UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

EFEITOS DOS DERIVADOS VEGETAIS DE CONDIMENTOS NAS CARACTERÍSTICAS DE QUALIDADE E VIDA DE PRATELEIRA DE LINGUIÇA DE FRANGO FRESCA.

LIDIANE NUNES BARBOSA

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, Unesp, para obtenção do título de Doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração Biomoléculas-Estrutura e função

Prof. Dr. Ary Fernandes Júnior

BOTUCATU - SP

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FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO DE BIBLIOTECA E DOCUMENTAÇÃO - CAMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE - CRB 8/5651

Barbosa, Lidiane Nunes.
Efeitos dos derivados vegetais de condimentos nas características de qualidade e vida de prateleira de linguiça de frango fresca / Lidiane Nunes Barbosa. - Botucatu, 2014

Tese (doutorado) - Universidade Estadual Paulista, Instituto de Biociências de Botucatu
Orientador: Ary Fernandes Júnior
Capes: 20100000

1. Frango de corte - Subprodutos. 2. Carne - Qualidade. 3. Produtos de ação antimicrobiana. 4. Especiarias. 5. Essências e Óleos essenciais.

Palavras-chave: Ação antibacteriana; Especiarias; Extratosetanólicos; Linguiça fresca; Óleos essenciais.

"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota".

Madre Teresa de Calcutá

__AGRADECIMENTOS

*Primeiramente agradeço **à Deus** pela vida e por todas as oportunidades de crescimento e amadurecimento. Por permitir a realização desse trabalho e por estar sempre comigo.*

*Agradeço à minha mãe **Marina** por todo o amor, incentivo e dedicação. Por me ensinar a viver com fé e a esperar sempre o melhor da vida. Saudades imensas!*

*A minha irmã **Márcia** que soube viver de forma intensa e lutar pelos seus objetivos.*

*Agradeço ao meu pai **Luiz Carlos** que nunca mediu esforços para me ajudar e impulsionar e, mesmo à distância sempre esteve presente em cada passo dessa caminhada. Ao meu irmão **Lincon** para quem eu tento ser um bom exemplo e que ao mesmo tempo tem tanto a me ensinar...*

*À toda a **minha família** que direta ou indiretamente me ajudou e sempre torceu pelo meu sucesso.*

*À todos os meus **amigos** por estarem presentes nos momentos alegres e também tristes. Vocês foram fundamentais para que eu permanecesse de pé e não desistisse da luta.*

*Agradeço à **CAPES** pela bolsa de doutorado e a **FAPESP** pelo auxílio financeiro.*

*Agradeço as amigas e companheiras de laboratório **Isabella**, **Priscilla**, **Bruna**, **Fernanda** e **Mariana** por me ajudarem e muito com todos os experimentos. Agradeço especialmente a Bruna, Fernanda e Mariana por toda a amizade, dedicação, apoio, paciência e por sempre estarem presentes na minha vida.*

*Agradeço também ao pessoal do **Laboratório de Microbiologia de Alimentos** em especial a Ivana Giovannetti Castilho, **Laboratório de Nutrição e Dietética** e **Laboratório de Tecnologia dos Produtos de Origem Animal.** Graças ao auxílio desses colaboradores foi possível a execução do trabalho.*

*Agradeço à todos os **funcionários e amigos do Departamento de Microbiologia e Imunologia**. Sempre me lembrarei de todos com muito carinho e gostaria de agradecer por todo apoio e pela ótima convivência. Agradeço especialmente ao técnico **Lula** que foi peça fundamental na logística do trabalho e, além disso, foi um grande amigo.*

Agradeço o auxílio dos professores ***Júlio Toshimi Doyama*** *nas análises químicas dos óleos essenciais,* ***Luciano Barbosa*** *e* ***Lídia Raquel de Carvalho*** *nas análises estatísticas,* ***Roberto de Oliveira Roça*** *e* ***Cristiane Mengue Feniman Moritz*** *no planejamento experimental e aos doutores* ***Leonardo Noboru Seito*** *e* ***Juliana Aparecida Severi*** *com as análises fitoquímicas dos extratos etanólicos.*

Agradecimento muito especial a ***profª Dra Vera Lúcia Mores Rall*** *por me auxiliar com todo o seu conhecimento, competência e sensibilidade. Muito obrigada por sempre me acompanhar e espero que nossa parceria ainda renda muitos frutos!*

E finalmente, agradeço ao meu orientador ***profº Dr. Ary Fernandes Júnior*** *pelos onze anos de orientação, ensinamentos, dedicação, amizade e paciência. Foi muito bom trabalhar com uma pessoa tão especial e que dificilmente perde ternura e o bom humor. Lembro-me das aulas de microbiologia dadas no laboratório quando eu ainda não tinha realizado a disciplina no primeiro semestre de 2003. De quando começamos a trabalhar com os óleos essenciais e de quando me convidou para fazer mestrado. E agora é chegada a defesa de Tese. Palavras são insuficientes para demonstrar toda a minha gratidão e respeito. Muito obrigada professor Ary!*

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LISTA DE ABREVIAÇÕES

a: intensidade de vermelho

ANVISA: Agência Nacional de Vigilância Sanitária

ATCC: American Type Culture Collection

b: intensidade de amarelo

BHA: Butil-hidroxianisol

BHT: Butil- hidroxitolueno

BPF: Boas Práticas de Fabricação

CBP: Coluna capilar de sílica fundida

CFU: Colony Forming Units

CIE: Comitê Internacional de Iluminação

CIM: Concentração Inibitória Mínima

CoPS: Coagulase-positive staphylococci

D: Density/ densidade

DFD: Darck, Firm, Dry

e.g.: por exemplo

EC: broth for *E. coli*

Eet(s): extrato(s) etanólico(s)

EO(s): essential oil(s)

FAO: Food and Agriculture Organization of the United Nations

FDA: Food and Drug Administration

FIESP: Federação das Indústrias do Estado de São Paulo

Fig: Figura

FIOCRUZ: Fundação Oswaldo Cruz

GC-MS: Gas chromatography- mass spectrometry

GRAS: Generally Recognized as Safe

i.e.: ou seja

IAL: Instituto Adolfo Lutz

IDA: Ingestão Diária Aceitável

IFAC: International Food Additives Council

JECFA: Expert Committee on Food Additives

L: luminosidade

LEB: *Listeria* enrichment broth

M: molar

MBC: Minimal Bactericidal Concentration

MDA: malonaldeído

MET: Microscopia Eletrônica de Transmissão

mg: miligrama

MHB: Mueller Hinton Broth

MIC: Minimal Inibitory Concentration

MPN: Most Probable Number

NIST: National Institute of Standards and Technology

nm: nanômetro

NMP: Número Mais Provável

OE: óleo essencial

P: Peso

PBS: tampão fosfato

PCA: Plate Count Agar

pH: potencial Hidrogeniônico

PSE: Pale, Soft,Eexudative

SHMP: Hexametafosfato de sódio

SPS: agar sulfite polymyxin sulfadiazine

srC: *Sulphite-reducing* clostridia

T_1: Linguiça controle/ Sausage control

T_2: linguiça com 0,03% OE *O. basilicum*/ sausage with 0.03% of *O. basilicum* EO

T_3: linguiça com 0,3% OE *O. basilicum*/ sausage with 0.3% of *O. basilicum* EO

T_4: linguiça com SHMP/ sausage with SHMP

T_5: linguiça com SHMP mais 0,03% OE *O. basilicum/* sausage with SHMP plus 0.03% of *O. basilicum* EO

T_6: linguiça com SHMP mais 0,3% OE *O. basilicum/* sausage with SHMP plus 0.3% of *O. basilicum* EO

TBA: ácido 2-tiobarbitúrico

TBHQ: terc-butil hidroquinona

TC: termotolerant coliform

TCA: ácido tricloroacético

TEM: Transmission Electron Microscopy

TPA: Análise do Perfil de Textura

TSA: trypticase soy agar

TSAYE: trypticase soy agar supplemented with yeast extract

UFC: Unidade Formadora de Colônia

USDA: United States Departament of Agriculture

V: volume

W: weight

W_1: weight of Eppendorf vials

W_2: weight of 1 ml essential oil

µg: micrograma

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RESUMO

Resumo

A carne de frango e seus subprodutos têm aumentado a sua popularidade nos últimos anos devido ao desenvolvimento do setor e a mudanças significativas no padrão de consumo de alimentos. A linguiça fresca é provavelmente uma das formas mais antigas de processamento de carnes, no entanto, é um produto altamente perecível que necessita de agentes que contribuam para a extensão da sua vida de prateleira. As pesquisas com os produtos naturais de origem vegetal, sobretudo as especiarias, tem revelado que além da contribuição para o sabor, estes produtos possuem atividades antimicrobianas e antioxidantes que poderiam ser exploradas pela indústria de alimentos. O objetivo do trabalho foi avaliar o uso de derivados vegetais como possível aditivo em linguiça de frango. Para tanto, o estudo foi dividido em três etapas. Na primeira foi caracterizada a ação antibacteriana *in vitro* (metodologia da microdiluição em meio de Mueller Hinton Caldo-MHC e determinação da concentração inibitória mínima-CIM) de doze derivados vegetais na forma de óleos essenciais (OEs) e extratos etanólicos (Eet) obtidos de seis plantas condimentares (louro-*Laurus nobilis*, manjericão- *Ocimum basilicum*, manjerona-*Origanum majorana*, orégano-*Origanum vulgare*, alecrim -*Rosmarinus officinalis* e tomilho-*Tymus vulgaris*) frente a 9 cepas bacterianas (*Salmonella* Enteritidis-*ATCC* 13076, *Escherichia coli* O157:H7-*ATCC* 43895, *Pseudomonas aeruginosa –ATCC* 27853, *Listeria monocytogenes-ATCC* 15313, *Enterococcus faecalis-ATCC* 10100, *Aeromonas hydrphila-ATCC* 7966, *Lactobacillus rhamnosus-ATCC* 9595 e *Staphylococcus xylosus-ATCC* 29979). Todos os derivados vegetais apresentaram ação antibacteriana, entretanto, a atividade dos Eet foi inferior a dos OEs, mesmo quando da mesma planta. *T. vulgaris* e *O. vulgare* apresentaram as melhores atividades verificadas pelos valores de CIM de 1,5 e 1,6 mg/mL para OEs e de 2,9 e 4,2 mg/mL para Eet, respectivamente. Os óleos dessas plantas apresentaram o timol como composto majoritário e os extratos foram positivos para a presença de flavonoides, fenóis, triterpenos e esteroides. Numa segunda etapa foram testados dois produtos vegetais, escolhidos a partir dos testes *in vitro* (OE de *O. vulgare* e *O. basilicum)* em concentrações de 0,3; 1,0 e 1,5% v/g, para verificar a redução na contagem de *L. monocytogenes* e *S.* Enteritidis adicionadas artificialmente em linguiça de frango fresca (produzida por nossa equipe) após períodos de 0, 5 e 24 horas de estocagem a 4 °C. Foi verificada a redução na contagem bacteriana utilizando a metodologia do número mais provável (NMP). Também foi utilizada microscopia eletrônica de transmissão (MET) para visualização dos danos causados por estes OEs na estrutura das bactérias. Os resultados da MET mostraram que os óleos de *O. vulgare* e *O. basilicum*

danificaram as células das duas bactérias. Nos testes de recuperação bacteriana, apenas na concentração 1,5% os dois OEs foram eficazes frente a *L. monocytogenes* após 5 e 24 horas de contato. O melhor efeito inibitório sobre *S.* Enteritidis foi verificado por 1,0% de *O. vulgare* durante 5 horas de contato. *O. basilicum* mostrou redução significativa na mesma concentração, porém, após 24 horas. Na terceira etapa do estudo foram avaliados os efeitos do OE de *O. basilicum* e do aditivo polifosfato (hexametafosfato de sódio - SHMP) em amostras de linguiça. Foram preparados seis tratamentos: T_1- linguiça controle, T_2- linguiça com 0,03% de OE, T_3- linguiça com 0,3% de OE, T_4- linguiça com polifosfato, T_5- linguiça com polifosfato e 0,03% de OE e T_6- linguiça com polifosfato e 0,3% de OE. Foram verificados os aspectos de qualidade, vida de prateleira e aceitação global do produto (análise sensorial) num período de armazenamento de quinze dias (4°C). Os resultados mostraram aumento do pH, aumento da força de compressão e redução da perda de peso por cocção nas amostras com SHMP. Nas amostras com 0,3 (T_2) ou 0,03% (T_3) do óleo de *O. basilicum* os coliformes foram inibidos durante todo o período do estudado, o que não foi observado nas amostras com OE mais SHMP (T_5 e T_6), demonstrando assim que o estabilizante bloqueou a ação antibacteriana do óleo. Contudo, confirmou-se atividade antibacteriana dos produtos vegetais e o seu potencial para uso em alimentos, bem como as atividades já relatadas dos polifosfatos em produtos cárneos. O uso dos OE foi limitado devido ao impacto nas características organolépticas que pode levar a não aceitação do produto. No entanto, deve ser dada maior atenção ao uso concomitante dos conservantes sintéticos e naturais, e aos efeitos que esta combinação pode provocar.

Palavras chave: Especiarias, linguiça fresca, ação antibacteriana, óleos essenciais, extratos etanólicos.

ABSTRACT

Abstract

The chicken meat and their subproducts have increased its popularity in recent years due to the development of the sector and the significant changes in the pattern of food consumption. Fresh sausage is probably one of the oldest forms of meat processing, however, is a highly perishable product that requires agents to contribute to the extension of shelf life. Research with natural products of plant origin, especially the spices, has revealed that beyond of the contribution to flavor; these products have antimicrobial and antioxidant activities which could be explored by the food industry. The aim of this study was to evaluate the use of plant derivatives as a possible additive in chicken sausage. Thus, the study was divided into three phases. The first was characterized in vitro antibacterial action (microdilution methodology in Mueller Hinton Broth medium-MHC and determination of minimum inhibitory concentration-MIC) twelve plant derived in the form of essential oils (EOs) and ethanol extracts (Eet) obtained from six plants condiments (laurel-*Laurus nobilis*, basil-*Ocimum basilicum*, marjoram-*Origanum majorana*, oregano-*Origanum vulgare*, rosemary -*Rosmarinus officinalis* e thyme-*Tymus vulgaris*) against nine bacterial strains (*Salmonella* Enteritidis-*ATCC* 13076, *Escherichia coli* O157:H7-*ATCC* 43895, *Pseudomonas aeruginosa –ATCC* 27853, *Listeria monocytogenes-ATCC* 15313, *Enterococcus faecalis-ATCC* 10100, *Aeromonas hydrphila-ATCC* 7966, *Lactobacillus rhamnosus-ATCC* 9595 e *Staphylococcus xylosus-ATCC* 29979). All plant derivatives showed antibacterial action, however, the activity of Eet was less than the EOs, even when the same plant. *T. vulgaris* and *O. vulgare* showed the best activities verified by the MIC values of 1.5 and 1.6 mg/ml for EOs and 2.9 and 4.2 mg/ml for Eet, respectively. The oils of these plants showed thymol as major compound and the extracts were positive for the presence of flavonoids, phenols, triterpenes and steroids. In a second step were tested two plant products chosen from in vitro tests (*O. vulgare* and *O. basilicum* EO) at concentrations of 0.3, 1.0 and 1.5% v / g, to verify the reduction in the count of *L. monocytogenes* and *S.* Enteritidis artificially added to fresh chicken sausage (produced by our team) after periods of 0, 5 and 24 hour storage at 4°C. Was verified reduction in bacterial count using the methodology the most probable number (MPN). Was also used transmission electron microscopy (TEM) to visualize the damage caused by these bacteria in the structure of EOs. The results of the TEM showed that *O. vulgare* and *O. basilicum* oils damaged cells of both bacteria. In bacterial recovery tests, only concentration 1.5% both EOs were effective against *L. monocytogenes* after 5 and 24 hours of contact. The best inhibitory effect on *S.* Enteritidis was observed in 1.0% of *O. vulgare* after 5 contact hours. *O. basilicum* showed significant reduction at the same concentration, however, after 24 hours. In the third

phase of the study evaluated the effects of EO *O. basilicum* and polyphosphate (sodium hexametaphosphate - SHMP) in samples of sausages. Six treatments were prepared: T_1-sausage control, T_2-sausage with 0.03% EO, T_3-sausage with 0.3% EO, T_4- sausage with polyphosphate, T_5-sausage with polyphosphate and 0.03% EO, T_6- sausage with polyphosphate and 0.3% of EO. The aspects of quality, shelf life and overall acceptability of the product (sensory evaluation) were evaluated over a period of fifteen days storage (4°C). The results showed increased pH, increased compressive force and reduced cook loss at the samples with SHMP. In samples with 0.3 (T_2) or 0.03% (T_3) of the *O. basilicum* EO thermotolerant coliforms were inhibited during the entire period of the study, which was not observed in samples with EO plus SHMP (T_5 and T_6), demonstrating that the stabilizing block the antibacterial activity of the oil. Nevertheless, it was confirmed antibacterial activity of plant products and their potential for use in foods as well as the activities previously reported of polyphosphates in meat products. The use of EO was limited due to the impact on the organoleptic characteristics which may lead to rejection of the product. However, more attention to the concomitant use of synthetic and natural preservatives and the effects should be given that this combination can result.

Keywords: spices, fresh sausage, antibacterial action, essential oils, ethanol extracts.

INTRODUÇÃO

Introdução

1. Conservação de alimentos

Com poucas exceções, todos os alimentos perdem qualidade e vida útil em alguma proporção após a colheita, abate ou fabricação. Essa perda é muito dependente do tipo de alimento, composição, condições de formulação, embalagem, microbiota e armazenamento. A deterioração ou outras alterações que levam a perda de vida de prateleira podem ocorrer em qualquer um dos diversos estágios entre a aquisição de matérias-primas até o eventual consumo de um produto acabado (GOULD, 1996).

A deterioração pode ser definida como qualquer processo que leve a perda da segurança, da qualidade sensorial ou valor nutricional do alimento. Entre os tipos pode-se citar: 1) Deterioração microbiana: devido à presença ou atividade de micro-organismos; 2) deterioração enzimática: alterações indesejáveis devido a reações catalisadas por enzimas; 3) deterioração química: entre os componentes de alimentos ou entre os alimentos e seu ambiente e 4) deterioração física: alterações indesejáveis na estrutura dos alimentos. No entanto, o principal tipo de deterioração é a microbiana que pode afetar tanto a qualidade quanto a segurança dos alimentos (BERK, 2009).

O número e tipos de micro-organismos presentes nos alimentos pode ser um indicativo do potencial de deterioração. Isto não significa, necessariamente, que se relacionam com intoxicação ou infecção de origem alimentar. Os perfis microbianos de alimentos crus e produtos processados são completamente diferentes e representam diferentes conjuntos de problemas em termos de potencial de deterioração e questões de segurança alimentar. Em geral, os alimentos crus tendem a ter uma população heterogênea, enquanto os produtos processados geralmente contêm micro-organismos que podem sobreviver a condições de transformação e armazenamento subsequentes (FUNG, 2009).

