), only the MSEO in a effective anti-yeast concentration of 1.875 μL/mL was selected for use in physicochemical and sensory analyses of juices.

Physicochemical and Sensory Characteristics of Fruit Juices

Considering that during the storage time fruit juice may present alterations in their physicochemical parameters impacting negatively on their quality aspects and market value, this study evaluated changes in selected physicochemical characteristics, namely °Brix, pH and titratable acidity, in cashew, guava, mango and pineapple juices with and without MSEO (1.875 μL/mL). These physicochemical characteristics were evaluated immediately after the essential oil addition and after 72 h of refrigerated storage (Supplementary Table S1). Overall, no difference ($p > 0.05$) in values of physicochemical parameters of juices with or without MSEO was observed, as well as at time zero and after 72 h of refrigerated storage. Juice samples with and without MSEO attended the criteria determined by the current Brazilian standard for unsweetened cashew, guava, mango, and pineapple juices, which determines titratable acidity (grams of citric acid per 100 g) values ≥0.15, ≥0.30, ≥0.30, and ≥0.16 and °Brix ≥ 5.0, ≥6.0, ≥10, and ≥6.0, respectively (Anonymous, 2003). These results are important because the physicochemical stability confirms that the studied fruit juices containing a effective anti-yeast dose of MSEO remain similar to those newly manufactured even after storage.

Results of the sensory analysis of cashew, guava, mango, and pineapple juice with 1.875 μL/mL MSEO are presented in **Table 3**. Considering the different sensory attribute terms established by panelists to describe the studied fruit juices in CATA test, some positive characteristics were observed in fruit juices with MSEO, such as the characteristic juice color and pleasant taste. Perception of the panelists indicated that most of the fruit juices tested presented high percent scores for non-characteristic fruit aroma, mint odor and taste and refreshing sensation. These sensory characteristics in juices could be associated with the presence of carvone, the majority constituent identified in the tested MSEO, which may impose these specific sensory characteristics in juices (Jakli and Saupe, 2006).

Overall, the results of CATA test showed that the incorporation of 1.875 μL/mL MSEO in cashew, guava, mango,

TABLE 3 | Attribute terms percentage of the check-all-that-apply (CATA) questions of cashew, guava, mango, and pineapple juices with *Mentha spicata* L. essential oil (1.875 μL/mL).

Attribute terms	Juices			
	Cashew (%)	Guava (%)	Mango (%)	Pineapple (%)
Characteristic color of the fruit juice	99^{ab}	100^{a}	98^{ab}	96^{b}
Uncharacteristic color of the fruit juice	1^{ac}	0^{b}	3^{ab}	5^{c}
Characteristic odor of the fruit juice	36^{a}	19^{b}	47^{a}	14^{b}
Uncharacteristic odor of the fruit juice	45^{b}	57^{c}	39^{b}	64^{a}
Mint odor	85^{b}	88^{ab}	73^{c}	96^{a}
Sweet	60^{a}	34^{b}	54^{a}	42^{b}
Not sweet	27^{b}	55^{c}	32^{bc}	42^{c}
Strange flavor	20^{b}	35^{c}	21^{b}	25^{b}
Refreshing sensation	89^{a}	86^{a}	60^{b}	84^{a}
Fruit flavor	42^{a}	29^{b}	19^{c}	22^{bc}
Mint flavor	88^{a}	87^{a}	69^{b}	88^{a}
Bitter flavor	13^{bc}	27^{a}	18^{b}	7^{c}
Pleasant flavor	67^{a}	37^{c}	52^{b}	54^{b}
Unpleasant flavor	9^{b}	19^{a}	16^{a}	19^{a}

$^{a-c}$Different superscript letters within a row are significantly different ($p \leq 0.05$), based on Cochran's Q test.

and pineapple juice did not affect negatively their sensory characteristics enough to imply on potential rejection by consumers. These results are noteworthy because a previous study observed that the incorporation of an effective antibacterial dose (1.25 μL) of *M. piperita* essential oil in cashew, mango, pineapple, and guava juice affected negatively the taste, aftertaste and overall acceptance of juices, although no negative effects have been observed in appearance, flavor and odor (de Sousa Guedes et al., 2016).

CONCLUSION

Results of this study showed that MSEO (3.75, 1.875, and 0.937 μL/mL) or MVEO (15.0, 7.5, and 3.75 μL/mL) are capable to inactivate *C. albicans*, *C. tropicalis*, *P. anomala*, and *S. cerevisiae* in laboratorial media and in cashew, guava, mango and pineapple juice over a 72 h-refrigerated storage. Overall, MSEO showed stronger anti-yeast effects than MVEO; *S. cerevisiae* and *C. tropicalis* were the most and less sensitive yeasts, respectively, to both studied essential oils; and the highest yeast count reductions were verified in cashew and guava juice. An effective anti-yeast dose of MSEO (1.875 μL/mL) did not affect the physicochemical parameters comprising the identity and quality characteristics for unsweetened juices, as well as did not induce negative alterations to cause possible sensory rejection of tested fruit juices. These results indicate MSEO and MVEO, primarily MSEO, as potential antimicrobials to comprise preservation strategies used to control spoilage yeasts in fruit juices.

AUTHOR CONTRIBUTIONS

ES, EC, and MM: conceived and designed the experiments and drafted the paper. EC, IM, ES, MM, JB-F, and JT: performed the experiments. EC, IM, ES, MM, and JT: analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01111/full#supplementary-material

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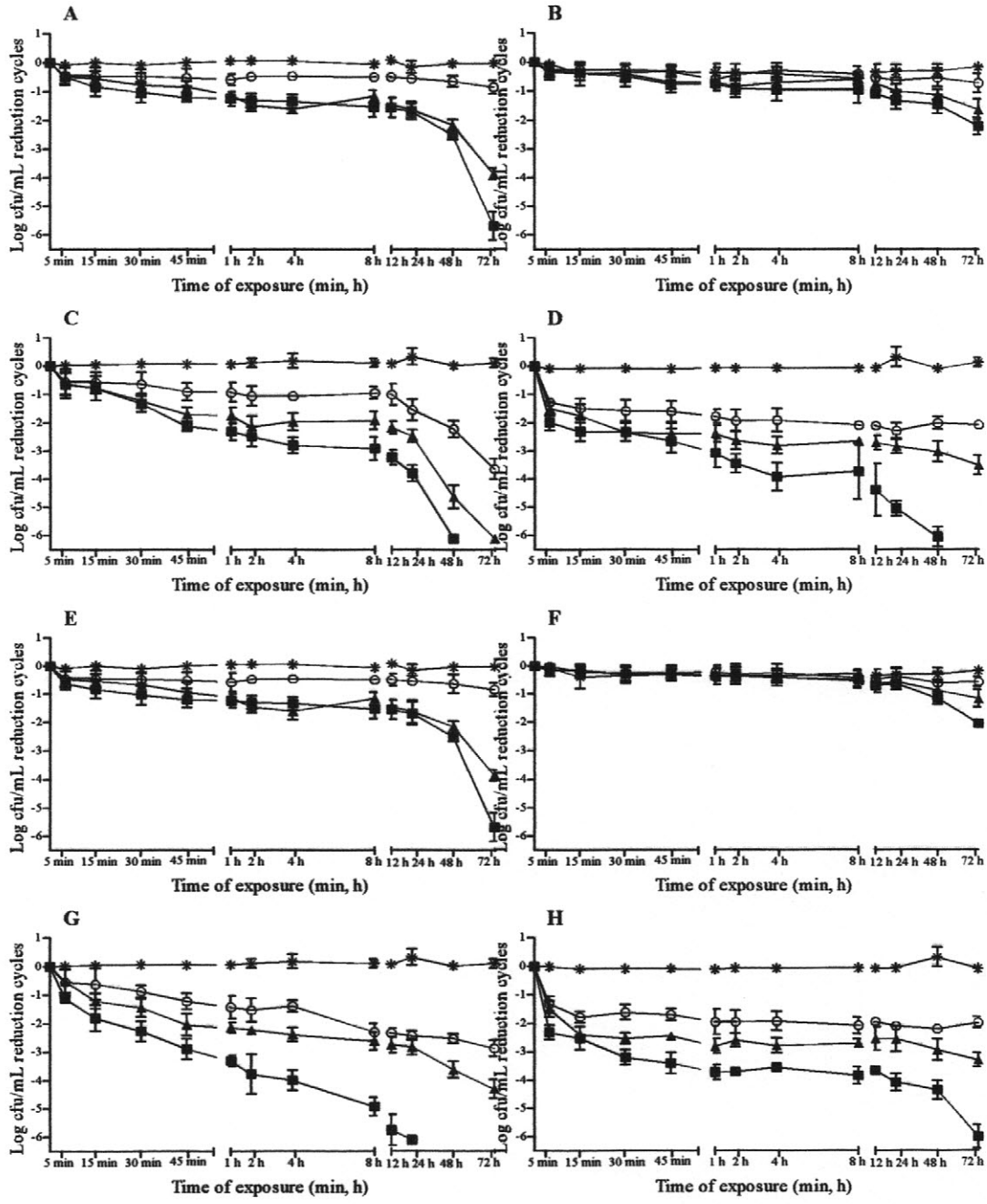
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.



