UNIVERSIDADE FEDERAL DE PERNAMBUCO

JOSSANA PEREIRA DE SOUSA

EFICÁCIA DE ÓLEOS ESSENCIAIS DE *Mentha spp.* NO CONTROLE DE BACTÉRIAS PATOGÊNICAS EM SUCOS DE FRUTAS

RECIFE

2017

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Tese apresentada ao Programa de Pós-Graduação em Nutrição do Centro de Ciências da Saúde da Universidade Federal de Pernambuco, como parte dos requisitos para obtenção do título de Doutor em Nutrição, com área de concentração em Ciência dos Alimentos.

Orientador: Prof. Dr. Evandro Leite de Souza

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Tese aprovada em: 11 de agosto de 2017.

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À minha família e ao meu marido Rhavy, companheiro de todas as horas.

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(Fl 4,13)

RESUMO

Sucos de frutas são bastante consumidos devido às suas características de sabor, aspecto refrescante e por ser reconhecidamente um alimento saudável. No entanto, também são susceptíveis à deterioração microbiana e representam risco para o consumidor quando patógenos estão presentes no produto. Por isso, necessitam de aplicação de tecnologias de conservação eficazes que possibilitem a extensão da sua vida de prateleira. Para tal, estes sucos são pasteurizados e adicionados de conservantes, porém, estes tratamentos apresentam desvantagens, como por exemplo, a destruição de importantes constituintes alimentares. Assim, faz-se necessário o emprego de tecnologias de conservação que não apresentem tais efeitos, a citar o uso de óleos essenciais. Diante deste contexto, objetivou-se avaliar a eficácia da aplicação dos óleos essenciais de *Mentha arvensis* L. e *M. piperita* L. no controle de bactérias patogênicas (*E. coli*, *L. monocytogene*s e *Salmonella* Enteritidis) em sucos de frutas (abacaxi, caju, goiaba e manga). Foram determinadas as Concentrações Inibitórias Mínimas (CIM) de cada óleo essencial frente ao inóculo misto das cepas. Os efeitos dos óleos essenciais na redução de 5 $\log_{10}$ UFC/mL, em meio laboratorial e nos sucos, e sobre características físico-químicas e sensoriais dos produtos foram avaliados. Além disso, foram investigados os danos causados pelos óleos essenciais nas células bacterianas. Os valores da CIM dos óleos essenciais de *M. arvensis* L. e *M. piperita* L. foram 5 e 10 µL/mL, respectivamente. A incorporação de 5, 2,5, 1,25 ou 0,625 µL/mL de *M. arvensis* L. em caldo BHI causou uma redução > 5 $\log_{10}$ UFC/mL nas contagens de *E. coli* e *Salmonella* Enteritidis após 24 h de armazenamento refrigerado, enquanto apenas a concentração de 5 µL/mL foi capaz de causar esta redução de *L. monocytogenes*. Apenas 10 µL/mL de *M. piperita* L. causou redução > 5 $\log_{10}$ UFC/mL na contagem das três cepas após 24 h de armazenamento refrigerado em caldo BHI. Resultados similares foram observados no suco de manga. Porém, nos sucos de abacaxi, caju e goiaba, em todas as concentrações testadas, os óleos essenciais foram capazes de reduzir as contagens microbianas (> 5 $\log_{10}$ UFC/mL) em apenas 1 h de armazenamento refrigerado. Os óleos essenciais não alteraram o °Brix, o pH e a acidez dos sucos, mas afetaram negativamente o sabor e a aceitação global dos produtos. Na análise de citometria de fluxo verificou-se que os óleos essenciais inativam as células de *E. coli*, *L. monocytogenes* e *Salmonella* Enteritidis por meio de um mecanismo multialvo, que aumenta a permeabilidade da membrana citoplasmática, despolariza a membrana, inibe a bomba de efluxo e a atividade respiratória. Estes resultados indicam que os óleos essenciais de *Mentha* spp., em baixas concentrações, apresentam-se como alternativa promissora para conservação de sucos de frutas. Mas, o uso combinado com outras tecnologias de conservação é necessário para reduzir os seus impactos negativos sobre os parâmetros sensoriais.

Palavras-chave: Antibacteriano. Bactérias. *Mentha*. Suco de fruta.

ABSTRACT

Fruit juices are widely consumed due to characteristics as flavor, refreshing aspects and for being admittedly a healthy food. Nevertheless, they are also susceptible to microbial deterioration and pose a risk to the consumer when pathogens are present. Therefore, the application of effective conservation technologies in fruit juices has been required to allow the extension of their shelf life. In general, fruit juices are pasteurized and preservatives are added to their compositions. However, these treatments have as drawback the destruction of important food constituents. Thus, it is necessary the application of conservation technologies that do not present such effects, to mention the use of essential oils. The objective of this study was to evaluate the efficacy of the essential oils of *Mentha arvensis* L. and *M. piperita* L. in the control of pathogenic bacteria (*E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis) in fruit juices (pineapple, cashew, guava and mango). The Minimum Inhibitory Concentrations (MIC) of each essential oil were determined against the mixed inoculum of the strains. The effects of the essential oils in the reduction of 5 $\log_{10}$ CFU/mL, in laboratory medium and in the juices, and on physical-chemical and sensorial characteristics of the products were evaluated. In addition, the damage caused by essential oils in bacterial cells has been investigated. The MIC values of the essential oils of *M. arvensis* L. and *M. piperita* L. were 5 and 10 µL/mL, respectively. The incorporation of 5, 2.5, 1.25 or 0.625 µL/mL *M. arvensis* L. into BHI broth caused a reduction > 5 $\log_{10}$ CFU/mL in the counts of *E. coli* and *Salmonella* Enteritidis after 24 h of refrigerated storage, whereas only the concentration of 5 µL/mL was able to cause this reduction of *L. monocytogenes*. Only 10 µL/mL *M. piperita* L. caused a reduction > 5 $\log_{10}$ CFU/mL in the count of the three strains after 24 h of refrigerated storage in BHI broth. Similar results were observed in mango juice. However, in pineapple, cashew and guava juices, at all concentrations tested, essential oils were able to reduce microbial counts (> 5 $\log_{10}$ CFU/mL) in only 1 h of refrigerated storage. The essential oils did not change the °Brix, pH and acidity of the juices, but negatively affected the taste and the overall acceptance of the products. In flow cytometric analysis it was found that the essential oils inactivated the *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis cells by means of a multi-target mechanism, which increases the permeability of the cytoplasmic membrane, depolarizes the membrane, inhibits the efflux and respiratory activity. These results indicate that the essential oils of *Mentha* spp., in low concentrations, are presented as a promising alternative for the preservation of fruit juices. Nevertheless, it is suggested that the combined use of the *Mentha* spp. essential oil with other conservation technologies may be necessary to reduce its negative impacts on sensory parameters.

Keywords: Anti-bacterial. Bacteria. *Mentha*. Fruit juices.

LISTA DE ILUSTRAÇÕES

Artigo 2

Artigo 3

LISTA DE TABELAS

Artigo 1

Artigo 2

Artigo 3

SUMÁRIO

1 INTRODUÇÃO

O Brasil é o terceiro maior produtor mundial de frutas, com cerca de 43 milhões de toneladas de frutas frescas colhidas no ano de 2015, em aproximadamente de dois milhões de hectares, distribuídos por todas as regiões do país, sendo um dos segmentos mais importantes da agricultura brasileira (TREICHEL et al., 2016). Concomitante ao aumento da produção de frutas no Brasil, ocorre a falta de adequação dessa produção, problemas logísticos de armazenamento, transporte e comercialização, como também alterações climáticas, que geram perdas pós-colheita na ordem de 20 a 50%. Além disso, ocorre o gasto de valiosos recursos na produção, como água e energia, emissões desnecessárias de dióxido de carbono, além de perda do valor econômico dos alimentos produzidos (FAO, 2011).

Diante deste panorama, acredita-se que a industrialização de frutas possa ser o caminho para a redução dos desperdícios e também para agregação de valor, alcançados pelo processamento em produtos derivados, como doces, geleias, e, principalmente, sucos, cuja produção ocupa papel de relevância no agronegócio mundial (TREICHEL et al., 2016). Apesar de não haver estatísticas oficiais que permitam estabelecer com precisão as perdas e a parte destinada para o processamento industrial, o Instituto Brasileiro de Frutas (IBRAF) estimou que em 2015, 47% da produção de frutas foi destinado ao mercado de frutas processadas. Dentre os produtos provenientes do processamento de frutas, destacam-se os sucos, os quais tem apresentado boa aceitação nos mercados interno e externo (IBRAF, 2016).

Os sucos de frutas são consumidos e apreciados em todo o mundo, por todas as faixas etárias, devido às suas características de sabor, aspecto refrescante e por ser um alimento reconhecidamente saudável (LUCKOW; DELAHUNTY, 2004; NEVES et al., 2011; SINGH et al., 2015). A crescente procura dos consumidores por produtos com estas características tem impulsionado o consumo de sucos prontos para o consumo. No entanto, estes alimentos também são susceptíveis à deterioração microbiana, necessitando de aplicação de tecnologias de conservação eficazes, que possibilitem a extensão da sua vida de prateleira (CARMO; DANTAS; RIBEIRO, 2014).

A alteração microbiológica de sucos de frutas ocorre principalmente devido à contaminação por micro-organismos deteriorantes, que comumente são encontrados em sucos de frutas em contagens variando de 3 a 5 $\log_{10}$ UFC/mL, os quais podem produzir aroma e sabor desagradáveis (STRATFORD; HOFMAN; COLE, 2000; TOURNAS; HEERES; BURGESS, 2006). Não obstante, mesmo apresentando pH desfavorável ao crescimento de bactérias potencialmente patogênicas, os sucos de frutas podem veicular micro-organismos como *Escherichia coli*, *Listeria* spp. e *Salmonella* spp. (CDC, 2011; EFSA, 2013, 2015; PARISH, 2009; RAYBAUDI-MASSILIA et al., 2009). Consequentemente, a *Food and Drug Administration* (FDA) recomendou que os sucos devem ser processados para atingir uma redução de cinco ciclos logarítmicos (5 $\log_{10}$ UFC/mL, 99,999%) na população de patógenos relevantes em saúde pública (USFDA, 2001), porém, não especifica o método.

Geralmente, os sucos de frutas são pasteurizados e adicionados de conservantes. Porém, o tratamento térmico destrói importantes constituintes alimentares, os quais conferem a qualidade nutricional e sensorial ao produto (USFDA, 2004). Além disso, conservantes como benzoato de sódio, sorbato de potássio e dióxido de enxofre, podem ser tóxicos e responsáveis pela ocorrência de alergias e câncer (AMIRPOUR et al., 2015; TONIOLO et al., 2010). Assim, faz-se necessário o emprego de novos conservantes que não apresentem tais efeitos, como por exemplo, o uso de óleos essenciais (BURT 2004; USFDA, 2015). As principais vantagens dos óleos essenciais referem-se a sua origem natural e sua composição complexa de constituintes, que apresentam diferentes mecanismos de ação, tornando mais difícil à adaptação por parte dos micro-organismos (SOUZA, 2016). Óleos essenciais de diversas espécies têm sido utilizados devido ao seu potencial antimicrobiano contra micro-organismos deteriorantes e patógenos de alimentos, com destaque para aqueles extraídos de espécies de *Mentha* (ISCAN et al., 2002; KARAMAN; SAGDIC; YILMAZ, 2016; TYAGI; MALIK, 2013).

Considerando os aspectos acima citados e o reconhecido potencial antimicrobiano dos óleos essenciais, acredita-se que o uso de óleos essenciais de *Mentha* spp. como conservantes de sucos de frutas represente um avanço na tecnologia de preservação destes produtos, em consonância ao atendimento das exigências dos consumidores por alimentos produzidos com baixos níveis ou ausência de conservantes sintéticos, porém com a conveniência de apresentarem-se nutritivos, seguros e possuidores de longa vida de prateleira.

2 REVISÃO DA LITERATURA

2.1 SUCOS DE FRUTAS

O suco é definido como uma bebida não fermentada, não concentrada e não diluída, obtida da fruta madura e sã, ou parte do vegetal de origem, por processamento tecnológico adequado, submetida a tratamento que assegure sua apresentação e conservação até o momento do consumo (BRASIL, 2009). O sabor do produto é resultado da interação entre as características de odor, gosto e sensações táteis. O tipo de fruta, bem como de sua variedade, maturidade, condições climáticas, práticas de cultura e processamento, influenciam em sua composição e, consequentemente, em suas características sensoriais (TEIXEIRA, 2009).

O crescimento do mercado de sucos de frutas caracteriza-se por uma série de fatores, dentre os quais, a demanda dos consumidores por alimentos saudáveis, com pouco ou nenhum aditivo químico, em decorrência da preocupação com a saúde (SANTOS et al., 2008b). No Brasil, onde existem mais de 20 polos de fruticultura distribuídos nas regiões Norte (frutas nativas da Amazônia, com destaque para o açaí e o cupuaçu), Sul (frutas de clima temperado) e Nordeste (culturas irrigadas no semiárido), segue-se a mesma tendência mundial, e por isso as indústrias se beneficiam da abundante disponibilidade de matéria-prima (OLIVEIRA et al., 2009).

O aumento da demanda dos consumidores por alimentos naturais é um fenômeno mundial, pois os consumidores têm modificado seus hábitos alimentares tornando-se conscientes da relação entre dieta e prevenção de doenças (FRANÇA, 2012). Neste contexto, os sucos de frutas prontos para o consumo têm ganhado destaque, face às suas características de conveniência, boa qualidade nutritiva, fonte de carboidratos, vitaminas e minerais, e aspecto refrescante. Mas, apesar da demanda por produtos de fruta ter aumentado, a elaboração de sucos a partir da fruta *in natura* tornou-se um inconveniente ao ritmo de vida acelerado da sociedade. Por isso, os consumidores demonstram interesse crescente em consumir sucos prontos para o consumo (CARMO; DANTAS; RIBEIRO, 2014).

No Brasil, a demanda por sucos prontos para o consumo este tipo de produto começou de maneira incipiente na década de 50, recebendo grande impulso e

investimentos no início da década seguinte, quando fenômenos climáticos adversos nos Estados Unidos geraram a procura do suco de laranja brasileiro. A falta do produto no mercado possibilitou ao Brasil assumir um papel de liderança na produção, com destaque para os derivados da laranja (MONTEIRO, 2006). Na década de 80, especialmente na região nordeste, a fruticultura esteve presente no desenvolvimento agroindustrial do litoral com a produção de sucos de caju, laranja, goiaba e acerola (LACERDA; LACERDA, 2004), impulsionando, a partir da década de 90, o surgimento de diversas marcas de sucos de frutas industrializados e a ampliação desse setor no mercado nacional.

Estimativas do IBRAF apontaram para a evolução da produção de sucos, néctares e drinques à base de frutas no Brasil, que passaria de 140 milhões de litros em 2000 para 350 milhões em 2004. Nesse intervalo, o mercado de sucos industrializados prontos para o consumo cresceu em uma taxa 15,6%, superior ao dos refrigerantes, que foi apenas de 6,54% (MONTEIRO, 2006). Entre os anos de 2011 e 2016 houve um aumento nas vendas de sucos de, aproximadamente, 1,5 para 2,3 milhões de litros (EUROMONITOR INTERNATIONAL, 2017a). Em 2016, a indústria de bebidas faturou R$ 117 bilhões, o que correspondeu a 1,9% do Produto Interno Bruto brasileiro e a 4,8% do valor bruto da produção da indústria de transformação (ASSOCIAÇÃO DAS INDÚSTRIAS DA ALIMENTAÇÃO, 2017).

Apesar das maiores produções estarem concentradas nas regiões Sul e Sudeste do país (CUNHA, 2009), a região Nordeste produziu e exportou sucos concentrados de laranja (Sergipe e Bahia), abacaxi (Paraíba e Sergipe) e maracujá (Bahia e Sergipe) no triênio 2003/2005 e aumentou suas vendas externas em 117% (SANTOS; BRAINER, 2007). Em ordem decrescente, os principais estados produtores de sucos na região Nordeste são Bahia, Pernambuco, Ceará e Paraíba. Dados do ano de 2012 apontaram que o suco de laranja permaneceu na primeira posição dentre os mais exportados (1,9 milhão t) (IBRAF, 2011). Em 2015, a exportação do suco de abacaxi surpreendeu e cresceu quase 371% em relação a 2014, atingindo US$ 17,4 milhões (TREICHEL et al., 2016).

Em relação ao consumo, o volume total consumido de sucos, néctares e drinques a base de frutas no Brasil passou de 115 milhões para 399 milhões de litros entre os anos de 1999 e 2005, incremento de quase 247% (FERNANDES; DANTAS, 2006). No mesmo ano, os consumidores da região Nordeste consumiram 13% do total de sucos prontos para beber. Portanto, verifica-se que houve um incremento no

consumo de sucos prontos no período analisado, de 13 e 11%, em receitas geradas e volume comercializado, respectivamente (SANTOS et al., 2008b). É importante destacar que desde 2005 tem ocorrido um contínuo crescimento da produção e do consumo de bebidas prontas para o consumo, sendo consumidos 550 milhões de litros de sucos no ano de 2010 (IBRAF, 2011). Nos últimos cinco anos esse número tem crescido na casa dos dois dígitos (TREICHEL et al., 2016). Estima-se que o crescimento no consumo de sucos no Brasil entre os anos de 2016 e 2020 seja em média de 3% (EUROMONITOR INTERNATIONAL, 2017b).

A demanda atual é crescente para sucos de frutas tropicais, principalmente de abacaxi e manga. Estes sucos, assim como os de caju e goiaba, podem ser considerados os mais populares no Brasil e, principalmente, no Nordeste, região onde estas frutas são produzidas em grandes quantidades (CUNHA, 2009). Em 2006, os Estados Unidos e países africanos importaram do Brasil néctares de abacaxi, acerola, caju, goiaba, manga e maracujá. No mesmo ano, sucos concentrados de abacaxi, maracujá e manga foram exportados para 26 países da Europa, África, Oriente Médio, Oceania, Ásia e América (SANTOS et al., 2008b).

A Associação das Indústrias Processadoras de Frutos Tropicais (ASTN) estabeleceu estratégias e definiu um plano de exportação contemplando inicialmente os sabores de abacaxi, acerola, caju, goiaba, manga, pitanga e maracujá (SANTOS et al., 2008b). O Brasil ainda participa timidamente do comércio internacional de suco de abacaxi, apesar do aumento de 371% na exportação no ano de 2015 e de ser um dos maiores produtores da fruta (TREICHEL et al., 2016). Em julho de 2017, a produção alcançou 1,6 bilhão de frutos, aumento de 3,4% em relação ao mês anterior. Em Pernambuco, a produção aumentou em 64,5% (IBGE, 2017).

2.2 CONTAMINAÇÃO MICROBIANA EM SUCOS DE FRUTAS

Os micro-organismos podem contaminar as frutas no campo, na colheita, no tratamento pós-colheita ou durante o armazenamento e distribuição (BARTH et al., 2009). Naturalmente, as frutas frescas possuem uma barreira protetora (casca) que age de forma eficaz na inibição da penetração da maior parte dos micro-organismos. Contudo, tal proteção é eliminada durante as etapas de transformação para obtenção de produtos derivados. A retirada da casca expõe a polpa do fruto a uma possível contaminação microbiana (BALLA; FARKAS, 2006). Por isso, em 1998, a

FDA publicou o *Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables*, que fornece diretrizes para uma série de práticas de segurança alimentar, por meio de medidas rigorosas de controle na cadeia produtiva destes alimentos. O objetivo deste guia é minimizar os riscos microbianos em frutas e hortaliças frescas e em seus produtos derivados (USFDA, 1998).

Os principais fatores que determinam a colonização de frutas e seus derivados por micro-organismos são fatores intrínsecos, como a atividade de água, pH, potencial redox, e outros. Mas também estão associados os tratamentos tecnológicos, condições ambientais e do manejo das matérias-primas e do produto durante o processamento e armazenamento (MONTVILLE; MATTHEWS, 2001). O pH destaca-se por se um dos fatores de maior influência na microbiologia dos sucos de frutas. A acidez elevada era reconhecida como sendo suficiente para evitar a proliferação de micro-organismos. Porém, este conceito foi modificado a partir dos relatos de deterioração de suco de maçã pasteurizado por bactérias tolerantes a acidez (CERNY; HENNICH; PORALLA, 1984).

Atualmente, sabe-se que a deterioração microbiana de frutas e produtos derivados ocorre principalmente devido à contaminação por fungos filamentosos (ex. *Penicillium, Aspergillus, Alternaria, Cladosporium, Botrytis* e *Rhizopus*), leveduras (ex. *Saccharomyces, Candida, Torulopsis, Pichia, Rhodotorula* e *Hansenula*), bactérias lácticas e acéticas (ex. *Acetobacter* e *Gluconobacter*), assim como bactérias do gênero *Pseudomonas, Bacillus* e *Alicyclobacillus* (RAYBAUDI-MASSILIA et al., 2009; TOURNAS; HEERES; BURGESS, 2006). As leveduras são responsáveis pelo sabor fermentado e produção de dióxido de carbono (CO_2), enquanto as bactérias lácticas, principalmente as pertencentes aos gêneros *Lactobacillus* (ex. *L. fermentum* e *L. Plantarum*) e *Leuconostoc* (ex. *L. mesenteroides*) podem produzir aroma e sabor desagradáveis, devido à formação de ácido láctico, etanol, acetato, CO_2, diacetil e acetoína. Bactérias pertencentes ao gênero *Acetobacter* podem crescer na presença de oxigênio dissolvido, utilizando o álcool para produção de ácido acético (ANEJA et al., 2014).

Desde o início da década de 1990 tem havido um aumento no consumo de frutas e hortaliças, assim como, na incidência de doenças de origem alimentar causadas por estes alimentos e seus produtos derivados. Contudo, o número de surtos associados ao consumo sucos de frutas pasteurizados ou não aumentou de 0 a 1 surto por ano para até 5 surtos por ano (CDC, 2007; EFSA 2013, 2015; HARRIS

et al., 2003; POWELL; LUEDTKE, 2000). Portanto, é evidente a possível sobrevivência e crescimento de micro-organismos como *E. coli* O157:H7, *Salmonella* spp., *Shigella* spp., *L. monocytogenes*, *Staphylococcus aureus, Campylobacter jejuni* e Cryptosporidium spp. nestes produtos (MIHAJLOVIC et al., 2013; RAYBAUDI-MASSILIA et al., 2009; VANTARAKIS et al., 2011).

Os sucos de frutas não eram considerados como alimentos de risco no que diz respeito à agentes patogênicos devido à sua natureza ácida (TAUXE, 1997). No enatnto, sucos ácidos (pH ≤ 4,6) contendo patógenos bacterianos entéricos têm causado graves surtos de doenças de origem alimentar. Há relatos da ocorrência de óbtios de uma criança e um homem idoso após o consumo de suco contaminado. Por isso, em decorrência do uso de matéria-prima contaminada e da capacidade de alguns desses agentes patogênicos de sobreviver em alimentos ácidos, juntamente ao processamento insuficiente e/ou de condições de armazenagem inadequadas, os sucos de frutas podem representar um risco à saúde dos consumidores (UDFDA, 2004).

Bactérias patogênicas causadoras de gastroenterites, tais como *E. coli* O157:H7, *Listeria* spp. e *Salmonella* spp., podem contaminar estes produtos e permanecer viáveis por um longo período de tempo (≥ 30 dias) (RAYBAUDI-MASSILIA et al., 2009), devido ao desenvolvimento de resistência ao meio ácido e ao calor. Destaca-se *E. coli* O157:H7, que tem sido considerado o patógeno mais resistente a estas condições (MAZZOTTA, 2001). Cepas desses micro-organismos já demonstraram ser ácido-tolerantes em sucos de laranja e maçã (ALBASHAN, 2009). Acredita-se, que estas cepas sejam relativamente resistentes ao pH ácido devido à sua capacidade de produzir algumas proteínas, chamadas de proteínas do estresse, cuja produção é induzida pelo ambiente ácido (CEBRIÁN et al., 2009; KIM; CHO, 2010).