A importância da limpeza e da higiene na produção de alimentos demorou muito para ser reconhecida. Foi somente por volta do século XIII, na Europa, que surgiram as primeiras normas de inspeção de carnes e de abatedouros de animais. Em 1658, Athanasius Kircher sugeriu a existência de relação entre a deterioração de carnes e leite e a presença de "vermes" invisíveis a olho nu, reconhecendo o significado das bactérias e de outros "micróbios" nos processos patogênicos. Apesar de muitas tentativas anteriores, foi o médico francês Louis Pasteur o primeiro cientista a compreender o papel dos micro-organismos nos alimentos. Em 1837, ele demonstrou que o azedamento do leite era provocado por micro-organismos e, em

1860, empregou o calor para destruir micro-organismos indesejáveis em alimentos (FRANCO e LANDGRAF, 2003). Hoje, sabe-se que os alimentos contaminados com micro-organismos patogênicos podem ter aspecto, odor e sabor normais e não precisam ser associados a alimentos em estado de deterioração (BRASIL, 2005).

Dentre as principais tecnologias de conservação estão a preservação por calor, onde micro-organismos e enzimas são destruídos a temperaturas elevadas. Preservação por baixas temperaturas, redução da atividade de água e radiação ionizante, que diminuem a atividade de micro-organismos e enzimas bem como a velocidade das reações químicas e a preservação química (BERK, 2009).

Devido aos impactos negativos de alguns processos de conservação na qualidade dos alimentos, limitações das técnicas e novos padrões de exigência dos consumidores, têm surgido outras tecnologias para este fim. Dentre os objetivos destaca-se a obtenção de alimentos com maior qualidade nutricional e organoléptica, frescos e com uma vida de prateleira adequada (ROSS et al. 2003). Nessa linha, tem ganhando espaço as tecnologias não térmicas como alta pressão hidrostática (LÓPEZ-PEDEMONTE et al. 2007) e campo elétrico pulsado (MARSELLÉS-FONTANET et al. 2009), diferentes sistemas de embalagens (APPENDINI e HOTCHKISS, 2002), produtos antimicrobianos naturais tais como micro-organismos desejáveis e/ou seus metabólitos (bacteriocinas) (MILLS et al., 2011; ROSS et al. 2003), sistema lactoperoxidase, lisozima, quitosana e os derivados de plantas (DEVLIEGHERE, VERMEIREN e DEBEVERE, 2004).

2. Antimicrobianos derivados de plantas

Agentes antimicrobianos naturais têm sido utilizados há séculos na conservação de alimentos. Óleos essenciais e especiarias já eram utilizados pelos antigos egípcios e também em países asiáticos, como China e Índia. No entanto, apenas recentemente, a ação conservante das especiarias e seus óleos essenciais têm sido focada na redução ou eliminação de bactérias patogênicas e aumento da qualidade dos produtos alimentares. Existem mais de 1340 plantas com compostos antimicrobianos definidos e mais de 30.000 compostos já detectados em óleos essenciais de plantas (TAJKARIMI, IBRAHIM e CLIVER, 2010).

De acordo com a forma tradicional de uso, a quantidade de especiarias e ervas utilizada nos alimentos não evita a deterioração por micro-organismos bem como podem servir como substrato para o crescimento microbiano e produção toxinas (CEYLAN e FUNG, 2004).

A análise de 236 amostras de 14 marcas diferentes de especiarias comercializadas no Brasil mostrou que 21% das amostras apresentaram excesso de coliformes termotolerantes e 5,6% foram positivas para a presença de *Salmonella* estando, portanto em desacordo com os padrões da legislação brasileira (MOREIRA et al. 2009). Uma série de surtos de salmonelose, causados por ervas e especiarias, ocorreram nos Estados Unidos, Reino Unido, Dinamarca, Holanda e Alemanha (ZWEIFEL e STEPHAN, 2012). No período 1973 a 2010 foram relatados 14 surtos atribuídos ao consumo de especiarias contaminadas por patógenos em países da América do Norte, Europa e Oceania. Juntos, esses surtos resultaram em 1946 casos, 128 hospitalizações e duas mortes. Foram identificados agentes etiológicos como *Salmonella enterica* e *Bacillus* spp. E, assim com no estudo de Zweifel e Stephan (2012), foi mostrada a ligação dos surtos com o consumo de alimentos onde especiarias não tratadas foram colocadas na etapa final de produção dos alimentos (VAN DOREN et al. 2013).

Durante a utilização das especiarias para a obtenção de extratos e óleos essenciais é possível concentrar os compostos ativos e com isso obter maior eficácia no controle de micro-organismos além de eliminar qualquer risco de contaminação presente na matéria prima fresca ou desidratada.

O uso de extratos de plantas, bem como outras formas alternativas de tratamento teve sua popularidade aumentada a partir da década de 1990. A escolha de plantas para verificação da possível atividade antimicrobiana inicia-se com o uso de extratos aquosos ou alcoólicos e são seguidas por vários métodos de extração orgânica (COWAN, 1999).

Os óleos essenciais são derivados do metabolismo secundário das plantas aromáticas. Na natureza, desempenham um papel importante na proteção das plantas como agentes antibacterianos, antivirais, antifúngicos, inseticidas e também contra herbívoros. São produtos líquidos, voláteis, límpidos e raramente coloridos, lipossolúveis e solúveis em solventes orgânicos, com uma densidade geralmente mais baixa do que a da água. Eles podem ser sintetizados em vários órgãos das plantas como brotos, flores, folhas, caules, galhos, sementes, frutos, raízes, madeira ou cascas e são armazenados em células secretoras, cavidades, canais, células da epiderme ou tricomas glandulares (BAKKALI et al. 2008; BURT, 2004).

Esses óleos podem ser extraídos por hidrodestilação, destilação a vapor, extração por solvente, CO_2 supercrítico (GUAN et al. 2007), entre outros métodos. São misturas naturais complexas que podem conter cerca de 20 a 60 de componentes em diferentes concentrações. Apresentam principalmente terpenos e terpenóides e outros constituintes aromáticos e alifáticos caracterizados por baixo peso molecular (BAKKALI et al. 2008).

A concentração de metabólitos secundários pode variar de acordo com a sazonalidade, ritmo circadiano, estágio de desenvolvimento e idade, temperatura, disponibilidade de água, a radiação UV, nutrientes do solo, altitude e composição atmosférica (GOBBO-NETO e LOPES, 2007). Além disso, o método de extração também pode influenciar a composição (GUAN et al. 2007; Holley e Patel, 2005) e consequentemente as atividades biológicas apresentadas. Quanto a atividade antimicrobiana em alimentos, a presença de gordura, carboidratos, proteínas, sal e pH podem influenciar a eficácia destes agentes (HOLLEY e PATEL, 2005) havendo a necessidade de uso de maiores concentrações do que aquelas verificadas nos testes realizados em meios de cultura (SMITH-PALMER, STEWART e FYFE, 2001).

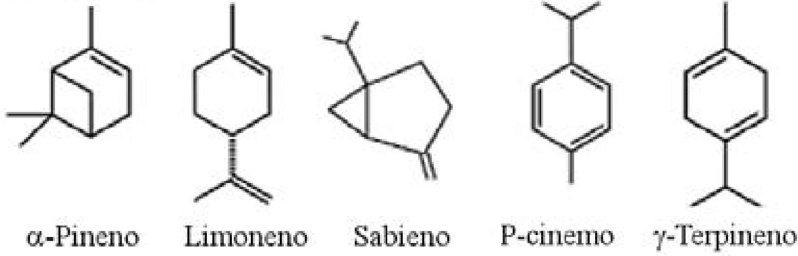
Os compostos ativos dos óleos essenciais podem ser divididos em quatro grupos de acordo com sua estrutura química: terpenos, terpenóides, fenilpropanóides, e "outros". **Terpenos** são hidrocarbonetos produzidos a partir de combinação de várias unidades de isopreno (C_5H_8). Os terpenos são sintetizados no citoplasma das células das plantas, através da via do ácido mevalônico. Os **terpenóides** são terpenos que sofreram modificações bioquímicas, via enzimas, que adicionam as moléculas de oxigênio e movem ou removem grupos metil. Podem ser subdivididos em álcoois, ésteres, aldeídos, cetonas, éteres, fenóis e epóxidos. **Fenilpropanóides** são sintetizados nas plantas a partir do aminoácido fenilalanina e constituem uma parte relativamente pequena dos óleos essenciais. Além desses grupos, os óleos essenciais contêm uma certa quantidade de **diferentes produtos de degradação,** provenientes de ácidos graxos insaturados, lactonas, terpenos, glicosídeos e compostos contendo enxofre e nitrogênio (CABALLERO, TRUGO e FINGLAS, 2003; HYLDGAARD, MYGIND e MEYER, 2012). Alguns exemplos desses compostos estão na Figura 1.

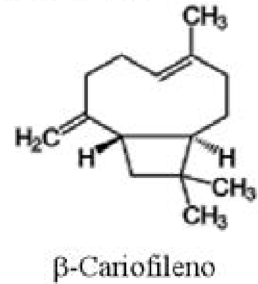
Embora o mecanismo de ação dos óleos essenciais sobre micro-organismos não tenha sido completamente elucidado, é consenso na literatura que estes compostos não só atuam na membrana citoplasmática, alterando sua permeabilidade e liberando constituintes intracelulares, mas também causam disfunção da membrana no que diz respeito ao transporte de elétrons, a absorção de nutrientes, a síntese de ácidos nucleicos, etc. A ação bactericida/ bacteriostática dos compostos é mostrada por alterações da parede celular e integridade de membrana e pelo estado fisiológico das bactérias. As alterações podem ser observadas por medição do vazamento do material celular, alterações na fluidez da membrana e a variação do teor de fosfolipídio, alterações nas funções da membrana, tais como transporte de elétrons e a absorção de nutrientes e por monitoramento do efeito destes compostos sobre as enzimas ligadas à membrana (NYCHAS, 1999).

Terpenos

Monoterpenos

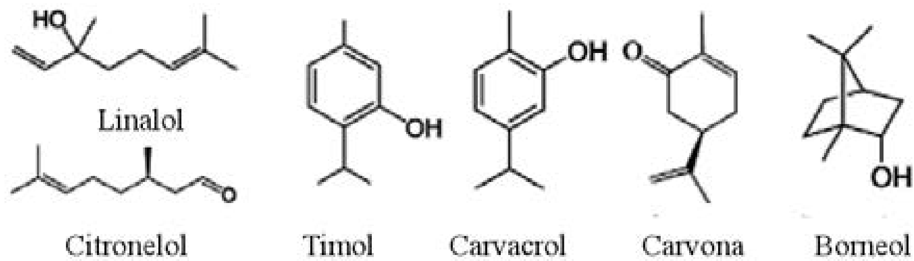
Sesquiterpenos



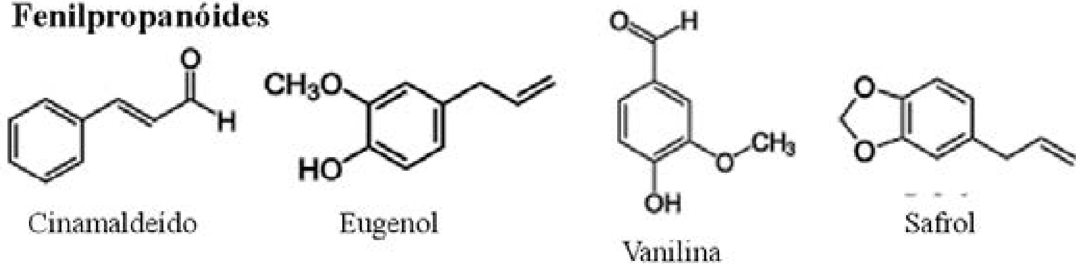


Terpenóides

Monoterpenóides



Fenilpropanóides



Outros

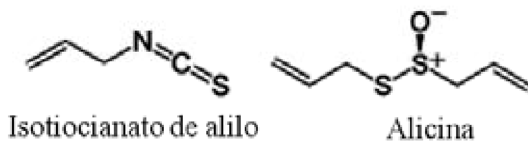


Fig. 1. Estrutura química de alguns constituintes dos óleos essenciais.

De acordo com a agência americana que regulamenta alimentos e medicamentos as especiarias (FDA), óleos essenciais de *Rosmarinus officinalis, Laurus nobilis*, *Ocimum basilicum*, *Origanum majorana*, *Origanum vulgare* e *Thymus vulgaris* são considerados substâncias GRAS, ou seja, “Substâncias Geralmente Reconhecidas como Seguras” para o consumo humano (FDA, 2013a).

3. Aditivos alimentares

Os aditivos alimentares têm sido utilizados durante séculos para dar sabor, cor, prolongamento da vida de prateleira, bem como a promoção da segurança dos alimentos. Eles podem ser divididos em cinco grandes categorias de acordo com a sua função: 1) potenciadores de sabor, 2) antioxidantes, 3) conservantes, 4) estabilizantes e emulsificantes e 5) corantes. Nos Estados Unidos existem mais de 3000 aditivos aprovados para uso em alimentos (RANGAN e BARCELOUX, 2009).

A Organização das Nações Unidas para Alimentação e Agricultura (FAO) definiu aditivo alimentar como uma substância não nutritiva adicionada intencionalmente ao alimento, geralmente em quantidades pequenas para melhorar a aparência, sabor, textura e propriedades de armazenamento (FAO, 1995). De acordo com a legislação brasileira, os aditivos alimentares são ingredientes adicionados intencionalmente aos alimentos, sem propósito de nutrir, com o objetivo de modificar as características físicas, químicas, biológicas ou sensoriais, durante a fabricação, processamento, preparação, tratamento, embalagem, acondicionamento, armazenagem, transporte ou manipulação de um alimento (BRASIL, 1997).

Os aditivos podem ser utilizados no Brasil, segundo as Boas Práticas de Fabricação (BPF). A Ingestão Diária Aceitável (IDA) deve ser "não especificada", ou seja, substância não representa risco à saúde nas quantidades necessárias para obter o efeito tecnológico desejado. A IDA é a quantidade estimada pelo "Comitê de Experts em Aditivos Alimentares" (JECFA) de aditivo alimentar, expressa em miligrama por quilo de peso corpóreo (mg/Kg), que pode ser ingerida diariamente, durante toda a vida, sem oferecer risco apreciável à saúde, à luz dos conhecimentos científicos disponíveis na época da avaliação (CODEX ALIMENTARIUS, 2013; BRASIL, 2002, 2014).

O fósforo é um nutriente essencial para a vida animal e vegetal. Ele é necessário ao crescimento, manutenção e reparação de todos os tecidos do corpo e (juntamente com o cálcio e magnésio) para a formação e crescimento adequado dos ossos, ocorrendo naturalmente nos alimentos, água e organismos. Os fosfatos são os derivados úteis do elemento fósforo, utilizados para melhorar as características e valorizar os alimentos em que são usados (IFAC, 2014).

Os fosfatos utilizados em indústrias de processamento de carne são sais de ácido fosfórico, de sódio ou de potássio. Dependendo do número de átomos de P na molécula o nome usual irá mudar: um átomo de fósforo $(PO_4)^3$ monofosfatos; dois átomos de fósforo

$(P_2O_7)^{4-}$ difosfatos; três átomos de fósforo $(P_3O_{10})^{5-}$ tripolifosfatos; e mais do que três átomos de fósforo $(P_nO_{3n+1})^{(n+2)-}$, polifosfatos (HOURANT, 2004; LONG, GÁL e BUŇKA, 2011). Os fosfatos alcalinos aumentam a capacidade de ligação da água nas proteínas musculares, preservando a suculência e aumentando o rendimento (SAMS, 2001). Tem sido relatado que também podem atuar na oxidação lipídica, textura e na cor da carne e produtos avícolas (MOLINS, 1991). Os polifosfatos são classificados como estabilizantes, cuja principal função é estabilizar uma mistura e não permitir que ocorram modificações físico-químicas no produto e são largamente utilizados em alimentos processados, incluindo carne vermelha, frango, frutos do mar e produtos lácteos (FAO, 1995; JURIATTO, 2003). A Tabela 1 lista os fosfatos comumente usados em produtos cárneos.

Quase todos os fosfatos bem como as misturas deles usadas nas carnes são alcalinos. Esses fosfatos têm várias funções tais como o ajuste de pH, propriedade tamponante, sequestro de cátions, alteração das distribuições das cargas iônicas, alteração da força iônica e efeito bacteriostático. Apesar de serem ligeiramente bacteriostáticos, os fosfatos não são considerados como conservantes (LONG et al. 2011).

O hexametafosfato de sódio também conhecido como tetrapolifosfato de sódio, polifosfato de sódio ou sal de Graham, pode ser utilizado até o limite máximo de 0,5% adicionado ao produto final de acordo com a legislação brasileira (BRASIL, 2006).

Entretanto, devido a critérios de saúde e estilo de vida saudável, uma crescente parcela de consumidores tem preferido produtos com menor adição de aditivos sintéticos (BUSATTA et al. 2008; DEVLIEGHERE et al. 2004).

A alergia e a intolerância são algumas das reações adversas aos aditivos alimentares. A alergia é uma reação onde ocorre o envolvimento do sistema imunológico (MADSEN, 1997), enquanto a intolerância é uma hipersensibilidade não mediada por IgE e induzida através de ativação direta de mastócitos. Os aditivos alimentares mais comuns a que os pacientes são intolerantes são os sulfitos, benzoato de sódio e corantes alimentares. Estima-se que 0,1-1,5% da população pode sofrer de intolerância a aditivo alimentar (WORM, 2011).

Os estudos também mencionam reações adversas crônicas aos aditivos alimentares, como alterações no comportamento (déficit de atenção e hiperatividade) e o desenvolvimento de alguns cânceres como o de estômago, esôfago e cólon, reto, mama e ovário, observados principalmente em longo prazo ou quando a IDA foi excedida (POLÔNIO e PERES, 2009).

Tabela 1. Lista dos fosfatos comumente utilizados em produtos cárneos e algumas das suas propriedades.