Supplementary Figure S1. Reduction cycles (log cfu/mL) of the 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) in sabouraud dextrose broth at 4 ± 0.5 °C as a function of the concentration of *M. spicata* L. essential oil (A – D) at (■): 3.75 µL/mL, (▲): 1.875 µL/mL, (○): 0.9375 µL/mL or *M. villosa* Huds. essential oil (E – H). (■): 15.0 µL/mL, (▲): 7.5 µL/mL, (○): 3.75 µL/mL, (*) control: 0 µL/mL. Detection limit of the test: 1 log cfu/mL.

Supplementary Table S1. Physicochemical parameters (average ± standard deviation; n = 6) of cashew, guava, mango and pineapple juices with and without 1.875 µL/mL of *Mentha spicata* L. essential oil (MSEO) immediately after the essential oil incorporation (time zero) and after 72 h of refrigerated storage (4 ± 0.5 °C).

Juices	Treatments	Physicochemical parameters (storage time interval)					
		Total soluble solids (°Brix)		pH		Titratable acidity (g/100 g citric acid)	
		Time zero	72 h	Time zero	72 h	Time zero	72 h
Cashew	MSEO (1.875 µL/mL)	5.70 (±0.30)^{Aa}	5.80 (±0.20)^{Aa}	4.86 (±0.15)^{Aa}	4.89 (±0.23)^{Aa}	0.17 (±0.08)^{Aa}	0.18 (±0.02)^{Aa}
	Control (0 µL/mL)	5.60 (±0.20)^{Aa}	5.70 (±0.30)^{Aa}	4.88 (±0.32)^{Aa}	4.84 (±0.20)^{Aa}	0.17 (±0.03)^{Aa}	0.19 (±0.02)^{Aa}
Guava	MSEO (1.875 µL/mL)	6.10 (±0.30)^{Aa}	6.10 (±0.20)^{Aa}	4.08 (±0.35)^{Aa}	4.12 (±0.25)^{Aa}	0.34 (±0.06)^{Aa}	0.33 (±0.03)^{Aa}
	Control (0 µL/mL)	6.20 (±0.30)^{Aa}	6.30 (±0.20)^{Aa}	4.14 (±0.45)^{Aa}	4.12 (±0.21)^{Aa}	0.35 (±0.08)^{Aa}	0.34 (±0.02)^{Aa}
Mango	MSEO (1.875 µL/mL)	10.20 (±0.10)^{Aa}	10.30 (±0.10)^{Aa}	44.74 (±0.25)^{Aa}	44.69 (±0.25)^{Aa}	0.32 (±0.06)^{Aa}	0.32 (±0.02)^{Aa}
	Control (0 µL/mL)	10.20 (±0.20)^{Aa}	10.30 (±0.20)^{Aa}	44.75 (±0.17)^{Aa}	44.71 (±0.24)^{Aa}	0.32 (±0.07)^{Aa}	0.34 (±0.02)^{Aa}
Pineapple	MSEO (1.875 µL/mL)	6.60 (±0.30)^{Aa}	6.60 (±0.20)^{Aa}	4.63 (±0.24)^{Aa}	4.58 (±0.20)^{Aa}	0.23 (±0.03)^{Aa}	0.22 (±0.02)^{Aa}
	Control (0 µL/mL)	6.70 (±0.20)^{Aa}	6.60 (±0.20)^{Aa}	4.65 (±0.14)^{Aa}	4.59 (±0.30)^{Aa}	0.24 (±0.02)^{Aa}	0.24 (±0.01)^{Aa}

Control (0 µL/mL): fruit juice without the incorporation of MSEO.
Similar superscript capital letters in the same row for the same fruit juice and essential oil concentration indicate no significant difference ($p > 0.05$), based on student t test.
Similar superscript small letters in the same column for the same fruit juice indicate no significant difference ($p > 0.05$), based on student t test.

4.2 ARTIGO DE RESULTADOS E DISCUSSÃO 2:

Incorporation of *Mentha piperita* L. essential oil in fruit juices causes inactivation and different physiological damages in spoilage yeasts

Running title: Anti-yeast effects of mint essential oil

Abstract

This study evaluated the efficacy of the essential oil from *Mentha piperita* L. (MPEO) to inactivate cells of the 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 caused by MPEO in *S. cerevisiae* in cashew and guava juices were investigated using flow cytometry analysis. The dyes propidium iodide, bis-1,3-dibutylbarbutiric acid, fluorescein diacetate and ethidium bromide were used to measure membrane integrity, membrane potential, enzymatic activity and efflux activity, respectively. 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 containing 7.5 and 3.75 µL/mL MPEO. Tested MPEO concentrations (i.e., 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 over time. 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. These results show the efficacy of MPEO to inactivate spoilage yeasts in fruit juices through a multi-target mechanism that causes alterations in different physiological functions in yeast cells.

Keywords: mint, essential oil, antimicrobial activity, flow cytometry, cell damage.

Introduction

The consumption of healthy, 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). Fruit juices framed in this trend are primarily spoiled by yeasts. *Candida*, *Pichia*, *Rhodotorula* and *Saccharomyces* are yeast genera commonly involved in fruit juices contamination and spoilage (Aneja et al., 2014), causing unpleasing 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 (EOs), which are generally recognized as safe (GRAS) and considered "green" antimicrobials for using in beverages (USFDA, 2015). EOs from *Mentha* species (Lamiaceae family) are widely recognized because 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 (Perricone et al., 2015). Early studies have shown inhibitory effects of MPEO against spoilage and pathogenic foodborne microorganisms (de Oliveira et al., 2017; de Sousa Guedes et al., 2017), but no previous study exploited the efficacy of MPEO to inhibit 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 that evaluated 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) and none have assessed the effects of EOs on spoilage yeasts in fruit juice.

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. Possible mechanisms underlying the anti-yeast effects of MPEO incorporated in cashew and guava juices were investigated through the measurement of damage in different physiological functions of *S. cerevisiae* cells using FC.

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 to 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 the 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

C. albicans (ATCC 90028), *C. tropicalis* (ATCC 28707), *P. anomala* (ATCC 40101) and *S. cerevisiae* (ATCC 2601), which were gently supplied by Collection of Reference

77 Microorganisms, National Institute of Quality Control in Health, Oswaldo Cruz Foundation
78 (Rio de Janeiro, Brazil), were used as test organisms. Stocks were kept in criovials containing
79 Sabouraud Dextrose broth (SDB; pH 5.6; Acumedia Manufacturers Inc., Michigan, USA)
80 with glycerol (15 g/100mL) stored at -20 °C. Working cultures, obtained by streak onto
81 Sabouraud Dextrose agar (SDA; pH 5.6, Acumedia Manufacturers Inc., Michigan, USA) were
82 maintained at 4 °C and transferred monthly to fresh SDA. Each yeast strain was cultured in
83 SDB and incubated at 30 °C for 48 h (to reach stationary growth phase), harvested by
84 centrifugation (4500 *g* x 15 min, 4 °C), washed twice and resuspended in sterile saline
85 solution (NaCl 0.85 g/100 mL) to obtain cell suspensions with OD reading at 625 nm (OD_{625})
86 of 0.75 for *C. albicans*, *C. tropicalis* and *P. anomala*, and of 0.95 for *S. cerevisiae*, which
87 corresponded to viable counts of approximately 7 log colony forming units per milliliter
88 (cfu/mL) when plated onto SDA.
89
90 *2.3 Preparation of fruit juices*
91 Cashew (*Anacardium occidentale* L.), guava (*Psidium guajava* L.), mango (*Mangifera*
92 *indica* L.) and pineapple (*Ananas comosus* L. Merrill) fruit in commercial maturation stage
93 with absence of mechanical damage and visible signs of infection were purchased from a
94 local wholesale distributor (João Pessoa, Brazil). Each fruit surface was disinfected by
95 immersion in a sodium hypochlorite solution (150 ppm, pH 7.2 adjusted using 1 M NaOH)
96 for 5 min, washed with sterile distilled water and dried for 30 min in a biosafety cabinet. Fruit
97 were aseptically peeled, chopped and mixed with sterile distilled water (50 g of fruit in 100
98 mL of sterile distilled water for guava juice and 60 g of fruit in 100 mL of sterile distilled
99 water for cashew, mango and pineapple; Anonymous, 2003) using a domestic blender (for 3
100 min). Fruit juices were double filtered using a triple-cheesecloth layer and sterilized using
101 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) and 50 µL-aliquots of this emulsion were dispensed into wells of a 96-well microplate (first line) containing 50 µL of double concentrate SDB and homogenized. Then, 50 µL-aliquots contained in the wells of the first line were transferred to the following wells (second line), and through geometric dilutions of the reason two, the MPEO concentration varied from 120 - 0.469 µL/mL. Subsequently, 50 µL-aliquots of the yeast suspensions were added to each well (approximately 7 log cfu/mL). Final MPEO concentration varied from 60 - 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 concentration of MPEO (µL/mL) required to prevent visible yeast growth.