2.3 MÉTODOS PARA CONSERVAÇÃO DE SUCOS DE FRUTAS

Os alimentos de origem vegetal caracterizam-se por se deteriorar com facilidade logo após a colheita. Por isso, toda alteração advinda dos micro-organismos, enzimas e outras causas deteriorantes deve ser evitada. Assim, os processos de conservação são baseados na eliminação total ou parcial dos agentes que alteram os produtos ou na modificação (ou supressão) de um ou mais fatores

essenciais, de modo que o meio se torne impróprio a qualquer atividade bioquímica ou manifestação vital (GAVA, 2009). Estes métodos podem ser classificados, de maneira geral, em três grupos: aqueles que inibem parcial ou completamente o crescimento microbiano (ex. refrigeração, congelamento, desidratação, salga, controle da atividade de água e uso de conservante); os que inativam os micro-organismos (ex. esterilização e pasteurização); e os que restringem seu acesso aos alimentos (ex. embalagem e processamento asséptico) (FRANCO; LANDGRAF, 2008).

Para melhorar a segurança e a vida de prateleira, os sucos de frutas são, geralmente, tratados pelo calor e pela ação de aditivos químicos da classe dos conservantes. O tratamento térmico tem por objetivo inativar enzimas e reduzir o número de micro-organismos, ambos indesejáveis e que podem deteriorar o produto, sendo a pasteurização, o método comumente utilizado. No entanto, apesar do aquecimento ser o método mais simples e confiável para eliminar a quase totalidade da carga microbiana dos alimentos, este procedimento muitas vezes altera características sensoriais e nutricionais do produto (PENG et al., 2017; VEGARA et al., 2013). O tratamento pelo calor excessivo causa a desnaturação indesejável das proteínas, escurecimento não enzimático (Reação de Maillard), perda de vitaminas e compostos de aroma (LADO; YOUSEF, 2002). Além disso, a pasteurização por si só não é considerada um método eficaz para conservação de alimentos, sendo necessárias outras tecnologias, como o uso de aditivos químicos, para manutenção da qualidade do alimento durante as etapas de armazenamento, distribuição e comercialização.

A incorporação de aditivos aos alimentos possui a finalidade de conservá-los ou de melhorar suas características gerais, produzindo um impacto favorável à sua aceitação pelo consumidor. Devido aos avanços da indústria química, o setor produtor de alimentos tem se beneficiado de novas substâncias que podem ser adicionadas aos seus produtos, não só para conservar, mas também para conferir melhor aparência, aroma, sabor e textura. Os conservantes são utilizados nos alimentos para eliminar, total ou parcialmente, os micro-organismos ou para criar condições desfavoráveis ao seu crescimento. Estes aditivos podem ser utilizados individualmente ou associado a métodos físicos, o que proporciona melhores resultados e otimização das características sensoriais dos alimentos, sempre em conformidade com os limites estabelecidos pela legislação (GAVA, 2009).

Os conservantes mais utilizados em alimentos são os bacteriostáticos e fungistáticos, que atuam inibindo o crescimento dos micro-organismos, porém mantendo suas características por um maior período de tempo. Para sucos de frutas, os mais utilizados e permitidos pela legislação brasileira são o ácido benzoico e seus sais de sódio, cálcio e potássio, com concentração máxima permitida de 0,05 g/100 mL; ácido sórbico e seus sais de sódio, potássio e cálcio, com concentração máxima permitida de 0,08 g/100 mL para bebidas sem gás; e dióxido de enxofre, com concentração máxima permitida de 0,004 g/100 mL (BRASIL, 2007). No entanto, os conservantes utilizados, como benzoato de sódio, sorbato de potássio e dióxido de enxofre, podem ser tóxicos e responsáveis pela ocorrência de alergias e câncer (AMIRPOUR et al., 2015; TONIOLO et al., 2010).

Como regra geral, os melhores métodos de conservação são aqueles que garantem sua conservação e que causam mínimas alterações às características do produto. Além disso, a FDA recomendou que os sucos devem ser processados para atingir uma redução de pelo menos cinco ciclos logarítmicos (99,999%) na população de patógenos relevantes para a saúde (USFDA, 2001), mas não especifica o método para alcançar este nível de inativação. Portanto, diante das desvantagens e consequências do uso dos métodos tradicionais na conservação deste tipo de produto, há uma busca por tecnologias alternativas que possibilitem preservar suas características. Além disso, a utilização de aditivos químicos tem gerado uma ampla discussão acerca das vantagens e desvantagens deste método, principalmente, devido à demanda atual dos consumidores por alimentos saudáveis, seguros e isentos de substâncias químicas (FRANÇA et al., 2012).

O processo de conservação de alimentos vem assumindo grande complexidade diante da perspectiva de os consumidores exigirem alimentos de melhor qualidade, tendo sido submetidos ao menor processamento possível, com um mínimo de alterações nutricionais, físico-químicas e sensoriais, mas que apresentem a conveniência de possuir larga vida útil, além de mostrarem-se inócuos ao consumidor (MORALES-DE LA PEÑA et al., 2011). Neste cenário, surge uma nova discussão sobre opções inovadoras e emergentes para o alcance da segurança microbiológica dos alimentos, a citar o uso de embalagens ativas, antimicrobianos naturais (óleos essenciais), campo elétrico pulsado, radiação ultravioleta e altas pressões (HYLDGAARD; MYGIND; MEYER, 2012; MOHAMED; EISSA, 2012; MOSQUEDA-MELGAR; RAYBAUDI-MASSILIA; MARTÍN-BELLOSO,

2012; PATRIGNANI et al., 2013). Estas tecnologias, consideradas como não térmicas, estão sendo pesquisadas objetivando-se avaliar o seu potencial como um processo alternativo ou complementar aos métodos tradicionais de preservação dos alimentos (ESPINA et al., 2013; LADO; YOUSEF, 2002; MOSQUEDA-MELGAR; RAYBAUDI-MASSILIA; MARTÍN-BELLOSO, 2008). Desta forma, são esperadas perdas mínimas de nutrientes e vitaminas, e alterações de sabor quase imperceptíveis (AIT-OUAZZOU et al., 2013; KHANDPUR; GOGATE, 2016; TYAGI et al., 2014).

2.4 USO DE ÓLEOS ESSENCIAIS PARA CONSERVAÇÃO DE SUCOS DE FRUTAS

Dentre as tendências em tecnologia de alimentos, com as características relacionadas anteriormente, pode-se destacar os antimicrobianos naturais, como os óleos essenciais (CALO et al., 2015). Óleos essenciais são compostos voláteis, naturais e complexos, caracterizados por um forte odor e por fazerem parte de plantas aromáticas como metabólitos secundários. São extraídos de várias espécies de plantas aromáticas por destilação a vapor, processos mecânicos ou destilação seca, e apresentam-se líquidos, límpidos e raramente coloridos, lipossolúveis e solúveis em solventes orgânicos, com densidade geralmente menor que a da água (ISO 9235, 2013). Podem ser sintetizados por todos os órgãos da planta (broto, flores, folhas, caules, galhos, sementes frutos, raízes ou casca) e são armazenados em células secretoras, cavidades, canais, células epidérmicas ou tricomas glandulares (BURT, 2004). Os óleos essenciais contêm compostos orgânicos de diferentes classes (hidrocarbonetos terpênicos, alcoóis simples, aldeídos, cetonas, fenóis, ésteres e ácidos orgânicos), em diferentes concentrações (BAKKALI et al., 2008).

As propriedades biológicas dos óleos essenciais atreladas a uma baixa toxicidade para humanos têm viabilizado o seu uso, especialmente, pelas indústrias farmacêuticas, sanitárias, cosméticas e de alimentos, particularmente com a exploração de suas propriedades antimicrobianas (BAKKALI et al., 2008). Suas principais vantagens como agentes antimicrobianos são o seu amplo espectro de atividade e seu baixo risco de desenvolver resistência microbiana, pois são constituídos por uma grande variedade de compostos, com diferentes mecanismos

de atividade antimicrobiana, tornando mais difícil uma possível adaptação dos micro-organismos frente a sua ação (GOMES NETO et al., 2012; LUZ et al., 2012; SOUZA, 2016). Caracterizam-se ainda como produtos naturais, Geralmente Reconhecidos como Seguros (GRAS), que apresentam eficácia no controle de micro-organismos patogênicos e deteriorantes de importância em alimentos (BASSOLÉ; JULIANI, 2012; LÓPEZ et al., 2005; MITH et al., 2014).

A efetividade antimicrobiana dos óleos essenciais depende do tipo da planta, da concentração do óleo essencial, do tipo e concentração do micro-organismo alvo, da composição e pH do substrato e da temperatura (AIT-OUAZZOU et al., 2012; HYLDGAARD; MYGIND; MEYER, 2012; MANSO et al., 2015). Alguns autores, sugerem que a atividade antimicrobiana se deve também ao equilíbrio entre os componentes majoritários e os que aparecem em menor proporção (RIAHI et al., 2013). Acredita-se que o possível mecanismo de ação seja pela presença de constituintes que alteram a bicamada lipídica da membrana celular microbiana, aumentando sua permeabilidade, com posterior liberação de constituintes intracelulares vitais, ou ainda por causar danos em seus sistemas enzimáticos (SOUSA et al., 2015; TURINA et al., 2006).

O sucesso dos testes antimicrobianos conduzidos *in vitro* tem sugerido a aplicação de óleos essenciais em diferentes matrizes alimentares (CARVALHO et al., 2015; GUERRA et al., 2015; HONORIO et al, 2015; OLIVEIRA et al., 2015; SMITH-PALMER; STEWART; FYFE, 2001). Em alimentos de origem vegetal, a atividade antimicrobiana de óleos essenciais pode ser beneficiada pelas baixas temperaturas empregadas nestes produtos e/ou pelo baixo pH do alimento (< 4,5) (GUTIERREZ; BARRY-RYAN; BOURKE, 2009). Ainda, o baixo conteúdo de lipídios pode contribuir para o sucesso da aplicação dos óleos essenciais, pois acredita-se na presença de grande quantidade de gordura, os compostos terpênicos hidrofóbicos estariam menos disponíveis para interagir com as células bacterianas (BURT, 2004).

Óleos essenciais já testados em alimentos de origem vegetal e seus produtos derivados demonstraram ser eficazes contra micro-organismos patógenos e deteriorantes (AZERÊDO et al., 2011; BARBOSA et al., 2016; GUTIERREZ et al., 2008; LEITE et al., 2016; RAYBAUDI-MASSILIA et al., 2006). No entanto, para o sucesso da aplicação em alimentos, é fundamental considerar o impacto que podem causar sobre os aspectos gerais de qualidade dos produtos, já que podem promover

características desagradáveis, com implicações no sabor e odor dos produtos, impactando diretamente na sua aceitação (ESPINA; GARCÍA-GONZALO; PAGÁN, 2014a,b). O potencial antimicrobiano de óleos essenciais e/ou seus constituintes e os possíveis impactos sobre indicadores de qualidade de sucos já foram relatados na literatura (SOUSA GUEDES et al., 2016).

Dentre as plantas aromáticas que têm demonstrado potencial propriedade antibacteriana, pode-se citar as do gênero *Mentha* spp., o qual pertence à família Lamiaceae e consiste em 18 espécies, com destaque para *M. piperita* L. (Figura 1) e *M. arvensis* L. (Figura 2) (ARRUDA et al., 2006; ISCAN et al., 2002; SINGH; SHUSHNI; BELKHEIR, 2011; TYAGI; MALIK, 2011). As espécies do gênero menta, conhecidas no Brasil como hortelã, geralmente são usadas para fins medicinais e nos alimentos como condimentos (GRISI et al., 2006). Os óleos essenciais são produzidos e armazenados em tricomas glandulares, os quais estão presentes principalmente nas folhas e flores e em menores densidades nos caules (DESCHAMPS et al., 2013).

Figura 1 – *Mentha piperita* L.

Fonte: Berg; Schmidt (1891-1893)

Figura 2 – *Mentha arvensis* L.

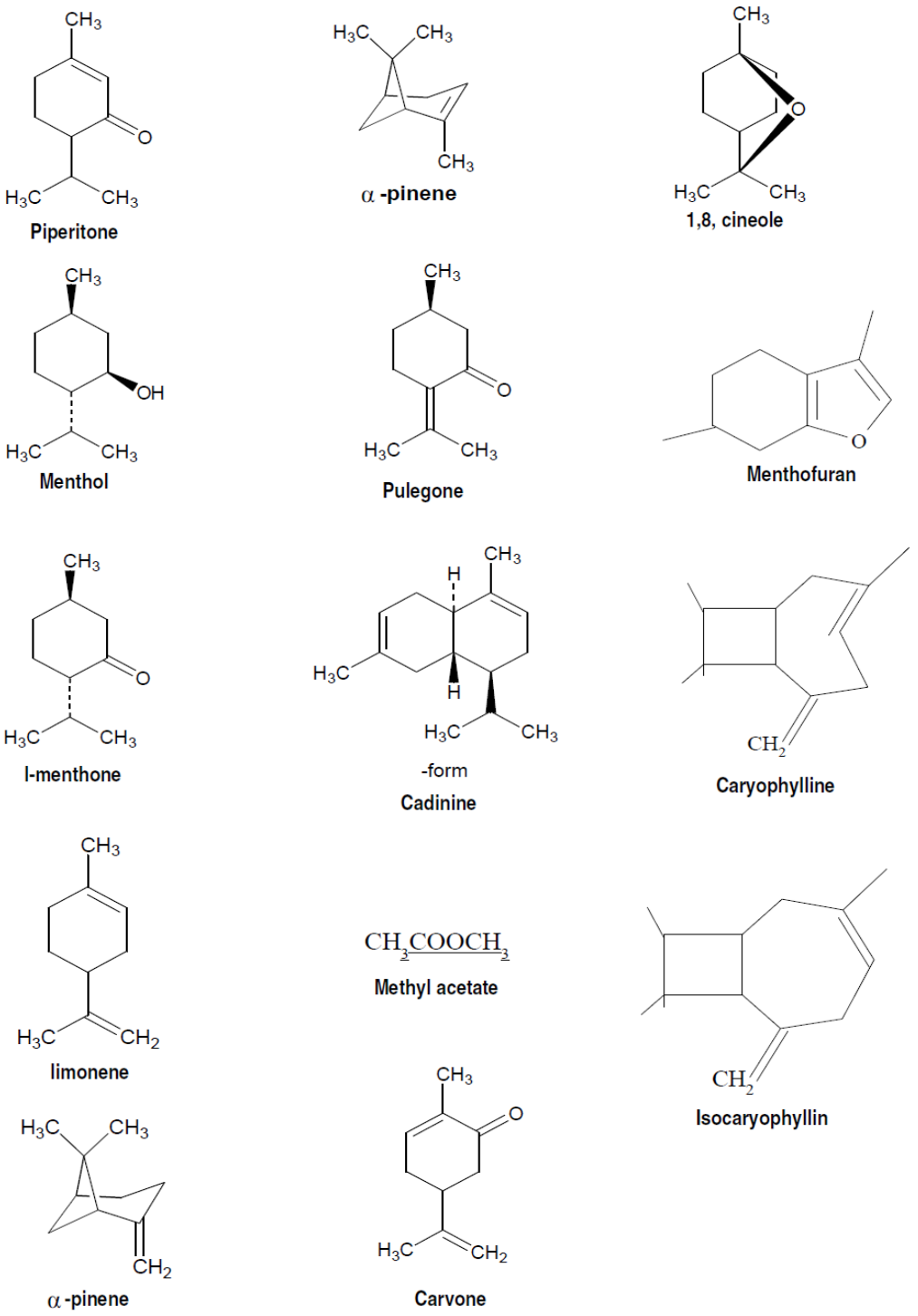
Fonte: van Eeden (1877)

M. piperita L. (hortelã-pimenta) é uma planta herbácea rizomatosa, de espalhamento rápido, perene e dura, cultivada na maior parte do mundo. Atinge de 30 a 90 cm de altura, com hastes lisas. As folhas ficam transversalmente opostas uma à outra na haste, são alongadas, ovais com um ápice agudo e margens grosseiramente dentadas. Tradicionalmente, a *M. piperita* L. tem sido utilizada na medicina popular. As folhas são usadas em chá de ervas e para fins culinários (NEERAJ; PRAKASH; SEEMA, 2013; PRAMILA et al., 2012). *M. arvensis* L. (hortelã-doce) é uma planta herbácea estolonífera, de caule quadrangular com folhas opostas, ovaladas e serradas. É também uma das espécies de hortelã mais cultivadas no Brasil e no mundo (WATANABE et al., 2006).

As espécies de hortelã diferem entre si morfologicamente e em relação à variedade e quantidade dos constituintes (Figura 3) de seus óleos essenciais (SILVA et al., 2006). O óleo essencial de *M. piperita* L. é um dos mais populares e amplamente utilizados devido aos seus componentes majoritários, mentol e mentona, que correspondem a 30-50% e 14-32% do óleo essencial,

respectivamente (GUERRA et al., 2015; TYAGI et al., 2013). O óleo essencial de *M. arvensis* L. também possui um grande número de compostos, como por exemplo, mentol, neomentol, isomentol, isomentona, cineol, p-cymene, piperitona, carvomentona, pineno, carvona e linalool (VERMA et al., 2010).

Figura 3 – Alguns constituintes dos óleos essenciais de *Mentha* spp.



Fonte: Adaptado de Alankar (2009).

O reconhecido efeito antimicrobiano dos óleos essenciais de *Mentha* spp. está associado às elevadas quantidades de mentol, o qual, provavelmente, ocasiona perturbação na membrana plasmática, induzindo alterações de permeabilidade e liberação de materiais intracelulares. Isto ocorre, principalmente, devido ao fato do mentol apresentar a capacidade de migrar em ambientes aquosos e interagir com os fosfolipídios de membrana (SCHELZ; MOLNAR; HOHMANN, 2006; TROMBETTA et al., 2005).

3 HIPÓTESE

Os óleos essenciais de *M. arvensis* L. ou de *M. piperita* L. em concentrações inibitórias e subinibitórias apresentam efetividade no controle do crescimento das bactérias patogênicas *E. coli*, *L. monocytogenes* e *Salmonella* Enteritidis quando adicionados em sucos de abacaxi, caju, goiaba e manga, assegurando a manutenção ou melhora dos seus aspectos de qualidade físico-química e sensorial quando armazenados em temperatura de refrigeração.

4 OBJETIVOS

4.1 OBJETIVO GERAL

Avaliar a eficácia dos óleos essenciais de *M. arvensis* L. e *M. piperita* L. no controle de bactérias patogênicas em sucos de abacaxi, caju, goiaba e manga, bem como os efeitos desta aplicação sobre a qualidade destes sucos.

4.2 OBJETIVOS ESPECÍFICOS

- Caracterizar quimicamente os óleos essenciais de *M. arvensis* L. e *M. piperita* L.;
- Avaliar o potencial dos óleos essenciais de *M. arvensis* L. e *M. piperita* L. na inibição do crescimento de cepas de bactérias patogênicas em inóculo misto cultivadas em meio laboratorial e em sucos de frutas;
- Identificar os danos causados pelos óleos essenciais de *M. arvensis* L. e *M. piperita* L. nas células bacterianas;
- Verificar a influência dos óleos essenciais de *M. arvensis* L. e *M. piperita* L. sobre indicadores físico-químicos de qualidade e atributos sensoriais dos sucos de frutas.

5 MÉTODOS

Os experimentos foram conduzidos no Laboratório de Microbiologia e Bioquímica de Alimentos e no Laboratório de Técnica Dietética, pertencentes ao Departamento de Nutrição, Centro de Ciências da Saúde, e também no IPeFArM – Instituto de Pesquisas em Fármacos e Medicamentos/NUCAAL – Núcleo de Caracterização e Análises, todos localizados no Campus I da Universidade Federal da Paraíba, João Pessoa – PB.

5.1 OBTENÇÃO DOS ÓLEOS ESSENCIAIS

Os óleos essenciais de *M. arvensis* L. (hortelã japonesa; lote 134; densidade a 20 °C, 0,897; índice de refração a 20 °C, 1,459; pH 5,76) e *M. piperita* L. (hortelã-pimenta; lote 181; densidade a 20 °C, 0,901; índice de refração a 20 °C, 1,460; pH 5,17), extraídos por destilação a vapor, foram obtidos da empresa Ferquima Indústria e Comércio de Óleos Essenciais Ltda (São Paulo, Brasil). As soluções dos óleos foram preparadas em caldo Brain Heart Infusion – BHI (HiMedia, Índia), nas concentrações de 80 (pH 7,49), 40 (pH 7,50), 20 (pH 7,51), 10 (pH 7,52), 5 (pH 7,53), 2,5 (pH 7,53), 1,25 (pH 7,53), 0,625 (pH 7,54) e 0,312 (pH 7,54) µL/mL, acrescidos de Tween 80 (1% v/v; Sigma-Aldrich, USA) como agente emulsificante (LEITE et al., 2016). Na concentração ensaiada (1%, v/v), o Tween 80 não apresentou efeito inibidor contra as cepas bacterianas testadas.

5.2 MICRO-ORGANISMOS E CONDIÇÕES DE CRESCIMENTO

Cepas tipo padrão de *E. coli* (UFPEDA 224), *L. monocytogenes* (ATCC 7644) e *Salmonella* Enteritidis (UFPEDA 414) foram utilizadas como micro-organismos teste nos ensaios antimicrobianos. As culturas de *E. coli* (UFPEDA 224) e *Salmonella* Enteritidis (UFPEDA 414) foram obtidas da Coleção de Cultura de Micro-organismos do Departamento de Antibióticos, Centro de Ciências Biológicas, Universidade Federal de Pernambuco (UFPE) (ANEXO A), e a cultura de *L. monocytogenes* (ATCC 7644) da Coleção de Micro-organismos de Referência do Instituto Nacional de Controle de Qualidade em Saúde (INCQS), Rio de Janeiro – RJ, e estocadas no Laboratório de Microbiologia e Bioquímica de Alimentos do

Departamento de Nutrição (Centro de Ciências da Saúde, Universidade Federal da Paraíba) em criotubos contendo caldo Brain Heart Infusion (BHI) (HiMedia, Índia) adicionado de glicerol (15 g/100 mL) a -20 °C.

Os inóculos das cepas foram obtidos a partir de culturas *overnight E. coli* e *Salmonella* Enteritidis incubadas a 37 °C e *L. monocytogenes* incubadas a 30 °C por 18–24 h (fase estacionária) em caldo BHI (HiMedia, Índia). Após o período de incubação, as culturas foram centrifugadas (4.500 x *g*, 15 min, 4 °C), lavadas duas vezes em solução salina estéril (NaCl a 0,85% p/v) e ressuspendidas em caldo BHI (HiMedia, Índia). As suspensões foram ajustadas em espectrofotômetro para uma densidade óptica de 1,0 (OD_{625}), correspondente a um inóculo de aproximadamente 8 $\log_{10}$ UFC/mL (MCMAHON et al., 2008). Para a preparação do inóculo misto, após o procedimento supracitado, as suspensões de todas as cepas foram misturadas na proporção de 1:1:1 (contagem final de cada cepa de aproximadamente 7 $\log_{10}$ UFC/mL) e o *pool* obtido foi utilizado nos ensaios de inibição do crescimento bacteriano (OLIVEIRA et al., 2015). Esta concentração de inóculo foi utilizada para que fosse possível quantificar uma redução de 5 $\log_{10}$ UFC/mL na contagem de células viáveis, recomendada pela USFDA (USFDA, 2001).