Nome comum	**Abreviação**	**Fórmulas**	**pH (solução 1%)**	**Solubilidade ($g/100g\ H_2O$)**	**INS***
Fosfato monossódico	MSP	NaH_2PO_4	4,4	85 (20°C)	339i
Fosfato dissódico	DSP	Na_2HPO_4	8,8	7,7 (20°C)	339ii
Fosfato trissódico	TSP	Na_3PO_4	12	13 (20°C)	339iii
Difosfato de sódio	TSPP	$Na_4P_2O_7$	10,2	6 (20°C)	450iii
Difosfato dissódico	SAPP	$Na_2H_2P_2O_7$	4,2	12 (20°C)	450i
Tripolifosfato de sódio	STPP	$Na_5P_3O_{10}$	9,8	15 (20°C)	451i
Hexametafosfato de sódio	SHMP	$(NaPO_3)_n$		Alta solubilidade	452i
		n = 10-15	6,2		
		n = 50-100	7,0		
Monofosfato de potássio	MKP	KH_2PO_4	4,4	20 (20°C)	340i
Fosfato dipotássico	DKP	K_2HPO_4	9,5	120 (20°C)	340ii
Fosfato tripotássico	TKP	K_3PO_4	12	51 (20°C)	340iii
Difosfato de potássio	TKPP	$K_4P_2O_7$	10,4	180 (20°C)	450v
Tripolifosfato de potássio	KTPP	$K_5P_3O_{10}$	9,6	178 (20°C)	451ii

Fonte: (LONG et al. 2011)

*INS: Sistema Internacional de Numeração.

A Comissão Especial (JECFA) reconhece muitas variáveis a serem consideradas em relação à segurança para o público dos usos atuais dos fosfatos nos alimentos. Estas incluem: (a) a variedade e as características diferentes de fosfatos e suas possibilidades de utilização, (b) as estreitas inter-relações metabólicas entre a vitamina D, cálcio e fósforo, e (c) as possíveis variações entre os diferentes segmentos da população no nível de fosfato consumido tanto em alimentos quanto em bebidas. O hexametafosfato de sódio é considerado uma substância GRAS e, portanto, não é considerado nocivo e o uso em alimentos não constitui um perigo se a quantidade total de fósforo ingerido e as ingestões de cálcio, magnésio, vitamina D e outros nutrientes são satisfatórios. No entanto, a possibilidade de que aumentos exagerados do uso destes fosfatos em alimentos reduziria significativamente a proporção cálcio:fósforo na alimentação e deve ser considerada na avaliação da probabilidade de um perigo de saúde (FDA, 2013b).

4. Carne de frango e linguiça

A carne de frango é uma fonte importante de proteínas de alto valor biológico e, além disso, as gorduras também são de boa qualidade, visto que se tratam em grande parte de mono e poli-insaturadas. Essa carne ainda é rica em ferro e fonte importante de vitaminas do complexo B, principalmente, B_2 e B_{12} (VENTURINI, 2007).

Os grandes avanços da pesquisa no desenvolvimento de novas tecnologias no setor da avicultura vêm tornando o Brasil um dos maiores produtores e exportadores mundiais de carne de frango. Aliado a esse fato, tem havido mudanças não desprezíveis no padrão alimentar fazendo da carne de frango uma das mais consumidas principalmente nas últimas duas décadas. Outro fator que também contribuiu para isso refere-se à abertura dos mercados resultante do processo da globalização econômica. Isso fez com que ocorresse uma elevação na escala de produção com redução de custos e, portando, um aumento no consumo (VOILA, 2013).

Os EUA ocupam historicamente a liderança mundial na produção de carne de frango, seguidos por China e Brasil. De acordo com o Departamento de Agricultura dos Estados Unidos (USDA), em 2012 foram produzidas 16,6 milhões de toneladas pelos EUA ante 13,7 milhões de toneladas pela China e 12,6 milhões de toneladas pelo Brasil. Nas exportações de carne de frango, o Brasil detém a liderança mundial desde 2004, e têm hoje um dos maiores consumos *per capita* do mundo totalizando 46 quilos por ano. Em 2012, as exportações brasileiras de carne de frango renderam US$ 7,2 bilhões, com 3,7 milhões de toneladas embarcadas. O valor arrecadado representou 3% das vendas externas do país e 7,5% do

agronegócio, o que leva o produto a figurar como o terceiro item de exportação do setor (FIESP, 2013).

A produção de frangos no Brasil está majoritariamente concentrada no Sul, que correspondeu a 60% dos abates em 2012. O estado do Paraná destacou-se como o principal produtor, com 28% do total, seguido por Santa Catarina (17%) e Rio Grande do Sul (14%). Apesar da falta de tradição, a Região Centro-Oeste é a que tem apresentado o maior crescimento, favorecida especialmente pela disponibilidade de grãos, fato que tem direcionado os investimentos da indústria para essa região (FIESP, 2013).

Com a crescente popularidade da carne de frango, aumentou também o consumo dos seus subprodutos, sendo os embutidos como salsicha e linguiça, os mais populares entre eles (BARBUT, 2001). O processamento de carnes surgiu da necessidade de conservar este alimento no passado. Nem toda a carne disponível podia ser consumida de uma só vez e a parte remanescente era processada para um consumo posterior (VANDENDRIESSCHE, 2008). A linguiça é uma das mais antigas formas de carne processada de que se tem conhecimento (ROMANS, 1994).

Não se sabe como e quando a primeira linguiça foi produzida. Existem inúmeros documentos atestando que as civilizações antigas fabricavam e consumiam esses alimentos há alguns milhares de anos atrás. Os romanos fizeram "circelli", "tomacinae", "butuli" e outros tipos de linguiças que eram consumidos durante as festas e sacrifícios. Linguiças feitas com tripas e outros subprodutos eram particularmente consumidos pelas classes mais pobres da população romana e a Igreja cristã primitiva proibiu seu consumo em Roma durante muitos anos (FAO, 1985).

De acordo com o Ministério da Agricultura, Pecuária e Abastecimento (MAPA), a linguiça é um produto cárneo industrializado, obtido de carnes de animais de açougue, adicionados ou não de tecidos adiposos e ingredientes, embutido em envoltório natural ou artificial e submetido ao processo tecnológico adequado. Conforme a tecnologia de fabricação pode ser um produto fresco, seco, curado e/ou maturado, cozido, entre outros. A composição deve possuir obrigatoriamente carne das diferentes espécies de animais de açougue e sal. Como ingredientes opcionais, a linguiça pode conter gordura, água, proteína vegetal e/ou animal, açúcares, plasma, aditivos intencionais, aromas, especiarias e condimentos (BRASIL, 2000).

Apesar da tecnologia de fabricação dos embutidos ser muito diversificada, composição química, microbiológica e métodos de processamento se desenvolveram mais nos últimos 20 anos que nos 3000 anos anteriores. As linguiças podem ser divididas em dois grupos: cruas e

tratadas termicamente. De acordo com os métodos aplicados na sua fabricação, as linguiças cruas podem ainda ser subdivididas em duas categorias: frescas e fermentadas. Da mesma forma, as linguiças tratadas termicamente são classificadas em defumadas pré-cozidas, do tipo emulsão (salsichas) e linguiças cozidas (FAO, 1985). Linguiças frescas são feitas a partir de cortes selecionados de carne e devem ser armazenadas sob-refrigeração ou congelamento até o preparo para consumo (LIU et al. 2009; ROMANS, 1994).

5. Análises de alimentos

5.1. Vida de prateleira

Os alimentos industrializados ou não, mantêm-se em constante atividade biológica, manifestada por alterações de natureza química, física microbiológica ou enzimática, o que os leva a deterioração da qualidade. Esta se caracteriza pela inaptidão dos produtos para o consumo humano, como resultado da existência de contaminação, da perda de atributos específicos, como cor, sabor, textura e viscosidade. A vida útil varia com o tipo de alimento, temperatura de estocagem, microbiota contaminante e embalagem utilizada. No planejamento de uma análise é muito importante determinar o critério que irá determinar o ponto final da vida de prateleira. São três tipos de ponto final (DUTCOSKY, 2011):

a) Ocorreu alteração no perfil sensorial global do produto;
b) Ocorreu alteração no atributo específico conhecido como atributo “chave” para percepção do consumidor de que o produto mudou;
c) A aceitabilidade do produto está muito baixa.

A vida de prateleira é o período durante o qual um produto alimentar mantém a sua segurança microbiológica e adequação a uma determinada temperatura de armazenamento e, em condições de armazenamento e manuseio especificadas (CODEX ALIMENTARIUS, 2009). O estudo de vida de prateleira dos alimentos consiste em submeter várias amostras a uma série de testes e examiná-las durante um período de tempo até o limite de aceitação. São observadas as alterações na qualidade do produto e o tempo que ele leva para se deteriorar até o limite que o torna impróprio para o consumo (MORI, MOURA e GERMER, 2004).

A carne de frango é um produto altamente perecível pelo fato de ser um excelente meio de crescimento microbiano por apresentar pH adequado e água livre em abundância. As carnes podem ser contaminadas com micro-organismos durante o abate ou processo de fabricação, embora os tecidos de animais saudáveis sejam naturalmente estéreis no momento do abate. Mesmo com a manutenção de uma cadeia de frio apropriada, o prolongamento da

vida de prateleira do frango depende também das condições microbiológicas, tipo de embalagem, atmosfera, entre outras diversas atividades metabólicas do produto (GILL, 1979; MEAD, 2004).

Juntamente com a com contaminação microbiológica, a oxidação lipídica é uma das principais causas da deterioração de qualidade da carne e seus derivados. Alterações indesejáveis na cor, sabor e valor nutritivo ocorrerem quando os lipídios oxidados da carne interagem com os outros componentes do alimento como pigmentos e proteínas, carboidratos e vitaminas (LOVE e PEARSON, 1971).

5.1.1. Oxidação lipídica e teste TBA (ácido 2-tiobarbitúrico)

O ranço oriundo da oxidação lipídica é um dos defeitos mais importantes de qualidade de carne ou produtos cárneos durante a armazenagem (LIU et al. 2009). A oxidação lipídica é iniciada pela abstração de um átomo de hidrogênio em ácidos graxos insaturados e se propaga como uma reação em cadeia mediada por radicais livres (VAYALIL, 2002). Os produtos secundários da oxidação, como os aldeídos, cetonas e ésteres são responsáveis pelo aumento da deterioração e o sabor rançoso durante o armazenamento congelado (NOLLET, 2008).

Carnes processadas como as linguiças estão mais sujeitas a oxidação. A moagem da carne altera a integridade das membranas musculares e expõe as membranas lipídicas a íons metálicos, o que facilita as interações entre pró-oxidantes e ácidos graxos insaturados (GILL, 1979).

O teste de TBA quantifica o malonaldeído (MDA), um dos principais produtos de decomposição dos hidroperóxidos de ácidos graxos poliinsaturados, formado durante o processo oxidativo. A reação envolve o ácido 2-tiobarbitúrico com o malonaldeído, produzindo um composto de cor vermelha, medido espectrofotometricamente a 532 nm de comprimento de onda. A quantificação de MDA é feita a partir de curvas de calibração construídas com concentrações conhecidas de MDA (OSAWA, FELÍCIO e GONÇALVES, 2005).

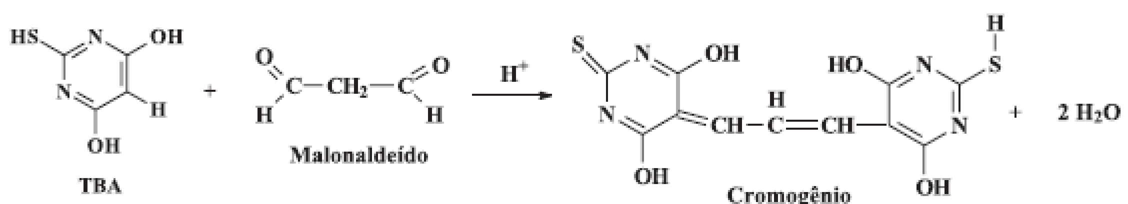


Fig. 2. Reação do teste de TBA entre o ácido 2-tiobarbitúrico e o malonaldeído, formando o composto colorido, medido espectrofotometricamente a 532 nm.

As determinações podem ser feitas diretamente na amostra; no lipídio extraído da amostra; no extrato ácido-aquoso e na porção do destilado da amostra. (OSAWA et al. 2005).

Agentes naturais que possuem propriedades antioxidantes e antimicrobianas têm a vantagem de serem facilmente aceitos pelos consumidores (BARBUT, 2001). Esses agentes naturais têm sido empregados em carnes devido aos seus benefícios potenciais para a saúde e segurança, em comparação com conservantes sintéticos, tais como butil-hidroxianisol (BHA), butil- hidroxitolueno (BHT) e terc-butil hidroquinona (TBHQ).

A atividade antioxidante irá depender da concentração dos compostos ativos presentes no produto capazes de eliminar os radicais livres formados durante o período de armazenamento (NOLLET, 2008).

5.1.2. Parâmetros microbiológicos para linguiças

Durante a produção de linguiças ocorre uma série de etapas de manipulação, o que eleva as possibilidades de contaminação por uma gama de espécies de micro-organismos, sejam eles patogênicos ou deteriorantes (TUTENEL et al. 2003). Além disso, a linguiça fresca não sofre tratamento térmico para redução da microbiota e possui elevada atividade de água, o que leva este alimento a ter uma curta vida de prateleira, estando diretamente sujeito à carga microbiana presente (TERRA, 1998).

Os parâmetros microbiológicos para embutidos frescais (linguiças cruas e similares) constantes na legislação brasileira são: ausência de *Salmonella* sp em 25g, Estafilococos coagulase positiva e Coliformes a 45°C tolerados até o limite de 5.10^3 UFC/g, respectivamente, por amostra e *Clostrídios* sulfito redutores até o limite de 3.10^3 UFC/g por amostra. A denominação de "coliformes a 45ºC" é equivalente à denominação de "coliformes de origem fecal" e de "coliformes termotolerantes". A determinação de clostrídio sulfito redutor a 46°C tem por objetivo a indicação de *Clostridium perfringens* e *C. botulinum*. A enumeração de estafilococos coagulase positiva tem por objetivo substituir a determinação de *Staphylococcus aureus*. Já o resultado da determinação de *Salmonella* sp, deve ser expresso como presença ou ausência na alíquota analisada (BRASIL, 2001).

Salmonella

A ocorrência e a quantidade de *Salmonella* presente na carne varia de acordo com as condições de manejo durante a criação e com os cuidados higiênicos nas operações de abate dos animais e posterior manipulação das carcaças. Apesar dos avanços tecnológicos, a carne de frango ainda é passível de contaminação bacteriana, especialmente por micro-organismos do gênero *Salmonella* que se encontram albergados no trato intestinal podendo contaminar as carcaças bem como outros produtos caso o processo de abate não seja realizado com cuidados higiênicos. Desta maneira os alimentos de origem animal, principalmente a carne de frango, representam papel fundamental na epidemiologia das salmoneloses humanas, podendo tornar-se um problema potencial na determinação de quadros de infecção alimentar em seus consumidores (CARVALHO e CORTEZ, 2005).

Estafilococos coagulase positiva

Os surtos de intoxicação alimentar estafilocócica na maioria das vezes estão associados com carnes vermelhas processadas, aves, molhos, produtos lácteos, e produtos de panificação contendo creme. A maioria dos surtos de contaminação dos alimentos com *Staphylococcus aureus* resulta da manipulação insalubre e manutenção do alimento na temperatura incorreta, permitindo assim o crescimento e síntese de enterotoxina pelo patógeno. O crescimento do *S. aureus* é acompanhado pela produção de compostos extracelulares como hemolisinas, nucleases, coagulase, lipase, e enterotoxinas. As enterotoxinas causadoras de intoxicação alimentar são produzidas por cerca de um terço das linhagens coagulase-positiva de *S. aureus* e a produção apropriada delas é afetada pela qualidade nutricional, pH do substrato, temperatura, atmosfera, teor de cloreto de sódio (e, portanto, atividade de água), outros produtos químicos e micro-organismos competidores (HALPIN-DOHNALEK e MARTH, 1989).

***Clostrídios* sulfito redutores**

Clostridium perfringens é uma das principais causas de infecções bacterianas de origem alimentar em países, onde o consumo de carne e aves é elevado. A doença é causada por uma enterotoxina produzida durante a esporulação e os veículos de infecção são tipicamente à base de carne e de aves (LIN e LABBE, 2003).

Essa bactéria é um importante patógeno humano e veterinário, Gram-positivo e formador de esporos. *C. perfringens* pode expressar pelo menos 15 toxinas diferentes, no entanto, os isolados expressam individualmente apenas algumas delas. Dessa forma, esses

isolados são classificados em cinco grupos (A-E) de acordo com as toxinas produzidas Os isolados do tipo A, por exemplo, estão associados com várias doenças gastrointestinais humanas, incluindo diarreia esporádica e diarreia associada a antibióticos (FISHER et al. 2005).

Coliformes termotolerantes

Os coliformes são bastonetes gram-negativos, que possuem, como habitat natural, o trato intestinal do homem e de animais. Pertencem à família *Enterobacteriaceae Salmonella* e *Shigella*. Podem ser divididos em coliformes totais e termotolerantes, dependendo do habitat do micro-organismo (SOUSA, 2006).

Coliformes termotolerantes são definidos como coliformes capazes de fermentar a lactose com produção de gás em 48h a 45ºC. *Escherichia coli*, juntamente com algumas cepas de *Enterobacter* e *Klebsiella*, podem apresentar essas características. Entretanto, apenas a presença de *Escherichia coli* em alimentos indica contaminação fecal por ser encontrada em grande quantidade no trato gastreintestinal do homem e animais de sangue quente, não sendo isolada normalmente em outros nichos. A relação direta da presença de coliformes termotolerantes em alimentos e água com contaminação de origem fecal não é correta, assim, a denominação clássica de coliformes fecais foi alterada para coliformes a 45ºC na legislação brasileira (SILVA, CAVALLI e OLIVEIRA, 2006).

Nos alimentos processados, a presença de níveis elevados de bactérias do grupo coliforme indica tratamento inadequado e/ou, contaminação pós-processamento ocorrida, principalmente, pelo contato do produto acabado com matérias-primas e equipamentos contaminados ou falta de higiene durante manipulação (RASZL et al. 2001).

Deteriorantes comuns em carne de frango

O metabolismo bacteriano produz uma mistura complexa de ésteres voláteis, alcoóis, cetonas e compostos contendo enxofre, os quais formam em conjunto, os odores indesejáveis. O primeiro indício de deterioração da carne de frango fresca é a produção desses odores, que se tornam aparentes quando os números microbianos atingem cerca de 10^7 UFC/cm^2. Neste ponto, os micro-organismos já esgotaram os níveis de glicose na carne e passam a utilizar os aminoácidos como substrato de crescimento. Quando a carga microbiana atinge níveis de cerca de 10^8 UFC/cm^2, mais um indicativo de deterioração torna-se evidente como a limosidade superficial visível (ADAMS e MOSS, 2000).