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

Effects of different concentrations of MPEO on yeast counts were evaluated in fruit juices (cashew, guava, mango and pineapple) along 72 h of refrigerated storage (4 ± 0.5 °C) 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 yeast suspension (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

127 homogenization, 5, 15, 30, 45 min and 1, 2, 4, 8, 12, 24, 48 and 72 h), a 100 µL-aliquot of
128 each sample was collected and serially diluted (10^{-1} - 10^{-6}) in sterile saline solution (NaCl
129 0.85 g/100 mL, w/v) for inoculation onto SDA. Inoculated juices without MPEO were
130 assayed as negative controls. After incubation at 30 °C for 48 h, the visible colonies were
131 counted and the results were obtained as the log of colony forming units per mL (log cfu/mL).
132 Plates inoculated with aliquots collected from juice samples with MPEO were incubated for
133 an additional period of 24 h at 30 °C compared with the samples collected from control juices.
134 The results were expressed as log cycle reductions in yeast counts (log N_0 - N; where N_0 and
135 N were the initial count and count after incubation for indicated storage time interval,
136 respectively).
137
138 *2.6 Staining procedure*
139 Flow cytometry was used to monitor the physiological responses of *S. cerevisiae* cells
140 (approximately 7 log cfu/mL) in cashew and guava juices containing an effective anti-yeast
141 dose (i.e., 1.875 µL/mL) of MPEO following a 45 min-exposure time period. After exposure
142 to MPEO in cashew and guava juices, yeast cells were harvested by centrifugation (4500 *g* x
143 10 min, 4 °C), washed twice and resuspended in phosphate-buffered saline (PBS; 8.0 g/L
144 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
145 with the fluorochromes: propidium iodide (PI, Sigma-Aldrich, St. Louis, MO, USA) for
146 membrane integrity; bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$,
147 Molecular Probes, Invitrogen, OR, USA) for membrane potential; fluorescein diacetate (FDA;
148 ThermoFisher Scientific, Molecular Probes™, F1303) for enzymatic activity; and ethidium
149 bromide (EB; Sigma-Aldrich, St. Louis, MO, USA) for efflux activity. The same staining
150 procedures were performed with positive (ethanol-treated cells at 70% v/v) and negative

(inoculated juices without MPEO and PBS) controls (Carrillo et al., 2018; Kim et al., 2017; Silva et al., 2011).

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* x 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* x 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* x 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 populations 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 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. For the yeast count assays, the statistical analysis was performed to determine significant differences ($p \leq 0.05$) based on Student's t-test or ANOVA followed by post-hoc Tukey 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 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 – 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. 1 A-D), guava (Fig. 1 E-H), mango (Fig. 2 A-D) and pineapple (Fig. 2 E-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

226 tested yeasts in fruit juices over time. Tested yeasts presented a linear growth in fruit juices
227 without MPEO (control) over the measured storage time period, with counts varying from 7 –
228 8 log cfu/mL.

229 Cashew juice with 7.5 μL/mL MPEO presented > 5 log reductions in counts of *P.*
230 *anomala* after 72 h of storage (Fig. 1C); the same reduction against *S. cerevisiae* was achieved
231 in cashew juice with 7.5 and 3.75 μL/mL MPEO after 12 and 48 h of storage, respectively
232 (Fig. 1D). Cashew juice with 7.5 and 3.75 μL/mL presented > 5 log reductions in counts of *C.*
233 *albicans* after 72 h of storage (Fig. 1A). Lowest counts reductions caused by 1.875, 3.75 and
234 7.5 μL/mL MPEO in cashew juice were observed against *C. tropicalis*, which were in the
235 range of 1.25 – 1.97 log (Fig. 1B).

236 Guava juice with 7.5 μL/mL MPEO presented 2.44 log and 0.87 log reductions in
237 counts of *C. albicans* (Fig 1E) and *C. tropicalis* (Fig. 1F) after 72 h of storage, respectively.
238 Otherwise, 7.5, 3.75 and 1.875 μL/mL MPEO in guava juice caused > 5 log reductions in
239 counts of *P. anomala* after 24 h of storage (Fig. 1G); this same reduction level was achieved
240 by 7.5 and 3.75 μL/mL MPEO against *S. cerevisiae* in guava juice after 72 h of storage (Fig.
241 1H).

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

250 Data of time-kill studies in fruit juices showed the efficacy of MPEO to inactivate
251 yeast cells over time, although the inactivation rates varied with EO concentration and target
252 yeast strain. The following general ranking of sensitivity to MPEO was observed: *S.*
253 *cerevisiae/P. anomala* > *C. albicans* > *C. tropicalis*. There are few reports on the anti-yeast
254 effects of *Mentha* EOs in fruit juices. Previous studies detected 2-log reductions in counts of
255 *S. cerevisiae* in a mixed fruit juice (orange and apple) containing 1.13 mg/mL MPEO after
256 eight days of storage at room temperature (Tyagi et al., 2013). Another study observed that 1
257 μL/mL MPEO in apple juice caused ≥5-log reductions in counts of *Zigosaccharomyces rouxii*
258 and *Z. bailii* after 15 days of refrigerated storage (Karaman et al., 2016).
259
260 *3.4 Flow cytometry study*
261 In order to investigate possible mechanisms underlying the inactivation of yeast cells
262 caused by MPEO in fruit juices, four fluorescent probes (PI, $DIBAC_4(3)$, FDA and EB) were
263 used to measure populations of *S. cerevisiae* cells with altered physiological functions after a
264 45-min exposure to 1.875 μL/mL MPEO in cashew and guava juices. These juices were
265 selected because overall the highest yeast inactivation rates were observed when MPEO was
266 incorporated into them. *S. cerevisiae* was used as target organism because this yeast species
267 has been used as a model organism to study the anti-yeast action modes of different
268 antimicrobial compounds and procedures (Ferrario and Guerrero, 2017; Ling et al., 2013;
269 Schenk et al., 2011).
270 Membrane potential is generated due to differences in electrical state of internal and
271 external sides of cell membrane (Comas-Riu and Rius, 2009). $DiBAC_4(3)$ is an anionic
272 molecule and fluorescent dye capable of indicating membrane potential alterations. Anionic
273 molecules are typically excluded by polarized cells, while they are accumulated by
274 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. Cells treated with 70% (v/v/) ethanol (positive control) showed ruptured and depolarized membranes (100%; Fig. 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 (Fig. 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 (92.6%).

Although the membrane potential strongly contributes to homeostasis because the balance between the amounts of ions inside and outside cell, reduced membrane potential alone indicate 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 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 (Shenk et al., 2011). 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 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.