5.3 OBTENÇÃO DA MATRIZ ALIMENTAR EXPERIMENTAL

Os sucos utilizados no estudo como matrizes experimentais foram os de abacaxi (*Ananas comosus* L. Merril), caju (*Anacardium occidentale* L), goiaba (*Psidium guajava* L.) e manga (*Mangifera indica* L.), considerando a produção abundante destas frutas na região nordeste (CUNHA, 2009). As frutas maduras foram adquiridas em um distribuidor local de João Pessoa – PB e selecionadas de acordo com tamanho, forma, aparência e ausência de danos mecânicos ou sinais visíveis de infecção. Para seleção das frutas foram considerados os seguintes estádios de maturação: abacaxi – parcialmente alaranjado; caju – pedúnculo laranja escuro; goiaba – amarela; manga – mais de 70% da fruta amarela (CAVALINI et al., 2006; COSTA, 2009; LOPES et al., 2011; SANTOS et al., 2008a). Para a preparação de cada suco, os frutos foram imersos durante 5 min em uma solução de hipoclorito de sódio (150 ppm, pH 7,2 ajustado com NaOH a 1 M), para a desinfecção da superfície, lavados com água destilada estéril e secos durante 1 h em cabine de biossegurança. Em seguida, as frutas foram descascadas, picadas e misturadas

com água destilada (1:1) usando um liquidificador doméstico (3 min). Os sucos obtidos foram centrifugados (12.500 rpm, 15 min, 4 °C) para separar a polpa do líquido sobrenadante, os quais foram filtrados usando uma camada tripla de gaze e esterilizados em autoclave (121 °C, 1,1 atm, 15 min). Os sucos filtrados e esterilizados foram utilizados nos ensaios de determinação da concentração inibitória mínima, nos testes de viabilidade das cepas e na análise de citometria de fluxo. Os sucos foram armazenados em alíquotas de 50 mL à temperatura de -20 °C, e, quando necessário, uma alíquota foi descongelada sob refrigeração (4 ± 1 °C) e utilizada para os ensaios subsequentes (LEITE et al, 2016; RAYBAUDI-MASSILIA et al., 2006).

5.4 IDENTIFICAÇÃO DOS CONSTITUINTES DOS ÓLEOS ESSENCIAIS

Os componentes individuais de cada óleo essencial foram identificados através de cromatografia em fase gasosa acoplada a espectrometria de massa (GC/MS), usando um cromatógrafo CGMS-QP2010 Ultra Shimadzu (Kyoto, Japan), sob as seguintes condições analíticas: coluna capilar RTX-5MS (30 m x 0,25 mm x 0,25 mm); programação de temperatura: 60-240 °C (3 °C/min); temperatura do injetor: 250 °C; temperatura do detector: 220 °C; gás de arraste: hélio; taxa: 0,99 mL/min de fluxo; impacto de elétrons: 70 eV; e faixa de massa (*m/z*): 40-500. Cada componente foi identificado após a comparação dos espectros de massa de cada composto com a base de dados GC/MS (NIST/EPA/NIH de Massa Espectral Database Versão 1.7). Os componentes foram quantificados, depois normalizadas as áreas e expressos em porcentagem total de área (%) (ADAMS, 2001). A caracterização química dos óleos essenciais foi necessária pois a empresa fornece apenas os dados de concentração dos constituintes principais.

5.5 ENSAIOS DE ATIVIDADE ANTIMICROBIANA

5.5.1 Determinação da concentração inibitória mínima (CIM) dos óleos essenciais

Os valores da CIM dos óleos essenciais de *M. arvensis* L. e *M. piperita* L. foram determinados pelo método de microdiluição em caldo, de acordo com a

padronização do Clinical and Laboratory Standards Institute (CLSI, 2015). Para esta análise, 50 µL de cada uma das emulsões de cada óleo essencial (80 – 0,312 µL/mL) foram distribuídos em poços de uma microplaca de 96 poços contendo 100 µL de caldo BHI (HiMedia, Índia). Subsequentemente, alíquotas de 50 µL da suspensão bacteriana mista foi adicionado a cada poço (contagem viável final de aproximadamente 6 $\log_{10}$ UFC/mL). A microplaca foi envolvida com filme plástico para evitar a desidratação bacteriana e a volatilização dos óleos essencias e incubada a 37 °C durante 24 h. Cada placa incluía também poços controle (sem os óleos essenciais) e poços não-inoculados (sem os micro-organismos). A CIM foi definida como a mais baixa concentração de cada óleo essencial capaz de inibir o crescimento bacteriano visível (NOSTRO et al., 2001).

5.5.2 Ensaio de viabilidade das cepas bacterianas frente aos óleos essenciais

O efeito de diferentes concentrações (CIM, CIM/2, CIM/4 e CIM/8) de cada óleo essencial nas contagens do inóculo misto de bactérias patogênicas, em meio laboratorial e nos sucos de abacaxi, caju, goiaba e manga, armazenados por 24 h em temperatura de refrigeração (4 ± 1 °C), foi avaliado pelo método de contagem de células viáveis. Inicialmente, 150 µL da suspensão bacteriana mista foi inoculada em 5850 µL de caldo BHI (HiMedia, Índia) e de cada suco de fruta (contagem viável final de aproximadamente 6 $\log_{10}$ UFC/mL), contendo o óleo essencial de *M. arvensis* L. ou de *M. piperita* L. nas concentrações finais desejadas. As diferentes misturas foram suavemente agitadas à mão durante 30 segundos e posteriormente incubadas a 4 ± 1 °C (temperatura de armazenamento adequada dos sucos) durante 24 h. Nos intervalos de 0 (imediatamente depois da homogeneização), 1, 2, 4, 8, 12 e 24 h após a incubação, uma alíquota de 100 µL de cada mistura foi diluída em série em solução salina estéril (NaCl a 0,85% p/v), e, subsequentemente, alíquotas de 20 µL de cada diluição foram inoculados em ágar seletivo correspondente a cada espécie formadora do inóculo bacteriano misto: ágar Eosina Azul de Metileno (HiMedia, Índia) para *E. coli*, ágar seletivo para *Listeria* spp. + Listeria Selective Supplement II para *L. monocytogenes* (HiMedia, Índia) e Salmonella-Shigella (HiMedia, Índia) para *Salmonella* Enteritidis (SOUSA et al., 2012), utilizando a técnica de microgotas (HERIGSTAD; HAMILTON; HEERSINK, 2001). Amostras controle, sem adição dos óleos essenciais foram ensaiadas de maneira semelhante. As placas foram

incubadas durante 24-48 h a 37 °C para *E. coli* e *Salmonella* Enteritidis e 30 °C para *L. monocytogenes*. Os resultados foram expressos como log_{10} UFC/mL. O limite de detecção da contagem de células viáveis foi de 1 log_{10} UFC/mL para todas os tratamentos e cepas testadas.

5.5.3 Avaliação dos danos causados pelos óleos essenciais às células bacterianas

A investigação dos danos causados pelos óleos essenciais de *M. arvensis* L. (CIM/8) e *M. piperita* L. (CIM/8) nas células bacterianas foi realizada por citometria de fluxo. Antes da análise de citometria de fluxo, as células foram expostas aos óleos essenciais de *M. arvensis* L. e *M. piperita* L. nos sucos de abacaxi e manga e armazenados por 15 min em temperatura de refrigeração. Considerando os resultados semelhantes dos ensaios de viabilidade nos sucos de abacaxi, caju e goiaba, optou-se por utilizar apenas os sucos de abacaxi e manga na análise de citometria de fluxo. Em seguida, os sucos foram centrifugados (4.500 x *g*, 15 min, 4 °C), as células bacterianas lavadas duas vezes, ressuspendidas em PBS (50 mM K_2HPO_4/KH_2PO_4; pH 7.4; Sigma-Aldrich, St. Louis, USA) e marcadas com diferentes fluorocromos (SILVA et al., 2011; SINGH; BARNARD, 2016). Foram utilizados: laranja de tiazol (TO, Sigma-Aldrich, St. Louis, MO, USA) e iodeto de propídio (PI, Sigma-Aldrich, St. Louis, MO, USA) para avaliação da integridade da membrana; ácido bis-1,3-dibutilbarbitúrico Trimethine Oxonol (BOX, Molecular Probes, Invitrogen, part of Life Technologies, Eugene, OR, USA) para o potencial da membrana; brometo de etídio (EB, Sigma-Aldrich, St. Louis, MO, USA) para atividade da bomba de efluxo; e cloreto de 5-ciano-2,3-ditolil tetrazólio (CTC, Polysciences, Warrington, PA, USA) para atividade respiratória. Células fixadas em etanol foram utilizadas como controle positivo para coloração com PI, BOX e EB e como controle negativo para coloração com CTC (SILVA et al., 2010).

As análises foram realizadas usando um citômetro de fluxo BD Accuri C6 (BD Accuri C6, New Jersey, EUA) equipado com um laser azul (488 nm) e um vermelho (640 nm), dois detectores de dispersão de luz (FSC e SSC) e quatro detectores de fluorescência. A fluorescência verde foi coletada no canal FL1 (533 nm ± 30 nm) e a vermelha no canal FL3 (> 670 nm). Os sinais de dispersão e a fluorescência das células individuais que passaram através do laser foram coletados como sinais

logarítmicos. O sinal de fluorescência (medições da área de pulso) dos fluorocromos utilizados foi coletado pelos filtros óticos nos canais FL1 (TO e BOX) e FL3 (PI, EB e CTC). O *threshold* foi ajustado em FSC (12.000) para eliminar ruídos ou partículas (de detritos celulares) muito menores que as células intactas. As células bacterianas foram identificadas por parâmetros FSC/SSC. A aquisição da amostra foi realizada em uma taxa de fluxo baixa (12 mL/min) e um total de 10.000 eventos foram adquiridos por amostra. A análise dos dados foi realizada no software BD Accuri C6.

Gráficos de densidade que representam luz de dispersão direta (FSC) versus luz de dispersão lateral (SSC) foram obtidos durante as medições. O FSC fornece informações relativas ao tamanho da célula e o SSC fornece informações sobre granulosidade celular. A análise de FL1 versus FL3 foi aplicada para determinar as propriedades de fluorescência das populações celulares marcadas com dois flurocromos simultaneamente. Células TO^+ PI^- representam células com membranas intactas e as células TO^+ PI^+ correspondem a células com membrana celular danificada ou ligeiramente permeabilizada. As células TO^- PI^+ representam células com membrana permeabilizada e as células TO^- PI^- representam células com DNA ou RNA danificado, enquanto a célula ainda pode estar intacta (SUROWSKY et al., 2014). Células BOX^+ PI^- representam células despolarizadas e não permeabilizadas; as células BOX^+ PI^+ e BOX^- PI^+ correspondem à populações de células despolarizadas e permeabilizadas com diferentes graus de dano; e as células BOX^- PI^- representam populações de células intactas, polarizadas e não permeabilizadas (HAMMER; HEEL, 2012). A análise de SSC versus FL3 foi realizada para determinar as propriedades de fluorescência das populações EB^+ e CTC^+, que reprsentam células com atividade de bomba de efluxo alterada e função respiratória não prejudicada, respectivamente (LÉONARD et al., 2016; MORISHIGE; FUJIMORI; AMANO, 2015). Essas populações encontram-se nos retângulos localizados no lado direito dos gráficos.

5.6 AVALIAÇÃO DE ASPECTOS DE QUALIDADE DOS SUCOS

5.6.1 Análises físico-químicas

Amostras dos diferentes sucos com ou sem a adição dos óleos essenciais (CIM/4 e CIM/8) de *M. arvensis* L. e *M. piperita* L. foram avaliadas quanto ao °Brix,

pH e acidez titulável logo após a adição dos óleos essenciais. Esses parâmetros incluem os atuais padrões físico-químicos brasileiros de sucos de frutas tropicais sem adição de açúcar (BRASIL, 2003). O teor de sólidos solúveis (°Brix) foi determinado utilizando um refratômetro digital (modelo HI 96801, Hanna Instruments, São Paulo, Brasil), os valores de pH foram determinados usando um potenciômetro digital (modelo Q400AS, Quimis, São Paulo, Brasil) e a acidez titulável foi determinada por titulometria utilizando NaOH 0,1 N na presença de fenolftaleína como indicador e os resultados foram expressos em gramas por 100 gramas (equivalentes de ácido cítrico; AOAC, 2012). As amostras de suco utilizadas nas análises físico-químicas não foram autoclavadas, pois a autoclavagem não interferiu nos parâmetros avaliados.

5.6.2 Análise sensorial

As análises sensoriais foram realizadas com a aprovação do Comitê de Ética em Pesquisa sob nº de protocolo 1.125.993/2015 (ANEXO B), utilizando o teste de aceitação (APÊNDICE C). Para isso, 60 provadores não treinados (18 a 60 anos de idade) foram pré-selecionados de acordo com o interesse e frequência de consumo de sucos de frutas. Os provadores realizaram as análises em cabines individuais com temperatura e iluminação adequadas, ausência de sons ou ruídos e livre de odores estranhos. Cada participante recebeu duas amostras de suco contendo diferentes concentrações (CIM/4 e CIM/8) dos óleos essenciais e o suco controle. Os óleos essenciais foram incorporados em amostras de suco 24 h antes do teste sensorial, as quais foram armazenadas a 4 ± 1 °C. Amostras de sucos sem os óleos essenciais de *M. arvensis* L. e *M. piperita* L. foram testadas como controles. Foram servidas 30 mL de cada amostra, em copos descartáveis brancos, codificados com um número aleatório de três dígitos. Os provadores foram orientados a consumir bolachas de baixo teor de sal e água para limpar os seus paladares entre as amostras avaliadas. Os atributos aparência, odor, viscosidade, sabor, sabor residual e avaliação global foram avaliados por meio de uma escala hedônica de 9 pontos, variando de 1 (desgostei muito) a 9 (gostei muito) (STONE; SIDEL, 1993; LEITE et al., 2016). Previamente, foram realizadas análises microbiológicas para garantir que as amostras utilizadas nos testes sensoriais se encontravam em conformidade com a legislação vigente (BRASIL, 2001).

5.7 ANÁLISE ESTATÍSTICA

Todos os ensaios foram realizados em triplicata, em três momentos independentes (repetições), sendo os resultados expressos como médias dos ensaios. Para os ensaios de determinação de CIM, os resultados foram expressos como valores modal, pois as CIMs foram as mesmas em todas as repetições. Para os ensaios de contagem de bactérias e parâmetros físico-químicos e sensoriais, as análises estatísticas foram realizadas para determinar diferenças significativas ($p \leq 0,05$) com base no teste t de Student ou ANOVA, seguida do pós teste de Tukey. O software Sigma Stat 3.5 (Software Científico Jandel, San Jose, Califórnia) foi utilizado para a análise estatística dos dados.

6 RESULTADOS E DISCUSSÃO

Os resultados e discussão produzidos durante o desenvolvimento da tese estão expostos em formato de artigos científicos submetidos ou publicados (Apêndices) em revistas científicas indexadas, e formatados de acordo com as normas dos periódicos. Por sua vez, as comprovações de submissão e aceite dos artigos científicos estão no item Anexos.

Artigo 1: The potential of the incorporation of essential oils and their individual constituents to improve microbial safety in juices: a review, foi publicado no periódico **Comprehensive Reviews in Food Science and Food Safety** (ISSN 1541-4337), v. 15, n. 4, p. 753–772, 2016, doi: 10.1111/1541-4337.12208. Esta revista tem fator de impacto de 5.974 (JCR 2016), sendo classificada como Qualis A1 na área de Nutrição (Classificação de Periódicos Quadriênio 2013–2016).

Artigo 2: The efficacy of *Mentha arvensis* L. and *M. piperita* L. essential oils in reducing pathogenic bacteria and maintaining quality characteristics in cashew, guava, mango, and pineapple juices, foi publicado no periódico **International Journal of Food Microbiology** (ISSN 0168-1605), v. 238, p. 183–192, 2016, doi: 10.1016/j.ijfoodmicro.2016.09.005. Esta revista tem fator de impacto de 3.339 (JCR 2016), sendo classificada como Qualis A1 na área de Nutrição (Classificação de Periódicos Quadriênio 2013–2016).

Artigo 3: Investigation of damage to *Escherichia coli*, *Listeria monocytogenes* and *Salmonella* Enteritidis exposed to *Mentha arvensis* L. and *M. piperita* L. essential oils in pineapple and mango juice by flow cytometry foi submetido para publicação no periódico **Food Microbiology** (ISSN 0740-0020). Esta revista tem fator de impacto de 3.759 (JCR 2016), sendo classificada como Qualis A1 na área de Nutrição (Classificação de Periódicos Quadriênio 2013–2016).

Patente depositada: Natureza da Patente – Patente de Invenção (PI). Título da Invenção – **Processo para conservação de sucos de frutas com Óleos essenciais**. Número do Processo – BR 10 2017 006417 4.

7 CONSIDERAÇÕES FINAIS

Os resultados obtidos demonstraram que a exposição de *E. coli*, *L. monocytogenes* e *Salmonella* Enteritidis aos óleos essenciais de *M. arvensis* L. e *M. piperita* L. nos sucos de abacaxi, caju e goiaba, proporcionou uma redução > 5 $\log_{10}$ UFC/mL nas contagens microbianas, corroborando com a recomendação da USFDA para conservação de sucos. Verificou-se ainda que a adição dos óleos essenciais de *Mentha* ssp. aos sucos de frutas não alterou os valores de °Brix, pH e acidez, mas afetou negativamente o sabor e a aceitação global dos sucos estudados. Além disso, os óleos essenciais de *M. arvensis* L. e *M. piperita* L. são capazes de causar aumento da permeabilidade de membrana, despolarização da membrana, inibição da atividade da bomba de efluxo e, ainda, alteração da atividade respiratória.

Estes achados revelam o potencial antimicrobiano dos óleos essenciais de *M. arvensis* L. e *M. piperita* L. quando considerada a sua capacidade de inibição do crescimento das cepas utilizadas no estudo. No entanto, considerando o impacto causado nos atributos sensoriais dos sucos testados, deve-se ressaltar a necessidade de estratégias para alcançar a eficácia antimicrobiana e a aceitação sensorial. A associação de concentrações reduzidas dos óleos essenciais de *Mentha* spp. com outras tecnologias de conservação de alimentos poderia potencializar a ação destes óleos e minimizar as alterações sensoriais. Ainda, o estudo dos danos causados à célula bacteriana e dos seus mecanismos de ação pode subsidiar a escolha destas tecnologias.

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APÊNDICES

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

UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM NUTRIÇÃO

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO (TCLE)

Convidamos o (a) Sr. (a) para participar como voluntário (a) da pesquisa *Aplicação combinada de óleos essenciais, temperaturas moderadas e campo elétrico pulsado na conservação de sucos de frutas tropicais*, que está sob a responsabilidade do (a) pesquisador (a) Jossana Pereira de Sousa, com endereço na Rua José Gomes de Sá Filho, nº 85, CEP: 58037-345, Jardim Oceania, João Pessoa-PB, Telefone: (83) 98882-1506, E-mail: jossanasousa@gmail.com, para contato com o pesquisador responsável (inclusive ligações a cobrar), e está sob a orientação do Prof. Dr. Evandro Leite de Souza, telefone para contato: (83) 3216-7807, e-mail: evandroleitesouza@ccs.ufpb.br.

Este Termo de Consentimento pode conter informações que o/a senhor/a não entenda. Caso haja alguma dúvida, pergunte à pessoa que está lhe entrevistando para que o/a senhor/a esteja bem esclarecido (a) sobre sua participação na pesquisa. Após ser esclarecido (a) sobre as informações a seguir, caso aceite em fazer parte do Estudo, rubrique as folhas e assine ao final deste documento, que está em duas vias. Uma delas é sua e a outra é do pesquisador responsável. Em caso de recusa o (a) Sr. (a) não será penalizado (a) de forma alguma. Também garantimos que o (a) Senhor (a) tem o direito de retirar o consentimento da sua participação em qualquer fase da pesquisa, sem qualquer penalidade.

INFORMAÇÕES SOBRE A PESQUISA:

Esta pesquisa tem como objetivo geral avaliar a aplicação combinada de óleos essenciais, tratamentos térmicos moderados e campos elétricos pulsados na conservação de sucos de frutas tropicais e busca informações sobre a conservação dos sucos de abacaxi, caju, goiaba e manga, pelo uso dos óleos essenciais de menta, incluindo o impacto do seu uso nas características sensoriais (sabor, aroma, aparência) do produto.

Serão considerados critérios de exclusão: pessoas menores de 18 anos, indivíduos que não tenham o hábito de consumir sucos de frutas, tabagistas, dependentes de álcool e gestantes. O período de participação é de aproximadamente 10 minutos. Serão oferecidas três amostras dos sucos de frutas (abacaxi, caju, goiaba ou manga) tratados para degustação e avaliação de suas características sensoriais (aparência, aroma, viscosidade, sabor, sabor residual e avaliação global), com posterior preenchimento dos formulários dos Testes de Aceitação e Intenção de Compra. Por fim, o provador deverá ordenar em ordem decrescente de preferência geral (amostra mais preferida para a amostra menos preferida) as amostras dos sucos (teste de Ordenação-preferência). Será também oferecido um copo

com água, bolacha, uma caneta e formulários para o registro de suas opiniões, em forma de escala de nove pontos, que irá do gostei muitíssimo ao desgostei muitíssimo. Após provar os sucos, os formulários serão devolvidos à equipe de pesquisadores e o Sr. (a) estará liberado (a) para suas atividades particulares.

O teste sensorial não apresenta riscos previsíveis ou mensuráveis, pois de acordo com a literatura os óleos essenciais de menta adicionados aos sucos de frutas não apresentam efeitos tóxicos significativos in vivo nas concentrações utilizadas no estudo. Inicialmente, será oferecida uma prova mínima, antes da degustação oficial, para verificar a sua aceitabilidade orgânica ao produto. 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 qualidade higiênico-sanitária dos produtos elaborados, sendo descartados os produtos que apresentaram valores acima dos permitidos pela legislação específica, garantindo que os provadores receberão amostras sem nenhum risco de contaminação microbiológica. Ainda, os participantes da pesquisa serão avisados de que há substâncias que possam causar alergia ou danos à saúde, e caso apresente alguma reação alérgica, não antes vivenciada, durante ou após o teste sensorial, comunique à equipe, que prontamente chamará o Serviço de Atendimento Móvel de Urgência – SAMU, por meio do número 192, assim como, na ausência deste, irá orientá-lo a ir ao hospital de referência mais próximo.

A condução deste plano de trabalho poderá contribuir para disponibilização de informações para a sociedade acerca das tecnologias empregadas para obtenção de um produto de qualidade, seguro e saudável. Acredita-se que um estudo dessa natureza trará subsídios para o efetivo aproveitamento de novas tecnologias de conservação como uma alternativa de inserção em sistemas de conservação de alimentos, em especial, sucos de frutas tropicais, na medida em que interfere no tipo e na quantidade de micro-organismos presentes nestes substratos.

As informações desta pesquisa serão confidenciais e serão divulgadas apenas 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. Os dados coletados nesta pesquisa (notas atribuídas às amostras dos sucos de frutas) ficarão armazenados em pastas de arquivo e computador pessoal, sob a responsabilidade do pesquisador, no endereço acima informado pelo período mínimo de cinco (5) anos.

O (a) senhor (a) não pagará nada para participar desta pesquisa. Se houver necessidade, as despesas para a sua participação serão assumidas pelos pesquisadores (ressarcimento de transporte e alimentação). Fica também garantida indenização em casos de danos, comprovadamente decorrentes da participação na pesquisa, conforme decisão judicial ou extrajudicial.