O micro-organismo mais comumente associado com a deterioração das carnes refrigeradas armazenadas sob condições aeróbicas é a *Pseudomonas* spp. Além de ser um micro-organismo psicotrófico, a *Pseudomonas* é altamente oxidativa, o que a torna capaz de usar compostos nitrogenados com fonte de energia. Essas características dão a esta bactéria vantagem competitiva na deterioração de carnes. Outro deteriorante frequentemente associado com a deterioração de carnes é o *Lactobacillus* spp. que pode ser encontrado tanto em condições aeróbicas quanto anaeróbias. Apesar de comum em muitos alimentos os *Lactobacillus* estão presentes em pequena quantidade na carne recém-abatida, no entanto, seu crescimento é estimulado por baixas temperaturas e embalagem à vácuo. As leveduras também são muito comuns na carne de frango. Espécies proteolíticas e/ou lipolíticas como *Candida zeylanoides* e *Yarrowia lipolytica* são as espécies mais comumente encontradas nesses alimentos (NOLLET, 2008).

5.2. Análises físico-químicas

As análises físico-químicas são de grande importância na avaliação da qualidade dos alimentos. Com elas, obtêm-se informações qualitativas e quantitativas dos nutrientes presentes nos alimentos, sobre aspectos toxicológicos e até mesmo microbiológicos. Os padrões de identidade de qualidade dos produtos alimentícios são atributos físico-químicos, microbiológicos e sensoriais que os caracterizam. Tais características são descritas no *Codex Alimentarius* e em diversas leis, decretos, resoluções, portarias e outros instrumentos técnicos e legais. As análises físico-químicas são primordiais para confirmação dessas características (GOMES, 2012).

5.2.1. Determinação de pH

Um processo de decomposição, seja por hidrólise, oxidação ou fermentação, altera quase sempre a concentração dos íons de hidrogênio. Os processos que avaliam o pH são colorimétricos ou eletrométricos. Os primeiros usam certos indicadores que produzem ou alteram sua coloração em determinadas concentrações de íons de hidrogênio. São processos de aplicação limitada, pois as medidas são aproximadas e não se aplicam as soluções intensamente coloridas ou turvas, bem como as soluções coloidais que podem absorver o indicador, falseando os resultados. Nos processos eletrométricos, empregam-se aparelhos que são potenciômetros especialmente adaptados e permitem uma determinação direta, simples e precisa do pH (IAL, 2008).

Para a medida potenciométrica do pH usam-se, geralmente, aparelhos construídos a partir do eletrodo de vidro sensível a íons H^+, situado na extremidade de um tubo de vidro resistente. No eletrodo, o bulbo é preenchido com uma solução aquosa diluída de ácido clorídrico; em contato com esta solução encontra-se um eletrodo de prata; o tubo de vidro é preenchido com solução saturada de cloreto de potássio. O condutor metálico, mergulhado no bulbo, é geralmente constituído de um fio de prata. A solução HCl 0,1N possui $[H^+]$ constante. Quando a concentração de H^+ é maior ou menor na solução na qual se está determinando o pH do que dentro do eletrodo, uma diferença de potencial maior ou menor existirá na extremidade do vidro (GOMES, 2012).

Um músculo vivo possui o valor do pH de 7,2. Ocorrido o abate, a carne continua em processo bioquímico, no qual o condutor energético do músculo é transformado em glicogênio lático através da ação de várias enzimas. O pH da carne de frango diminui devido à formação ácida, onde a carne de peito deve apresentar pH final entre 5,7 e 5,9. Passado 24 horas, se o pH estiver superior a 6,2, a carne de frango irá se encontrar com grande retenção de água, o que implica em curto tempo de conservação e o estabelecimento da coloração escura, caracterizando a carne DFD (dark, firm, dry – escura, dura e seca). Caso o pH se encontre abaixo de 5,8 em menos de 4 horas, teremos a carne PSE (pale, soft, exudative – pálida, mole e exsudativa) caracterizado pela má retenção de água além do aspecto pálido e mole (VENTURINI, 2007).

5.2.2. Análise de cor

Cor e aparência de um produto são atrativas para o consumidor. As cores que não são apropriadas para o item indicam perda de frescor ou sugerem falta de maturação podendo gerar a recusa do produto. Em geral, produtos alimentares específicos estão associados com os atributos de cor específicos. Medição da cor de produtos alimentares tem sido utilizada como uma medida indireta de outros atributos de qualidade, tais como aroma e teores de pigmentos porque é simples, rápida e correlaciona-se bem com outras propriedades físico-químicas (PATHARE, OPARA e AL-SAID, 2013).

A cor da carne está relacionada com as fibras musculares, o pigmento mioglobina e a hemoglobina presente no sangue. Estas duas substâncias são proteínas associadas ao ferro e têm a possibilidade de reagir com oxigênio, alterando a cor da carne. A quantidade de mioglobina varia com a espécie, sexo, idade, localização anatômica do músculo e atividade física, por exemplo, um animal qual a sangria foi boa a mioglobina constitui 80 a 90% do

total de pigmentos. A cor da carne e frango varia da tonalidade cinza a vermelho pálido (HEDRICK, 1994; VENTURINI, 2007).

Por se tratar de um atributo complexo, a cor é o principal componente da aparência em carne de frango ou seus produtos. Os métodos instrumentais para mensurar a cor são baseados numa fonte de luz e um detector. Os objetos/ alimentos absorvem e refletem comprimentos de onda de luz que são detectados por um instrumento ou um observador (SAMS, 2001).

O homem possui visão tridimensional. Dessa forma, para que se tenha uma representação satisfatória da cor, a curva espectrofotométrica deve ser reduzida a três números. O Comitê Internacional de Iluminação (CIE) recomenda o uso do sistema-padrão fundamentado em um “observador-padrão”, que simula o olho humano e consiste em três filtros primários de cor. A curva espectrofotométrica é especificada em termos de X, Y e Z, em que X está relacionado com a cor vermelha; Y com a luminosidade e a cor verde; e Z, com a cor azul. Hunter desenvolveu um colorímetro fotoelétrico triestímulo que ganhou bastante aceitação na indústria de alimentos. O equipamento consiste basicamente de três circuitos separados, filtros cuidadosamente selecionados e fotocélulas que fornecem estreitas aproximações de X, Y e Z. A coordenada L* (luminosidade) está correlacionada com a grandeza Y do sistema CIE. A coordenada a* é mensurável em termos de intensidade de vermelho e verde, e a coordenada b* está relacionada com a intensidade de amarelo a azul (GOMES, 2012; PATHARE et al. 2013).

O parâmetro a* correlaciona valores positivos a cores avermelhadas e valores negativos aos esverdeados, enquanto que b* toma valores positivos para as cores amareladas e valores negativos para as azuladas. L* é uma medição aproximada de luminosidade, e pode ser considerada como equivalente da escala de cinza, entre o preto e o branco, tendo os valores dentro do intervalo de 0-100 (MACDOUGALL, 2002).

5.2.3. Análise de perda de peso por cocção

Perdas de peso durante o cozimento afetam o custo de fabricação dos produtos cárneos processados. É importante controlar essas perdas, pois as alterações no rendimento podem resultar em mudanças na composição dos produtos acabados e consequentemente podem afetar as características de palatabilidade (PIETRASIK, 1999).

A perda de peso por cocção pode ser determinada pela diferença do peso (P) do produto antes (P antes) e após o tratamento térmico (P após) que corresponde à perda de peso devido ao aquecimento (BERRY, 1994). A porcentagem de perda de peso durante o tratamento térmico pode ser calculada como:

$$\% \text{ Perda de peso por cocção} = \frac{(\text{P antes} - \text{P após})}{\text{P antes}} \times 100$$

Com os dados de peso também é possível calcular também o rendimento do processo (CANDOGAN e KOLSARICI, 2003).

5.3. Textura instrumental

A importância da textura na aceitabilidade geral dos alimentos varia muito, dependendo do tipo de alimento. Pode ser *Crítica* nos alimentos onde a textura é a característica de qualidade dominante; por exemplo, carne, batatas e milho. *Importante* nos alimentos em que a contribuição da textura é equivalente ao sabor e a aparência; por exemplo, frutas, vegetais, queijos e pães e, *Secundária* em alimentos onde a contribuição da textura é muito pequena como bebidas e sopas ralas (BOURNE, 2002).

Em 1963 Friedman, Whitney e Szczesniak, publicaram um procedimento para fazer medições de textura de alimentos (FRIEDMAN, WHITNEY e SZCZESNIAK, 1963). Cinco anos mais tarde Bourne adaptou o método e ao fazê-lo, ele superou algumas limitações instrumentais (BOURNE, 1968). Depois desses dois trabalhos pioneiros uma série de pesquisadores têm utilizado estes protocolos bem como variações deles (ROSENTHAL, 2010).

Os métodos instrumentais de análise de textura avaliam propriedades mecânicas a partir de forças aplicadas ao alimento. A Análise do Perfil de Textura (TPA) instrumental aplica sucessivas forças deformantes, numa simulação da ação de compressão e corte dos dentes durante a mastigação. A dureza, elasticidade e coesividade são parâmetros mecânicos primários que podem ser utilizados para caracterizar as propriedades de textura de embutidos (LI, CARPENTER e CHENEY, 1998).

5.4. Teste de aceitação

Embora a indústria de alimentos sempre tenha reconhecido a importância da qualidade sensorial de seus produtos, os métodos utilizados para medi-la variaram em função do estágio de evolução tecnológica da indústria. Distinguiram-se quatro fases na metodologia de avaliação da qualidade sensorial (DUTCOSKY, 2011):

1ª fase (antes de 1940): época artesanal/ pré-científica da indústria de alimentos. A qualidade sensorial era determinada pelo proprietário da empresa.

2ª fase (1940-1950): época da expansão da indústria de alimentos e incorporação de pessoal técnico, geralmente vindo da área química e farmacêutica. Conceitos de controle de processos e de produto final foram introduzidos, porém, os métodos utilizados eram químicos e instrumentais, não sensoriais.

3ª fase (1950-1970): foi nessa fase da indústria alimentícia que se considerou seriamente a utilização do homem como instrumento de medida das características sensoriais dos alimentos. Os principais avanços nesse período foram:

a) A definição dos atributos sensoriais primários que integram a qualidade sensorial dos alimentos e os órgãos sensoriais a eles relacionados;
b) O entendimento de que o homem tem uma habilidade natural de comparar, diferenciar e quantificar atributos sensoriais, mas que era preciso normalizar a forma e as condições em que a pergunta era feita, bem com dar um tratamento estatístico aos dados obtidos;
c) Desenvolvimento de pesquisas básicas sobre o processo pelo qual o homem percebe um estímulo, como a sensação provocada pelo estímulo é elaborada e como o homem verbaliza essa sensação, dentro de áreas como Fisiologia, Psicologia e Sociologia.

No fim da terceira fase, desenvolveram-se muito os métodos de avaliação sensorial. Paralelamente, instrumentos capazes de imitar ou duplicar a ação humana foram desenhados e métodos estatísticos capazes de correlacionar medidas sensoriais e instrumentais foram desenvolvidos.

4ª fase (após 1970): definiu-se que a qualidade sensorial de um alimento não é uma característica própria do alimento, mas sim o resultado da interação entre o alimento e o homem. Reconheceu-se que qualidade sensorial é função tanto de estímulos procedentes dos alimentos como também das condições fisiológicas, psicológicas e do indivíduo ou de um grupo que avalia o alimento. Definiu-se que medidas instrumentais são úteis tão somente quando apresentam boa correlação com as medidas sensoriais. As técnicas para avaliação sensorial objetiva (discriminação e descrição de atributos) são distintas das técnicas utilizadas para estudos subjetivos (preferência e aceitabilidade). Para se obter máxima sensibilidade, reprodutibilidade e confiabilidade dos resultados, os testes devem ser realizados com equipe de julgadores devidamente selecionados e treinados (análise objetiva) ou público-alvo muito bem definido (análise subjetiva), em ambientes apropriado, sob condições rigorosamente controladas, aplicando-se técnicas estatísticas para a avaliação dos resultados (DUTCOSKY, 2011).

Os métodos de análise sensorial podem ser divididos em analíticos (discriminativos e descritivos) e afetivos. Os métodos discriminativos são aqueles que estabelecem diferenciação qualitativa e/ou quantitativa entre amostras. Nos testes discriminativos, os provadores de uma equipe atuam como instrumentos para detectar pequenas diferenças (NASSU, 2007).

Nos testes afetivos o julgador expressa seu estado emocional ou reação afetiva ao escolher um produto pelo outro. É a forma usual de se medir a opinião de um grande número de consumidores com respeito as suas preferências, gostos e opiniões. As escalas mais empregadas são: de intensidade, a hedônica, do ideal e de atitude ou de intenção. Os julgadores não precisam ser treinados bastando serem consumidores frequentes do produto em avaliação. Com o teste da escala hedônica, o indivíduo expressa o grau de gostar ou de desgostar de um determinado produto, de forma globalizada ou em relação a um atributo específico. As escalas mais utilizadas são as de 7 e 9 pontos, que contêm os termos definidos situados, por exemplo, entre "gostei muitíssimo" e "desgostei muitíssimo" contendo um ponto intermediário com o termo "nem gostei; nem desgostei"(IAL, 2008). Quando a escala hedônica é usada com o objetivo de verificar a aceitação, a preferência de um protótipo em relação aos demais é inferida a partir do valor médio da aceitação. Produtos que apresentem média significativamente maior do que os outros ($p \leq 0,05$) são considerados preferidos (BERGARA-ALMEIDA, APARECIDA e SILVA, 2002).

Dessa maneira, devido ao potencial antimicrobiano e antioxidante dos produtos vegetais, espera-se que eles atuem em parâmetros microbiológicos, de qualidade, e de aceitação de linguiça de frango fresca, podendo contribuir positivamente na extensão da vida de prateleira desses alimentos e agir conjuntamente com métodos tradicionais de conservação.

Objetivos

Objetivo geral

Verificar a atividade antibacteriana de óleos essenciais e extratos de plantas condimentares sobre bactérias de interesse em alimentos e a partir desses ensaios, escolher um derivado vegetal para os testes no modelo alimentar (linguiça de frango fresca) envolvendo parâmetros de qualidade e vida de prateleira.

Objetivos específicos

- Verificação *in vitro* da atividade antimicrobiana de doze derivados vegetais com potencial de uso em alimentos sobre nove espécies bacterianas distintas;

- Verificação da atividade antimicrobiana de dois derivados vegetais sobre *Listeria monocytogenes* e *Salmonella* Enteritidis, inoculadas artificialmente em linguiça de frango;

- Estudo dos efeitos de um derivado vegetal e polifosfato nos parâmetros de qualidade e vida de prateleira de linguiça de frango.

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___________CAPÍTULO I

**Manuscrito submetido à Journal of Medicinal Food*

CHEMICAL CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS AND ETHANOL EXTRACTS FROM SPICE

ABSTRACT

There are frequent reports about the use of aromatic plants in cookery over the centuries, as well as of their antimicrobial effects when added to foods. To allow the use of plant products as agents of food safety is essential understand its potential antimicrobial beyond its chemical composition. Thus, the aim of this study was to verify the *in vitro* antibacterial activity of six essential oils (EOs) and six ethanol extracts (Eet) from *Rosmarinus officinalis*-rosemary, *Laurus nobilis*-laurel, *Ocimum basilicum*-basil, *Origanum majorana*-marjoram, *Origanum vulgare*-oregano, and *Thymus vulgaris*-thyme samples against bacteria of interested in foods, as well as to characterize chemically these plant antimicrobial products. Twelve antimicrobial products were prepared from plant and assays using the broth microdilution method were performed and Minimal Inhibitory Concentration (MIC) were established. Antimicrobial products from thyme and oregano showed the highest antibacterial activity with MIC of 1.5 and 1.6 mg/ml for EOs and 2.9 and 4.2 mg/ml for Eet, respectively. The EOs of these plants had thymol as the main compound, while flavonoids, phenols, triterpenes and steroids were present in the extracts. Thus, the results showed strong antibacterial activity of these plant-derived and its potential for application in the food industry. However, further testing should be performed as cytotoxicity and acceptability tests.

Keywords: foodborne pathogens, minimal inhibitory concentration, aromatic plants, essential oil, ethanol extracts.

INTRODUCTION

The use of plants as flavoring in foods is not a recent activity and several species synthesize active antimicrobial compounds against bacteria, fungi and yeasts. On the other hand, foodborne diseases have a vital interest in public health and *Salmonella* spp, *Listeria monocytogenes* and *Escherichia coli* are responsible for a large number of cases, outbreaks and deaths among the human population.[1]

With the aim of reducing risks to the health and economic losses generated by the microorganisms that contaminate foods, the use of natural antibacterial compounds has been a promising alternative. [2,3] In nature, the compounds present in essential oils play an important role in plant protection as antibacterial, antiviral and antifungal agents, as insecticides and

against herbivores. [4,5] Numerous studies have reported the antimicrobial action of EOs [6-11] and plant extracts [12-16] with potential use in foods.

The mechanisms of action of these plant antimicrobials depend not only on the chemical composition but also on the structural configuration, functional groups and possible synergistic interactions among the present compounds.[17] Thus, chemical composition is studied to better understand these mechanisms and cell targets preferred by these compounds.[18]

This study aimed to verify in vitro the antibacterial activity of twelve plant derivatives of potential use in foods against nine bacterial strains, as well as to chemically characterize these twelve plant derivatives.

MATERIALS AND METHODS

Aromatic plants and preparation of oils and extracts

Fresh samples of *Rosmarinus officinalis*-rosemary, *Laurus nobilis*-laurel, *Ocimum basilicum*-basil, *Origanum majorana*-marjoram, *Origanum vulgare*-oregano, and *Thymus vulgaris*-thyme were purchased in the market from Botucatu, São Paulo State, Brazil and were prepared the respective EOs and Eet of each plant, totaling 12 antimicrobial products. EOs were obtained according to the steam distillation methodology [19] in a Marconi device, Model M480. The density values of EOs were calculated by using the following formula, adapted from Fonseca and Librand: [20]

$$D = \frac{W_1 - W_2}{V} = \frac{mg}{mL}$$

Where:

W_1= Weight of Eppendorf vials

V= Volume of EO (1 ml)

W_2= Weight of 1 ml EO

D= Density

Extracts were prepared with 300 gram of fresh plants samples (ground in an industrial blender) plus 2 liters of 70% ethanol[13] and kept at refrigerator temperature (± 4°C) during 48 hours, followed by filtration in filter paper. The solvent elimination was carried out in a rotary evaporator (Phoenix) with total ethanol extraction, followed by determination of the extract weight, using 1ml volumes (5 replicates) of the extract subjected to residual water evaporation. The density values (mg/ml) of EO and the dry weight (mg/ml) of extracts were

established and used to MIC values expressed as mg/ml. Samples of each plant were stored at the Herbarium "Irina Delanova Gemtchujnicov" of the Department of Botany, Institute of Biosciences/UNESP, Botucatu, São Paulo State, Brazil, and the respective voucher numbers were recorded as: *R. officinalis*-BOTU 26038, *L. nobilis*- BOTU 26039, *O. basilicum* -BOTU 26037, *O. majorana* -BOTU 26288, *O. vulgare* -BOTU 26287 and *T. vulgaris* -BOTU 26286.