Membrane efflux pumps are integral membrane proteins that exert important role in yeast tolerance to antimicrobials. Efflux pump activity causes 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., 2009). 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 (Fig. 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 counts reduction, 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 trough the partition into cell membranes, causing inhibition of plasma membrane H+-ATPase and intracellular acidification, as well as through 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

325 implicated with the altered physiological functions observed in *S. cerevisiae* cells in cashew
326 and guava juice with MPEO. However, other components detected in lower amounts in tested
327 MPEO (e.g., isomenthone, eucalyptol and limonene) could also potentiate these effects in
328 yeasts cells (de Sousa Guedes et al., 2017)
329
330 **4. Conclusion**
331 The results of this study showed that 7.5, 3.75 and 1.875 μL/mL MPEO were effective to
332 inactivate *C. albicans*, *C. tropicalis*, *P. anomala* and *S. cerevisiae* in cashew, guava, mango
333 and pineapple juices over a 72 h-refrigerated storage, although variations in inactivation rates
334 were evident. FC analysis showed that an effective anti-yeast dose of MPEO (i.e., 1.875
335 μL/mL) in cashew and guava juices sharply compromised membrane permeability, membrane
336 potential, enzymatic activity and efflux pump in *S. cerevisiae* cells. These results show that
337 MPEO exerts inhibitory effects against spoilage yeasts in fruit juices through a multi-target
338 mechanism that affects simultaneously different physiological functions in yeast cells.
339
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347
348
349

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447

448 **Fig. 1.** Reduction cycles (log cfu/mL) of the initial counts of *C. albicans* ATCC 90028 (A, E),
449 *C. tropicalis* ATCC 28707 (B, F), *P. anomala* ATCC 40101 (C, G) and *S. cerevisiae* ATCC
450 2601 (D, H) as a function of the concentration of *M. piperita* L. essential oil in cashew juice
451 (A – D) and guava juice (E – H) stored at 4 ± 0.5 °C. (■): 7.5 μL/mL, (▲): 3.75 μL/mL, (○):
452 1.875 μL/mL, (*) control: 0 μL/mL. Detection limit of the test: 1 log cfu/mL.
453
454 **Fig. 2.** Reduction cycles (log cfu/mL; n = 6) of the initial counts of *C. albicans* ATCC 90028
455 (A, E), *C. tropicalis* ATCC 28707 (B, F), *P. anomala* ATCC 40101 (C, G) and *S. cerevisiae*
456 ATCC 2601 (D, H) as a function of the concentration of *M. piperita* L. essential oil in mango
457 juice (A – D) and pineapple juice (E – H) stored at 4 ± 0.5 °C. (■): 7.5 μL/mL, (▲): 3.75
458 μL/mL, (○): 1.875 μL/mL, (*) control: 0 μL/mL. Detection limit of the test: 1 log cfu/mL.
459
460 **Fig. 3** Fluorescence density plots of *S. cerevisiae* in response to staining with PI and
461 $DiBAC_4(3)$, FDA and EB after a 45-min exposure to 1.875 μL/mL MPEO in cashew juice
462 stored at 4 ± 0.5 °C. The vertical axis indicates the fluorescence intensity of $DiBAC_4(3)$ and
463 side-light scatter intensity; the horizontal axis indicates the fluorescence intensity of PI, FDA
464 and EB. The negative stain subpopulation was gated in the left rectangles; the positive stain
465 subpopulation was gated in the right rectangles. Percentage of cell populations that fell in
466 each gate are shown in each plot.
467
468 **Fig. 4** Fluorescence density plots of *S. cerevisiae* in response to staining with PI and
469 $DiBAC_4(3)$, FDA and EB after a 45-min exposure to 1.875 μL/mL MPEO in guava juice
470 stored at 4 ± 0.5 °C. The vertical axis indicates the fluorescence intensity of $DiBAC_4(3)$ and
471 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.

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

Retention time	Kovats index	Constituents	Amounts (%)*
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	Ą429	β-gurjunene	0.10
27.023	1454	α-Caryophyllene	0.10
32.401	1584	Caryophyllene oxide	0.13

* Constituents detected in amounts ≥ 0.1%.

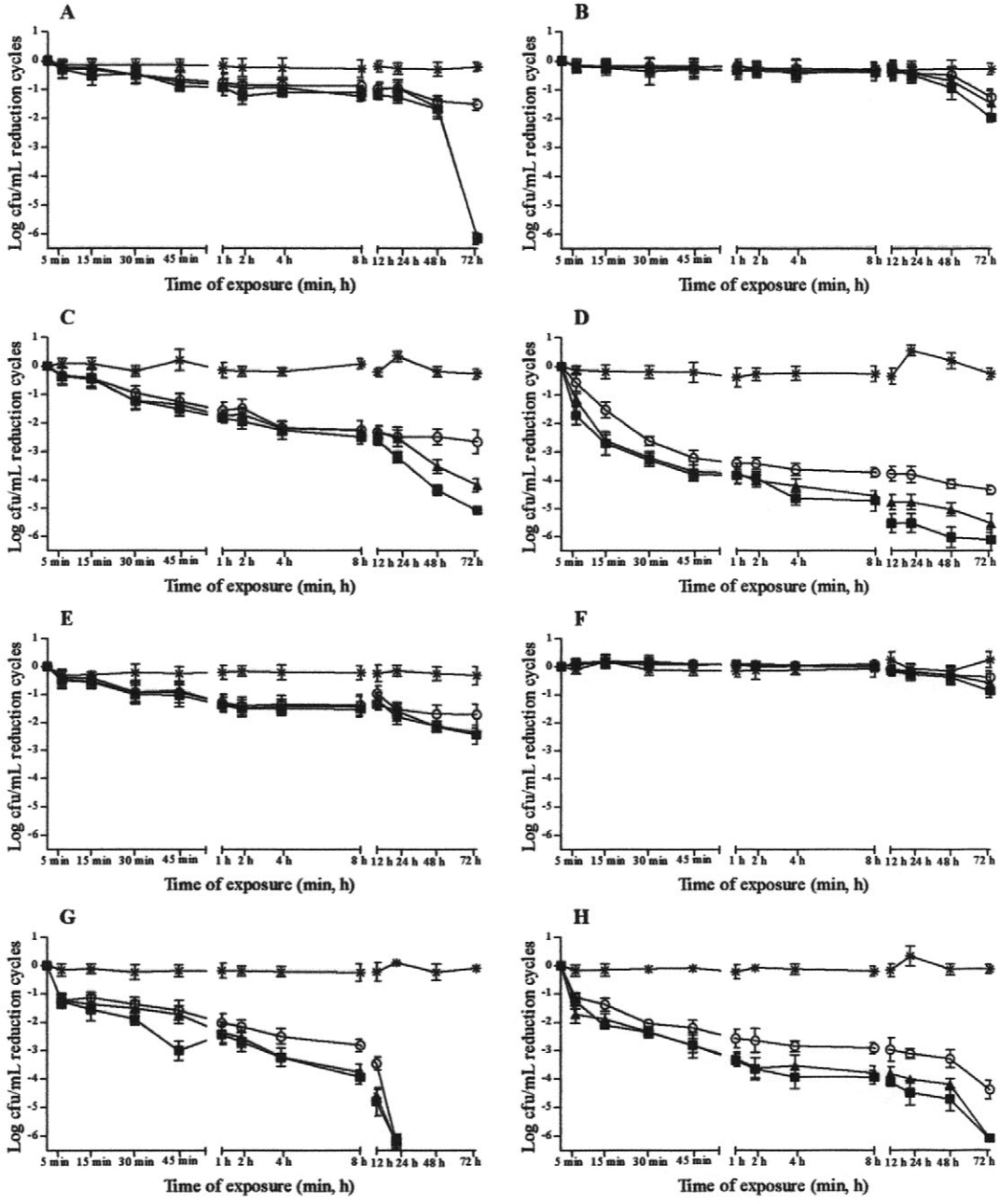

A
B
C
D
E
F
G
H
Log cfu/mL reduction cycles
Time of exposure (min, h)
5 min 15 min 30 min 45 min 1 h 2 h 4 h 8 h 12 h 24 h 48 h 72 h