Em caso de dúvidas relacionadas aos aspectos éticos deste estudo, você poderá consultar o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da UFPE no endereço: Avenida da Engenharia s/n – 1º Andar, sala 4 - Cidade Universitária, Recife-PE, CEP: 50740-600, Telefone: (81) 2126.8588. E-mail: cepccs@ufpe.br

Josseana Pereira de Sousa

Assinatura do Pesquisador

APÊNDICE B – Consentimento da participação da pessoa como voluntário

CONSENTIMENTO DA PARTICIPAÇÃO DA PESSOA COMO VOLUNTÁRIO (A)

Eu, _____,
CPF_____, abaixo assinado, após a leitura (ou a escuta da leitura) deste documento e de ter tido a oportunidade de conversar e ter esclarecido as minhas dúvidas com o pesquisador responsável, concordo em participar do estudo *Aplicação combinada de óleos essenciais, temperaturas moderadas e campo elétrico pulsado na conservação de sucos de frutas tropicais*, como voluntário (a). Fui devidamente informado (a) e esclarecido (a) pelo (a) pesquisador (a) sobre a pesquisa, os procedimentos nela envolvidos, assim como os possíveis riscos e benefícios decorrentes de minha participação. Foi-me garantido que posso retirar o meu consentimento a qualquer momento, sem que isto leve a qualquer penalidade (ou interrupção de meu acompanhamento/assistência/tratamento).

João Pessoa, _____/_____/_____

_____
Assinatura do Participante da Pesquisa

Presenciamos a solicitação de consentimento, esclarecimentos sobre a pesquisa e o aceite do voluntário em participar (duas (02) testemunhas não ligadas à equipe de pesquisadores):

Nome: _____

Assinatura: _____

Nome: _____

Assinatura: _____

APÊNDICE C – Formulário para Análise Sensorial

UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM NUTRIÇÃO

TESTE DE ACEITAÇÃO

Nome: _____ Idade: _____
E-mail: _____ Fone: _____
Escolaridade: _____ Data: _____

1. Você está recebendo 03 amostras codificadas de suco de abacaxi adicionado de diferentes concentrações do óleo essencial de hortelã-pimenta (*Mentha piperita* L). Prove as amostras da esquerda para direita, avalie-as sensorialmente e use a escala abaixo para indicar o quanto você gostou ou desgostou de cada atributo. Antes de cada avaliação, você deverá fazer uso da água e da bolacha.

9 – gostei muitíssimo
8 – gostei muito
7 – gostei moderadamente
6 – gostei ligeiramente
5 – nem gostei/nem desgostei
4 – desgostei ligeiramente
3 – desgostei moderadamente
2 – desgostei muito
1 – desgostei muitíssimo

Atributos	Amostras (código)		
Aparência			
Aroma			
Viscosidade			
Sabor			
Sabor residual			
Avaliação global			

OBRIGADA!

APÊNDICE D – Artigo publicado no periódico Comprehensive Reviews in Food Science and Food Safety (ISSN 1541-4337).

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 mesophilic 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.	Donsì 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 $\mu g/g$	NA	Sensory evaluation	The EO did not affect the sensory properties of juice.	Liu and Yang (2012)
Apple (3.7)	*Citrus lemon* L.	200 $\mu L/mL$	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 ρ-cymene	20, 100, and 200 $\mu L/L$	NA	Sensory evaluation	Pennyroyal mint or lemon EO at 20 $\mu L/L$ 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 $\mu L/mL$	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 $\mu L/mL$	NA	pH, soluble solids, and titratable acidity	The EO at 2.5 and 1.25 $\mu L/mL$ 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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The efficacy of *Mentha arvensis* L. and *M. piperita* L. essential oils in reducing pathogenic bacteria and maintaining quality characteristics in cashew, guava, mango, and pineapple juices

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ABSTRACT

This study evaluated the ability of the essential oil from *Mentha arvensis* L. (MAEO) and *M. piperita* L. (MPEO) to induce ≥5-log reductions in counts (CFU/mL) of *E. coli*, *L. monocytogenes*, and *Salmonella enterica* serovar Enteritidis in Brain-Heart Infusion broth (BHIB) and cashew, guava, mango, and pineapple juices during refrigerated storage (4 ± 0.5 °C). The effects of the incorporation of these essential oils on some physicochemical and sensory parameters of juices were also evaluated. The incorporation of 5, 2.5, 1.25, or 0.625 μL/mL of MAEO in BHIB caused a ≥5-log reduction in counts of *E. coli* and *Salmonella* Enteritidis after 24 h of storage; but only 5 μL/mL was able to cause the same reduction in counts of *L. monocytogenes*. The incorporation of 10 μL/mL of MPEO in BHIB caused a ≥5-log reduction in counts of *E. coli*, *Salmonella* Enteritidis, and *L. monocytogenes* after 24 h of storage; smaller reductions were observed in BHIB containing 5, 2.5, and 1.25 μL/mL of MPEO. Similar reductions were observed when the MAEO or MPEO was incorporated at the same concentrations in mango juice. The incorporation of MAEO or MPEO at all tested concentrations in cashew, guava, and pineapple juices resulted in a ≥5-log reduction in pathogen counts within 1 h. The incorporation of MAEO and MPEO (0.625 and 1.25 μL/mL, respectively) in fruit juices did not induce alterations in °Brix, pH, and acidity, but negatively affected the taste, aftertaste, and overall acceptance. The use of MAEO or MPEO at low concentrations could constitute an interesting tool to achieve the required 5-log reduction of pathogenic bacteria in cashew, guava, mango, and pineapple fruit juices. However, new methods combining the use of MAEO or MPEO with other technologies are necessary to reduce their negative impacts on specific sensory properties of these juices.

1. Introduction

Fruit juice is consumed and appreciated worldwide because of its attractive flavor characteristics and refreshing aspect, as well as because it is considered a natural source of beneficial health components (Neves et al., 2011; Singh et al., 2015). Reports have cited that approximately 40% of fresh fruit is converted into juices and that inappropriate manipulation and storage condition result in contamination of these products with pathogenic bacteria, posing a potential risk to consumers (Chen et al., 2010; Mattioli et al., 2013; Simforian et al., 2015). The pathogenic bacteria in fruit juices are from microflora normally present on the fruit surfaces or that were contaminated during harvest, postharvest handing, and distribution, which invade the fruit pulp during the processing, or from post-processing contamination (Aneja et al., 2014; Raybaudi-Massilia et al., 2009; Tribst et al., 2009; Vantarakis et al., 2011).

Generally, the common high acidity of fruit juice is enough to avoid the proliferation of pathogenic bacteria by creating an unfavorable environment for their survival. However, several foodborne outbreaks caused by pathogenic bacteria have been associated with fruit juices (CDC, 2011; EFSA, 2013, 2015). Reports of the incidence, survival, and growth of pathogenic bacteria, such as *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* spp., in acidic (pH ≤ 4.6) and low-acid (pH > 4.6) fruit juices have changed the lack of concern that these foods may harbor pathogenic bacteria (Raybaudi-Massilia et al., 2009; USFDA, 2001). Therefore, the United States Food and Drug Administration (USFDA) issued its final rule on processing fruit and vegetable juices in 2001 (Juice Hazard Analysis and Critical Control Point) requiring a ≥ 5-log reduction (99.999%) of the pathogen of concern in juices, but without specifying the use of any particular method (USFDA, 2001).

Traditionally, the safety and stability of juices have been achieved through thermal processing and the use of chemical preservatives (Amirpour et al., 2015; Rawson et al., 2011; Toniolo et al., 2010).

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The most commonly used heat treatment is pasteurization; however, the high temperature destroys many of the desirable constituents of juice that provide nutritional and sensorial qualities to the product. The chemical compounds (e.g., benzoic and sorbic acids, and sulfur dioxide) used to preserve juices may be toxic and responsible for the occurrence of allergies (Polônio and Peres, 2009; Vally et al., 2009). Thus, it is necessary to use preservation technologies, or a combination of methods, that have no or minimal adverse effects on quality characteristics of juices (Khandpur and Gogate, 2016; Leite et al., 2016; Mohamed and Eissa, 2012).

Some nonthermal pasteurization methods, such as pulsed electric fields, high-pressure homogenization, high hydrostatic pressure, and ultrasound, has been considered emerging technologies to use in juices preservation (Rupasinghe and Yu, 2012). In addition, the interest in using nonsynthetic substances or compounds to prevent microbiological spoilage in juices while assuring safety and maintaining quality characteristics has increased in recent years (Espina et al., 2014b; Kapoor et al., 2014; Leite et al., 2016; Raybaudi-Massilia et al., 2009). This demand could be achieved by the use of essential oils (EOs) (Bassolé and Juliani, 2012; Calo et al., 2015; Perricone et al., 2015; Souza et al., 2016). The EOs have been known for their antimicrobial activity, safe use, and current status as "generally recognized as safe" (GRAS) food ingredient, and approval by USFDA for use in foods and drinks (Burt, 2004; USFDA, 2015).

The EOs have been extensively described to have antimicrobial activities against several spoilage microorganisms and foodborne pathogens in different types of foods, although these activities have varied with the type of the assayed food matrix (Burt, 2004; Goñi et al., 2009; López et al., 2005; Manso et al., 2015; Mith et al., 2014; Perricone et al., 2015). The studies available in the literature reveal that EOs and their constituents are promising alternatives to achieve microbial safety in apple, pear, melon, carrot, orange, watermelon, pineapple, tomato, strawberry, and apricot juices (Souza et al., 2016). However, there are no investigations about the use of EOs or their constituents in juices produced from cashew, guava, and mango, which are some of the main fruit species cultivated in Brazil (FAO, 2015).

Early studies showed that *Mentha* spp. EOs possess interesting antimicrobial activities against spoilage microorganisms and foodborne pathogens (Iscan et al., 2002; Tyagi and Malik, 2011; Tyagi et al., 2013). The *Mentha* genus belongs to the Lamiaceae family and consists of 18 species, including peppermint (*Mentha piperita* L.) and Japanese mint (*Mentha arvensis* L.). In the food industry, the *Mentha* spp. EOs are primarily used as flavoring agents in foods and beverages and are commonly exploited because of their antioxidant, antimicrobial, and sensory properties (Espina et al., 2014a; Nguyen and Mittal, 2007; Singh et al., 2011); however, there is no study to date on the efficacy of the EOs from *M. arvensis* (MAEO) and *M. piperita* (MPEO) to inhibit pathogenic bacteria in fruit juices or their possible impacts on quality attributes of these products. Fruit products, such as frozen pulp, and unpasteurized juices, containing *Mentha* spp. leaves are available in market, and these products are often well accepted because of their characteristic fresh-like taste. Thus, *Mentha* spp. EOs could represent potential antimicrobial substances to be incorporated in fruit juices.

In this context, this study evaluated the efficacy of MAEO and MPEO to induce ≥5-log reduction of a mixed composite of the potentially pathogenic bacteria *E. coli*, *L. monocytogenes*, and *Salmonella enterica* serovar Enteritidis in synthetic broth and in cashew, guava, mango, and pineapple juices. Moreover, the effects of the incorporation of these EOs on some physicochemical and sensory quality parameters of the tested juices were evaluated.

2. Materials and methods

2.1. MAEO and MPEO

The MAEO (batch 134; density at 20 °C, 0.897; refractive index at 20 °C, 1.459; pH 5.76) and MPEO (batch 181; density at 20 °C, 0.901; refractive index at 20 °C, 1.460; pH 5.17) extracted through steam distillation were purchased from Ferquima Ind. Com. Ltd. (São Paulo, Brazil). Emulsions of MAEO and MPEO were prepared in Brain Heart Infusion Broth (BHIB) (pH 7.54; HiMedia, Mumbai, India) at concentrations of 80 (pH 7.49), 40 (pH 7.50), 20 (pH 7.51), 10 (pH 7.52), 5.0 (pH 7.53), 2.5 (pH 7.53), 1.25 (pH 7.53), 0.625 (pH 7.54), and 0.312 (pH 7.54) μL/mL using Tween 80 (1%, v/v; Sigma–Aldrich, USA) as an emulsifier (Leite et al., 2016). At the highest assayed concentration (1%, v/v), Tween 80 presented no inhibitory effect against the tested bacterial strains.

2.2. Microorganisms and growth conditions

The *L. monocytogenes* (ATCC 7644) strain used in the present study was obtained from the Collection of Reference Microorganisms, National Institute of Quality Control in Health, Oswaldo Cruz Foundation (Rio de Janeiro, Brazil), and the *E. coli* (UFPEDA 224) and *Salmonella* Enteritidis (UFPEDA 414) strains were obtained from the Collection of Microorganisms, Department of Antibiotics, Federal University of Pernambuco (Recife, Brazil). The stock cultures were maintained in cryovials with BHIB containing glycerol (15 g/100 mL) at −20 °C. Pure suspensions of each strain were cultured in a sterile saline solution (0.85% NaCl, w/v) from overnight cultures grown in Brain Heart Infusion agar) (BHIA; HiMedia, Mumbai, India) at 37 °C for *E. coli* and *Salmonella* Enteritidis, and at 30 °C for *L. monocytogenes* for 18–24 h (to stationary growth phase), which were harvested through centrifugation (4500*g*, 15 min, 4 °C), washed twice in a sterile saline solution (0.85% NaCl, w/v) and re-suspended in BHIB to obtain standard cell suspensions. The optical density (OD) reading at 625 nm (OD_{625}) of these suspensions was 0.1 and provided viable counts of approximately 8 log colony forming units per milliliter (CFU/mL) when pour-plated on BHIA (McMahon et al., 2008). The mixed composite of the three species tested was obtained by mixing the bacterial suspensions at a ratio of 1:1:1 immediately after the preparation of each pure suspension (final count of each strain approximately 7 log CFU/mL) (Oliveira et al., 2015). This level of inoculum was used because the HACCP for the safe and sanitary processing of juices requires a high number of viable cells in the inoculum in order to measure the ≥5-log cycle reduction in CFU/mL (USFDA, 2001).

2.3. The identification of EOs constituents

The constituents of MAEO and MPEO were identified using a gas chromatograph coupled with a mass spectrometer (CGMS-QP2010 Ultra Shimadzu, Kyoto, Japan). Analysis through GC–MS was performed under the following conditions: 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 speed; ionizing energy: 70 eV; and mass range (*m*/*z*): 40–500. The NIST/EPA/NIH Mass Spectral Database (Version 1.7, National Institute of Standards Technology, Norwalk, CT) was used to identify the EOs constituents. The quantification of the constituents was obtained after normalizing the areas of each detected constituent, expressed as a percentage of area (%).

2.4. Preparation of fruit juices

The pineapple (*Ananas comosus* L. Merrill), mango (*Mangifera indica* L.), cashew (*Anacardium occidentale* L.), and guava (*Psidium guajava* L.) fruit were purchased in the commercial maturation stage from a local wholesale distributor (João Pessoa, Brazil) and were selected for uniformity in size, form, appearance, and the absence of mechanical injuries or visible signs of infection. For each juice preparation, the fruit were surface disinfected through a 5-min immersion in a sodium hypochlorite solution (150 ppm, pH 7.2 adjusted using 1 M NaOH), washed with sterile distilled water, and dried for 30 min in a safety cabinet.

Next, the fruit were aseptically peeled, chopped, and mixed with distilled water (1:1 ratio) using a domestic blender (for 3 min). The obtained juices were each centrifuged (12,500 rpm for 15 min, 4 °C) to separate the pulp from the remaining liquid. The supernatants were filtered using a triple-cheesecloth layer and sterilized by autoclaving (121 °C, 1.1 atm, for 15 min). The juices were stored in 50-mL aliquots at −20 °C, and, when required, an aliquot was thawed under refrigeration (4 ± 0.5 °C) and used for subsequent assays (Leite et al., 2016; Raybaudi-Massilia et al., 2006).

The juice samples assessed for physicochemical parameters were not submitted to autoclaving because previous experiments revealed that the monitored physicochemical parameters in juices were not changed after autoclaving. The non-inoculated juice samples used in the sensory analysis were prepared 24 h before assessing acceptability, and microbiological analysis determined the compliance of current Brazilian standards for these products (Brazil, 2001).

2.5. Determination of the minimum inhibitory concentration (MIC)

A microtiter plate assay was used to determine the MIC of MAEO and MPEO according to a previously described procedure (CLSI, 2015), with minor modifications related to the cultivation media. For this analysis, approximately 50 μL of each of the tested EO emulsions (80–0.312 mL/mL) was dispensed into each well of a 96-well microplate containing 100 μL of BHIB. Subsequently, 50 μL aliquots of the bacterial suspension were added to each well (final viable counts were approximately 6 log CFU/mL). The microplate was loosely wrapped with cling wrap to prevent bacterial dehydration and EO volatilization. Each plate included either a control (without EO), inoculated (positive control), or non-inoculated (negative control) sample. The microplate was incubated at 37 °C for 24 h. The MIC was defined as the lowest concentration of each EO required to prevent visible bacterial growth (Nostro et al., 2001).

2.6. Effects of MAEO and MPEO on bacterial counts in BHIB and fruit juices

The effects of different MAEO (5, 2.5, 1.25, and 0.625 μL/mL) and MPEO (10, 5, 2.5, and 1.25 μL/mL) concentrations on the counts of the bacterial mixed composite inoculated in BHIB and juices under refrigerated storage (4 ± 0.5 °C) were assessed over 24 h using a viable cell count method. Initially, 150 μL of the tested bacterial mixed composite was inoculated into 5850 μL of separated BHIB or fruit juice samples (final viable counts of approximately 6 log CFU/mL), containing MAEO or MPEO at the desired final concentrations. The different mixtures were gently hand-shaken for 30 s and subsequently incubated at 4 ± 0.5 °C (proper storage temperature of juices) for 24 h. At intervals of 0 (just after homogenization), 1, 2, 4, 8, 12, and 24 h post-incubation, an aliquot of 100 μL of each mixture was serially diluted in sterile saline solution (0.85% NaCl, w/v), and subsequently, 20 μL-aliquots of each dilution were inoculated onto selective agar, as follows: eosin-methylene blue agar (HiMedia, India) for *E. coli*, *Listeria* selective agar + *Listeria* Selective Supplement II (HiMedia, India) for *L. monocytogenes*, and *Salmonella-Shigella* agar (HiMedia, India) for *S.* Enteritidis (Sousa et al., 2012), using the microdrop technique (Herigstad et al., 2001). Control mixtures without the addition of MAEO or MPEO were similarly assayed. The plates were incubated for 24–48 h at 37 °C for *E. coli* and *S.* Enteritidis and 30 °C for *L. monocytogenes*. Plates inoculated with aliquots collected from BHIB or juice samples with EOs were incubated for 24 h longer than the samples collected from juice without EOs. The results are expressed as log CFU/mL. The detection limit of the viable cell count procedure was 1 log CFU/mL for all experiments and strains tested.

2.7. Physicochemical analyses of fruit juices

Samples of the different juices with or without the addition of MAEO or MPEO were assessed for °Brix, pH and titratable acidity just after the addition of MAEO and MPEO. These parameters were selected because they comprise the current Brazilian physicochemical standard to unsweetened fruit juices (Brazil, 2003). The soluble solids content (°Brix) was determined using a digital refractometer (model HI 96801, Hanna Instruments, São Paulo, Brazil). The pH values were determined using a digital potentiometer (model Q400AS, Quimis, São Paulo, Brazil). The titratable acidity was determined using phenolphthalein as an indicator with 0.1 N NaOH, and the results were expressed as grams per 100 g (equivalents of citric acid; AOAC, 2012).

2.8. Sensory analyses of fruit juices

The sensory analyses were performed with approval from an Ethics Research Committee (protocol 1.125.993/2015). Sensory evaluation was performed using an acceptability test. For this, sixty untrained panelists (18 to 60 years old) were preselected according to interest in and frequency of fruit juice consumption. Panelists worked in individual booths with controlled temperature and lighting. Each panelist received three juice samples containing the different concentrations of MAEO or MPEO tested. The EOs were incorporated into juice samples 24 h prior to the sensory test, and the samples were then stored at 4 ± 0.5 °C. Samples of juices without EO were tested as controls. The different juice samples were served in 30-mL aliquots in white disposable cups coded with a randomized three-digit number. Immediately after removal from refrigerated storage (4 ± 0.5 °C), the samples were served simultaneously using a blind method of random sequence. The panelists were asked to use low-salt crackers and water to cleanse their palates between the assessed samples. The appearance, odor, viscosity, taste, aftertaste, and overall acceptance attributes were evaluated on a 9-point hedonic scale ranging from 1 (dislike very much) to 9 (like very much) (Stone and Sidel, 1993; Leite et al., 2016).

2.9. Statistical analysis

All assays were performed in three independent experiments in triplicate. The three measurements for each data point in each independent repetition were first used to calculate average values; then, these values from the three independent repetitions were used for calculating the average values and standard deviations presented in results and to do statistical analysis. Different fruit juices batches and standardized inoculum from a single bacterial suspension prepared from two independent cultures of each of the tested bacterial strains were used in each independent experiment. The viable counts in each monitored time point were obtained from a single mixture comprising each of the tested fruit juice, the MAEO or MPEO concentration and the target bacterial inoculum. For MIC determination assays, the results are expressed as modal values because the MICs were the same in all repetitions. For the bacterial count assays and physicochemical and sensory parameters, statistical analyses were performed to determine significant differences ($p \leq 0.05$) based on Student's *t*-test or ANOVA, followed by a post hoc Tukey test. Sigma Stat 3.5 software (Jandel Scientific Software, San Jose, California) was used for the statistical analysis of the data.

3. Results and discussion

3.1. Identification of EOs constituents

The analysis of MAEO and MPEO identified the presence of 26 and 36 different constituents, respectively (Table 1). The constituents detected at highest amounts in MAEO were menthol (56.85%), isomenthone (21.13%), menthyl acetate (4.62%), limonene (4.07%), and isoisopulegol (3.71%). In MPEO, the major constituent was also menthol (59.73%),

followed by isomenthone (18.45%), methyl acetate (6.02%), neomenthol (2.43%), and isopulegol (2.15%). A variety of constituents were detected in amounts < 1% in MAEO (0.06–0.49%) and MPEO (0.02–0.56%). Previous investigations on the composition of *Mentha* spp. EOs similarly found menthol and menthone as the major constituents (Guerra et al., 2014; Tyagi et al., 2013; Watanabe et al., 2006). In *Mentha* spp. EOs, the presence of oxygenated monoterpenes (e.g., menthol, and isomenthone), monoterpene hydrocarbons (e.g., α-pinene, β-pinene, and limonene), and sesquiterpene hydrocarbons (e.g., E-caryophyllene) has been commonly verified (Tyagi and Malik, 2011; Verma et al., 2010).

3.2. Inhibitory effects on pathogenic bacteria in BHIB and fruit juices

The MIC displayed by MAEO and MPEO against the mixed composite of *E. coli*, *L. monocytogenes*, and *Salmonella* Enteritidis were 5 and 10 μL/mL, respectively. Regarding the detected MICs, the MAEO was more active than MPEO to inhibit the tested strains in the mixed inoculum. The MICs of MAEO and MPEO against the mixed composite of pathogens were higher than those verified in previous studies with the same EOs against *E. coli* (MIC 1.25–2.5 mg/mL), *S. typhimurium* (MIC 1.25–2.5 mg/mL), and *L. monocytogenes* (MIC 0.156–0.625 mg/mL), but all as single inoculum (Iscan et al., 2002). The observed inhibitory effects of MAEO and MPEO may be correlated primarily with the high amounts of menthol and isomenthone in their compositions, as these constituents have already proven effective in inhibiting *E. coli* (with MIC of 1.25 for MAEO, and 5.0 mg/mL for MPEO), *S. typhimurium* (with MIC of 0.625 for MAEO, and 5.0 mg/mL for MPEO), and *L. monocytogenes* (with MIC of 0.625 for MAEO, and 1.25 mg/mL for MPEO) (Iscan et al., 2002). In addition to the major constituents, other compounds detected in MAEO and MPEO, such as neomenthol, pulegone, isopulegol, and piperitone, have demonstrated inhibitory effects against a variety of potentially pathogenic bacteria (Lawrence, 2007).