Chemical analysis

The qualitative chemical analysis of extracts aimed at verifying the presence or absence of steroids, triterpenes, saponins, flavonoids, phenols, tannins, cyanogenic glycosides, quinones, coumarins and alkaloids.[21] The EOs underwent chemical analysis through gas chromatography-mass spectrometry (GC-MS), Shimazu, model QP5050A, using a capillary column, CBP-5, of 50m length, with internal diameter of 0.25mm, 0.25µm film thickness, and He as the carrier gas. The EO compounds were identified based on NIST library (National Institute of Standards and Technology), analysis of mass spectra and data in the literature.[22]

Bacterial strains

Standard ATCC strains of *Salmonella* Enteritidis-13076, *Escherichia coli* O157:H7-43895, *Pseudomonas aeruginosa*-27853, *Listeria monocytogenes*-15313, *Enterococcus faecalis*-10100, *Aeromonas hydrophila*-7966, *Lactobacillus rhamnosus*-9595, *Staphylococcus xylosus*-29979 and *Staphylococcus aureus*-25923. The strains were stored at -80°C before antimicrobial assays.

***In vitro* antibacterial activity of plant antimicrobials**

The activities of each antimicrobials were performed to MIC determination against standard ATCC strains, using microdilution tests [23] when the antimicrobials were mixed in Mueller Hinton Broth (MHB) and the MIC values were established for each bacterial strain. The microdilution test is the most economic method as to time and resources and is more appropriate to select a great number of combinations of different bacteria and plant extracts.[24]

MHB was supplemented with Tween 80 at 0.5% and antimicrobials were placed into 96-well microplates and concentrations from 0.2 to 45 mg/ml for EOs and extracts were performed. These concentrations were chosen according preliminary tests for each of the plant

antimicrobials. Each bacterial strains were grew (37°C/24h) in Brain Heart Infusion and after patterning by using the 0.5 MacFarland scale, the wells received volume of standadized suspension to make bacterial concentration around 10^5 to 10^6 CFU/ml (Colony Forming Units). The microplates were incubated (37°C/24h) and MIC results were read with the oxi-reduction indicator resazurine at 0.01%. The bacterial growths were indicated by the color change from violet (no growth) to pink (growth). [25] The lowest concentration at which there was no color change was considered the MIC. The obtained MIC values were converted based on the density values; thus, MIC results of plant derivatives were expressed as mg/ml. All assays were carried out in triplicates.

Statistical analysis

Data were subjected to Kruskal-Wallis test (analysis of variance) and Student-Newman-Keuls method (multiple comparisons), considering 5% significance level. The software used was SigmaStat version 3.5.

RESULTS AND DISCUSSION

The values of Eet dry weight, EO density and EO yield are shown in Table 1. *L. nobilis* EO had the highest value (mean of 0.62%), while *O. majorana* EO showed the lowest yield value (mean of 0.13%). Results of phytochemical analysis of Eet are shown in Table 2. All Eet were positive for the presence of phenols and flavonoids and negative for cyanogenic glycosides, alkaloids and quinones. Only *L. nobilis* extract was positive for tannins and only *O. majorana* extract was positive for coumarins. The geographic location, harvest period, cultive and age of plants, climate and storage conditions can influence the yield and the chemical profile of EOs/Eet of plants. [26, 27] However, phytochemical screening is important for knowledge of the active principles of plant antimicrobials and subsequently contributing to establish quality and efficacy standards for plant products.

The mean MIC values obtained for EOs and Eet against nine bacterial strains of interest in foods are shown in Table 3.

Mean values obtained for six EOs and Eet against nine bacterial strains and the comparison among twelve plant derivatives are shown in Table 4. The highest antimicrobial activities were verified with EOs from *T. vulgaris*, *O. vulgare* and *O. majorana* and with extracts from *T. vulgaris* and *O. vulgare*.

Table 1. Mean values of ethanol extract (Eet) density, essential oil (EO) density and EO yield obtained from aromatic plants.

Aromatic Plant	**Eet dry weight (mg/ml)**	**EO density (mg/ml)**	**EO yield (%)**
Rosmarinus officinalis	47.2	853	0.23
Laurus nobilis	93.7	871	0.62
Ocimum basilicum	16.4	875	0.20
Origanum majorana	18.5	870	0.13
Origanum vulgare	15.1	917	0.17
Thymus vulgaris	11.6	900	0.14

Table 2. Qualitative phytochemical analysis of ethanol extract of aromatic plants.

Classes of compounds	***Rosmarinus officinalis***	***Laurus nobilis***	***Ocimum basilicum***	***Origanum vulgare***	***Origanum majorana***	***Thymus vulgaris***
Flavonoids	+	+	+	+	+	+
Quinones	-	-	-	-	-	-
Coumarins	-	-	-	-	+	-
Triterpenes	+	-	-	+	+	+
Steroid	-	-	+	+	-	+
Alkaloids	-	-	-	-	-	-
Saponins	+	+	-	+	+	-
Cyanogenic glycosides	-	-	-	-	-	-
Tannins	-	+	-	-	-	-
Phenols	+	+	+	+	+	+

Table 3.MIC values (mg/ml) obtained for six essential oil (EOs) and ethanol extract (Eet) against nine bacterial strains.

Microorganisms	*Rosmarinus officinalis*		*Laurus nobilis*		*Ocimum basilicum*		*Origanum majorana*		*Origanum vulgare*		*Thymus vulgaris*		Mean	
	EOs	Eet	EOs	Eet	EOs	Eet	EOs	Eet	EOs	Eet	EOs	Eet	EOs	Eet
S. Enteritidis	8.5	21.0	3.0	43.0	3.0	7.5	3.0	8.0	3.0	5.0	3.0	5.0	3.6	15.1
E. coli O157:H7	7.0	21.0	4.0	43.0	3.0	7.5	3.0	8.0	0.9	3.0	0.9	2.0	3.0	14.2
P. aeruginosa	43.0	21.0	44.0	43.0	44.0	7.5	35.0	7.0	0.2	4.0	0.4	2.0	27.7	14.2
L. monocytogenes	17.0	19.0	7.0	43.0	3.0	7.5	3.0	6.5	3.0	4.0	3.0	4.0	5.8	13.9
E. faecalis	43.0	21.0	26.0	43.0	4.5	7.5	3.0	8.0	3.0	1.5	0.9	0.6	13.3	13.6
A. hydrophila	17.0	21.0	3.0	43.0	4.5	7.5	3.0	6.5	0.2	4.0	0.9	2.0	4.6	14.0
S. xylosus	26.0	21.0	3.0	43.0	3.0	7.5	3.0	8.0	0.2	7.0	0.2	5.0	5.7	15.2
L. rhamnosus	3.0	19.0	3.0	43.0	3.0	7.5	3.0	8.0	0.9	5.0	3.0	2.0	2.3	14.1
S. aureus	17.0	19.0	9.0	33.0	3.0	7.5	3.0	6.5	3.0	4.5	0.9	3.5	5.8	12.3

Table 4. Mean values MIC for comparisons between essential oil (EOs) and ethanol extract (Eet) group and comparisons between these groups against nine bacterial strains.

Plants	EOs	Eet
Rosmarinus officinalis	20.2 ±14.6 c (6)	20,3 ±1.0 c (7)
Laurus nobilis	11.4 ± 14.3 b (4)	41.9 ±3.3 d (8)
Ocimum basilicum	7.9 ±13.6 b (3)	7.5 ±0.0 b (5)
Origanum majorana	6.6 ±10.7 a (2)	7.4 ±0.8 b (5)
Origanum vulgare	1.6 ±1.3 a (1,2)	4.2 ±1.5 a (3)
Thymus vulgaris	1.5 ±1.2 a (1)	2.9 ±1.5 a (2)

*Values followed by the same letter in the same column, i.e. for the same group (EOs or Eet), do not differ (p>0.05). Values followed by the same number, in both columns, i.e. for EOs and Eet, do not differ (p>0.05). **a** most efficient, **c** less efficient; **1** most efficient, **7** less efficient.

The found compounds in the EOs are shown in decreasing order in Table 5.

Table 5. Chemical analysis (GC-MS) of EOs of aromatic plants.

EOs	Chemical composition (GC-MS)
Rosmarinus officinalis	Camphor 22.51%, 1,8-cineol 15.95%, myrcene 10.56%, α-pinene 9.11%, β-caryophyllene 6.91%, β-pinene 4.38%, camphene 3.98%, borneol 2.24%, γ-terpinolene 2.18%, bornyl acetate 1.14%, linalool 0.86%, α-phellandrene 0.58%.
Laurus nobilis	Eucalyptol (1,8-cineol) 37.4%, linalool 25.29%, α-terpinyl acetate (α-terpinyl acetate) 10.56%, β-pinene 6.39%, o-methyl eugenol (eugenol ether) 5.37%, α- terpineol 3.58%, β-myrcene 1.94% ,α-pinene 1.56%, eugenol 0.94%, terpinen-4-ol 0.84%, β-caryophyllene 0.63%, β-ocimene 0.42%, γ-terpinene 0.28%.
Ocimum basilicum	Linalool 31.52%, eugenol 14.39%, eucalyptol 14.06%, camphor 11.31%, germacrene 6.64%, β-caryophyllene 2.13%, myrcene 0.73%, α-pinene 0.36%, β-pinene 0.31%.
Origanum vulgare	Thymol 48.62%, γ-terpinene 12.98%, ρ-cymene 7.48%, β-caryophyllene 5.31%, linalool 3.02%, myrcene 1.6%, α- terpinene 1.28%.
Origanum majorana	Linalool 16.95%, 3-ciclohexen-1-ol 4methyl-1-(isopropyl)13.51%, thymol 13.45%, γ-terpinene 8.22%, linalyl acetate 6.62%, germacrene D 6.53%, β-caryophyllene 4.59%, ocimene 3.94%, germacrene B 2.51%, β-pinene 1.94%, α-terpinene 1.79%, ρ-cymene 1.51%, β-myrcene 1.18%, limonene 0.74%, α-pinene 0.15%.
Thymus vulgaris	Thymol 49.09%, γ-terpinene 14.81%, ρ-cymene 7.3%, β-caryophyllene 4.53%, linalool 3.62%, α-terpinene 1.21%, germacrene 1.04%, β-pinene 0.58%, α-caryophyllene 0.54%.

Thymol was the main compound of *T. vulgaris* and *O. vulgare* EOs, reaching a value that corresponds to almost 50% of their composition. *O. basilicum* and *O. majorana* EOs had linalool as the main compound while camphor and eucalyptol were the compounds in the *R. officinalis* and *L. nobilis* EOs, respectively. In a chemical analysis of some EOs, [28] detected that *T. vulgaris* and *O. vulgare* EOs had thymol (≅63% in both samples), ρ-cymene (23.5 and 9.9%, respectively) and γ-terpinene (4.3 and 12.9%, respectively) as the major compounds. The genus *Origanum* has also been confirmed to be rich in thymol, carvacrol, *p*-cymene and α-terpinene. [29, 30] Our results also revealed that the linalool, was main compound found in *O.*

basilicum and *O. majorana* EOs (31.52% and 16.95%, respectively). Similarly, Gurbuz *et al.* [31] identified linalool as the main compound (41.2%) in *O. basilicum* EOs obtained by means of hydrodistillation. Linalool was also the most abundant compound (56.7–60.6%) in EOs of *O. basilicum* cultured in Pakistan.[32] On the other hand, *O. majorana* samples showed as major compounds terpinen-4-ol (29.13–32.57%), cis-sabinene hydrate (19.9–29.27%), trans-sabinene hydrate (3.5–11.61%), γ-terpinene (2.11–8.20%), bornyl acetate (1.52–2.94%) and linalool at only 1.05–1.39%. These compounds showed a wide spectrum of antibacterial activity against microorganisms.[33] A study carried out with EOs produced in Pakistan found that samples obtained in the winter and from autumn cultures showed higher antimicrobial activity, which was correlated to the high level of linalool and other oxygenated compounds in these samples. [32] In EOs extracted from *R. officinalis* cultured in Algeria, [34] identified 30 compounds. Those authors found 1,8-cineole (29.5%), 2-ethyl-4,5-dimethylfenol (12.0%), camphor (11.5%), borneol (9.4%), (+)-α-terpineol (9.2%), α-pinene (7.5%) and camphene (5%) as the major compounds, as expected for this plant. [35, 36]

In the chemical analysis by GC-MS of some Lauraceae EOs [37], *L. nobilis* showed as major compounds 1,8-cineol (41.86%), sabinene (9.12%), α-pinene (7.20%), linalool (7.02%), α-terpinyl acetate (5.49%) and β-pinene (5.23%). Our results for *L. nobilis* oil were similar: 1,8-cineol (37.4%) as the compound present at the largest quantity, followed by linalool (25.29%), α-terpinyl acetate (10.56%), β-pinene 6.39%. Those authors related the oil moderate antifungal activity to its high level of 1,8 cineol [37]. The 1,8-cineol (60.72%) was again found as the major compound of laurel. [38] *E. coli* O157: H7 inactivation by laurel oil increased with the applied levels. The initial count of *E. coli* O157: H7 was 8.02 log CFU/ml and reduced to 5.10, 4.02, 3.79, 2.90, 0.47, 0.00 and 0.00 log CFU/ml with levels of 5, 10, 20, 30, 40, 50 and 80μl/ml laurel EO, respectively ($P < 0.05$). Laurel EO was the most effective in inactivating *E. coli* O157: H7 at all levels, [38] differently from the results of this study, in which the oils most efficient in inhibiting *E. coli* O157:H7 were those of *T. vulgaris* and *O. vulgare* (MIC 0.9 mg/ml).

The efficiency of the six plants varied between EO and Eet (Table 4- letters). For EOs: *T. vulgaris*=*O. vulgare*=*O. majorana*>*O. basilicum*=*L. nobilis*>*R. officinalis*, of which *T. vulgaris*, *O. vulgare* and *O. majorana* showed the best efficiency and *R. officinalis* the worst efficiency. For the Eet group: *T. vulgaris*=*O. vulgare* > *O. basilicum* = *O. majorana* >*R. officinalis* > *L. nobilis*, of which *T. vulgaris* and *O. vulgare* were the most effective, whereas *L. nobilis* had the worst performance. In the comparison between EOs and Eet (Table 4- numbers), which indicated that *T. vulgaris* and *O. vulgare* EOs had the best performance,

followed by *T. vulgaris* Eet, *O. majorana* and *O. basilicum* EOs, and *O. vulgare* Eet. The better inhibitory effect of antimicrobials from *T. vulgaris* and *O.vulgare* could be verified more than once, which ranked them among the three most effective samples. *R. officinalis* and *L. nobilis* derivatives in turn were the least efficient ones.

The EO levels of 1, 10 and 15% were tested, indicating that the laurel EO levels capable of inactivating *E. coli*, *S. aureus*, *S.* Typhimurium, and *Y. enterocolitica* were superior (15%) to those of oregano, savory and cumin EOs, [39] similarly to our study in which *L. nobilis* was not the most efficient oil. Dadalioğlu and Evrendilek,[38] reported the inhibitory effect of laurel EOs against some pathogens, in increasing order: *E. coli* O157: H7>*S. aureus*> *S.* Typhimurium> *L. monocytogenes*.

The antimicrobial activity of Eet was inferior to that of EOs, even when obtained from the same plant, since the quantity of the Eet (mg/ml) needed to obtain the same antibacterial effect was larger, as also suggested by the density values of plant products.

Flavonoids have notable antimicrobial activity and several mechanisms of action, especially inhibition of nucleic acid synthesis, cytoplasmic membrane functions and energetic metabolism.[40] Phenolic compounds cause damages to the bacterial membrane and consequently release of intracellular constituents, leading to death or cell growth inhibition.[41, 42] Terpenes are classified into multiples of isoprene units like hemiterpenes (C_5H_8), monoterpenes ($C_{10}H_{16}$), sesquiterpenes ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$), sesterpenes ($C_{25}H_{40}$), triterpenes ($C_{30}H_{48}$), to which steroids are closely related [43]. However, the mechanism of action of triterpenoids is not completely understood, while some triterpenoids are suggested to block cell division by inhibiting the synthesis of DNA and macromolecules in *Bacillus subtilis*. Inhibition of macromolecule synthesis could be due to the cell membrane damage.[44]

EOs with the best antibacterial activity has in their composition thymol, γ-terpinene and ρ-cymene. These volatile terpenes of the *O. vulgare, T. vulgaris* and *O. majorana* EOs enables some biological activities, probably being responsible for the antimicrobial activity of the mentioned oils.[45-47] Thymol alone only shows antimicrobial activity,[48] however, it must be emphasized that this activity is complex and possibly due the interaction among the EO compounds, which suggests that even minor compounds can play an special role for this activity.

On a revision work about essential oils, whole EOs were reported to have higher antibacterial activity than the mixture of their main compounds,[4] although varied results are reported in the literature for this issue. The antibacterial activity of thyme and basil EOs and their major compounds (thymol, estragole, carvacrol, linalool and p-cymene) was determined

against *Shigella sonnei* and *S. flexneri*. The oil from basil had lower inhibitory activity against those strains, compared to thyme EO. Among the five main compounds found in EOs the carvacrol showed strong antibacterial activity against the microorganisms, followed by thymol, while estragole and linalool had limited antibacterial activity and p-cymene did not show antibacterial activity.[49] *O. basilicum* oil was also reported to have strong antimicrobial activity; however, linalool alone showed higher inhibitory activity against nine pathogens, compared to the whole oil, with MIC value of 0.3–1.9 mg/ml.[32]

It was concluded that all plant derivatives showed antimicrobial activity against the studied strains, but with different efficacy patterns. Although there was a difference between EOs and Eet, *T. vulgaris* and *O. vulgare* derivatives were most efficient against bacteria. As regards a possible use of these agents in foods, it must be emphasized that the plant compounds may also have antioxidant activities, which make them potentially useful as part of an efficient system of barriers for food preservation. Therefore, the choice of plant products that show considerable efficiency when incorporated to food and are economically viable remains a great challenge but has received attention by numerous researchers.