Fig. 1

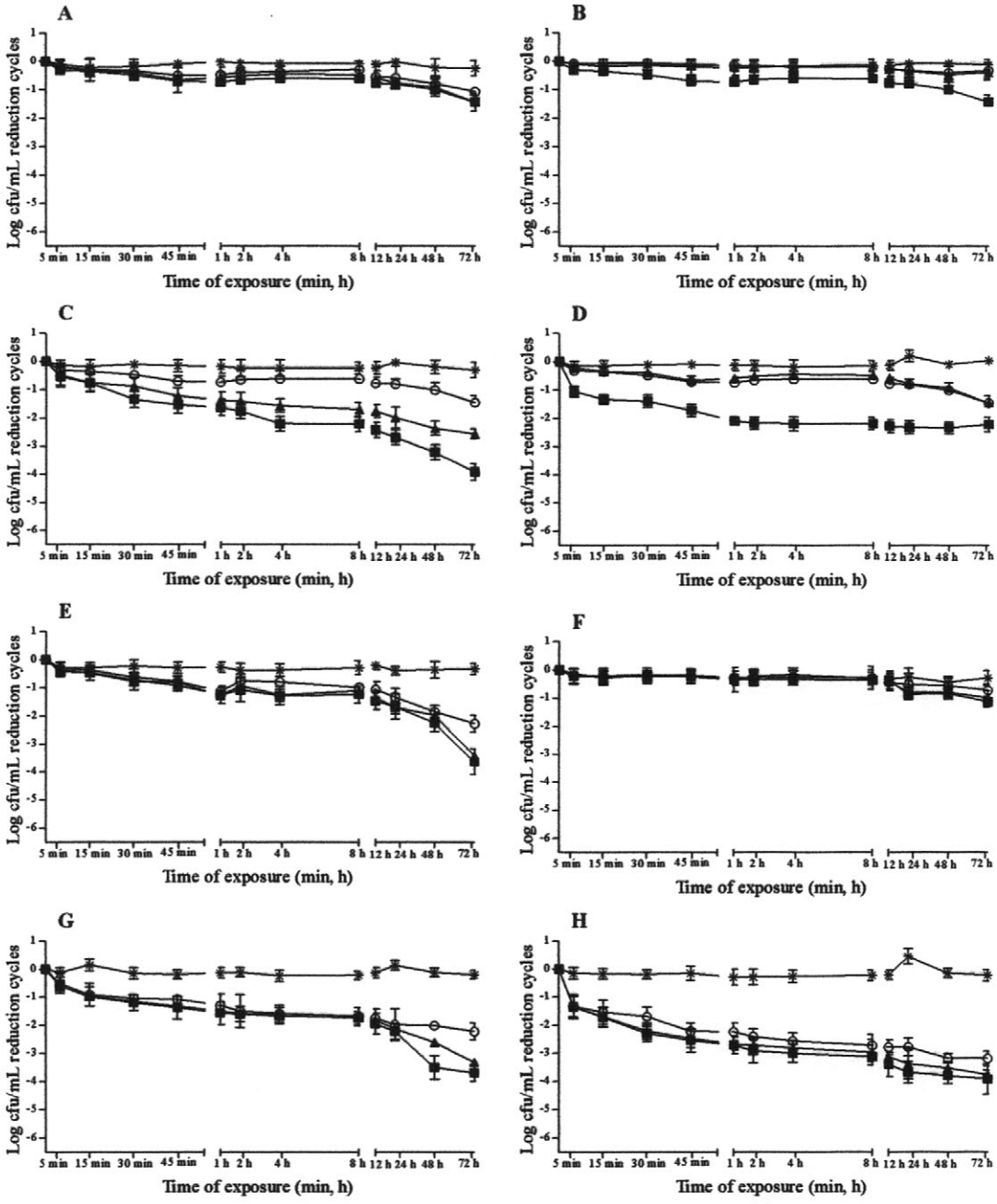
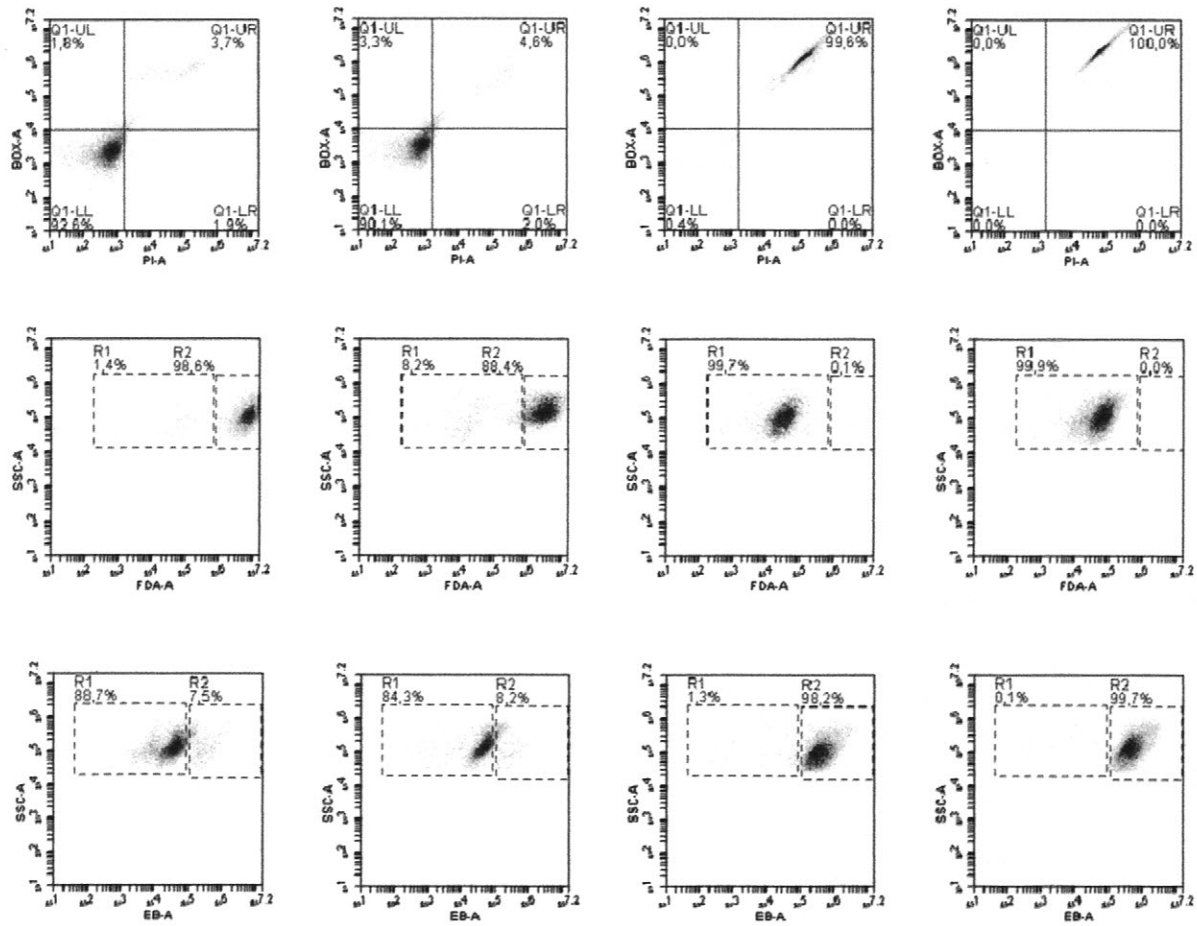

A
B
C
D
E
F
G
H
Log cfu/mL reduction cycles
Time of exposure (min, h)
5 min 15 min 30 min 45 min 1 h 2 h 4 h 8 h 12 h 24 h 48 h 72 h

Fig. 2



Inoculated PBS **Inoculated juice** **Inoculated juice + MPEO** **Ethanol-treated cells**