Antimicrobial effects of *Mentha* spp. EOs and menthol are associated with their ability to cause perturbation of the lipid fraction of the microbial plasma membrane, inducing alterations in permeability and leakage of intracellular materials (Schelz et al., 2006). The antibacterial efficacy of menthol in fruit juices should be related with both its water solubility and lipophilic characteristic that enable the compound to migrate across the aqueous medium and to interact with bacterial phospholipidic membranes, inducing damages in this cell structure (Trombetta et al., 2005).

The majority of the available literature has considered the inactivation effects of EOs on pathogens to determine potential doses to use in real foods. Considering that EOs may negatively affect the sensory characteristics of juices, the assays of bacterial inactivation were performed using BHIB (Fig. 1A–F) and mango (Fig. 2A–F), cashew, guava, and pineapple juices containing sub-MICs of MAEO or MPEO over 24 h of storage at refrigeration temperature. This approach was used to evaluate if the tested concentrations of MAEO and MPEO are able to induce the required ≥5-log cycle reduction (≥5-log reduction) in counts of target bacteria and to measure the time needed to reach this reduction level.

The MAEO and MPEO were effective in reducing ($p \leq 0.05$) the counts of *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis forming the mixed composite when inoculated in BHIB and in all tested fruit juices during the 24-h refrigerated storage period. The incorporation of 5, 2.5, 1.25 or 0.625 μL/mL of MAEO in BHIB caused ≥5-log reductions in counts of *E. coli* and *Salmonella* Enteritidis after 8 and 24 h of

Table 1
Constituents identified in the essential oils from *M. arvensis* L. (MAEO) and *M. piperita* L. (MPEO).

MAEO				MPEO			
Peak	Retention index	Constituent	%	Peak	Retention index	Constituent	%
1	5.735	α-Thujene	0.06	1	4.139	Hex-(3Z)-enol	0.08
2	5.948	α-Pynene	1.56	2	5.963	α-Pinene	0.15
3	6.150	Hept-(2E)-en-1-ol	0.21	3	6.167	Hept-(2E)-en-1-ol	0.05
4	6.402	3-Methyl-cyclohexanone	0.26	4	7.083	Sabinene	0.14
5	7.064	Sabinene	0.37	5	7.209	β-Pinene	0.36
6	7.192	β-Pinene	1.41	6	7.560	Myrcene	0.12
7	7.675	Ethyl-hexanol	0.49	7	7.693	Ethyl-hexanol	0.28
8	8.735	ρ-Cymene	0.12	8	8.750	ρ-Cymene	0.11
9	8.890	Limonene	4.07	9	8.907	Limonene	1.97
10	9.013	Eucalyptol	0.10	10	9.015	Eucalyptol	0.90
11	10.409	*n*-Octanol	0.28	11	10.014	γ-Terpinene	0.02
12	11.169	Terpinolene	0.10	12	10.354	*trans*-Sabinene hydrate	0.13
13	11.585	Linalool	0.17	13	10.434	*n*-Octanol	0.20
14	13.998	Isomenthone	21.13	14	11.613	Linalool	0.17
15	14.911	Menthol	56.85	15	13.611	Isopulegol	2.15
16	15.002	Isoisopulegol	3.71	16	14.048	Isomenthone	18.45
17	15.497	Neomenthol	1.27	17	15.016	Menthol	59.73
18	15.572	α-Terpineol	0.45	18	15.545	Neomenthol	2.43
19	17.719	Pulegone	1.40	19	15.618	α-Terpineol	0.56
20	18.386	Piperitone	0.10	20	16.425	Octyl-acetate	0.03
21	19.272	Neomenthyl acetate	0.25	21	16.661	4-Methyl-dec-3-en-5-ol	0.11
22	20.125	Menthyl acetate	4.62	22	17.514	*cis*-3-Hexenyl pentanoate	0.12
23	23.569	Ylangene	0.25	23	17.750	Pulegone	1.60
24	23.766	α-Cubebene	0.13	24	17.945	Carvone	0.07
25	24.165	β-Bourbonene	0.39	25	18.409	Piperitone	1.33
26	24.446	β-Elemene	0.07	26	19.129	Decyl alcohol	0.10
27	ND	ND	ND	27	19.294	Neomenthyl acetate	0.23
28	ND	ND	ND	28	20.794	Menthyl acetate	6.02
29	ND	ND	ND	29	20.997	Isopulegyl acetate	0.05
30	ND	ND	ND	30	22.056	Bicyclogermacrene	0.06
31	ND	ND	ND	31	24.185	β-Bourbonene	0.39
32	ND	ND	ND	32	24.473	β-Elemene	0.11
33	ND	ND	ND	33	25.697	E-Caryophyllene	1.24
34	ND	ND	ND	34	26.106	β-Cubebene	0.09
35	ND	ND	ND	35	27.142	ε-Muurolene	0.18
36	ND	ND	ND	36	28.317	β-Cubebene	0.14
Total			99.82	Total			99.87

ND: not detected.

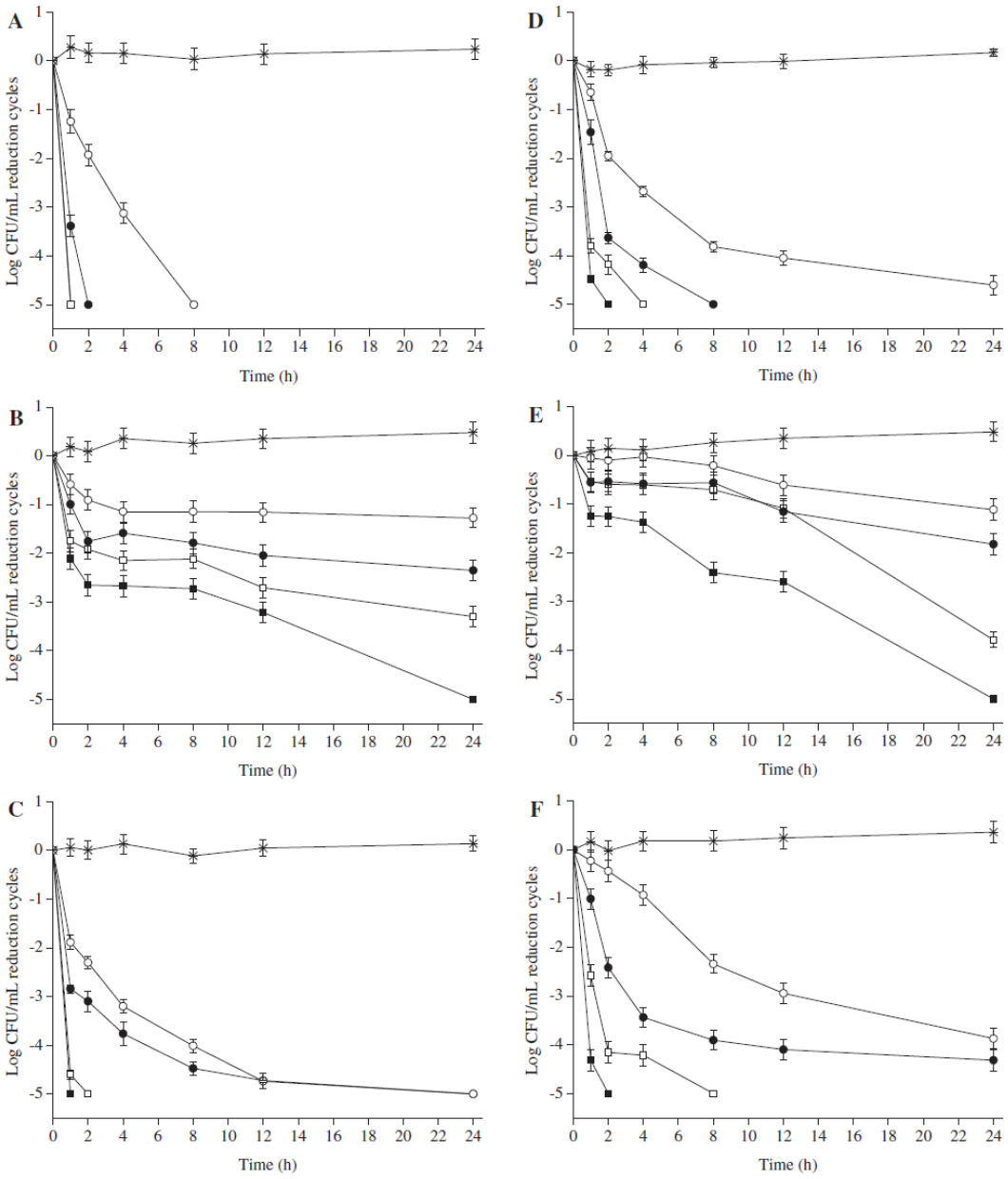

Fig. 1. Reduction cycles (log CFU/mL) of the initial counts of *E. coli* UFPEDA 224 (A, D), *L. monocytogenes* ATCC 7644 (B, E), and *Salmonella* Enteritidis UFPEDA 414 (C, F) in brain-heart infusion broth at 4 °C as a function of *M. arvensis* L. essential oil concentration (A, B, C) at (■) MIC: 5 μL/mL, (□) MIC/2: 2.5 μL/mL, (●) MIC/4: 1.25 μL/mL, (○) MIC/8: 0.625 μL/mL, (*) control: 0 μL/mL; and *M. piperita* L. essential oil concentration (D, E, F) at (■) MIC: 10 μL/mL, (□) MIC/2: 5 μL/mL, (●) MIC/4: 2.5 μL/mL, (○) MIC/8: 1.25 μL/mL, (*) control: 0 μL/mL. Detection limit of the test: 1.0 log CFU/mL.

refrigerated storage, respectively; however, only a ≤ 3.3-log reduction in counts of *L. monocytogenes* was observed when exposed to 2.5, 1.25 or 0.625 μL/mL of MAEO in BHIB over 24 h of refrigerated storage (Fig. 1A–C). The incorporation of 10 μL/mL of MPEO in BHIB resulted in a ≥ 5-log reduction in counts of *E. coli* and *Salmonella* Enteritidis after 2 h of storage and in *L. monocytogenes* after 24 h of storage (Fig. 1D–F). Similar reductions in counts of *E. coli* and *Salmonella* Enteritidis were observed in BHIB containing 5 and 2.5 μL/mL of MPEO; however, smaller reductions in counts of *E. coli* and *Salmonella* Enteritidis were observed in BHIB containing 1.25, 2.5, and 1.25 μL/mL of MPEO (Fig. 1D, F). The incorporation of 5, 2.5, and 1.25 μL/mL of MPEO in BHIB resulted in ≤3.8-log reductions in counts of *L. monocytogenes* after 24 h of storage (Fig. 1E).

In mango juice containing 10 μL/mL (MIC), 5 μL/mL (MIC/2), and 2.5 μL/mL (MIC/4) of MPEO, ≥5-log reductions in counts of *E. coli* and *Salmonella* Enteritidis were observed after 1 h of storage. However, the incorporation of 1.25 μL/mL (MIC/8) of MPEO in mango juice caused the same reduction in counts of *E. coli* and a ≤2.8-log reduction in counts

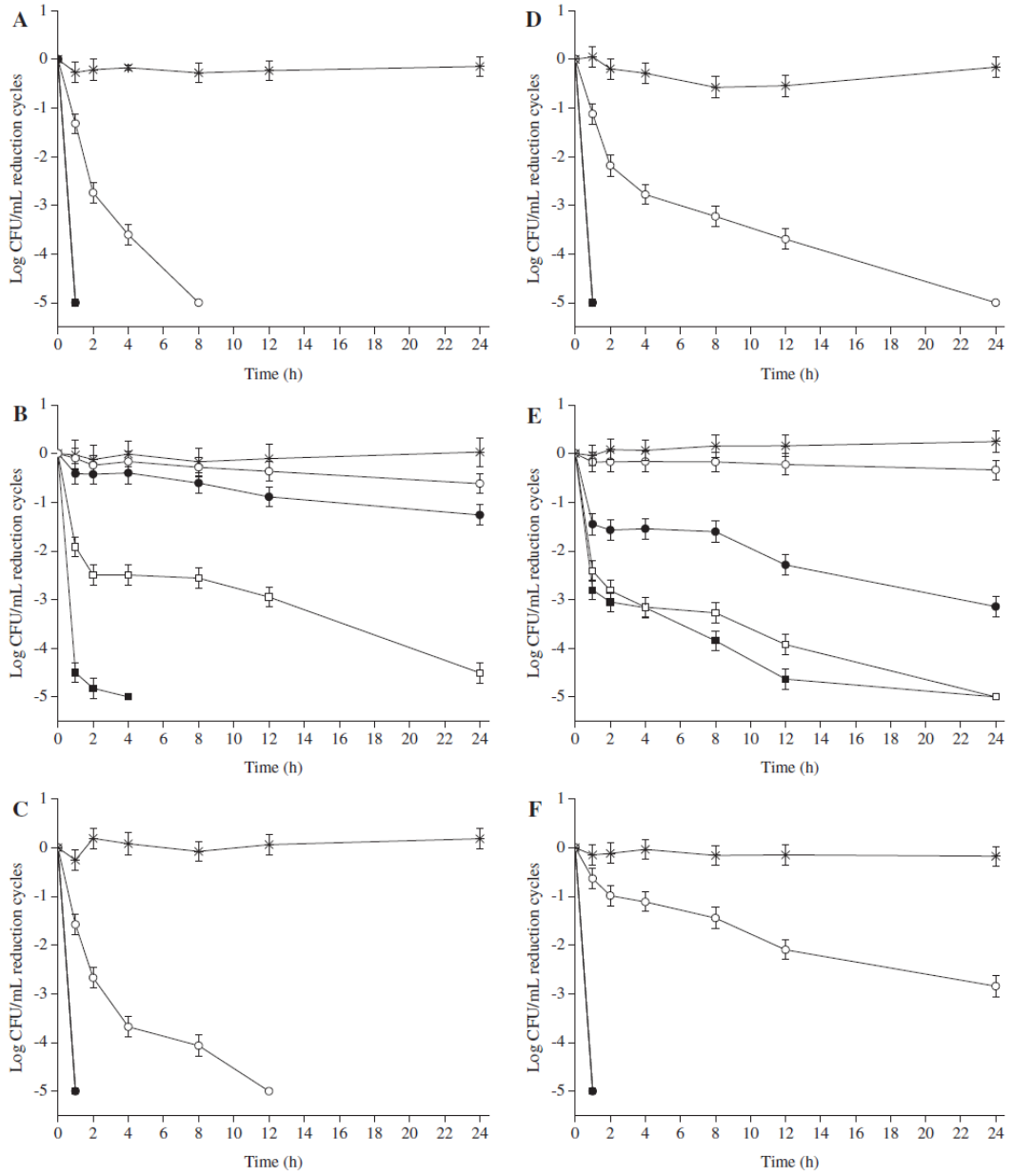

Fig. 2. Reduction cycles (log_{10} CFU/mL) of the initial viable cell counts of *E. coli* UFPEDA 224 (A, D), *L. monocytogenes* ATCC 7644 (B, E), and *Salmonella* Enteritidis UFPEDA 414 (C, F) in mango juice at 4 °C as a function of *M. arvensis* L. essential oil concentration (A, B, C) at (■) MIC: 5 μL/mL, (□) MIC/2: 2.5 μL/mL, (●) MIC/4: 1.25 μL/mL, (○) MIC/8: 0.625 μL/mL, (*) control: 0 μL/mL; and *M. piperita* L. essential oil concentration (D, E, F) at (■) MIC: 10 μL/mL, (□) MIC/2: 5 μL/mL, (●) MIC/4: 2.5 μL/mL, (○) MIC/8: 1.25 μL/mL, (*) control: 0 μL/mL. Detection limit of the test: 1.0 log CFU/mL.

of *Salmonella* Enteritidis after 24 h of storage (Fig. 2D, G). In mango juice containing 0.625 μL/mL (MIC/8) of MAEO, ≥5-log cycle reductions in counts of *E. coli* and *Salmonella* Enteritidis were observed after 8 and 12 h of exposure, respectively (Fig. 2A, C). The incorporation of MAEO or MPEO at all tested concentrations in cashew, guava, and pineapple juices resulted in ≥5-log reductions of *E. coli*, *L. monocytogenes*, and *Salmonella* Enteritidis within 1 h of storage (data not shown). In cashew juice not containing MAEO or MPEO, a ≥ 5-log reduction of L. *monocytogenes* was verified after 4 h, and a 2.26-log reduction of *E. coli* was noted after 8 h of storage. In guava juice not containing EO, a 3.01-log reduction of *L. monocytogenes* was verified after 8 h of storage.

Overall, when the highest reduction in counts of target bacteria in BHIB or in tested fruit juice containing either MAEO or MPEO was reached within a maximum storage period of 12 h, no recovery in bacterial counts was verified at the later evaluated storage time intervals (viable counts were not monitored for storage time intervals longer than 24 h). Notably, the MAEO and MPEO at sub-MICs were able to

induce ≥5-log reductions in counts of the tested pathogenic bacteria in cashew, guava, mango, and pineapple juice.

There is a lack of available studies on the antimicrobial efficacy of *Mentha* spp. EOs as applied alone against pathogenic bacteria in juices; however, their efficacy in reducing the counts of the juice-spoiling yeasts *Zygosaccharomyces rouxii* and *Z. bailii* (6–7 log reductions) in apple juice (Karaman et al., 2016), *Saccharomyces cerevisiae* (3 log reduction) in mixed fruit juice (Tyagi et al., 2013), and naturally occurring microorganisms in tomato juice (Nguyen and Mittal, 2007) has been already reported. An earlier study assessed the efficacy of using 0.2 μL/mL of pennyroyal mint (*M. pulegium* L.) EO in combination with a mild heat treatment (54 °C for 10 min) against *E. coli* O157:H7 VTEC (Phage type 34) in apple and orange juices. The incorporation of the EO into apple juice that was then submitted to heat treatment caused a ≥ 5 log reduction in counts of *E. coli* O157:H7, occurring faster than the heat treatment acting alone (Ait-Ouazzou et al., 2012). Nevertheless, other EOs and constituents such as lemongrass EO, geraniol, and trans-cinnamaldehyde have proven effective against *E. coli*, *Listeria* spp., and *Salmonella* spp. in apple, pear, melon, and pineapple juices (Baskaran et al., 2010; Leite et al., 2016; Raybaudi-Massilia et al., 2006).

In view of the data obtained in assays of bacterial count reductions, the general ranking of sensitivity to MAEO and MPEO in synthetic broth and mango juice was *E. coli* > *Salmonella* Enteritidis > *L. monocytogenes*. This result was somewhat surprising because the literature commonly cites gram-negative bacteria (e.g., *E. coli*, and *Salmonella* Enteritidis) as generally less sensitive to EOs than gram-positive bacteria (e.g., *L. monocytogenes*) because the outer membrane surrounding the cell wall (absent in gram-positive bacteria) may restrict the diffusion of hydrophobic constituents toward the inside of the cell (Burt, 2004, Mazzarrino et al., 2015, Mosqueda-Melgar et al., 2008). In agreement with the results of this study, MPEO (5–20 μL/mL) induced greater decreases in viable counts of *Salmonella* Enteritidis than of *L. monocytogenes* in Greek appetizers (pH 4.3, 4.9, and 6.8) at 4 and 10 °C during a 7-day storage period (Tassou et al., 1995). Otherwise, MPEO (~1 μL/mL) decreased the counts of *Staphylococcus aureus* 2-fold more than those of *Salmonella* Enteritidis after 48 h of incubation at 30 °C in synthetic broth (Tassou et al., 2000). These results suggest that the antibacterial effects of *Mentha* spp. EOs may depend not only on the target bacteria but also on the EO concentration, the characteristics of the media and the incubation (storage) temperature.

3.3. Physicochemical and sensory characteristics of fruit juices

Considering that the incorporation of MIC and MIC/2 of MAEO or MPEO could impact negatively on the sensory characteristics of the tested juices and that MIC/4 and MIC/8 were able to sharply reduce the counts of target pathogens in fruit juices (with the only exception being *L. monocytogenes* in mango juice), these concentrations were used in assays for the evaluation of physicochemical and sensory characteristics of fruit juices. The physicochemical characteristics, namely °Brix, pH, and titratable acidity (citric acid equivalent) in juices with or without MAEO (1.25 and 0.625 μL/mL) or MPEO (2.5 and 1.25 μL/mL) were evaluated immediately after the EO addition. No difference ($p > 0.05$) in physicochemical parameters of juices containing MAEO or MPEO at the two tested concentrations was observed (data not shown). Therefore, only the results of the physicochemical analysis of fruit juices with and without 0.625 μL/mL of MAEO or 1.25 μL/mL of MPEO are presented in Table 2. The °Brix, pH and titratable acidity of tested fruit juices was not affected ($p > 0.05$) by incorporation of MAEO or MPEO. Other studies evaluating the addition of limonene (1.0, 5.0, and 10 g/L), lemongrass (1.25 and 0.625 μL/mL), and MPEO (500 and 1000 ppm) to pear, orange, pineapple, or apple juices, respectively, similarly observed a pattern of no alteration in °Brix, pH and acidity of EOs-supplemented juices (Donsì et al., 2011; Kapoor et al., 2008, 2014; Karaman et al., 2016; Leite et al., 2016; Montero-Prado et al., 2011; Patrignani et al., 2013). Notably, juice samples with or without MAEO and MPEO met the current Brazilian standard for unsweetened cashew, guava, mango, and pineapple juices, which require 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 (Brazil, 2003).

Previous studies reported higher antibacterial effects of EOs at an acidic pH, where EOs may behave more hydrophobically and enter into cells more easily (Gutierrez et al., 2009; Negi, 2012; Smith-Palmer et al., 2001; Manso et al., 2015). In this study, the incorporation of MAEO and MPEO in juices with the lowest pH values, namely, cashew (4.3), guava (4.2), and pineapple (3.8), resulted in sharp reductions in bacterial counts in an exposure time as short as 1 h. In contrast, the incorporation of MAEO or MPEO in mango juice, which presented a higher pH (5.7), needed a more prolonged time to cause sharp reduction in bacterial counts. The lowest bacterial reductions induced by MAEO and MPEO in mango juice may be also associated with its already reported higher amounts of complex carbohydrates compared to the other juices tested in this study (Ramos et al., 2004).

The fruit juices containing 1.25 and 0.625 μL/mL of MAEO and 2.5 and 1.25 μL/mL of MPEO were subjected to acceptance tests after 24 h of refrigerated storage. No difference ($p > 0.05$) in sensory attributes of juices containing each MAEO or MPEO at the two tested concentrations was observed (data not shown). Thus, only the results of the sensory analysis of fruit juices with and without 0.625 μL/mL of MAEO or 1.25 μL/mL of MPEO are presented in Table 3, respectively. The attributes appearance, odor, and viscosity were not affected ($p > 0.05$) when 0.625 μL/mL of MAEO or 1.25 μL/mL of MPEO were incorporated in juices. All juices with or without MAEO or MPEO received scores that ranged between "like slightly" and "really enjoyed" for appearance and viscosity. In contrast, taste, aftertaste, and overall acceptance of juices containing MAEO or MPEO received lower scores ($p \leq 0.05$) than juices without EO. The negative impacts on taste, aftertaste, and overall acceptance of juices were probably related with the large

Table 2
Physicochemical parameters (average ± standard deviation) of different fruit juices with or without *M. arvensis* L. essential oil (MAEO) or *M. piperita* L. essential oil (MPEO).