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CAPÍTULO II

**Manuscrito submetido à Journal of Food Protection*

ESSENTIAL OILS FROM HERBS AGAINST FOODBORNE PATHOGENS IN CHICKEN SAUSAGE

ABSTRACT

Consumption of chicken meat and its products, especially sausage, have increased in recent years. However, this product is highly susceptible to microbial contamination during manufacturing, which compromises its shelf life. The flavoring and preservative activities of essential oils (EOs) have been recognised and the application of these antimicrobial agents as natural active compounds in food preservation has shown promise. The aim of this study was to evaluate the effect of *Ocimum basilicum* and *Origanum vulgare* EOs on *Listeria monocytogenes* and *Salmonella enterica* serotype Enteritidis strains in artificially inoculated samples of fresh chicken sausage. First, the minimal inhibitory concentration (MIC) of EO *in vitro* was determined. The sausage was prepared and kept at ± 4°C; then, the inoculation of individual bacteria was carried out. Then were added 0.3%, 1.0% and 1.5% v/w of EO. After 0, 5, and 24 hours, the most probable number method (MPN) was performed. Transmission electron microscopy (TEM) was used to view the damage caused by these EO on bacterial morphology and/or structure. Only the 1.5% concentration was effective in reducing *L. monocytogenes* for both EOs after 5 and 24 hours of storage. The 0.3% of *O. vulgare* EO was able to reduce the MPN/g of *Salmonella* Enteritidis (2 log) after 5 hours ($p<0.05$) trials. *O. basilicum* EO showed no effect after 5 hours, but decreased by 2 log after 24 hours. The 1% concentration of *O. vulgare* EO gave a greater reduction of *S.* Enteritidis at 5 hours, increasing or maintaining this effect after 24 hours. Thus, to the results confirmed the potential benefits of using EO in control of foodborne pathogens.

Keywords: *Ocimum basilicum, Origanum vulgare, Salmonella* Enteritidis, *Listeria monocytogenes.*

INTRODUCTION

Foodborne illnesses are a widespread and growing public health concern, both in developed and developing countries. *Salmonella* is the most common foodborne pathogen that is widely distributed, with high costs associated with treating *Salmonella*-related diseases *(57, 58)*. *Salmonella enterica* Enteritidis is considered the most important serovar of *Salmonella,* causing gastrointestinal disease of varying severity in humans *(23)*. This pathogen is commonly found in chicken *(36, 59)*, which is the primary vector for transmission to humans

(12). The Department of Agriculture of the United States reported that 45% of ground chicken samples contained *Salmonella (40)*. *Salmonella* spp. in Brazil accounted for about 42% of the etiological agents identified in outbreaks in the period from 2000 to 2011*(11)*.

Although *Listeria* presents a relatively low incidence, in some cases the severity makes it one of the most serious foodborne infections *(57, 58)*. *Listeria monocytogenes* is an opportunistic pathogen that mainly affects pregnant women, newborns, the elderly, and immunocompromised individuals. This pathogen emerged in the late 20th century and has caused many outbreaks with high mortality rates *(4, 24, 33)*. *Listeria* is common in dairy products and red meat, but it can also be found in chicken, adding to the health concerns of *Salmonella* and *Campylobacter (28)*.

Moreover, chicken meat and its products have increased in popularity and have become widespread throughout the world, with chicken sausage being one of the most popular categories among these products *(7)*. Sausage manufacture involves a number of handling steps, which increase the chances of contamination by pathogens or spoilage *(54)*. Fresh sausage, a very popular food in Brazil, does not undergo heat treatment and has a high water activity, giving this food a short shelf life and subjecting it directly to the action of the microorganisms present *(53)*.

Thus, the application of agents with adequate antimicrobial and antioxidant activities has significant potential to extend the shelf life of chicken products and prevent economic losses *(61)*. Due to the negative perception of chemical preservatives, consumers' attention is changing to natural alternatives and particular interest has been focused on the potential use of essential oils (EOs) from aromatic plants *(44)*. Thus, the antimicrobial compounds found in EO show potential in the goal of natural food preservation *(17)*.

It is well known that most species, especially those belonging to the Lamiaceae family, have different biological and pharmacological activities, which has meant that for a long time they have been used for improving the taste and organoleptic properties of different foods *(9)*.

Ocimum basilicum (basil) is a perennial herb and native to Asia, Africa, South America, and Mediterranean countries, but is widely cultivated in many other countries *(26)*. Basil and its essential oil are used as flavoring in tomato-based products and those that are prone to deterioration by acid-tolerant microbiota *(19, 22)*. Among the more than 150 species of the genus *Ocimum*, basil is considered the main species for the purpose of the production of essential oil, and is cultivated commercially in several countries *(47)*.

Origanum vulgare (oregano) is recognised as one of the most commonly used herbs in the world, and is abundant in Eastern Europe, Asia, and North America *(32, 55)*. Various

studies have revealed the potential use of the *O. vulgare* EO against yeasts *(52)*, *Staphylococcus aureus (8)*, *Yersinia enterocolitica*, *Aeromonas hydrophila, L. monocytogenes (5)*, *Escherichia coli*, and *Pseudomonas aeruginosa (15)*, among others.

Thus, our aim was to investigate the antimicrobial activities of *O. basilicum* and *O. vulgare* EOs against *Listeria monocytogenes* and *Salmonella* Enteritidis in artificially inoculated fresh chicken sausage samples.

MATERIALS AND METHODS

Essential oils. Fresh plants samples of *O. basilicum* and *O. vulgare* were purchased in the city of Botucatu, São Paulo, Brazil, and used in the preparation of EOs by steam distillation methodology in a Marconi device, Model M480*(6)*. The voucher plant was deposited in the Herbarium "Irina Delanova Gemtchujnicov" Department of Botany, Institute of Biosciences – IBB/ UNESP, whose numbers were: *O. basilicum* Botu 26037 and *O. vulgare* Botu 26287.

Chemical characterization. Chemical analysis of EOs was performed by gas chromatography-mass spectrometry (GC-MS) in a Shimadzu device, model QP5050A, using a capillary column, CBP-5, 50 m in length, with an internal diameter of 0.25 mm and 0.25 μm film thickness. The carrier gas was He and the identification of EOs compounds was was made on the basis of the National Institute of Standards and Technology (NIST) library, analysis of the mass spectra, and also data in the literature *(1)*.

Preparation of fresh chicken sausage samples. The formulation proposed by Silva et al. 2008 *(50)* was used, comprising 84.55% of boneless chicken breast, 10% lard, 3% water, 1.5% salt, 0.5% polyphosphate, 0.25% garlic, and 0.2% pepper. The mass was embedded in swine casings with a mean diameter of around 30 mm, and the buds produced were divided, separated by lots, and stored in a refrigerator at 4°C.

Bacterial strains. *Salmonella* Enteritidis (ATCC-13076) and *L. monocytogenes* (ATCC-15313) strains. The strains were stored at -80°C until their use in microbiological assays.

Enumeration of *L. monocytogenes* and *S. Enteritidis* in chicken sausage assays. Susceptibility tests of the EOs were performed with the inoculation of bacterial strains on chicken sausage samples (25 g) with suspensions standardized by a 0.5 MacFarland standard, aiming at a bacterial concentration of around 10^5 colony forming unit/g (around 5 log CFU/ g) *(6)*. After, volumes of *O. vulgare* and *O. basilicum* EO were added separately, to achieve concentrations of 0.3 [the minimum inhibitory concentration (MIC) obtained in previous microdilution *in vitro* assays *(16, 29)* – (data not shown), 1.0, and 1.5% in inoculated sausage samples. All phases of assays were performed in sterile Petri plates, all procedures were carried out at laminar flow, and handling of the bacteria and EO homogenization were performed using sterile cutlery (knife and fork) made of stainless steel. Following homogenization, sausage samples were kept at 4°C. After 0, 5, and 24 hours, quantification of the bacteria inoculated in the sausage samples was performed by the most probable number (MPN) method. Control tests were also prepared; using non-inoculated and inoculated sausage samples both without EO addition. Assays were performed in triplicate.

For the quantification of *Listeria* in sausage, the methodology proposed by Ryser and Donnelly *(46)* was used (Fig. 1).

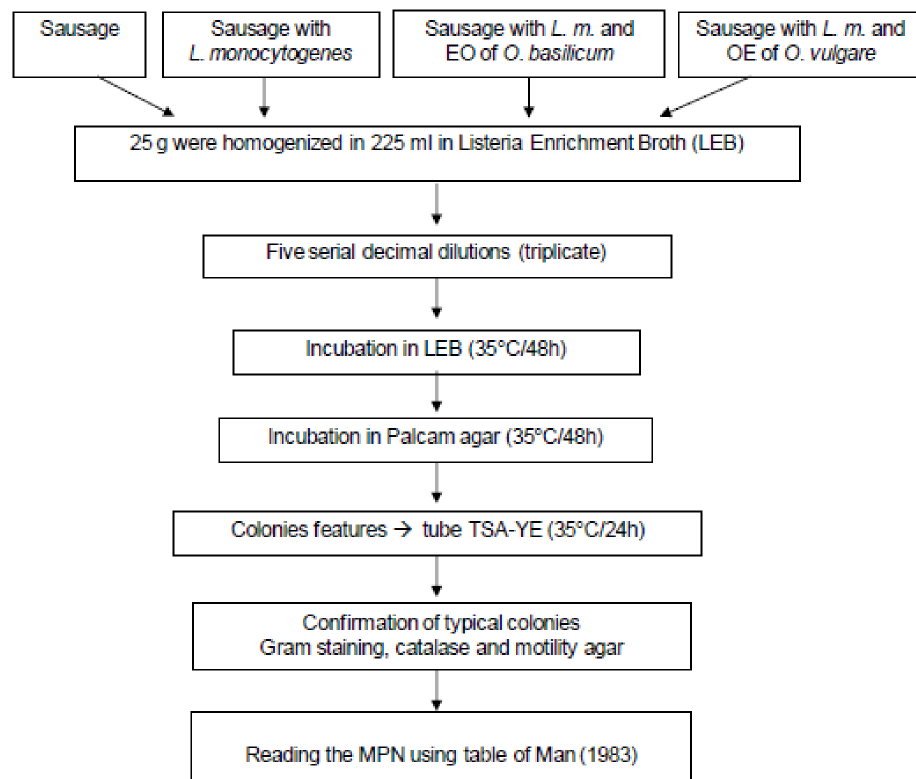

Fig. 1: Flowchart of verification tests of the action of essential oils on *Listeria monocytogenes* in artificially inoculated chicken sausage.

In tests with *Salmonella*, it is worth noting that although the methodology advocates the need for two means of enrichment, it was decided only by Rappaport Vassiliadis Broth according to recent studies *(35, 41)*. The analysis followed the methodology proposed by Andrews with modifications *(3)* (Fig. 2).

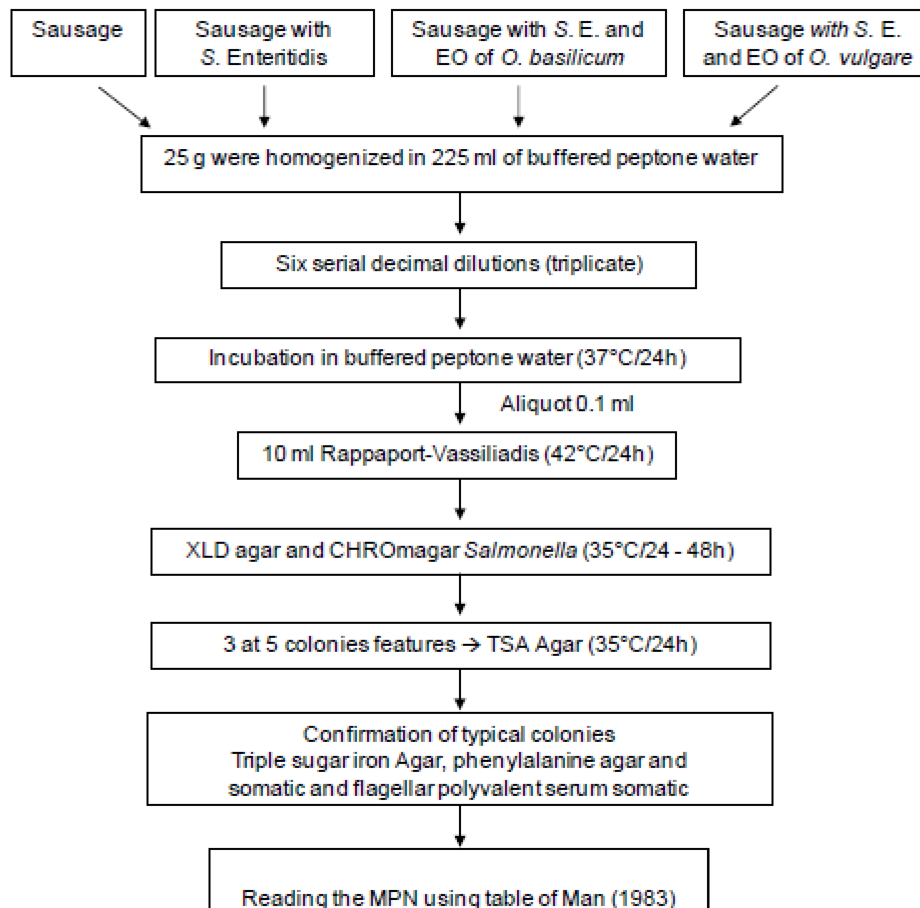

Fig. 2: Flowchart of verification tests of the action of essential oils on *Salmonella* Enteritidis in artificially inoculated chicken sausage.

Transmission electron microscopy (TEM). The overnight cultures of *S.* Enteritidis and *L. monocytogenes* (Brain Heart Infusion at 37°C/ 24h) received the *O. vulgare* or *O. basilicum* EO at 0.3%, the *in vitro* MIC found previously, and at three times this MIC value (1%). After 2 hours of contact with the EO *(21)*, the bacteria were prepared for TEM, as recommended by Moosavy et al. *(38)*. Pre-fixation of the bacterial samples was performed by adding 3 ml of 2.5% glutaraldehyde in 0.1 M phosphate buffer (PBS) (pH 7.2) for 24 hours, followed by centrifugation (1500 *g* for 20 minutes). The supernatants were discarded and 3 ml of glutaraldehyde was added to the pellet, which was stored at 4°C for 24 hours. The post-

fixation procedure was performed in an osmium tetroxide solution (1%) in 0.1 M phosphate buffer at pH 7.3 for 2 hours, followed by dehydration of the material in acetone and impregnation into blocks of Araldite® to obtain ultrafine sections. The sections were subsequently stained with uranyl acetate and lead citrate. The samples were analyzed and photographed with a transmission electron microscope (CM 100, Philips) operated at 80 kV. The images were analyzed with digital imaging software (Iten).

Statistical analysis. The results were analyzed by Kruskal-Wallis analysis of variance (ANOVA) and the Student-Newman-Keuls test (multiple comparisons), with the significance level of 5%. SigmaStat software version 3.5 was used.

RESULTS

The chemical composition of *O. basilicum* and *O. vulgare* EOs (Table 1) emphasized the terpenoids linalool (31.52%) and thymol (48.62%) respectively as the major compounds of these EOs. Considering the bacterial determinations of the sausage samples at time 0 for all treatments where *L. monocytogenes* was added (sausage with bacteria added and sausage with bacteria added and either EO at 0.3, 1.0, and 1.5%, there was a significant difference ($p \leq 0.05$) compared with the results of the negative control (sausage without addition of the bacteria or EO), with values of <3 MPN/g obtained in all assays.

Table 1: Chemical analysis of *Ocimum basilicum* and *Origanum vulgare* EOs.

Essential oil	Compound	%
Ocimum basilicum	linalool	31.52
	eugenol	14.39
	eucalyptol	14.06
	camphor	11.31
	germacrene	6.64
	β-caryophyllene	2.13
	α-pinene	0.36
	β-pinene	0.31
Origanum vulgare	thymol	48.62
	γ-terpinene	12.98
	ρ-cymene	7.48
	β-caryophyllene	5.31
	linalool	3.02
	myrcene	1.60
	α- terpinene	1.28

The concentrations of 0.3 and 1% were ineffective for *L. monocytogenes* control (p≥0.05). Therefore, we present only the results obtained with 1.5% (Fig. 3). There was no reduction in MPN/g values immediately after the addition of EO and *L. monocytogenes* compared with the untreated control. However, with 1.5% of *O. vulgare* EO reductions of 1.1 and 1.3 log MPN/g for *L. monocytogenes* were found after 5 and 24 hours of contact, respectively. In sausages treated with *O. basilicum* EO, there was a reduction of approximately 1.4 log MPN/g after 5 hours, which was maintained after 24 hours. All reductions were statistically significant compared with the control samples, sausage samples with bacteria and without EO, but there was no difference between the treatments with either EO.

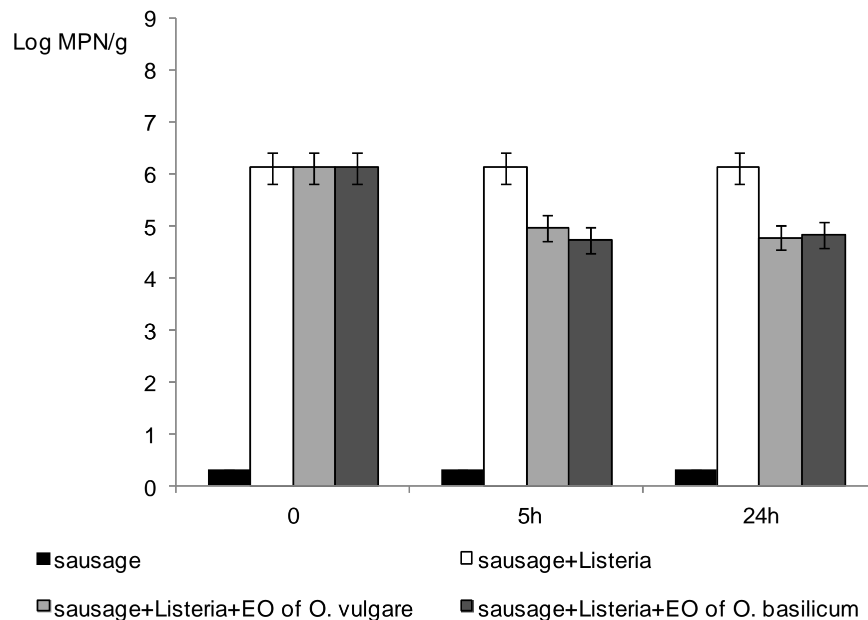

Fig. 3: Log of more probable number of bacteria per gram of sausage, for sausage inoculated with *Listeria monocytogenes,* sausage inoculated with *L. monocytogenes* and treated with 1.5% v/g of *Origanum vulgare* EO, and sausage treated with 1.5% v/g of *Ocimum basilicum* EO. Readings were taken at 0, 5, and 24 hours.

For *Salmonella* strain, a difference between negative control and treatments (p<0.05) was found at 0 hours, with values of <3 MPN/g in all assays. Regarding the other contact times, it was found that after 5 hours of contact with 0.3% of *O. vulgare* EO (Fig. 4A), there was reduction of *Salmonella* in 2 log MPN/g, and such a reduction was lower after 24 hours of storage (1.3 log MPN/g). On the other hand, *O. basilicum* EO had no effect after 5 hours of contact, but reduced the determination in 2.1 log MPN/g after 24 hours of contact (p≥0.05).