Fig. 3

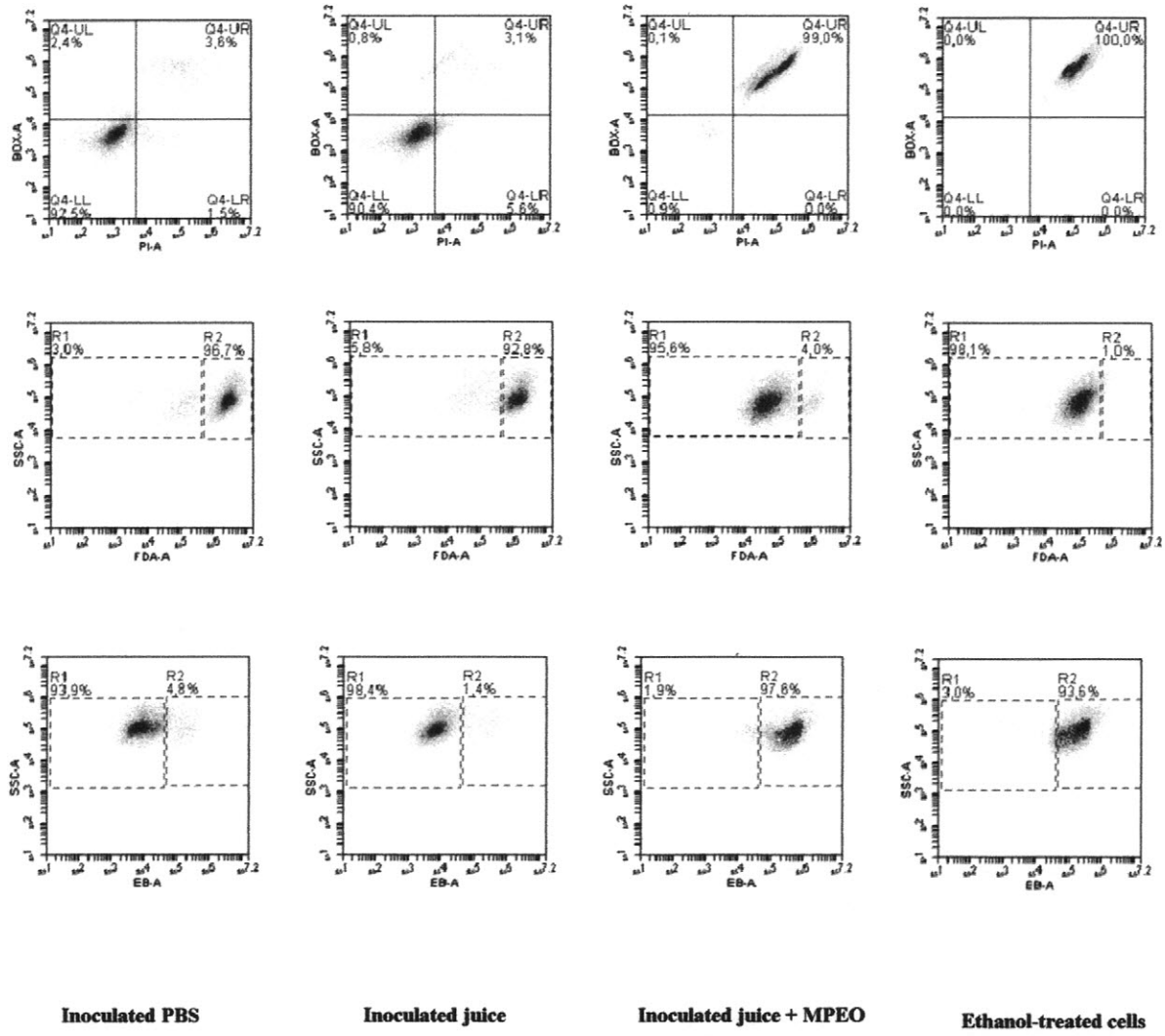

Q4-UL
2,4%
Q4-UR
3,6%
Q4-LL
92,5%
Q4-LR
1,5%
BOX-A
PI-A
Q4-UL
0,8%
Q4-UR
3,1%
Q4-LL
90,4%
Q4-LR
5,6%
Q4-UL
0,1%
Q4-UR
99,0%
Q4-LL
0,9%
Q4-LR
0,0%
Q4-UL
0,0%
Q4-UR
100,0%
Q4-LL
0,0%
Q4-LR
0,0%
R1
3,0%
R2
96,7%
SSC-A
FDA-A
R1
5,8%
R2
92,8%
R1
95,6%
R2
4,0%
R1
98,1%
R2
1,0%
R1
93,9%
R2
4,8%
EB-A
R1
98,4%
R2
1,4%
R1
1,9%
R2
97,6%
R1
3,0%
R2
93,6%
Inoculated PBS
Inoculated juice
Inoculated juice + MPEO
Ethanol-treated cells

Fig 4.

APÊNDICES

APÊNDICE A: Termo de Consentimento Livre e Esclarecido (TCLE)

UNIVERSIDADE FEDERAL DA PARAÍBA
CENTRO DE TECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E TECNOLOGIA DE ALIMENTOS

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

Prezado (a) Senhor (a)

Esta pesquisa é sobre a avaliação sensorial de sucos de frutas tropicais adicionada de óleo essencial de *Mentha spicata*, que está sob a responsabilidade de Erika Tayse da Cruz Almeida, doutoranda regularmente matriculada do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, da Universidade Federal da Paraíba, e sob orientação do Prof. Dr. Evandro Leite de Souza. Trata-se de uma pesquisa vinculada ao projeto *Aplicação combinada de óleos essenciais, temperaturas moderadas e campo elétrico pulsado na conservação de sucos de frutas tropicais*.

Solicitamos a sua colaboração em responder o questionário CATA (*Check-all-that-apply*) e os testes de aceitação e intenção de compra para amostras de sucos de abacaxi, caju, goiaba e manga adicionada de concentração específica de óleo essencial de *M. spicata*, com a finalidade de caracterizá-la sensorialmente. Além disso, solicitamos sua autorização para divulgar os resultados em eventos ou publicações científicas, não havendo identificação dos voluntários, a não ser entre os responsáveis pelo estudo, sendo assegurado o sigilo sobre a sua participação.

Informamos que o teste sensorial não apresenta riscos previsíveis ou mensuráveis, pois de acordo com a literatura os óleos essenciais adicionados aos sucos de fruta não apresentam efeitos tóxicos significativos in vivo nas concentrações utilizadas no estudo. Durante esta pesquisa, os voluntários serão avisados das substâncias empregadas, e caso apresente alguma reação alérgica, não antes vivenciada, durante ou após o teste sensorial, será acionado o Serviço de Atendimento Móvel de Urgência – SAMU, por meio do número 192.

Esclarecemos que sua participação será voluntária, e, portanto, o (a) senhor (a) não é obrigado (a) a fornecer informações e/ou colaborar com as atividades instruídas pela pesquisadora. Caso decida, não participar da pesquisa ou resolver a qualquer momento desistir da mesma, sem qualquer penalidade. A pesquisadora estará a sua disposição para qualquer esclarecimento que considere necessário.

Diante do exposto, declaro que fui devidamente informado (a) e esclarecido (a) e dou meu consentimento em participar da pesquisa e para a publicação de resultados.

João Pessoa, ____/_____/____

_____
Assinatura do Participante da Pesquisa

Caso necessite de maiores informações sobre a presente pesquisa, fazer contato com a pesquisadora responsável: Erika Tayse da Cruz Almeida
Endereço: Rua Professora Maria Lianza, nº 210, CEP: 58052-320, Cidade Jardim Universitária, João Pessoa-PB. Contato: (83) 99611-5777 ou (83) 98693-0133 e-mail: erika_yse@hotmail.com

_____
Assinatura da pesquisadora responsável

APÊNDICE B: Formulário para Análise Sensorial

Nome: _____ Data: ____/___/___
Idade: _____ Profissão: _____

Ficha de caracterização sensorial utilizando o método CATA

Por favor, avalie a amostra e dentre as características sensoriais citadas abaixo, assinale aquela(s) que melhor descreve(m) esse produto em sua opinião. Marque quantas opções julgar necessárias.

( ) Cor característica de suco de fruta
( ) Cor não característica de suco defruta
( ) Aroma característica de suco de fruta
( ) Aroma não característica de suco de fruta
( ) Aroma de menta
( )Viscoso
( ) Ralo
( ) Adocicado
( ) Não adocicado
( ) Sabor estranho
( ) Sensação refrescante
( ) Sabor da fruta
( ) Sabor de menta
( ) Sabor amargo
( ) Sabor agradável
( ) Sabor desagradável

MUITO OBRIGADA!

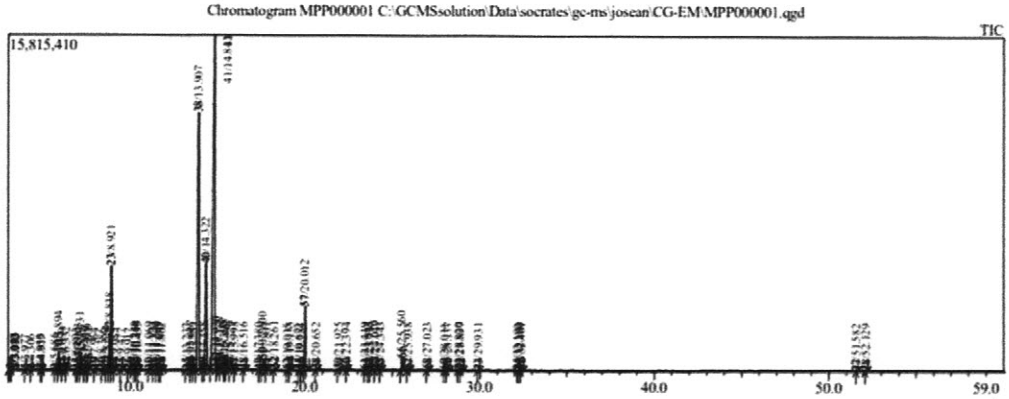
APÊNDICE C: CROMATOGRAMA DE *M. piperita* L.