Juices	Treatments	Physicochemical quality parameters		
		Total soluble solids (°Brix)	pH	Titratable acidity (g/100 g citric acid)
Cashew	MAEO (0.625 μL/mL)	5.87 (±0.51)[a]	4.27 (±0.41)[a]	0.22 (±0.06)[a]
	MPEO (1.25 μL/mL)	5.90 (±0.33)[a]	4.27 (±0.32)[a]	0.23 (±0.08)[a]
	Control (0 μL/mL)	6.01 (±0.48)[a]	4.27 (±0.36)[a]	0.23 (±0.04)[a]
Guava	MAEO (0.625 μL/mL)	6.42 (±0.35)[a]	4.15 (±0.31)[a]	0.32 (±0.08)[a]
	MPEO (1.25 μL/mL)	6.50 (±0.13)[a]	4.18 (±0.40)[a]	0.31 (±0.07)[a]
	Control (0 μL/mL)	6.30 (±0.29)[a]	4.17 (±0.34)[a]	0.30 (±0.06)[a]
Mango	MAEO (0.625 μL/mL)	10.71 (±0.28)[a]	5.44 (±0.36)[a]	0.34 (±0.05)[a]
	MPEO (1.25 μL/mL)	10.73 (±0.31)[a]	5.61 (±0.27)[b]	0.34 (±0.07)[a]
	Control (0 μL/mL)	10.71 (±0.15)[a]	5.68 (±0.25)[b]	0.34 (±0.06)[a]
Pineapple	MAEO (0.625 μL/mL)	6.64 (±0.37)[a]	3.74 (±0.45)[a]	0.26 (±0.05)[a]
	MPEO (1.25 μL/mL)	6.57 (±0.46)[a]	3.75 (±0.36)[a]	0.26 (±0.04)[a]
	Control (0 μL/mL)	6.31 (±0.28)[b]	3.77 (±0.42)[a]	0.26 (±0.07)[a]

Same superscript letters in a column for the same juice indicate no significant difference ($p > 0.05$) among treatments, based on Tukey's test.

Table 3
Scores (average ± standard deviation) for sensory attributes of juices with or without *M. arvensis* L. essential oil (MAEO) or *M. piperita* L. essential oil (MPEO) after 24 h-storage at 4 ± 0.5 °C.

Juices	Treatments	Sensory attributes					
		Appearance	Odor	Viscosity	Taste	Aftertaste	Overall
Cashew	MAEO (0.625 μL/mL)	7.86 (±1.59)[a]	7.16 (±1.73)[a]	7.00 (±1.73)[a]	4.52 (±1.68)[a]	4.46 (±2.05)[a]	4.82 (±1.96)[a]
	MPEO (1.25 μL/mL)	7.56 (±1.18)[a]	6.10 (±1.89)[a]	5.94 (±1.75)[a]	2.62 (±1.72)[a]	2.78 (±1.73)[a]	3.38 (±1.55)[a]
	Control (0 μL/mL)	7.96 (±1.28)[a]	7.64 (±1.38)[a]	6.96 (±1.63)[a]	6.38 (±1.92)[b]	6.45 (±1.78)[b]	6.83 (±1.59)[b]
Guava	MAEO (0.625 μL/mL)	8.26 (±0.92)[a]	8.00 (±1.05)[a]	7.60 (±0.76)[a]	4.06 (±2.46)[a]	4.00 (±2.49)[a]	4.58 (±2.19)[a]
	MPEO (1.25 μL/mL)	7.68 (±1.27)[a]	6.48 (±1.76)[a]	7.00 (±1.37)[a]	2.48 (±1.64)[a]	2.96 (±1.95)[a]	3.68 (±2.01)[a]
	Control (0 μL/mL)	8.02 (±1.10)[a]	7.86 (±1.02)[a]	7.22 (±1.16)[a]	6.49 (±1.34)[b]	6.60 (±1.32)[b]	7.02 (±1.19)[b]
Mango	MAEO (0.625 μL/mL)	7.72 (±1.41)[a]	7.20 (±1.85)[a]	7.18 (±1.52)[a]	4.32 (±2.68)[a]	4.42 (±2.68)[a]	5.06 (±2.54)[a]
	MPEO (1.25 μL/mL)	8.00 (±1.05)[a]	6.44 (±2.14)[a]	6.86 (±1.75)[a]	3.38 (±2.22)[a]	3.46 (±2.16)[a]	3.98 (±2.36)[a]
	Control (0 μL/mL)	7.83 (±1.38)[a]	7.60 (±1.62)[a]	7.16 (±1.66)[a]	6.38 (±2.02)[b]	6.53 (±1.99)[b]	6.84 (±1.76)[b]
Pineapple	MAEO (0.625 μL/mL)	6.78 (±1.43)[a]	6.96 (±1.62)[a]	6.62 (±1.38)[a]	3.94 (±2.08)[a]	4.02 (±2.07)[a]	4.46 (±2.22)[a]
	MPEO (1.25 μL/mL)	7.20 (±1.28)[a]	6.74 (±1.74)[a]	6.42 (±1.77)[a]	3.84 (±1.99)[a]	3.82 (±2.15)[a]	4.72 (±2.18)[a]
	Control (0 μL/mL)	6.48 (±1.56)[a]	7.28 (±1.57)[a]	6.32 (±1.65)[a]	5.83 (±1.94)[b]	5.88 (±1.61)[b]	6.32 (±1.58)[b]

Different superscript letters in the same column for each juice indicate significant difference ($p \leq 0.05$) among treatments, based on Tukey's test.

amounts of menthol in MAEO (56.85%) and MPEO (59.73%). Menthol is the main responsible by organoleptic characteristics ("like-mint flavor") of *Mentha* spp. EOs and can induce burning, stinging, and cold sensation that may be not well-accepted by some consumers (Davis et al., 2005; Green, 2005; Pramila et al., 2012). For these parameters, juices without EO received scores that ranged between "liked moderately" and "really enjoyed", juices containing MPEO received scores that ranged between "disliked moderately" to "neither liked nor disliked", and juices containing MAEO scored between "disliked slightly" and "neither liked nor disliked". The scores attributed to overall acceptance did not differ ($p > 0.05$) among juices containing MAEO or MPEO.

There are no studies on the impact of MPEO and MAEO addition on the sensory characteristics of fruit juices; however, the impacts of other EOs and constituents have been evaluated. The incorporation of 0.25 μL/mL of lemon EO positively affected the taste of apple juice, although the odor was perceived as unpleasant in some samples (Tserennadmid et al., 2011). In tomato juice, 0.02 μL/mL of pennyroyal mint or lemon EO did not affect the taste; however, higher concentrations of these EOs (0.1 or 0.2 μL/mL) or any tested concentration (0.02, 0.1, or 0.2 μL/mL) of thyme EO, rosemary EO, carvacrol, and *p*-cymene did affect the taste (Espina et al., 2014a). The incorporation of 1.25 or 2.5 μL/mL of lemongrass EO in pineapple juice did not affect the appearance, odor, and viscosity of the juice, although noticeable unsatisfactory changes were found in taste, aftertaste, and overall acceptability (Leite et al., 2016). These findings corroborate the results of sensory analysis obtained in this study and reinforce the point that possible negative impacts on taste and odor caused by the addition of EOs may limit their use to increase the safety and shelf life of fruit juices. The use of MAEO or MPEO as antimicrobials in cashew, guava, mango, and pineapple juices at the doses tested in this study should be carefully considered because of potential adverse effects on the sensory properties of these products; therefore, combinations of MAEO or MPEO with other preservation methods may be required to decrease the effective dose and hence negative impacts on odor and taste.

4. Conclusion

The MAEO and MPEO directly incorporated to cashew, guava, mango, and pineapple juices can effectively reach the required ≥5 log-reduction of *E. coli*, *L. monocytogenes*, and *Salmonella* Enteritidis during refrigerated storage, although the dose and exposure time needed to produce this reduction level has varied. In general, the MAEO and MPEO demonstrated the strongest inhibitory effects against *E. coli* and *Salmonella* Enteritidis, specifically in cashew, guava, and pineapple juices, which presented the lowest pH values. Moreover, EOs did not affect important physicochemical quality parameters, namely the °Brix, pH, and acidity, of all tested fruit juices. In contrast, MAEO and MPEO negatively affected the taste, aftertaste, and overall acceptance of fruit juices. Therefore, new studies combining the use of MAEO or MPEO at lower doses with other traditional or emerging technologies for preservation of cashew, guava, mango, and pineapple juices are necessary to exploit the antibacterial effects of these EOs and reduce the negative effects on particular sensory properties of these products.

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APÊNDICE F – Artigo submetido para publicação no periódico Food Microbiology (ISSN 0740-0020).

Investigation of damage to *Escherichia coli, Listeria monocytogenes* and *Salmonella* Enteritidis exposed to *Mentha arvensis* L. and *M. piperita* L. essential oils in pineapple and mango juice by flow cytometry

Running title: Damage in bacterial functions induced by essential oils

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Abstract

This study evaluated the effects of *Mentha arvensis* L. (MAEO; 0.625 µL/mL) and *M. piperita* L. (MPEO; 1.25 µL/mL) essential oil on viable cell counts and physiological functions in *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica* Serovar Enteritidis in pineapple and mango juice after a 15 min-exposure under refrigeration. The physiological functions of the bacterial cells were assessed by flow cytometry using the fluorochromes thiazole orange, propidium iodide, bis-1,3-dibutylbarbutiric acid, ethidium bromide, and 5-cyano-2,3-ditolyl tetrazolium chloride to investigate membrane integrity, membrane potential, efflux activity, and respiratory activity. MAEO and MPEO sharply reduced (> 5 $\log_{10}$ CFU/mL cycles) the counts of *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis in pineapple juice, and caused smaller reductions (0.61 – 1.58 $\log_{10}$ CFU/mL cycles) in mango juice. Bacterial cells exposed to MAEO and MPEO in pineapple and mango juice showed increased membrane permeability, membrane depolarization and changes in efflux pump and respiratory activity. More physiological damage occurred in bacterial cell populations exposed to MAEO or MPEO in pineapple juice than in mango juice. These results indicate that MAEO and MPEO inactivate *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis cells in pineapple and mango juice through a multi-target action mode that disrupts cytoplasmic membranes, increases permeability and potential depolarization, as well as inhibits efflux pump and respiratory activity.

Keywords: *Mentha* spp., pathogenic bacteria, antibacterial action mode, juice, flow cytometry.

1. Introduction

Several foodborne outbreaks have been associated with fruit juice (EFSA, 2013, 2015). The microorganisms that have been most frequently implicated in these outbreaks are

Escherichia coli O157:H7, *Listeria monocytogenes* and *Salmonella* spp. (USFDA, 2001). The use of essential oils (EOs) is considered an alternative emerging technology for preserving fruit juices (Aneja et al., 2014; Rupasinghe and Yu, 2012; Souza et al., 2016). EOs are "generally recognized as safe" (GRAS) food ingredients, possess antimicrobial properties and are approved by the USFDA for use in foods and drinks (Souza et al., 2016; USFDA, 2015).

EOs from *Mentha* species possess broad-spectrum antimicrobial activity against pathogenic and food-spoiling microorganisms and could be potential antimicrobial agents in fruit juices (Iscan et al., 2002; Sousa Guedes et al., 2016; Tyagi and Malik, 2011; Tyagi et al., 2013). The antimicrobial mechanisms of EOs have been associated with their ability to change the cell membrane permeability and structural characteristics of target organisms, often in a short timeframe (Sousa et al., 2015). However, studies on the antimicrobial mechanisms of EOs on microbial cells in food matrices are scarce (Clemente et al., 2016; Oussalah et al., 2006).

Flow cytometry (FC) has received increasing attention as a method of measuring the viability and physiological functions of bacteria. FC is a relatively new technique that enables fast and reliable detection of multiple parameters in both cultivable and non-cultivable cells (Pan et al., 2014). Conventional methods for cell population analysis are limited by the need to determine a single value for each cell parameter that is then considered representative of the whole cell population. In contrast, FC provides segregated data for different cell subpopulations (Paparella et al., 2012). Some studies have used FC to monitor the effects of EOs on food-related microorganisms (Bouhdid et al., 2009; Paparella et al., 2008; Zhang et al., 2016), but none have assessed the effects of EOs on bacteria in fruit juice.

In a previous study (Sousa Guedes et al., 2016), MAEO and MPEO directly incorporated into cashew, guava, mango, and pineapple juice were effective to cause the required ≥5 log reduction in counts of *E. coli*, *L. monocytogenes*, and *Salmonella enterica*

Serovar Enteritidis during refrigeration, although the doses and exposure times needed to produce this reduction level vary. In general, the MAEO and MPEO had a stronger inhibitory effect on *E. coli* and *Salmonella* Enteritidis than on *L. monocytogenes* and a stronger effect in pineapple juice than in mango juice. This study evaluated the effects of MAEO and MPEO on membrane integrity, membrane potential, efflux activity and respiratory activity in *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis in pineapple and mango juice using flow cytometry analysis.

2. Materials and methods

2.1. MAEO and MPEO

MAEO (batch 134; density at 20 °C, 0.897; refractive index at 20 °C, 1.459; pH 5.8) and MPEO (batch 181; density at 20 °C, 0.901; refractive index at 20 °C, 1.460; pH 5.2), extracted through steam distillation, were obtained from Ferquima Ind. Com. Ltd. (São Paulo, Brazil). The MAEO and MPEO used in this study present menthol as their majority constituent, corresponding to 56.85 and 59.73% of the EO's total mass, respectively (Sousa Guedes et al., 2016).

2.2. Bacterial strains and inoculum preparation

The *L. monocytogenes* (ATCC 7644) strain was obtained from the Collection of Reference Microorganisms, National Institute of Quality Control in Health, Oswaldo Cruz Foundation (Rio de Janeiro, Brazil), and the *E. coli* [UFPEDA 224, originally ATCC 25922, a surrogate for *E. coli* O157:H7 (Kim and Harrison, 2009)] and *Salmonella* Enteritidis (UFPEDA 414, originally MM 6247) strains were obtained from the Collection of Microorganisms, Department of Antibiotics, Federal University of Pernambuco (Recife, Brazil). Stocks were maintained in cryovials containing Brain Heart Infusion Broth (BHIB;

HiMedia, Mumbai, India) and glycerol (15 g/100 mL) at -20 °C. For inoculum preparation, a 3-mL aliquot from an overnight culture grown in BHIB at 37 °C for *E. coli* and *Salmonella* Enteritidis, and at 30 °C for *L. monocytogenes*, for 18–24 h (to reach stationary growth phase) were harvested (4,500 x *g*, 15 min, 4 °C), washed twice and re-suspended in PBS (50 mM K_2HPO_4/KH_2PO_4; pH 7.4; Sigma-Aldrich, St. Louis, USA) to obtain standard cell suspensions. The optical density (OD) reading at 625 nm (OD_{625}) of these suspensions was 0.1 and provided viable counts of approximately 8 $\log_{10}$ colony forming units per milliliter (CFU/mL) when pour-plated on Brain Heart Infusion agar (BHIA; HiMedia, Mumbai, India) (McMahon et al., 2008).

2.3. Preparation of fruit juices

Pineapple (*Ananas comosus* L. Merrill) and mango (*Mangifera indica* L.) fruit were purchased in the commercial maturation stage from a local wholesale distributor (João Pessoa, Brazil) and were selected for uniformity in size, form, appearance, and the absence of mechanical injuries or visible signs of infection. For each juice preparation, the fruit surface was disinfected by a 5-min immersion in a sodium hypochlorite solution (150 ppm, pH 7.2 adjusted using 1 M NaOH), washed with sterile distilled water, and dried for 30 min in a safety cabinet. Next, the fruit were aseptically peeled, chopped, and mixed with distilled water (1:1 ratio) using a domestic blender (for 3 min). The juices were centrifuged (12,500 x *g*, for 15 min, 4 °C) to separate the pulp from the remaining liquid. The supernatants were filtered using a triple-cheesecloth layer and sterilized by autoclaving (121 °C, 1.1 atm, for 15 min). The juices were stored in 50-mL aliquots at -20 °C, and when required, an aliquot was thawed under refrigeration (4 ± 0.5 °C) and used for subsequent assays (Sousa Guedes et al., 2016).

2.4. Exposure of bacteria to MAEO and MPEO in the juices

The effects of MAEO and MPEO on the test strains were evaluated in pineapple and mango juice under refrigerated storage. MAEO and MPEO were incorporated directly into the fruit juices to reach the final concentrations of 0.625 and 1.25 μL/mL, respectively, as previously described (Sousa Guedes et al., 2016). Initially, 150 μL of the tested bacterial suspension was inoculated into 5850 μL of separated juice samples (final viable counts of approximately 6 $\log_{10}$ CFU/mL) containing MAEO or MPEO. The different mixtures were gently hand-shaken for 30 s and subsequently incubated at 4 ± 0.5 °C for 15 min. The concentrations of 0.625 μL/mL MAEO and 1.25 μL/mL MPEO were selected based on our previous studies, which demonstrated that these concentrations reduced the counts of *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis in pineapple and mango juice, while also having less impact on the sensory characteristics of the juice (Sousa Guedes et al., 2016).

2.5. Bacterial viable cell counts in the juices

After the 15 min-exposure to MAEO or MPEO, a 100 μL-aliquot of each fruit juice sample was serially diluted in sterile PBS buffer (pH 7.4), and subsequently a 20 μL-aliquot of each dilution was inoculated onto BHIA using the microdrop technique (Herigstad et al., 2001). Juice samples without MAEO or MPEO were similarly assayed as controls. The plates were incubated for 24 – 48 h at 37 °C for *E. coli* and *Salmonella* Enteritidis, and at 30 °C for *L. monocytogenes*. Plates inoculated with aliquots from the juice samples with MAEO or MPEO were incubated for 24 h longer than the samples from the control juices. Data were generated from experimental data by plotting $\log_{10}$ N0-N (where N0 is the initial number of CFU/mL and N is the number of CFU/mL after 15 min). The results are expressed as a reduction of $\log_{10}$ CFU/mL cycles. The detection limit of the viable cell count procedure was 1 $\log_{10}$ CFU/mL.

2.6. Staining procedure

FC was used to investigate the effects of MAEO and MPEO on *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis membranes and cellular functions using the following fluorochromes: thiazole orange (TO, Sigma-Aldrich, St. Louis, MO, USA), a permeant dye that enters all cells, live and dead, to varying degrees; propidium iodide (PI, Sigma-Aldrich, St. Louis, MO, USA) for membrane integrity; bis-1,3-dibutylbarbutiric acid (BOX, Molecular Probes, Invitrogen, part of Life Technologies, Eugene, OR, USA) for membrane potential; ethidium bromide (EB, Sigma-Aldrich, St. Louis, MO, USA) for efflux activity; and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polysciences, Warrington, PA, USA) for respiratory activity. Prior to the FC analysis, cells from each test strain in the pineapple and mango juice with or without MAEO or MPEO were harvested (4,500 x *g*, 15 min, 4 °C), washed twice and re-suspended in PBS (pH 7.4) and labeled with the fluorochromes (Silva et al., 2011). Ethanol-fixed cells were used as a positive control for PI, BOX and EB staining, and as a negative control for CTC staining (Silva et al., 2010).

2.6.1. Membrane permeability

For TO, 1 µL of dye solution [10 µg/mL in DMSO (Sigma-Aldrich, St. Louis, USA)] was added to 1000 µL of each cell suspension in PBS (pH 7.4) and incubated for 5 and 15 min for *L. monocytogenes* and for *E. coli* and *Salmonella* Enteritidis, respectively, at room temperature (Singh and Barnard, 2016). For PI, *L. monocytogenes* suspensions were incubated for 5 min in PBS with 10 µg/mL PI, and *E. coli* and *Salmonella* Enteritidis suspensions were incubated for 15 min in PBS with PI 1 µg/mL at 37 °C (Silva et al., 2011).

2.6.2. Membrane potential

Suspensions of *L. monocytogenes*, and *E. coli* and *Salmonella* Enteritidis cells, were incubated in PBS (pH 7.4) with 0.5 mg/mL BOX and in PBS (pH 7.4) with 4 mM EDTA with 2.5 mg/mL BOX, respectively. *L. monocytogenes* suspensions were incubated for 5 min, and *E. coli* and *Salmonella* Enteritidis cells were incubated for 15 min at 37 °C (Silva et al., 2011).

2.6.3. Efflux activity

L. monocytogenes and *E. coli* and *Salmonella* Enteritidis suspensions were incubated in PBS (pH 7.4) with 1% (w/v) glucose and 5 mg/mL EB or in pure PBS with 10 mg/mL EB, respectively. *L. monocytogenes* suspensions were incubated for 5 min, and *E. coli* and *Salmonella* Enteritidis suspensions were incubated for 15 min at 37 °C (Silva et al., 2011).

2.6.4 Respiratory activity

Suspensions of *L. monocytogenes*, *E. coli* and *Salmonella* Enteritidis were incubated in PBS (pH 7.4) with 1% (w/v) glucose and 5 mM CTC for 30 min at 37 °C under stirring at 250 rpm (Silva et al., 2011).

2.7 *FC analysis*

FC measurements were performed using a BD Accuri C6 flow cytometer (BD Accuri C6, New Jersey, USA), with 488-nm excitation from a blue solid-state laser. Green fluorescence was collected in the FL1 channel (533 nm ± 30 nm) and red fluorescence in the FL3 channel (> 670 nm). 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 (TO and BOX) and FL3 (PI, EB and CTC) bandpass filters. Threshold level was adjusted for FSC (12,000) to eliminate noise or particles (of

cellular debris) that were much smaller than intact cells. Bacterial cells were gated per FSC/SSC parameters. Sample acquisition was operated at the low flow rate setting (12 mL/min), and a total of 10,000 events were acquired for each sample. Data analysis was performed using BD Accuri C6 Software.

2.8 Data analysis

Density plots representing forward scatter light (FSC) vs. side scatter light (SSC) were obtained during measurements. FSC is measured in the plane of the beam and gives relative information on cell size. SSC is measured at 90° to the beam and provides information on cell granularity. Density plot analysis of FL1 vs. FL3 was applied to determine the fluorescence properties of the cell populations. TO^+ PI^- cells (gate upper left; UL) represent intact cell membranes, and TO^+ PI^+ cells (gate upper right; UR) correspond to a damaged or slightly permeabilized cell membrane. TO^- PI^+ cells (gate lower right; LR) represent a permeabilized cell membrane, and TO^- PI^- cells (gate lower left; LL) represent damaged DNA or RNA, while the cell may still be intact (Surowsky et al., 2014). BOX^+ PI^- cells (gate UL) represent depolarized and nonpermeabilized cells; BOX^+ PI^+ and BOX^- PI^+ cells (gate UR and LR) correspond to a population of depolarized and permeabilized cells with different degrees of damage; and BOX^- PI^- cells (gate LL) represent unstained populations of intact cells, polarized and non-permeabilized (Hammer and Heel, 2012). Density plot analysis of SSC vs. FL3 was applied to determine the fluorescence properties of the EB^+ and CTC^+ populations, representing cells with altered efflux pump activity and non-impaired respiratory function, respectively (Léonard et al., 2016; Morishige et al., 2015). These populations were gated in the right rectangles.

2.9. Statistical analysis

The viable cell count assays were performed in three independent experiments in triplicate (from plating the same sample in threefold). The viable counts were obtained from a single mixture comprised of each fruit juice, the MAEO or MPEO concentration and the target bacterial inoculum. For these data, statistical analyses were performed to determine significant differences ($p \leq 0.05$) based on student's t-test or an ANOVA followed by a post hoc Tukey test. Sigma Stat 3.5 computational software (Jandel Scientific Software, San Jose, California) was used for the statistical analysis.