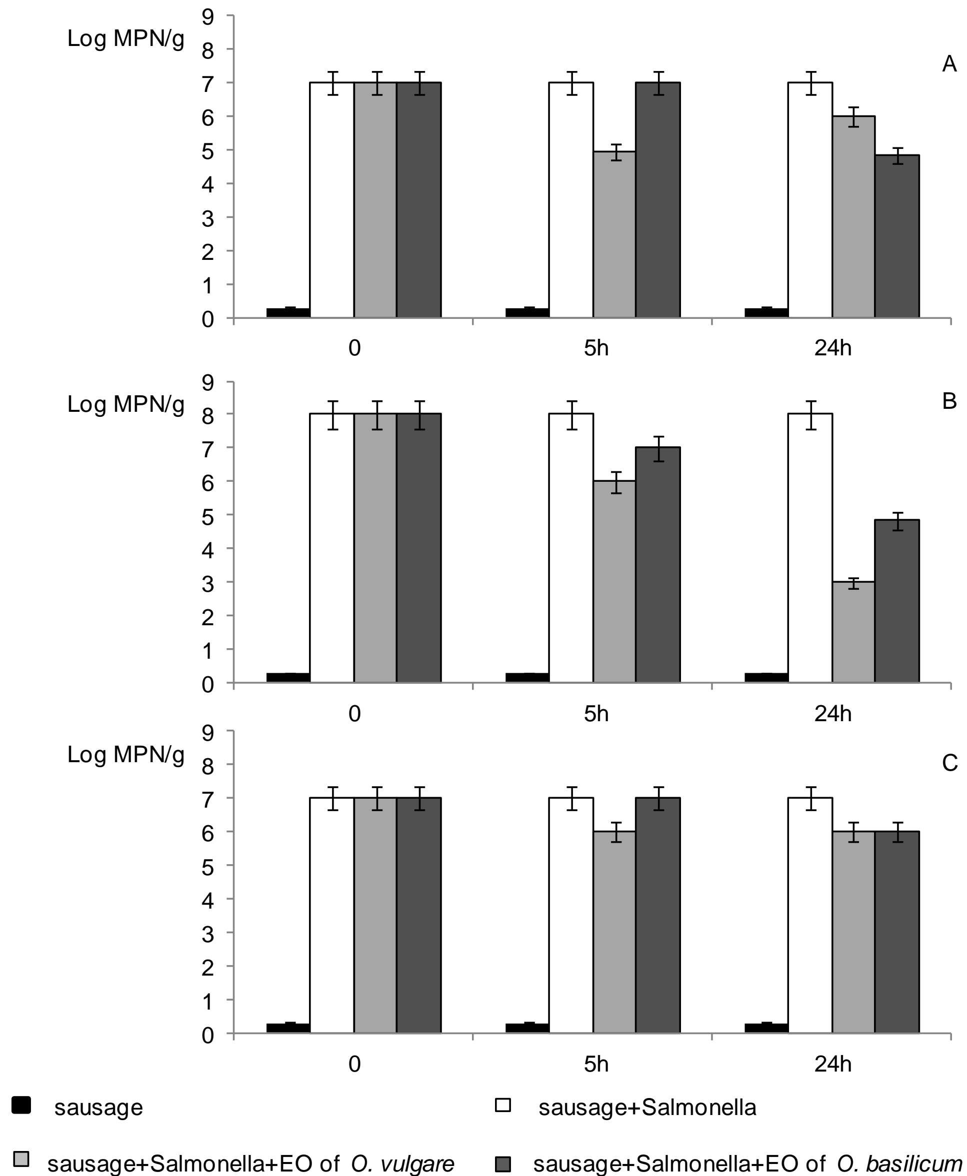

Fig. 4: Log of more probable number of bacteria per gram of sausage, for sausage inoculated with *Salmonella* Enteritidis, sausage inoculated with *Salmonella* Enteritidis and treated with *Origanum vulgare* or treated with *Ocimum basilicum* EO. EO were used at concentrations of 0.3% (A), 1% (B), and 1.5% (C). Readings were taken at 0, 5, and 24 hours.

At 1% of *O. vulgare* EO (Fig. 4B), it was found that 5 hours of contact between the bacteria and EO was sufficient to reduce the bacterial determination by 2 log MPN/g, and the was reduced by 5.1 log MPN/g after 24 hours of contact ($p \leq 0.05$). For *O. basilicum* EO, there was a reduction of 0.6 and 3.1 log MPN/g in *Salmonella* Enteritidis determination after 5 and 24 hours, respectively ($p \leq 0.05$).

At 1.5% (Fig. 4C), it was found that *O. vulgare* EO reduced the determination of *Salmonella* Enteritidis by 1.6 log MPN/g after 5 hours and it remained significantly reduced at 24 hours ($p<0.05$). At 1.5%, *O. basilicum* EO showed a significant reduction in *Salmonella* Enteritidis only after 24 hours of storage (1.4 log MPN/g).

The *L. monocytogenes* and *Salmonella* Enteritidis cells treated with 0.3% (MIC from *in vitro* assays) and 1.0% (around three times the MIC) and non-treated cells were observed by TEM. After the period of culture, untreated cells of *L. monocytogenes* and *Salmonella* Enteritidis showed a uniform cell structure with defined bacterial membranes and wall and cytoplasm with electron-dense material (Figs. 5A and 5B). On the other hand, in cells treated with EO, morphological changes were observed, including irregularities in the shape and loss of structural integrity of the cell wall and intracellular matrix. Also, we observed the presence of cell debris around the damaged cells (Figs. 5 A1–A4 and B1–B4).

DISCUSSION

Although this study had the objective of verifying the antimicrobial activities of EO against *L. monocytogenes* and *Salmonella* Enteritidis in chicken sausage, were performed tests for check previous contamination of samples sausage (i.e. before artificial contamination) arising out the raw material or handling during the preparation of these samples. Thus, it was verified that there was no contamination of sausage samples with the bacterial strains studied, with values corresponding to <3 MPN/g. This suggests to us that the bacteria recovered and identified during *Listeria* and *Salmonella* MPN assays were certainly the *Listeria* and *Salmonella* strains artificially inoculated in the sausage.

Despite the inherent characteristics of the MPN technique (e.g., large volume of material required, workload, and the time necessary to complete identification), this method proved to have high sensitivity and high reproducibility *(37)*. Thus, we considered the methodology of MPN adequate for this study, which relates to the recovery of bacteria inoculated artificially in sausage samples.

In research conducted in Brazil *(56)*, *L. monocytogenes* was found in 25% of sausages produced industrially. Also, according to this research, the pathogen was detected in all samples of raw material used in the preparation of sausages, revealing that contamination by *L. monocytogenes* was observed during the production process through contact with the environment, equipment, and handlers *(56)*. As the sausage samples used in this study were produced in compliance with good manufacturing practices and using good quality raw material, there was no problem of contamination by this bacterium, which is usually common

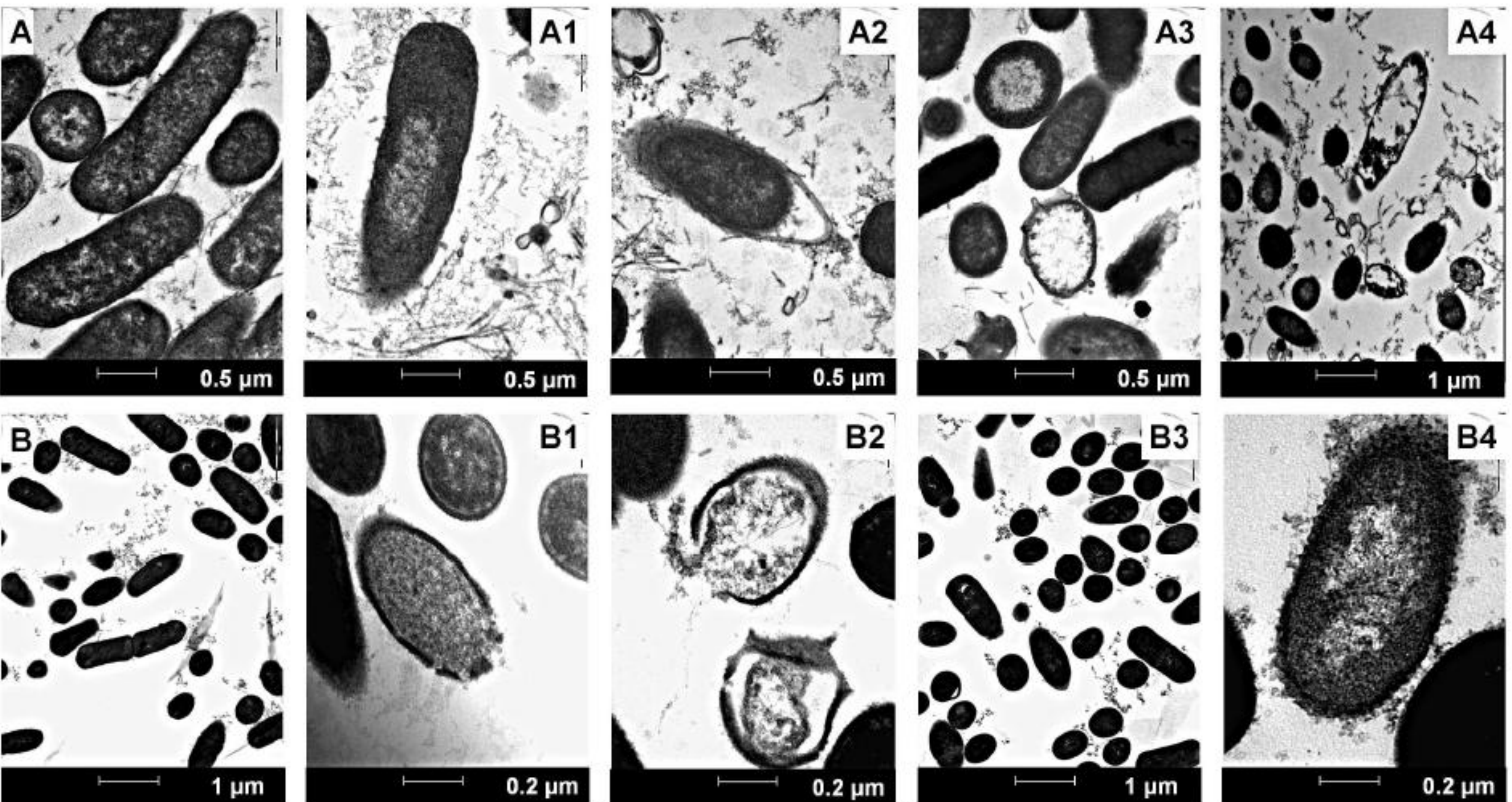

Fig. 5: Transmission Electron Microscope images of (A) untreated *Salmonella* Enteritidis; (A1, A2) *Salmonella* treated with *Origanum vulgare* EO at 0.3 and 1%, respectively; (A3, A4) *Salmonella* treated with *Ocimum basilicum* EO at 0.3 and 1%, respectively; (B) untreated *Listeria monocytogenes*; (B1, B2) *Listeria* treated with *O. vulgare* EO at 0.3 and 1%, respectively; (B3, B4) *Listeria* treated with *O. basilicum* EO at 0.3 and 1%, respectively.

in refrigerated products. In another study conducted in Brazil, the percentage of *Salmonella* isolated by the traditional method was 9.3% of sausages marketed in the city of Botucatu, São Paulo state *(43)*. *Salmonella* positivity (16%) was also found in chicken sausages from the north-east of São Paulo state *(14)*.

In chicken carcasses from the Brazilian state of Goiás, 52 samples, i.e., around 14.32% of total samples collected, were contaminated with *Salmonella* and 11 serovars were identified; *Salmonella* Enteritidis was the second most frequently found (13.5%) *(39)*. Although the frequency of contamination by *Salmonella* in Brazilian sausages was high, samples of sausages were used in the tests were in accordance to legislation. According to the National Health Surveillance Agency (ANVISA) *Salmonella* should be absent in 25 g of food *(10)*. Overall, In general, the literature shows variability in results which were usually influenced by the technique used for the recovery of bacteria.

The concentration of 1.5% showed the best anti-*Listeria* activity with both EOs after 5 hours of storage, and the reduction in bacterial determination hardly changed after 24 hours. For *Salmonella* Enteritidis, bacteria determinations in the samples treated with 0.3 and 1.5% of *O. vulgare* EO were reduced after 5 and 24 hours of storage. In samples treated with *O. basilicum* at the same concentrations, only 24 hours after the EO showed antimicrobial effect. At 1% of EO, reduction of *Salmonella* was observed after 5 hours, and the reduction was even greater after 24 hours of incubation with *O. vulgare* EO. However, *O. basilicum* EO (1%) was effective at reducing the CFU/g only after 24 hours.

The determination of *Listeria* was reduced by EO at the higher concentration, whereas *Salmonella* was inhibited at the lower concentrations (0.3%). Generally, Gram-negative bacteria are less sensitive to antimicrobial agents because of the bacterial wall and its outer membrane, which limit the diffusion of hydrophobic compounds. However, the literature has shown that this does not mean that the Gram-positive bacteria are always more susceptible *(13)*. Furthermore, this Gram-positive pathogens is able to adapt to several environmental conditions, such as refrigeration, acid foods, or foods with high salt contents, probably due the increased percentage of peptidoglycan in their wall *(45, 48)*.

As *L. monocytogenes* is a psychrotrophic bacteria that is able to survive and multiply in meat refrigerated products and ready for consumption which increases the risk of surviving cells in the finished product *(25, 62)*. It is possible that, at a low concentration of EO plus refrigeration, *Listeria* shows better survival against the antimicrobial agent, which was not seen to occur with *Salmonella*. Another fact to consider is that the antimicrobial activity of the EO is influenced by several factors that are extrinsic and intrinsic to the food (e.g., fat,

protein, and pH). With regard to the complex food matrix, the activity tends to be decreased compared with the results obtained with culture medium, since food can protect the bacteria from the action of EO. Smith-Palmer *(51)* studied cheeses with different fat contents and verified that the composition of the food influenced the efficiency of plant EO against *L. monocytogenes* and *Salmonella* Enteritidis. When considering the potential application of EO in foods, the authors found that *L. monocytogenes* was inhibited more readily with a low-fat content but that the composition was less influential with *S*. Enteritidis *(51)*. Thus, the lard or lipid content of the sausage may also have negatively affected *L. monocytogenes* inhibition.

Analysis of the chemical composition showed that linalool and thymol were the most abundant compounds in the EO. Linalool makes the membrane of the bacteria permeable *(31)*; the MIC observed in some studies was 1000 µg/ml for *Salmonella* Typhimurium and ranged from 1000–2145 µg/ml for *L. monocytogenes (30)*. In a study comprising the hydrophobic constituents of EOs, linalool showed high bacteriostatic activities, with an MIC of ≤0.2 mg/ml against *L. monocytogenes* and *E. coli* O157:H7, while the minimal bactericide concentrations (MBCs) were 0.2 mg/ml. The combination of mild heat (54°C/10 min) and 0.2 mg/ml of the antimicrobial showed a higher inactivation than the sum of the methods acting separately *(2)*. The antimicrobial activity of thymol is due structural and functional damage of the cytoplasmic membrane that causes the release of lipopolysaccharides present in the outer membrane of Gram-negative bacteria and rupture of the outer membrane *(27, 49)*. Thymol at 0.4 and 0.2 mg/ml, as wash solution, reduced by 5 log and 2 log, respectively, the amount of *Salmonella* on the surface of contaminated grape tomatoes *(34)*. The antimicrobial effect of thymol and nisin was also investigated, and the results showed a synergistic effect of a combination of sub-inhibitory concentrations of both, thereby reducing the growth of *L. monocytogenes* and *Bacillus subtilis*. Thymol increased bacterial permeability, allowing a greater amount of nisin to enter the cell *(20)*. Such a synergistic interaction allows the use of lower concentrations, and reduces the impact of EO on organoleptic characteristics of the food. Knowledge of the chemical composition of EO allows a better understanding of the sites of action in the bacterial cell and, at the same time, points to the use of these compounds alone or in combination with other preservation techniques for food safety.

Our results showed that *O. vulgare* and *O basilicum* EOs had damaged the cells of two bacterias causing cell death, and thus the there was no bacterial recovery by the MPN technique. This information is corroborated by the results of TEM, which showed morphological changes in the bacteria after 2-hour exposure to the EOs. These changes have been interpreted as being due to the effects of EO on the permeability of the membrane, and

causing their lysis as well as damage of the bacterial cell wall and loss of intracellular contents, as shown by the accumulation of materials on the surface of treated cells *(18)*. Structural changes of the cell wall of Gram-positive and Gram-negative bacteria may lead to different damage when exposed to antimicrobial compounds *(42)*. Cellular damage in foodborne pathogens treated with natural products has also been shown by TEM by other authors. Wu et al. *(60)* found that *L. monocytogenes* and *S. aureus* were not easily destroyed, even with injuries or channels in the cell wall, compared with *E. coli* O157 and *S.* Typhimurium. When using thyme EO, it was reported that in addition to all the degenerative changes in the cells of *L. monocytogenes* (e.g., loss of cytoplasm and uniform distribution of the agglomeration of intracellular material), with an increasing concentration of EO *Listeria* bacterial wall also lost its characteristics and uniformity *(44)*.

Thus, we concluded that *L. monocytogenes* was less sensitive to the action of EO than *Salmonella*. With respect to *Listeria* in fresh chicken sausage, a concentration of 1.5% was the most efficient for both EOs. The best inhibitory effect on *Salmonella* was shown by 1.0% of *O. vulgare* EO, for which 5 hours of contact was sufficient to promote a reduction in the bacterial count; in contrast, *O. basilicum* EO showed a significant reduction at the same concentration after 24 hours. Inhibitory effects of EOs on bacterial strains were not immediate. Our results showed an effect the first 5 hours that remained constant or increased after 24 hours. Both EOs have the potential to be explored by the food industry, and the combination of processes for food protection has shown a promising reduction of the factors limiting the use of these compounds, such as a negative impact on taste and high cost.

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CAPÍTULO III

**Manuscrito submetido à Journal of Food Protection*

EFFECTS OF *OCIMUM BASILICUM* LINN ESSENTIAL OIL AND SODIUM HEXAMETAPHOSPHATE ON THE SHELF LIFE OF FRESH CHICKEN SAUSAGE

ABSTRACT

While consumers and the food industry have an interest in reducing the use of synthetic additives, the consumption of embedded food in Brazil has been increasing because of the ease of preparation and low cost. Due to the antimicrobial and antioxidative properties of *Ocimum basilicum* essential oil (EO), it has potential applications in food products. Polyphosphates are already used in meat processing with the goal of improving the quality of the products. The aim of this work was to assess the effects of sodium hexametaphosphate (SHMP) and *O. basilicum* EO, when added separately or together, on physical, chemical, and microbiological parameters during the shelf life of chicken sausage. We also performed sensory analysis of the product prepared in this manner. Six different treatments were produced in which the substances were tested together or separately, and the content of EO was 0.3 or 0.03%. The samples were analyzed after 1, 7, and 15 days of storage at 4 °C. An increase in pH at day 7 and 15 in samples that contained SHMP was observed. In the samples that contained either 0.3 or 0.03% EO, coliforms inhibited throughout the study period ($p<0.05$), which was not observed in samples with EO plus SHMP, thus demonstrating that the stabilizer blocked the antibacterial action of EO. There was a reduction in the cook loss and increased compressive force in the samples with 0.5% SHMP, contributing to greater juiciness of the product. The EO had a substantial impact on the acceptibility of the samples, but did not influence the activities that have already been described for the polyphosphate.