IPeFarM
INSTITUTO DE PESQUISA EM FÁRMACOS E MEDICAMENTOS

Instituto de Pesquisa em Fármacos e Medicamentos da Universidade Federal da Paraíba
Unidade de Caracterização e Análise / UNICAL | www.ufpb.br/ipefarm | unical@ltf.ufpb.br | 09.2017
Arquivo: MPP000001.qgd / Data: 18/09/2017

Cromatógrafo Gasoso acoplado a Espectrômetro de Massas: Modelo: GCMS-QP2010 Ultra | Marca: Shimadzu
Coluna: marca: RTX-5MS capilar (5% Diphenyl / 95% dimethyl polysiloxane)
Tamanho: 30 m (comprimento) / 0.25 mm de Diâmetro Interno / 0.25 um df

Chromatogram MPP000001 C:\GCMSsolution\Data\socrates\gc-ms\josean\CG-EM\MPP000001.qgd



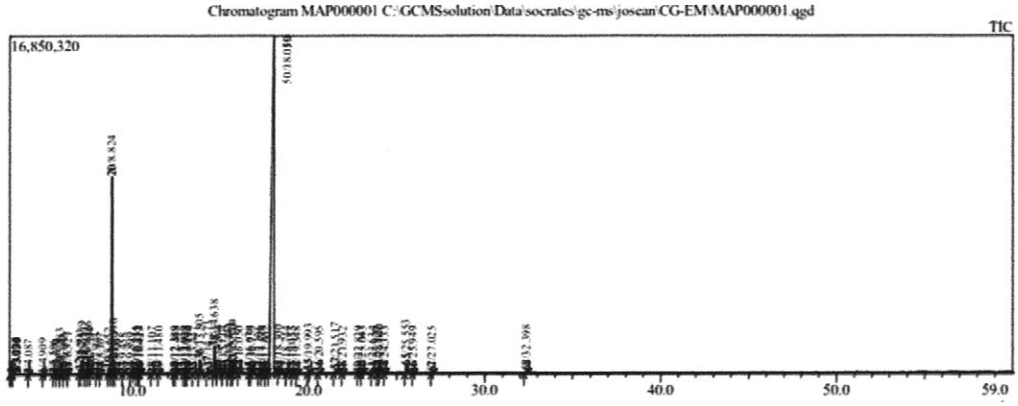
APÊNDICE D: CROMATOGRAMA DE *M. spicata* L.

IPeFarM
INSTITUTO DE PESQUISA EM FÁRMACOS E MEDICAMENTOS

Instituto de Pesquisa em Fármacos e Medicamentos da Universidade Federal da Paraíba
Unidade de Caracterização e Análise / UNICAL | www.ufpb.br/ipefarm | unical@ltf.ufpb.br | 09.2017
Arquivo: MAP000001.qgd / Data: 18/09/2017

Cromatógrafo Gasoso acoplado a Espectrômetro de Massas: Modelo: GCMS-QP2010 Ultra | Marca: Shimadzu
Coluna: marca: RTX-5MS capilar (5% Diphenyl / 95% dimethyl polysiloxane)
Tamanho: 30 m (comprimento) / 0.25 mm de Diâmetro Interno / 0.25 um df

Chromatogram MAP000001 C:\GCMSsolution\Data\socrates\gc-ms\josean\CG-EM\MAP000001.qgd



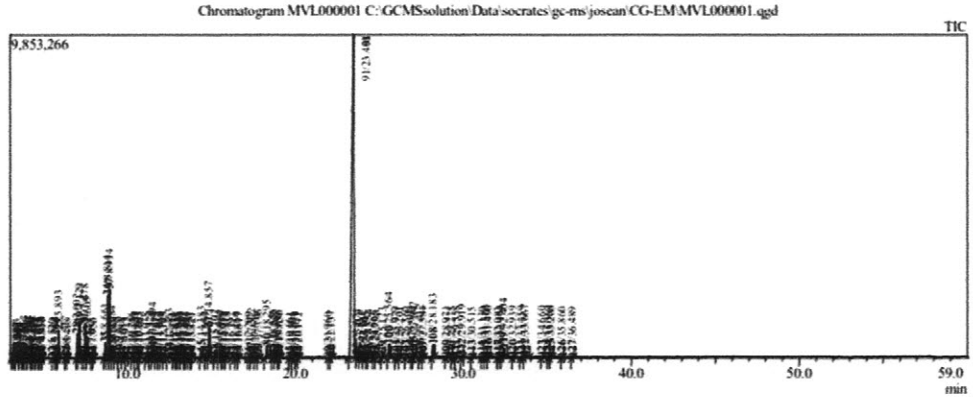
APÊNDICE E: CROMATOGRAMA DE *M. x villosa* HUDS.

IPeFarM
INSTITUTO DE PESQUISA EM FÁRMACOS E MEDICAMENTOS

Instituto de Pesquisa em Fármacos e Medicamentos da Universidade Federal da Paraíba
Unidade de Caracterização e Análise / UNICAL | www.ufpb.br/ipefarm | unical@ltf.ufpb.br | 09.2017
Arquivo: MVL000001.qgd / Data: 18/09/2017

Cromatógrafo Gasoso acoplado a Espectrômetro de Massas: Modelo: GCMS-QP2010 Ultra | Marca: Shimadzu
Coluna: marca: RTX-5MS capilar (5% Diphenyl / 95% dimethyl polysiloxane)
Tamanho: 30 m (comprimento) / 0.25 mm de Diâmetro Interno / 0.25 um df

Chromatogram MVL000001 C:\GCMSsolution\Data\socrates\gc-ms\josean\CG-EM\MVL000001.qgd



ANEXOS

ANEXO A: Parecer consubstanciado do Comitê de Ética em Pesquisa

Comitê de Ética em Pesquisa Envolvendo Seres Humanos CEP-CCS-UFPE

UNIVERSIDADE FEDERAL DE PERNAMBUCO CENTRO DE CIÊNCIAS DA SAÚDE / UFPE-

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Aplicação combinada de óleos essenciais, temperaturas moderadas e campo elétrico pulsado na conservação de sucos de frutas tropicais

Pesquisador: Jossana Pereira de Sousa

Área Temática:

Versão: 2

CAAE: 43653415.6.0000.5208

Instituição Proponente: CENTRO DE CIÊNCIAS DA SAÚDE

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.125.993

Data da Relatoria: 29/06/2015

Apresentação do Projeto:

Trata-se de estudo experimental cuja proposta é tratar quatro tipos de sucos de frutas tropicais da região nordeste com óleos essenciais de Mentha piperita L. (hortelã-pimenta) e Mentha arvensis L. (hortelã-doce), aplicação de Tratamentos Térmicos Moderados e de Campo Elétrico Pulsado(CEP). Os parâmetros de qualidade dos sucos de frutas adicionados ou não dos óleos essenciais e submetidos ou não aos tratamentos com temperaturas moderadas e CEP, serão mensurados em diferentes intervalos (1, 2, 3, 4, 5, 6 e 7 dias) de armazenamento refrigerado a 7 ºC (±1 °C). Os parâmetros físico-químicos avaliados serão pH, acidez, sólidos solúveis, vitamina C e açúcares totais de acordo com metodologia padrão (AOAC, 2006). Após estes tratamentos os sucos serão submetidos à análise sensorial, por meio do teste de aceitação, com 200 provadores voluntários.

Os provadores realizarão o teste sensorial em condições controladas de temperatura e iluminação e provarão porções dos sucos adicionados de diferentes concentrações dos óleos essenciais, servidas em copos descartáveis(50 ml). Cada grupo de 50 provadores fará o teste com um tipo de suco, totalizando os 200 provadores. Os provadores farão uso de bolacha salgada e água para limpar os seus paladares entre as amostras avaliadas.

A aceitação da (aparência, cor, aroma, sabor, sabor residual, doçura, consistência e avaliação global), serão avaliados em uma escala hedônica de 9 pontos, variando de 1 (não gostei

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Bairro: Cidade Universitária **CEP:** 50.740-600
UF: PE **Município:** RECIFE
Telefone: (81)2126-8588 **E-mail:** cepccs@ufpe.br

muitíssimo) a 9 (gostei muitíssimo). A intenção de compra será avaliada utilizando uma escala hedônica de 5 pontos, variando de 1 (certamente não compraria) a 5 (certamente compraria). Amostras dos sucos sem adição dos óleos essenciais serão testadas de forma semelhante como um controle. O desenvolvimento dos testes sensoriais obedecerão a metodologia descrita por Azerêdo et al. (2011).

As análises estatísticas serão realizadas utilizando-se testes de estatística descritiva e inferencial para determinação de diferenças significantes ($p<0,05$) entre os tratamentos aplicados. Para a análise estatística utilizar-se-á o software Sigma Stat. 2.03.