3. Results and discussion

3.1. Reduction in bacterial viable counts by MAEO and MPEO in juices

The reductions in bacterial viable counts of *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis in pineapple and mango juice containing 0.625 µL/mL MAEO or 1.25 µL/mL MPEO after a 15 min-exposure under refrigeration are shown in Table 1. Count reductions > 5 $\log_{10}$ cycles were observed for all target strains in pineapple juice with MAEO or MPEO. In mango juice with MAEO, the count reductions were 1.58 ± 0.15, 0.82 ± 0.11, and 1.57 ± 0.22 $\log_{10}$ cycles, and in mango juice with MPEO, the count reductions were 0.93 ± 0.14, 0.61 ± 0.16, and 0.98 ± 0.14 $\log_{10}$ cycles, for *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis, respectively. In the juices without MAEO or MPEO, count reductions in *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis were 0.15 ± 0.01, 0.05 ± 0.04, and 0.30 ± 0.08 $\log_{10}$ cycles, and 0.10 ± 0.05, 0.07 ± 0.01, and 0.11 ± 0.04 $\log_{10}$ cycles in the pineapple and mango juices, respectively. These count reductions in the juices without MAEO or MPEO could be due to the intrinsic characteristics of these juices, particularly their pH value (Manso et al., 2015; Sousa Guedes et al., 2016). MAEO and MPEO reduced the counts of target strains in pineapple juice more than ($p \leq 0.05$) in mango juice.

Since the non-growth of bacteria is not equivalent to cellular death, and the non-growing bacteria may still have metabolic activity (Bunthof and Abee, 2002), flow cytometric measurements were performed to detect the physiological functions of *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis following the exposure to MAEO or MPEO in pineapple and mango juice.

3.2. Flow cytometry study

To understand cell inactivation caused by a 15-min exposure to 0.65 µL/mL MAEO and 1.25 µL/mL MPEO, five fluorescent probes were used (TO, PI, BOX, EB and CTC) to monitor bacterial cellular functions, particularly the membrane integrity, membrane potential, efflux activity, and respiratory activity using FC. TO is a cell-permeant DNA dye used to identify DNA-containing particles and can enter all cells, while PI is an impermeant DNA dye that only penetrates cells with damaged membranes. Density plots corresponding to dual-parameters stained with TO and PI of *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis cells exposed to MAEO or MPEO in pineapple and mango juice are shown in Fig. 1. The percentage of the microbial populations that fall in each gate is presented in the four edges of each plot.

Bacterial cells exposed to MAEO or MPEO shifted cells from the gates LL and UL (cells with DNA damage and still intact and cells with intact membranes, respectively) to gates LR and UR (cells with permeabilized membrane and cells with damaged membrane or slightly permeabilized, respectively). The shifts to gate UR were from 64 to 94.4% (Fig. 1A), 21.8 to 39.1% (Fig. 1C), and 65.1 to 97.1% (Fig. 1E) for *E. coli*, *L. monocytogenes*, and *Salmonella* Enteritidis in pineapple juice, respectively. The shifts were from 50.5 to 55.5% (Fig. 1B), 0.0 to 0.2% (Fig. 1D), and 0.9 to 5.9% (Fig. 1F) for *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis in mango juice, respectively.

The percentage of TO^+ PI^+ stained cells in *E. coli* (Fig. 1A), *L. monocytogenes* (Fig. 1C) and *Salmonella* Enteritidis (Fig. 1E) exposed to MAEO or MPEO in pineapple juice were higher than the percentage of TO^+ PI^+ stained cells after exposure to the EOs in mango juice (Fig. 1B, D, and F), indicating that cytoplasmic membrane integrity was affected more by the EOs in the pineapple juice. The antibacterial effects of EOs at a more acidic pH (as occurs in pineapple juice) are probably higher because the EOs' constituents may behave more hydrophobically and enter cells more easily (Gutierrez et al., 2009). These results agree with the data that larger count reductions occurred in pineapple juice containing either MAEO or MPEO than in mango juice, probably due to the higher number of cells presenting a damaged membrane in the former. The rupture of the cytoplasmic membrane leading to increased permeability has been typically cited as the main antibacterial action mechanism of EOs (Gill and Holley, 2006; Sousa et al., 2015).

In the juice samples without MAEO or MPEO, there were less than 64, 21.8, and 65.1% of *E. coli*, *L. monocytogenes*, and *Salmonella* Enteritidis cells in pineapple juice (Fig. 1A, C, and E), respectively, and 50.5, 0.0 and 0.9% of these cells in mango juice (Fig. 1B, D, and F) were TO^+ PI^+. *E. coli* and *Salmonella* Enteritidis had a higher percentage of injured cells (TO^+ PI^+ and TO^- PI^+) than *L. monocytogenes* in both pineapple and mango juice. These results also agreed with the data from the viable counts assays, showing that the Gram-negative bacteria were more sensitive to MAEO and MPEO than *L. monocytogenes*, a Gram-positive bacterium. The available literature reports that menthol, the majority constituent of MAEO and MPEO used in this study, can interact with the bacterial phospholipidic membranes and induce damage to the cell structure by causing a perturbation of the lipid fraction, inducing alterations in permeability and leakage of intracellular materials (Trombetta et al., 2005).

The effects of MAEO and MPEO in pineapple and mango juice on the membrane cells of *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis are shown in Fig. 2. Using dual-parameter density plots of green fluorescence (y-axis) and red fluorescence (x-axis), it was possible to monitor the capability of *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis cells in pineapple (Fig. 2A, C, and E) and mango (Fig. 2B, D, and F) juice of accumulating and retaining BOX and PI, as indicators of membrane potential and membrane damage, respectively, following the exposure to MAEO or MPEO. Polarized cells can exclude anionic molecules (e.g., BOX) and depolarized cells allow the accumulation of BOX inside the cells (Silva et al., 2011). Representative membrane potential vs. permeability plots for *E. coli* (Fig. 2A and B), *L. monocytogenes* (Fig. 2C and D), and *Salmonella* Enteritidis (Fig. 2E and F) show loss of membrane potential in cells exposed to MAEO or MPEO in pineapple (Fig. 2A, C and E) and mango (Fig. 2B, D and F) juice.

For MAEO, the total percentages of depolarized and permeabilized cells were 88.2, 73.4, and 94.4% for *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis in pineapple juice, and 19.2, 2.1, and 5.1% in mango juice, respectively. The total percentage of depolarized and permeabilized cells of *E. coli*, *L. monocytogenes* and *Salmonella* Enteritidis were 86.4, 53.1, and 95.2% in pineapple juice with MPEO, and 14.8, 2.0, and 4.2% in mango juice with MPEO, respectively. For the three test strains, cells not exposed to MAEO or MPEO remained largely polarized and non-permeabilized in pineapple juice (32.3 - 46.6%), but in mango juice, the percentage (1.8 – 2.5%) was lower than in pineapple juice because most of the cells (83.4 – 97.6%) were in the UL gate, i.e., remained depolarized and non-permeabilized.

The percentages of BOX^+ PI^+ and BOX^- PI^+ cells, corresponding to populations of permeabilized cells with different degrees of damage, were higher in the pineapple juice containing MAEO or MPEO than the mango juice. *L. monocytogenes* (Fig. 2C and D)

presented a low percentage of BOX^+ PI^+ and BOX^- PI^+ cells. These data suggest that MAEO and MPEO affect bacterial cell membranes by decreasing polarity, in addition to increasing permeability over time. Furthermore, membrane depolarization seems to be an event prior to membrane permeabilization and damage (Díaz et al., 2010). Membrane depolarization presumably occurs when sufficient molecules have accumulated within the membrane, causing increased ion permeability and transmembrane ion gradient dissipation. Permeabilization may occur as an extension of these events due to the accumulation of additional molecules within the membrane from a higher concentration of EOs' constituents or a longer exposure time (Hammer and Heel, 2012).

Simultaneous double-staining and multiparametric FC could allow characterizing an intermediate state where cells show fluorescence with both probes, which could provide insight into the physiological functions of bacterial cells (Ferrario and Guerrero, 2017; Léonard et al., 2016; Singh and Barnard, 2016; Tracy et al., 2010). A higher percentage of cells was observed at gate UR, particularly in those exposed to MAEO or MPEO in pineapple juice (Fig. 1A, C and E, and 2A, C and E). As mentioned before, cells separated in this gate were stained by both TO or BOX and PI. The presence of these double-stained populations indicates that these cell membranes were damaged, allowing PI to penetrate the cells. These findings reveal that PI can diffuse into the cells because of the membrane damage caused by MAEO and MPEO (Ananta et al., 2004). However, membrane disruption alone could not have been responsible for bacterial cell inactivation in the pineapple and mango juice containing MAEO or MPEO; hence, efflux activity and respiratory activity in these cells were also evaluated.

Efflux activity was measured using EB, and the percentage of bacterial cell populations that fell in each gate is shown in Fig. 3. EB is a membrane-permeant that enters intact cell membranes but is actively pumped out of the cell via a non-specific proton anti-port

transport system. When the membrane is damaged and the efflux pump malfunctions, EB can stain the intracellular DNA in cells (Díaz et al., 2010; Kim et al., 2009). FC analysis using varying dyes with different targets in the bacterial cell suggests that the antibacterial activity of MAEO and MPEO is associated with their action toward several targets and not only in their direct membrane perturbation.

Exposure of cells to MAEO or MPEO led to a cell shift from the left gate (cells with efflux pump activity) to the right gate (cells with compromised efflux pump activity) compared to control cells, depending on the type of juice. In pineapple juice (Fig. 3A, C and E), the percentage of cells shifted from 10.1 – 46% in control juice samples, from 51.4 – 71.8% in juice samples containing MAEO or MPEO; in mango juice (Fig. 3B, D and F) shifted from 0.8 – 1.2% in control juice samples to 8.8 – 11.1% in juice samples containing MAEO or MPEO. The percentage of EB^+ cells was higher in juices containing MAEO or MPEO than in the control juices, indicating that efflux pump activity was compromised due to the MAEO and MPEO (Ferreira et al., 2014; Lechner et al., 2008).

The respiratory activity of bacterial cells was measured by using the CTC-reduction assay (Morishige et al., 2015). Density plots indicating the distribution of the respiratory-active subpopulation cells measured by FC are shown in Fig. 4. Respiratory-active cells, which have an active electron transport system, showed accumulation of fluorescent CTC-formazan particles. In contrast, the respiratory-inactive cells did not accumulate the formazan. Exposure of *E. coli* cells to MAEO or MPEO led to a shift in the cells from the right (cells with respiratory activity) to the left gate (cells with no respiratory activity) compared to control juice samples, depending on the type of juice (Fig. 4A and B). In pineapple juice (Fig. 4A), the percentage of cells with respiratory activity dropped from 59.1 (control) to 12.4 and 14.1% after exposure to MAEO and MPEO, respectively. In mango juice (Fig. 4B), the reduction was lower, dropping from 15.9 to 12 and 10% after exposure to MAEO and MPEO,

respectively. Similar behavior was observed for both *L. monocytogenes* (Fig. 4C and D) and *Salmonella* Enteritidis (Fig. 4E and F) in pineapple juice (38.4 to 16.6 and 3.1% and 42.3 to 10.1 and 9.6%, respectively) and in mango juice (65.1 to 1.5 and 0.6% and 12.6 to 9.4 and 8.5%, respectively).

The CTC dye is reduced by the respiratory electron transport chain to an insoluble fluorescent formazan, which accumulates in actively respiring cells. However, cells with low respiratory activity may not be detected as CTC positive, probably due to the relative toxicity of cellular CTC accumulation (Díaz et al., 2010; Ferreira et al., 2014). Hence, different viable cell count values (99.99% vs. 50.48%) can present similar percentages of CTC-positive cells (11.74% vs. 17.09%) (Bouhdid et al., 2009), as observed for *E. coli* in mango juice with MAEO and MPEO (Fig. 4B).

Considering the results of the FC analysis, MAEO and MPEO likely cause bacterial cell inactivation due to the impairment of several cellular functions, including cell wall permeabilization and efflux pump inhibitory activity (Saviuc et al., 2016). The multiparameter FC analysis using the double-staining method was a useful technique for real-time and quantitative detection of altered/non-altered physiological functions in *E. coli, L. monocytogenes* and *Salmonella* Enteritidis exposed to MAEO or MPEO in pineapple and mango juice, revealing more detail on the antibacterial mechanism of these EOs.

4. Conclusion

The results of this study showed that 0.625 µL/mL MAEO and 1.25 µL/mL MPEO incorporated directly into pineapple and mango juice reduce the counts of *E. coli, L. monocytogenes,* and *Salmonella* Enteritidis after an exposure time as short as 15 min. However, both MAEO and MPEO reduced the target bacteria in the pineapple juice more than in the mango juice. The results of the FC analysis indicate that MAEO and MPEO inactivate

E. coli, *L. monocytogenes* and *Salmonella* Enteritidis cells in pineapple and mango juice through a multi-target mechanism that disrupts the cytoplasmic membrane, increases permeability and potential depolarization, as well as inhibits the efflux pump and respiratory activity. Further studies with different microorganisms of concern in fruit juice are necessary for a better understanding of the microbial response to EO treatments in these products.

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Fig. 1. Fluorescence density plots of *E. coli* UFPEDA 224 (A and B), *L. monocytogenes* ATCC 7644 (C and D) and *Salmonella* Enteritidis UFPEDA 414 (E and F) in response to staining with TO and PI after a 15-min exposure to *M. arvensis* L. (MAEO; 0.625 µL/mL) or *M. piperita* L. essential oil (MPEO; 1.25 µL/mL) in pineapple (A, C and E) and mango (B, D and F) juice at 4 ± 0.5 °C. The vertical axis indicates the fluorescence intensity of the TO; the horizontal axis indicates the fluorescence intensity of the PI. The percentage of cell populations that fell in each gate are displayed in the four edges of each plot.

Fig. 2. Fluorescence density plots of *E. coli* UFPEDA 224 (A and B), *L. monocytogenes* ATCC 7644 (C and D) and *Salmonella* Enteritidis UFPEDA 414 (E and F) in response to staining with BOX and PI after a 15-min exposure to *M. arvensis* L. (MAEO; 0.625 µL/mL) or *M. piperita* L. essential oil (MPEO; 1.25 µL/mL) in pineapple (A, C and E) and mango (B, D and F) juice at 4 ± 0.5 °C. The vertical axis indicates the fluorescence intensity of BOX; the horizontal axis indicates the fluorescence intensity of the PI. The percentages of the cell populations that fell in each gate are displayed in the four edges of each plot.

Fig. 3. Fluorescence density plots of *E. coli* UFPEDA 224 (A and B), *L. monocytogenes* ATCC 7644 (C and D) and *Salmonella* Enteritidis UFPEDA 414 (E and F) in response to staining with EB after a 15-min exposure to *M. arvensis* L. (MAEO; 0.625 µL/mL) or *M. piperita* L. essential oil (MPEO; 1.25 µL/mL) in pineapple (A, C and E) and mango (B, D and F) juice at 4 ± 0.5 °C. The horizontal axis indicates the fluorescence intensity of EB; the vertical axis indicates the side-light scatter intensity. The negative stain subpopulation was gated in the left rectangles; the positive stain subpopulation was gated in the right rectangles. The percentages of the cell populations that fell in each gate are shown in each plot.

Fig. 4. Fluorescence density plots of *E. coli* UFPEDA 224 (A and B), *L. monocytogenes* ATCC 7644 (C and D) and *Salmonella* Enteritidis UFPEDA 414 (E and F) in response to staining with CTC after a 15-min exposure to *M. arvensis* L. (MAEO; 0.625 µL/mL) or *M. piperita* L. essential oil (MPEO; 1.25 µL/mL) in pineapple (A, C and E) and mango (B, D and F) juice at 4 ± 0.5 °C. The horizontal axis indicates the fluorescence intensity of CTC-formazan; the vertical axis indicates the side-light scatter intensity. The respiratory active cell subpopulation was gated in the right rectangles; the inactive cell subpopulation was gated in the rectangles. The percentages of the cell populations that fell in each gate are shown in each plot.

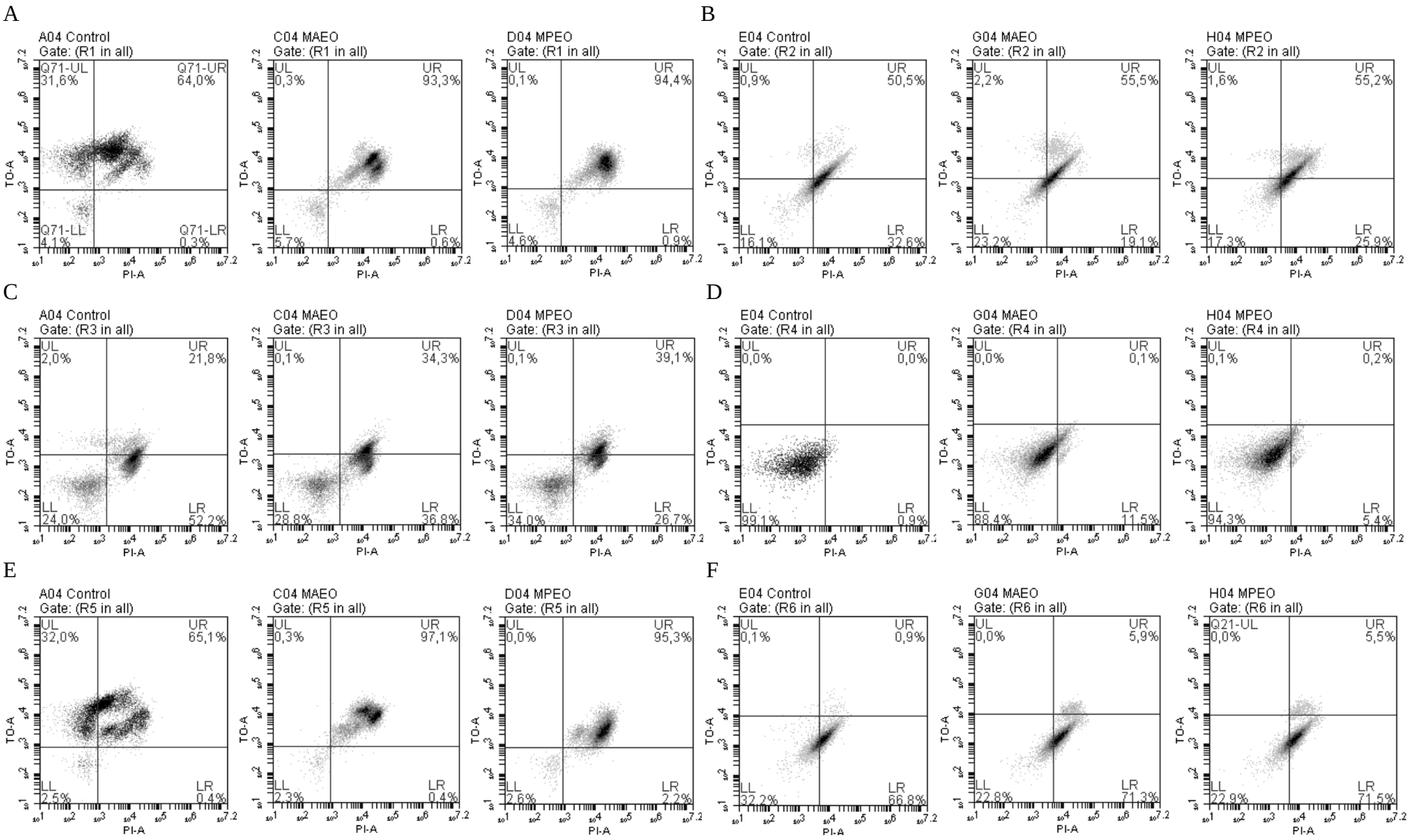
Table 1

Reduction of log_{10} CFU/mL cycles of *E. coli* UFPEDA 224, *L. monocytogenes* ATCC 7644, and *Salmonella* Enteritidis UFPEDA 414 exposed to *M. arvensis* L. (MAEO; 0.625 µL/mL) or *M. piperita* L. essential oil (MPEO; 1.25 µL/mL) in mango and pineapple juice after a 15 min-exposure at 4 ± 0.5 °C.

Strains	Treatments	Pineapple juice	Mango juice
		Reduction log_{10} cycle	Reduction log_{10} cycle
E. coli UFPEDA 224	Control	0.15 (± 0.01) ^{Ab}	0.10 (± 0.05) ^{Ac}
	MAEO (0.625 µL/mL)	5.10 (± 0.42) ^{Aa}	1.58 (± 0.15) ^{Ba}
	MPEO (1.25 µL/mL)	5.10 (± 0.46) ^{Aa}	0.93 (± 0.14) ^{Bb}
L. monocytogenes ATCC 7644	Control	0.05 (± 0.04) ^{Ab}	0.07 (± 0.01) ^{Ab}
	MAEO (0.625 µL/mL)	5.33 (± 0.39) ^{Aa}	0.82 (± 0.11) ^{Ba}
	MPEO (1.25 µL/mL)	5.33 (± 0.45) ^{Aa}	0.61 (± 0.16) ^{Ba}
Salmonella Enteritidis UFPEDA 414	Control	0.30 (± 0.08) ^{Ab}	0.11 (± 0.04) ^{Bc}
	MAEO (0.625 µL/mL)	5.15 (± 0.41) ^{Aa}	1.57 (± 0.22) ^{Ba}
	MPEO (1.25 µL/mL)	5.15 (± 0.51) ^{Aa}	0.98 (± 0.14) ^{Bb}

$^{A-B}$: Different superscript capital letters in the same row indicate significant difference ($p \leq 0.05$) among counts obtained for each strain in different fruit juices, based on student t test.

$^{a-c}$: Different superscript lowercase letters in the same column for each juice indicate significant difference ($p \leq 0.05$) among counts obtained for each strain after different treatments, based on Tukey's test.