Critério de Inclusão:

Os sujeitos participantes serão funcionários, alunos, professores e visitantes, maiores de 18 anos, que se encontrem no Campus I da Universidade

Federal da Paraíba nos dias de realização dos testes sensoriais, e que estejam interessados em participar voluntariamente da pesquisa. Serão

selecionados de acordo com seu interesse em participar da pesquisa e por possuir o hábito de consumir sucos de fruta (abacaxi, acerola, caju e

manga), ou seja, prováveis consumidores desse tipo de produto.

Critério de Exclusão:

Pessoas menores de 18 anos.Indivíduos que não tenham o hábito de consumir sucos de fruta.Tabagistas.

Objetivo da Pesquisa:

Objetivo Geral. avaliar o potencial da aplicação combinada de óleos essenciais, tratamentos térmicos moderados e campos elétricos pulsados na conservação de sucos de frutas tropicais,

Objetivos específicos:

a) avaliar a eficácia de óleos essenciais, tratamentos térmicos moderados e campos elétricos pulsados, quando aplicados isolados e em combinação, na inibição de cepas de bactérias contaminantes de sucos de frutas em inóculo misto, cultivadas em meio laboratorial e em sucos de frutas tropicais;

b) investigar a ocorrência de injúria subletal nas cepas teste utilizadas nos ensaios após aplicação dos óleos essenciais, tratamentos térmicos moderados e campos elétricos pulsados, isolados e combinados;

c) identificar os possíveis danos às células bacterianas, causados pelos tratamentos utilizados;
d) verificar a influência dessa aplicação, isolada ou combinada, sobre indicadores físico-químicos de qualidade e atributos sensoriais de sucos de frutas tropicais ao longo do período de armazenamento.

Avaliação dos Riscos e Benefícios:

O teste sensorial não apresenta riscos previsíveis ou mensuráveis, pois de acordo com a literatura os óleos essenciais adicionados aos sucos de fruta não apresentam efeitos tóxicos significativos in vivo nas concentrações utilizadas no estudo. Por ser considerado um alimento perecível, para controlar o fator contaminação, todo o procedimento de elaboração dos sucos foi conduzido de acordo com as Boas Práticas de Fabricação, de acordo com as legislações vigentes. Além disto, antes da aplicação das análises sensoriais, as amostras foram submetidas a análises microbiológicas que demonstraram a qualidade higiênico-sanitária dos produtos elaborados, sendo descartados os produtos que apresentaram valores acima dos permitidos pela legislação específica, garantindo que o Sr (a) está recebendo amostras sem nenhum risco de contaminação microbiológica. Caso apresente alguma reação alérgica, não antes vivenciada, a equipe estará preparada a chamar o Serviço de Atendimento Móvel
de Urgência – SAMU, por meio do número 192, assim como, na ausência deste, a encaminhá-lo ao hospital de referência mais próximo. Assim como, será oferecida uma prova mínima a cada avaliador, antes da degustação oficial, para verificar a sua aceitabilidade orgânica ao produto.
Benefícios: Considerando a propriedade antimicrobiana dos óleos essenciais de plantas aromáticas que serão estudados, bastante relatada na literatura, espera-se encontrar um potencial efeito antimicrobiano quando utilizados em combinação com outras tecnologias de conservação, como tratamentos térmicos moderados e pulsos elétricos, frente à microbiota natural e micro-organismos patógenos comumente encontrados em sucos de frutas tropicais

Comentários e Considerações sobre a Pesquisa:

sem comentários

Considerações sobre os Termos de apresentação obrigatória:

Apresentou todos os termos exigidos pela resolução 466/12

Recomendações:

Sem recomendações

Conclusões ou Pendências e Lista de Inadequações:

Sem pendências

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

As exigências foram atendidas e o protocolo está APROVADO, sendo liberado para o início da coleta de dados. Informamos que a APROVAÇÃO DEFINITIVA do projeto só será dada após o envio do Relatório Final da pesquisa. O pesquisador deverá fazer o download do modelo de Relatório Final para enviá-lo via "Notificação", pela Plataforma Brasil. Siga as instruções do link "Para enviar Relatório Final", disponível no site do CEP/CCS/UFPE. Após apreciação desse relatório, o CEP emitirá novo Parecer Consubstanciado definitivo pelo sistema Plataforma Brasil.

Informamos, ainda, que o (a) pesquisador (a) deve desenvolver a pesquisa conforme delineada neste protocolo aprovado, exceto quando perceber risco ou dano não previsto ao voluntário participante (item V.3., da Resolução CNS/MS Nº 466/12).

Eventuais modificações nesta pesquisa devem ser solicitadas através de EMENDA ao projeto, identificando a parte do protocolo a ser modificada e suas justificativas.

Para projetos com mais de um ano de execução, é obrigatório que o pesquisador responsável pelo Protocolo de Pesquisa apresente a este Comitê de Ética relatórios parciais das atividades desenvolvidas no período de 12 meses a contar da data de sua aprovação (item X.1.3.b., da Resolução CNS/MS Nº 466/12).

O CEP/CCS/UFPE deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (item V.5., da Resolução CNS/MS Nº 466/12). É papel do/a pesquisador/a assegurar todas as medidas imediatas e adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e ainda, enviar notificação à ANVISA – Agência Nacional de Vigilância Sanitária, junto com seu posicionamento.

Continuação do Parecer: 1.125.993

RECIFE, 26 de Junho de 2015

Assinado por:
LUCIANO TAVARES MONTENEGRO
(Coordenador)

Endereço: Av. da Engenharia s/nº - 1º andar, sala 4, Prédio do CCS
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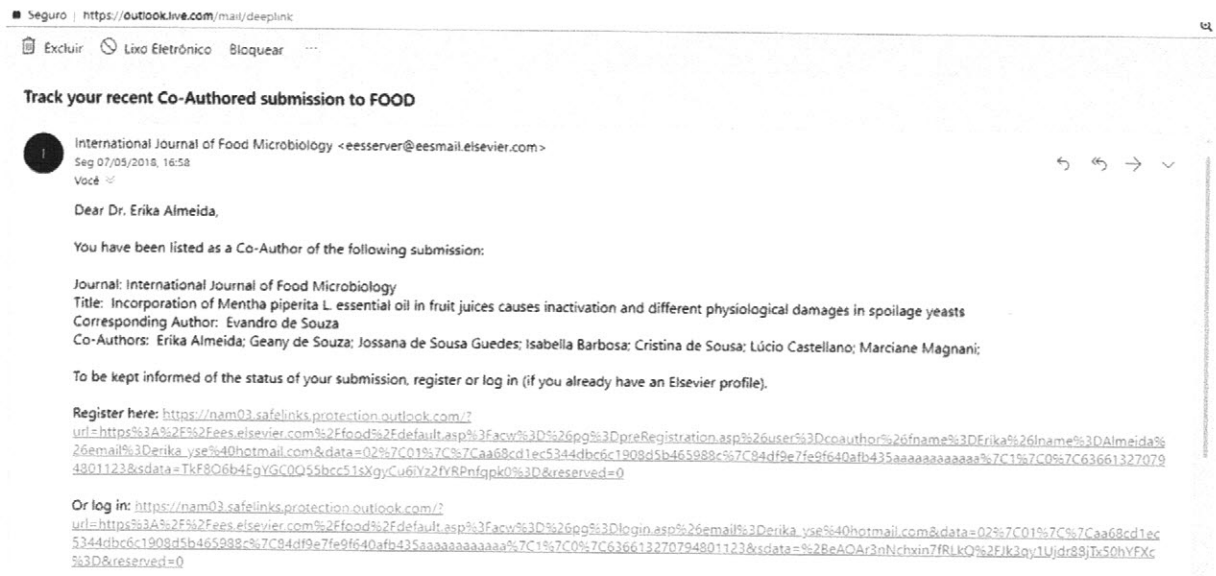
UF: PE **Município:** RECIFE
Telefone: (81)2126-8588 **E-mail:** cepccs@ufpe.br

ANEXO B: Aceite do artigo publicado no Periódico International Journal of Food Microbiology (ISSN 0168-1605).



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Track your recent Co-Authored submission to FOOD

International Journal of Food Microbiology <eesserver@eesmail.elsevier.com>
Seg 07/05/2018, 16:58
Você

Dear Dr. Erika Almeida,

You have been listed as a Co-Author of the following submission:

Journal: International Journal of Food Microbiology
Title: Incorporation of Mentha piperita L. essential oil in fruit juices causes inactivation and different physiological damages in spoilage yeasts
Corresponding Author: Evandro de Souza
Co-Authors: Erika Almeida; Geany de Souza; Jossana de Sousa Guedes; Isabella Barbosa; Cristina de Sousa; Lúcio Castellano; Marciane Magnani;

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