A
A04 Control
Gate: (R1 in all)
Q71-UL
31,6%
Q71-UR
64,0%
Q71-LL
4,1%
Q71-LR
0,3%
C04 MAEO
Gate: (R1 in all)
UL
0,3%
UR
93,3%
LL
5,7%
LR
0,6%
D04 MPEO
Gate: (R1 in all)
UL
0,1%
UR
94,4%
LL
4,6%
LR
0,9%
B
E04 Control
Gate: (R2 in all)
UL
0,9%
UR
50,5%
LL
16,1%
LR
32,6%
G04 MAEO
Gate: (R2 in all)
UL
2,2%
UR
55,5%
LL
23,2%
LR
19,1%
H04 MPEO
Gate: (R2 in all)
UL
1,6%
UR
55,2%
LL
17,3%
LR
25,9%
C
A04 Control
Gate: (R3 in all)
UL
2,0%
UR
21,8%
LL
24,0%
LR
52,2%
C04 MAEO
Gate: (R3 in all)
UL
0,1%
UR
34,3%
LL
28,8%
LR
36,8%
D04 MPEO
Gate: (R3 in all)
UL
0,1%
UR
39,1%
LL
34,0%
LR
26,7%
D
E04 Control
Gate: (R4 in all)
UL
0,0%
UR
0,0%
LL
99,1%
LR
0,9%
G04 MAEO
Gate: (R4 in all)
UL
0,0%
UR
0,1%
LL
88,4%
LR
11,5%
H04 MPEO
Gate: (R4 in all)
UL
0,1%
UR
0,2%
LL
94,3%
LR
5,4%
E
A04 Control
Gate: (R5 in all)
UL
32,0%
UR
65,1%
LL
2,5%
LR
0,4%
C04 MAEO
Gate: (R5 in all)
UL
0,3%
UR
97,1%
LL
2,3%
LR
0,4%
D04 MPEO
Gate: (R5 in all)
UL
0,0%
UR
95,3%
LL
2,6%
LR
2,2%
F
E04 Control
Gate: (R6 in all)
UL
0,1%
UR
0,9%
LL
32,2%
LR
66,8%
G04 MAEO
Gate: (R6 in all)
UL
0,0%
UR
5,9%
LL
22,8%
LR
71,3%
H04 MPEO
Gate: (R6 in all)
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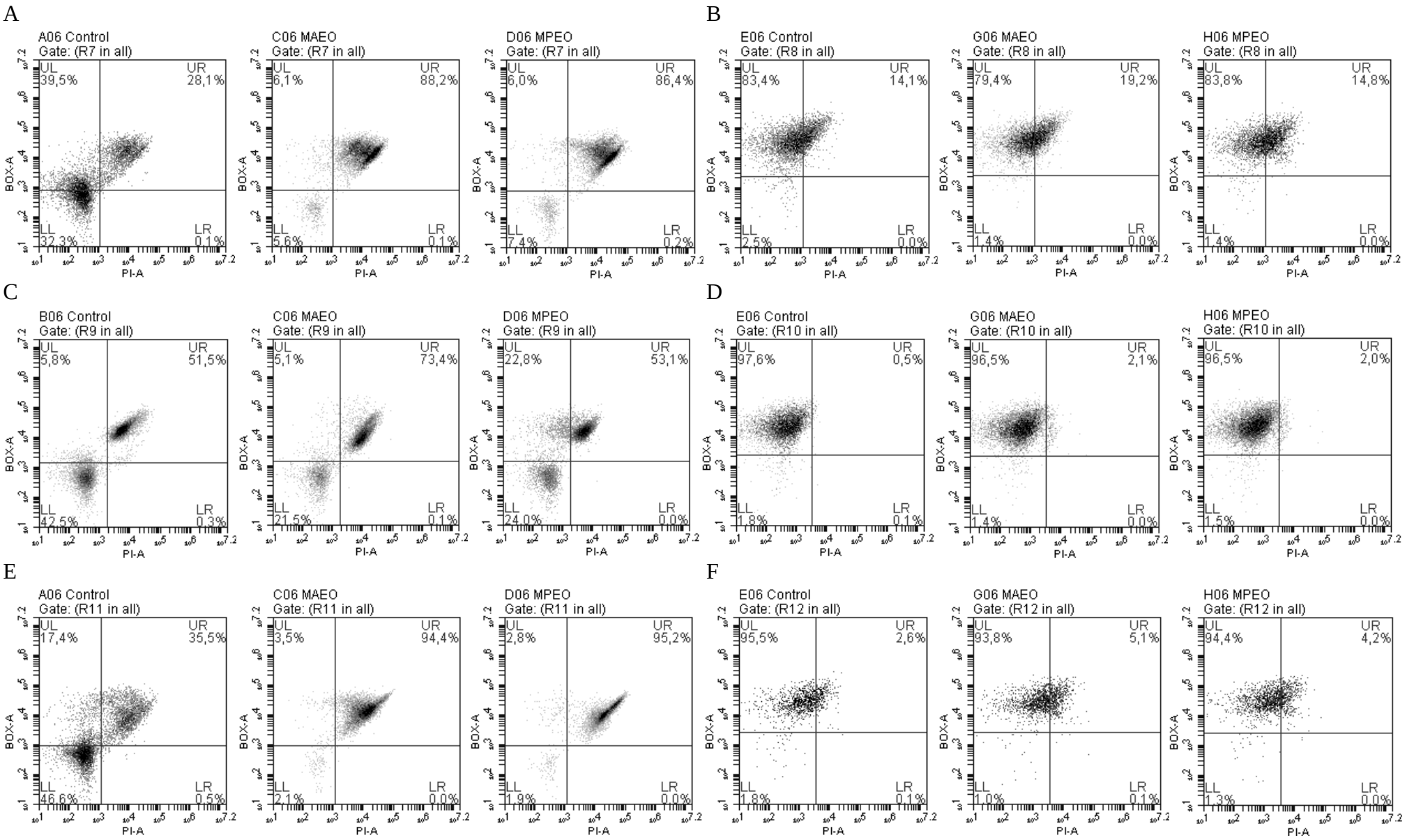
UR
5,5%
LL
22,9%
LR
71,5%
TO-A
PI-A

Fig. 1.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UR 28,1%
LL 32,3%
LR 0,1%
C06 MAEO
Gate: (R7 in all)
UL 6,1%
UR 88,2%
LL 5,6%
LR 0,1%
D06 MPEO
Gate: (R7 in all)
UL 6,0%
UR 86,4%
LL 7,4%
LR 0,2%
B
E06 Control
Gate: (R8 in all)
UL 83,4%
UR 14,1%
LL 2,5%
LR 0,0%
G06 MAEO
Gate: (R8 in all)
UL 79,4%
UR 19,2%
LL 1,4%
LR 0,0%
H06 MPEO
Gate: (R8 in all)
UL 83,8%
UR 14,8%
LL 1,4%
LR 0,0%
C
B06 Control
Gate: (R9 in all)
UL 45,8%
UR 51,5%
LL 42,5%
LR 0,3%
C06 MAEO
Gate: (R9 in all)
UL 25,1%
UR 73,4%
LL 21,5%
LR 0,1%
D06 MPEO
Gate: (R9 in all)
UL 22,8%
UR 53,1%
LL 24,0%
LR 0,0%
D
E06 Control
Gate: (R10 in all)
UL 97,6%
UR 0,5%
LL 1,8%
LR 0,1%
G06 MAEO
Gate: (R10 in all)
UL 96,5%
UR 2,1%
LL 1,4%
LR 0,0%
H06 MPEO
Gate: (R10 in all)
UL 96,5%
UR 2,0%
LL 1,5%
LR 0,0%
E
A06 Control
Gate: (R11 in all)
UL 17,4%
UR 35,5%
LL 46,6%
LR 0,5%
C06 MAEO
Gate: (R11 in all)
UL 3,5%
UR 94,4%
LL 2,1%
LR 0,0%
D06 MPEO
Gate: (R11 in all)
UL 2,8%
UR 95,2%
LL 1,9%
LR 0,0%
F
E06 Control
Gate: (R12 in all)
UL 95,5%
UR 2,6%
LL 1,8%
LR 0,1%
G06 MAEO
Gate: (R12 in all)
UL 93,8%
UR 5,1%
LL 1,0%
LR 0,1%
H06 MPEO
Gate: (R12 in all)
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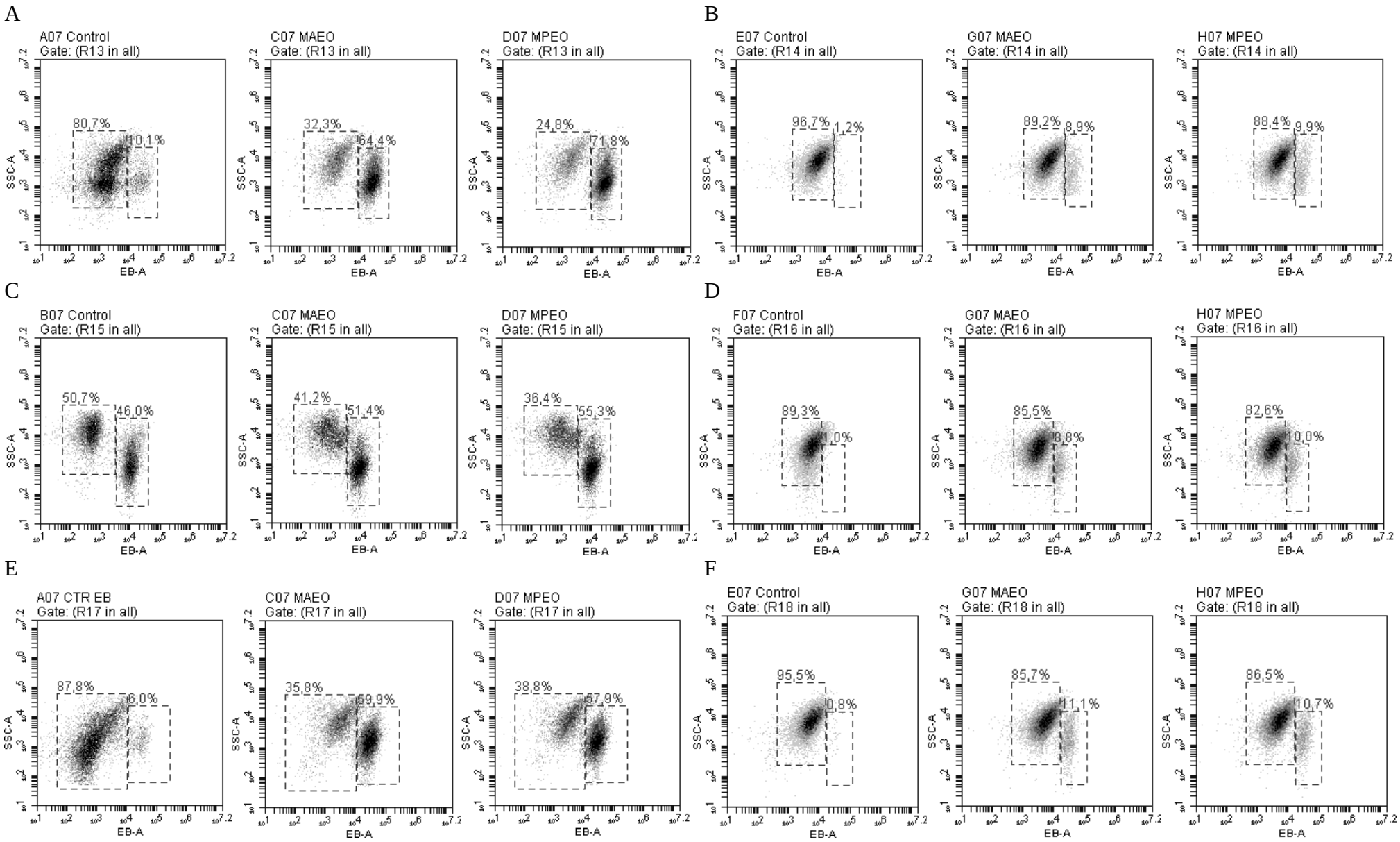
BOX-A
PI-A

Fig. 2.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C07 MAEO
Gate: (R13 in all)
32,3%
64,4%
D07 MPEO
Gate: (R13 in all)
24,8%
71,8%
B
E07 Control
Gate: (R14 in all)
96,7%
1,2%
G07 MAEO
Gate: (R14 in all)
89,2%
8,9%
H07 MPEO
Gate: (R14 in all)
88,4%
9,9%
C
B07 Control
Gate: (R15 in all)
50,7%
46,0%
C07 MAEO
Gate: (R15 in all)
41,2%
51,4%
D07 MPEO
Gate: (R15 in all)
36,4%
55,3%
D
F07 Control
Gate: (R16 in all)
89,3%
1,0%
G07 MAEO
Gate: (R16 in all)
85,5%
8,8%
H07 MPEO
Gate: (R16 in all)
82,6%
10,0%
E
A07 CTR EB
Gate: (R17 in all)
87,8%
6,0%
C07 MAEO
Gate: (R17 in all)
35,8%
59,9%
D07 MPEO
Gate: (R17 in all)
38,8%
57,9%
F
E07 Control
Gate: (R18 in all)
95,5%
0,8%
G07 MAEO
Gate: (R18 in all)
85,7%
11,1%
H07 MPEO
Gate: (R18 in all)
86,5%
10,7%
SSC-A
EB-A

Fig. 3.

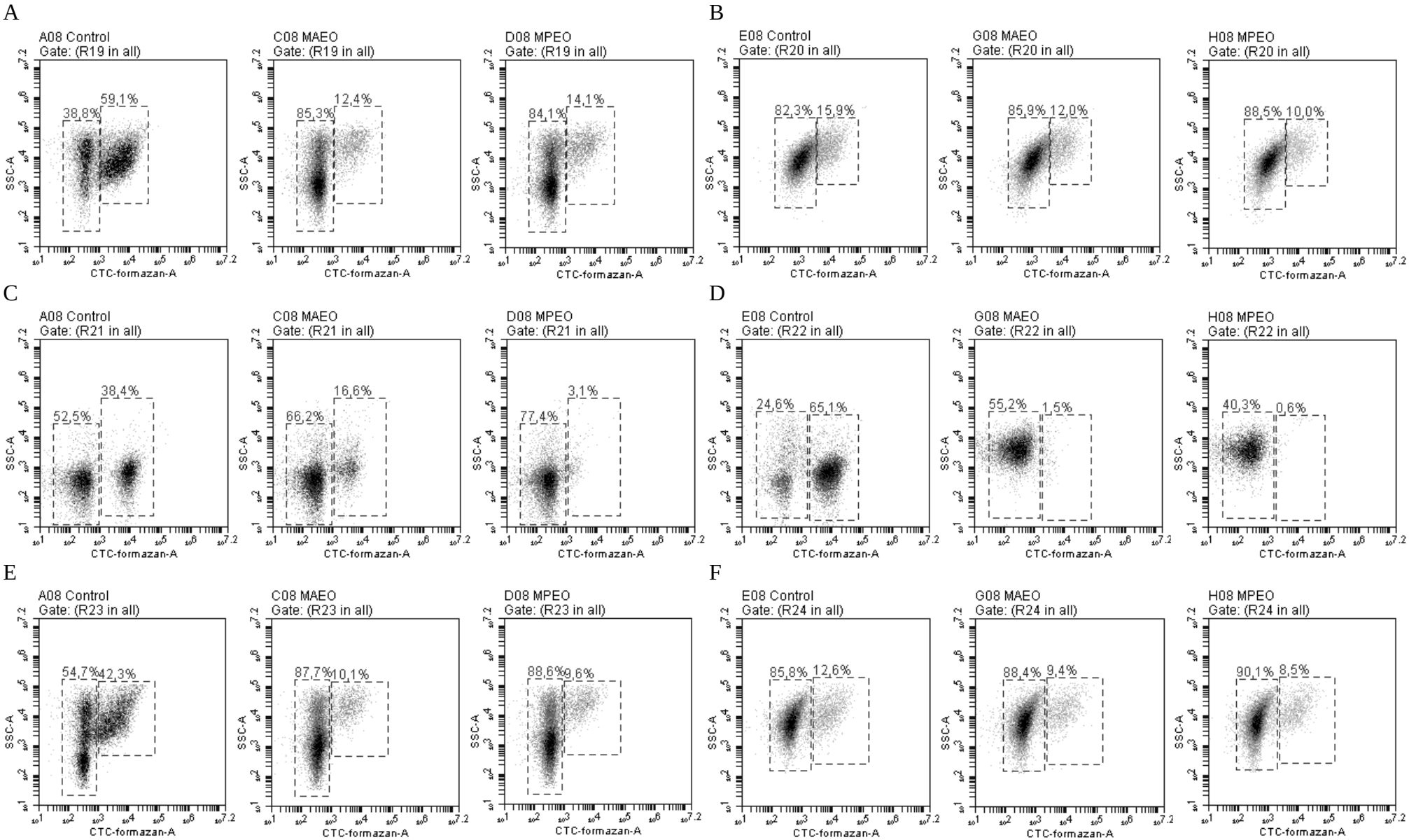
A
A08 Control
Gate: (R19 in all)
38,8%
59,1%
C08 MAEO
Gate: (R19 in all)
85,3%
12,4%
D08 MPEO
Gate: (R19 in all)
84,1%
14,1%
B
E08 Control
Gate: (R20 in all)
82,3%
15,9%
G08 MAEO
Gate: (R20 in all)
85,9%
12,0%
H08 MPEO
Gate: (R20 in all)
88,5%
10,0%
C
A08 Control
Gate: (R21 in all)
52,5%
38,4%
C08 MAEO
Gate: (R21 in all)
66,2%
16,6%
D08 MPEO
Gate: (R21 in all)
77,4%
3,1%
D
E08 Control
Gate: (R22 in all)
24,6%
65,1%
G08 MAEO
Gate: (R22 in all)
55,2%
1,5%
H08 MPEO
Gate: (R22 in all)
40,3%
0,6%
E
A08 Control
Gate: (R23 in all)
54,7%
42,3%
C08 MAEO
Gate: (R23 in all)
87,7%
10,1%
D08 MPEO
Gate: (R23 in all)
88,6%
9,6%
F
E08 Control
Gate: (R24 in all)
85,8%
12,6%
G08 MAEO
Gate: (R24 in all)
88,4%
9,4%
H08 MPEO
Gate: (R24 in all)
90,1%
8,5%
SSC-A
CTC-formazan-A

Fig. 4.

APÊNDICE G – Patente depositada no Instituto Nacional da Propriedade Industrial – INPI (BR 10 2017 006417 4).

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

29/03/2017 870170020625 09:03

00.000.2.2.17.0202833.4

Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2017 006417 4

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Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 29/03/2017 às 09:03, Petição 870170020625

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CPF: 88140113034

Nacionalidade: Brasileira

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CEP: 58051-900

País: BRASIL

Telefone: (83) 321 67576

Fax:

Email: magnani2@gmail.com

Documentos anexados

Tipo Anexo	Nome
Comprovante de pagamento de GRU 200	Comprovante de pagamento GRU.pdf
Relatório Descritivo	Relatório Descritivo.pdf
Reivindicação	Reivindicaçoes.pdf
Resumo	Resumo.pdf

Acesso ao Patrimônio Genético

☑ Declaração Negativa de Acesso - Declaro que o objeto do presente pedido de patente de invenção não foi obtido em decorrência de acesso à amostra de componente do Patrimônio Genético Brasileiro, o acesso foi realizado antes de 30 de junho de 2000, ou não se aplica.

Declaração de veracidade

☑ Declaro, sob as penas da lei, que todas as informações acima prestadas são completas e verdadeiras.

ANEXOS

ANEXO A – Comprovante de cessão das culturas microbianas

Departamento de Antibióticos

Recife, 11 de outubro de 2013.

Da: Profa Dra. Gláucia Manoella de Souza Lima
Diretora da Coleção de Microrganismos UFPEDA – Departamento de Antibióticos - UFPE

Para: Evandro Leite de Souza

Programa de Pós-Graduação em Nutrição.Departamento de Nutrição – CCS/UFPE

Em atendimento a sua solicitação, estamos encaminhando as cultura abaixo relacionadas.

Escherichia coli UFPEDA 224
Salmonella enteritidis UFPEDA 415

Atenciosamente.

Gláucia Manoella de Souza Lima
SIAPE:1736206

Departamento de Antibióticos - CCB/UFPE - Cidade Universitária - 50.670-901, Recife-PE. Brasil
Telefone: (081) 2126-8347/8866-8867| Fax: (081) 2126-8346 | e-mail: gmslima@yahoo.com.br

ANEXO B – 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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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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ANEXO C – Comprovante de aceite do artigo publicado no periódico Comprehensive Reviews in Food Science and Food Safety (ISSN 1541-4337).

Gmail Jossana Sousa <jossanasousa@gmail.com>

Decision on CRF3-2016-0152.R1

3 mensagens

kv7@email.psu.edu <kv7@email.psu.edu> 21 de março de 2016 17:10
Para: evandroleitesouza@hotmail.com
Cc: erika_yse@hotmail.com, jossanasousa@gmail.com

Dear Evandro de Souza:

We are pleased to inform you that your manuscript "The Potential of the Incorporation of Essential Oils and Their Individual Constituents to Improve Microbial Safety in Juices: A Review" (CRF3-2016-0152.R1) has been accepted for publication in Comprehensive Reviews in Food Science and Food Safety.

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A few comments of the Editor follow and, if applicable, please take appropriate action.

Editor's Comments to the Author:

Associate Editor: Kroger, Manfred
Comments to the Author:
As can be seen on pages 1-8 of the revised manuscript, the authors have satisfactorily replied to all comments and questions filed by the 3 peer reviewers and changes and additions (6 new references) have been made accordingly. All language and format deviations, as pointed out by the editor, have been meticulously corrected. The following further minor corrections can be made at the next best opportunity or during the final proofreading by the authors:
line 32: therein (1 word)
326: Arkansas Black apple juice
431, 625, 626: high-pressure homogenization (compound adjectives get a hyphen)
733: composition of nutrients
849: Author Contributions
page 56 (at bottom) and page 60: spore germination

During the latter part of the production process -- which should take about 1 to 2 months -- you will receive an e-mail with a link to access your PDF page proof. There are no page charges for Comprehensive Reviews in Food Science and Food Safety.

We greatly appreciate your choice of CRFSFS as an outlet for your fine work.

Sincerely,

Manfred Kroger, Ph.D.
Scientific Editor, Comprehensive Reviews in Food Science and Food Safety
Professor of Food Science Emeritus

ANEXO D – Comprovante de aceite do artigo publicado no periódico International Journal of Food Microbiology (ISSN 0168-1605).

Gmail **Jossana Sousa <jossanasousa@gmail.com>**

Enc: Your Submission

Evandro de Souza <evandroleitesouza@hotmail.com> 11 de setembro de 2016 11:26
Para: Jossana Sousa <jossanasousa@gmail.com>

De: ees.food.6f.3bebae.4852aab7@eesmail.elsevier.com <ees.food.6f.3bebae.4852aab7@eesmail.elsevier.com> em nome de Luca Cocolin <lcocolin@gmail.com>
Enviado: domingo, 11 de setembro de 2016 14:03:39
Para: evandroleitesouza@hotmail.com
Assunto: Your Submission

Ms. Ref. No.: FOOD-D-16-00591R3
Title: The efficacy of Mentha arvensis L. and M. piperita L. essential oils in reducing pathogenic bacteria and maintaining quality characteristics in cashew, guava, mango, and pineapple juices
International Journal of Food Microbiology

Dear Dr. Evandro de Souza,

I am pleased to inform you that your paper "The efficacy of Mentha arvensis L. and M. piperita L. essential oils in reducing pathogenic bacteria and maintaining quality characteristics in cashew, guava, mango, and pineapple juices" has been accepted for publication in International Journal of Food Microbiology.

Your accepted manuscript will now be transferred to our production department and work will begin on creation of the proof. If we need any additional information to create the proof, we will let you know. If not, you will be contacted again in the next few days with a request to approve the proof and to complete a number of online forms that are required for publication.

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Yours sincerely,

Chris W Michiels, PhD
Editor
International Journal of Food Microbiology

ANEXO E – Comprovante de submissão do artigo no periódico Food Microbiology (ISSN 0740-0020).

Gmail Jossana Sousa <jossanasousa@gmail.com>

Fwd: Successfully received: submission Investigation of damage to Escherichia coli, Listeria monocytogenes and Salmonella Enteritidis exposed to Mentha arvensis L. and M. piperita L. essential oils in pineapple and mango juice by flow cytometry for Food Microbiology

evandroleitesouza@ccs.ufpb.br <evandroleitesouza@ccs.ufpb.br> 7 de julho de 2017 14:45
Para: jossanasousa <jossanasousa@gmail.com>

De: "Food Microbiology" <EviseSupport@elsevier.com>
Para: evandroleitesouza@ccs.ufpb.br
Enviadas: Sexta-feira, 7 de julho de 2017 14:42:11
Assunto: Successfully received: submission Investigation of damage to Escherichia coli, Listeria monocytogenes and Salmonella Enteritidis exposed to Mentha arvensis L. and M. piperita L. essential oils in pineapple and mango juice by flow cytometry for Food Microbiology

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Ref: FM_2017_565
Title: Investigation of damage to Escherichia coli, Listeria monocytogenes and Salmonella Enteritidis exposed to Mentha arvensis L. and M. piperita L. essential oils in pineapple and mango juice by flow cytometry
Journal: Food Microbiology

Dear Dr. de Souza,

Thank you for submitting your manuscript for consideration for publication in Food Microbiology. Your submission was received in good order.

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Kind regards,

Food Microbiology

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