Keywords: sausage, essential oil, stabilizer, shelf life.

INTRODUCTION

Sausages are a traditional food in some countries, and were introduced in Brazil by European immigrants. The Brazilian population has a high frequency of consumption of embedded products, such as sausage *(32)*. In fact, during 2009, chicken sausage reached 28% of the sausage consumed in the country *(2)*.Fresh sausages are mixtures of meat, fat, and spices inside a casing, what makes it necessary cooking before consumption. While it is widely produced by mechanized industrial processes governed by health regulations, in many countries it is still prepared by manual procedures, which are capable of contamination *(3, 19, 37)*.

The use of food additives has become commonplace in recent years due to the increased production due to increased production of premade and processed foods. At the same time, consumers and researchers have raised questions about the necessity and safety of these substances *(66)*. This has lead to increased interest in the use of natural antimicrobials in recent years, especially in response to consumer demand for additives considered "green". Thus, the application of natural agents has grown sustainably because of the demand for food that has been less processed and contains natural ingredients for preservation *(64)*.

On the other hand, a growing number of consumers worldwide have come to prefer products without or with reduced content of synthetic additives. Furthermore, a report on the potential toxicity of synthetic antioxidants revealed the search for natural products, including by the poultry industry *(36)*. In this sense, the natural products, such as chitosan, nisin, and essential oils (EOs), have been studied for the purpose of use in foods, with the goal of safety and food preservation *(1, 13, 18, 23)*. In a recent review, Karre et al. *(38)* reported that ingredients, such as spices, can affect the flavor of meat and poultry products and may be viewed as negative or as positive results. Therefore, when selecting a natural product for use in a meat or poultry product, sensory and quality impacts on the product should be considered to ensure a product with the desired traits is achieved.

The shelf life of fresh sausage can be directly influenced by microbiota and/or the non-thermal processing. The shelf life can also decrease even in the absence of microorganisms because processes such as proteolysis, lipolysis, and oxidation can also reduce product quality during storage *(74)*. Thus, the use of agents with antimicrobial and antioxidant properties on the meat has the potential to increase the sausage shelf life and avoid significant economic losses *(71)*.

Polyphosphates are intentional additives that are classified as stabilizers, which function to stabilize the mixture and prevent the physical and chemical changes that occur in meat products, and are widely applied in processed foods, such as red meat, chicken, seafood, and dairy products *(19, 36)*. Sodium polyphosphate, or sodium hexametaphosphate (SHMP), is the most widely used, with the Brazilian and American legislations recommends a maximum concentration of up to 0.5% *(12, 65)*. The positive aspects of these phosphates are that they cause an increase in the binding capacity of water in muscle proteins, while maintaining the juiciness of a food *(59)*, they act positively on lipid oxidation, and improve the texture and color of meat *(48)*.

Although the polyphosphates are not classified as antimicrobial, this property has been reported against gram-positive (e.g. *Listeria monocytogenes, Bacillus subtilis*, and

Staphylococcus aureus) and against gram-negative bacteria (e.g. *Escherichia coli* K-12, *E. coli* O157:H7, and *Salmonella* Typhimurium) *(48, 54, 73)*.

The *Ocimum basilicum* Linn. (basil; Lamiaceae) is a very popular herb and has been widely used as an ingredient for food, especially as a flavoring agent in baked foods, particularly meats. There are also reports that it has analgesic, anti-inflammatory, antibacterial, hepatoprotective, and immunomodulatory properties *(8)*. The effects of EO as an alternative to the safety and preservation of food have been studied by several authors who showed their potential for use in food *(27, 49, 63)*. Regarding the chemical composition, Kwee and Niemeyer *(39)* reported that the contents of phenolic acids, especially rosmarinic, caffeic, chicoric acids, were detected in 15 cultivars of *O. basilicum* by the Folin–Ciocalteau assay. However, there was variation in these levels according to the cultivar. It is believed that agronomic procedures during the production of this plant can influence the composition of secondary compounds. According to Nguyen et al. *(51)*, the increased total phenolic concentration at the highest potassium rate are, therefore, likely to correlate with higher rosmarinic and chicoric acid levels.

There are few reports characterizing the effects of the combined use of *Ocimum basilicum* EO and polyphosphate despite the importance of studies on this topic.

Therefore, the aim of this work was verify the effects of the application of *O. basilicum* EO and SHMP, either alone or in a mixture, on the physical, chemical, and microbiological parameters fresh sausages samples prepared with chicken meat during a period of 15 days at 4°C of storage temperature.

MATERIALS AND METHODS

Materials. American Type Culture Collection (ATCC) bacterial strains were provided by Fundação Oswaldo Cruz-FIOCRUZ (Rio de Janeiro, Brazil). The culture media were from Difco (USA). Fresh chicken sausage was produced with boneless chicken breast, lard, salt, spices, and natural casings bought from local retailers. Distilled water was used (4 °C). The stabilizer SHMP (INS 452 (i)) and *O. basilicum* EO were purchased from Brazilian suppliers and according the EO supplier, the following compounds were identified by gas chromatography (GC/MS): linallol (46%), 1–8 cineol (26%), and camphor (13%), and the relative density varied from 0.873 to 0.943.

Minimal inhibitory concentration assays. These tests aiming the minimal inhibitory concentration (MIC) for *O. basilicum* EO against *Salmonella* Enteritidis (ATCC-13076), *Escherichia coli* O157:H7 (ATCC-43895), *Listeria monocytogenes* (ATCC-15313), and

Staphylococcus aureus (ATCC-25923) were performed according the microdilution method *(16)*. We used ELISA microplates containing Mueller Hinton broth supplemented with 0.5% Tween (200 µl) whose concentrations tested ranged from 0.025 to 5% v/v and inoculum values were approximately 10^5 Colony Forming Units (CFU)/ml using 0.5 MacFarland standardized bacterial suspensions. After incubating the samples at 35 ^oC for 24 hours, the MIC results were determined with the aid of the indicator stain resazurin redox 0.01% *(30)*. These assays were performed in triplicate. The addition of EO concentration directly in the sausage was calculated from the mean MIC against standard ATCC strains tested.

The microdilution revealed that the 0.3% v/v MIC was the average obtained for the four strains tested. Thus, obtaining this value further supported the validity for testing antimicrobial activity during the 15 days of storage.

Sausage samples preparation and treatments. The chicken sausage was produced with boneless chicken breast (84.55%), lard (10%), water (3%), salt (1.5%), garlic (0.25%), and pepper (0.2%) *(61)*. Chicken meat and lard were processed in a meat grinder (FunKitchen model CM-4005, Brazil) and all of the ingredients, except the stabilizer SHMP, were mixed in an industrial mixer. The mass of the sausage was divided into two separate lots, and one of them received the addition of 0.5% SHMP. Each batch was divided into three subgroups, resulting in six treatments: Sausage control ($\mathbf{T_1}$), sausage with 0.03% of *O. basilicum* EO ($\mathbf{T_2}$), sausage with 0.3% of *O. basilicum* EO ($\mathbf{T_3}$), sausage with SHMP ($\mathbf{T_4}$), sausage with SHMP plus 0.03% of *O. basilicum* EO ($\mathbf{T_5}$), and sausage with SHMP plus 0.3% of *O. basilicum* EO ($\mathbf{T_6}$).

Two concentrations of EO were chosen: 0.3% (v/w), which was the MIC value previously obtained by *in vitro* assays against four bacterial strains that are important food borne pathogens; and 0.03%, the value found after the acceptance test (details below).

The portions of the respective mixtures of treatments were incorporated into natural swine casings with a diameter around 30 mm, and the buds of sausages produced were separated by treatments and stored at 4°C.

Sensory analysis. The sensory analysis was performed on the first day of sausage storage and was performed with 60 untrained panel members. The samples were roasted in an electric grill and offered to volunteers while still hot. The acceptance degree was recorded using a nine-point hedonic scale, where 9 = like extremely, 8 = like moderately, 7 = like regular, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike regularly, 2 = moderate, and 1 = dislike extremely. The preference was obtained by inference *(31)* and expressed as degree of overall like or dislike. The samples were coded with three-digit

numbers and randomized. Previously, preliminary analyses were performed with samples of sausages containing 0.01 to 1% v/g of EO (data not show). From these data, the sensory analysis was made with six treatments (T_1, T_2, T_3, T_4, T_5 e T_6) as described in the "*Sausage samples preparation and treatments*".

The research was authorized by the Research Ethics Committee of the Faculty of Medicine of Botucatu, UNESP (protocol 3423-2010).

Microbiology analyses in fresh sausage and chicken meat. Microbiological analyzes on fresh sausage were performed on the first, seventh, and fifteenth day at 4°C. Each treatment was performed in five replicates and microbiological analyses were performed in duplicate. The same analyses were also performed on samples of ground chicken breast (raw material of the sausage preparation) in order to check the initial contamination of the chicken meat.

The presence of coagulase-positive *Staphylococcus*, *Salmonella* sp., sulphite-reducing clostridia, and coliforms at 45 °C was analyzed according to RDC No. 12 *(11)* for fresh embedded (raw sausages and similar). The mesophilic, psychrotrophic, yeast, and mould counts were also determined. For these organisms, excluding *Salmonella*, 25 g of each sample was placed in 225 ml of sterile buffered water and homogenized in a Stomacher 400 Lab Blender for two minutes. From this initial dilution (10^{-1}), we prepared the appropriate decimal dilutions of the samples using the same diluent and transferred each sample, in duplicate, to specific culture media (Table 1).

For analysis of *Salmonella*, the homogenization procedure was similar to that previously mentioned, but buffered peptone water was used. The homogenates were transferred to Erlenmeyer flasks and incubated at 35 ºC for 24 hours. Then, 0.1 ml was inoculated into a tube containing 10 ml of Rappaport Vassiliadis broth and incubated at 42 °C for 24 hours and also 1 ml was inoculated in 10 ml tetrathionate broth, added 0.2 ml of potassium iodide (at the time of use) and incubated at 35°C for 24 hours. After these periods of incubation, a loopful of each growth was seeded on agar Xylose Lysine Deoxycholate and CHROMagar *Salmonella*. After incubation at 35ºC for 24 hours, the characteristic colonies of *Salmonella* were isolated and peaked into tubes containing tryptic soy agar (TSA), inclined, and incubated at 35°C for 24 hours. Suspected colonies were subjected to biochemical tests with Triple Sugar Iron agar and agar Phenylalanine inclined, and incubated at 35°C for 18–24 hours. Strains that were positive in these tests were tested against serum polyvalent somatic and flagellar *(5)*.

Table 1. Culture media, incubation conditions, and references used during the microbiological analysis.

Microbiological group	Culture Media	Incubation	References
Coagulase-positive staphylococci [a,b]	Baird-Parker added potassium tellurite solution and egg yolk	35°C/48h	*(42)*
Sulphite reducing clostridia [a]	Sulfite polymyxin sulfadiazine agar (SPS)	43°C/48h	*(40)*
Yeasts and molds [a]	Potato dextrose agar (pH 3.5)	25°C/5 days	*(7)*
Psychrotrophic [a]	Plate count Agar (PCA)	7°C/ 10 days	*(50)*
Mesophilic [c]	Plate count Agar (PCA)	35°C/48h	*(50)*
Coliforms at 45°C or thermotolerant [d]	Sodium Lauryl Sulfate Broth (presumptive)	37°C /48h	*(21)*
	EC broth (confirmatory)	45°C/24h	*(44)*

a) Spread-plate technique (0.1 ml); b) The suspected Staphylococcus colonies were isolated and spiked into tubes with TSA and subjected to the catalase, Gram stain, and tube coagulase test with rabbit plasma; c)Pour-plate technique (1 ml); d) Most probable number (MPN) technique.

Physicochemical analyses. Sausages were kept under refrigeration at 4°C and samples were taken for analysis on days 1, 7, and 15 of storage to assess the color and lipid oxidation. The cook loss and texture were determined only on the first day of storage. Each of the six treatments had five replicates.

pH. The pH was determined with a TECNAL pH meter (TEC-3MP-Port, Piracicaba, São Paulo, Brazil) on a homogenate of 10 g of sausage with 100 ml of distilled water. *(31).*

Color measurement. The sausage buds had the casings removed and were homogenized with a mixer and placed in a Petri dish, where the readings were taken. The color of sausage was determined by averaging three different points using the Minolta Colorimeter (Model CR-410, USA). We used the CIELab system by light reflectance in three dimensions where L* represents the lightness, a* represents the color intensity from red to green, and b* the yellow to blue intensity, according to the methodology described by Honikel *(28).*

Lipid oxidation. Lipid oxidation was evaluated by measuring the malondialdehyde (MDA) content using the 2-thiobarbituric (TBA) test *(69)*. A standard curve was made from a serial solution of tetraethoxyphropane. With this curve, we obtained the equation for the linear regression, which was used to calculate the MDA content. The results were expressed in mg MDA/kg sample. Analyses were performed in duplicate.

Cook loss. Initiallym the sausage samples (buds) were weighed, vacuum packed, and placed in a water bath (80°C for 60 minutes). After this procedure, the samples were maintained at 4°C for 24 hours. The liquid contained in the packaging was fully drained, and then the samples were reweighed. The cook loss was expressed as the percentage of initial weight lost. These analyses were performed in duplicate.

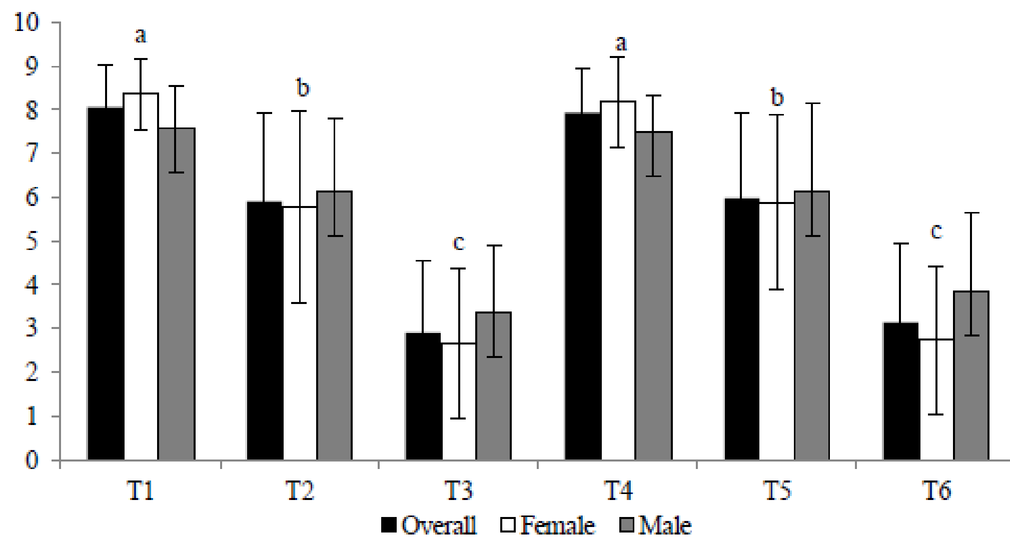
Texture measurements. Samples were prepared as described in the section above "*Cook loss*" After 24 hours of chilling at 4 °C, cylinders of 2 cm high were cut. The diameter of the sausage (about 30 mm) was determined by the natural casing. The texture analysis was performed according to Bourne *(9)* using a texturometer (Brookfield Engineering Labs, Inc., USA). Compression force was applied to 50% of the height of the sample. Was used the probe TA- BT-Kit and test speed was 1.0 mm/s. The parameter used was the compression force (g' hardness-peak force of the first compression cycle) and TexturePro CT V1.2 Build 9 software. The analyses were performed using eight units of each repetition.

Statistical analysis. For microbiological parameters, we used the non-parametric Kruskal-Wallis and Friedman tests. Other data were subjected to ANOVA and Tukey's test. We used a of 5% significance level. We used the SAS version 9.2 software.

RESULTS AND DISCUSSION

Sensory analyses. The acceptability of the sausage samples was not influenced by addition of SHMP (Fig. 1), while the samples with high EO levels (T_3 and T_6) were rejected by the panelists, with all of the assigned values being below 5.

The T_1 and T_4 samples, both without EO, received the best evaluations by the panelists, with values above 7.9, which indicates that the sausage control was well accepted. However, the T_2 and T_5 samples exhibited values higher than 5.0 (limit of rejection). The T_3 and T_6 treatments were rejected, with overall means of 2.9±1.7 and 3.1±1.8, respectively. The panel members could not distinguish between the sausage with or without SHMP, and there was no difference in the values assigned to each treatment when the sex of the tester was considered. Chouliara et al. *(14)* found that sensory results



Different letters indicate the difference of acceptability between treatments for the total number of panel (overall) as for sex.

Fig. 1. Sensory analysis performed with a 9-point hedonic scale for roasted chicken sausages treated with or without *O. basilicum* EO and/or SHMP on the first day of storage.

were not in agreement with the microbiological study of the effects of oregano EO and modified atmosphere packaging on chicken breast. Valero and Giner *(67)* evaluated the anti-*Bacillus cereus* effects of compounds from EO on carrot broth and found that, though the antibacterial activities of carvacrol, cinnamaldehyde, and thymol were evident, the concentrations tested had a significant effect on the odor and taste of the broth. The samples with greater acceptance contained 2 µl of cinnamaldehyde/100 ml of carrot broth, while all samples containing carvacrol and thymol were found to be unacceptable by the panelists *(67)*. The effect of hyssop and coriander EOs on the extension of shelf life of ground beef was also studied. Again, acceptance limited the highest concentration that could be tested *(46)*, and 0.02% v/w was chosen to be acceptable by the sensory evaluation panel. This value was very close to the concentration of *O. basilicum* EO with the best acceptance rate of this study (0.03%). Moreover, unlike the findings of the present study, Govaris et al. *(24)* found that much higher concentrations (0.6 and 0.9%) of oregano EO were organoleptically acceptable in minced sheep meat. The taste and overall acceptability of meat with 0.6% of the EO was higher than the control.

The use of EO in food can directly influence the acceptance of the product by the consumer due to its strong odor and flavor, and some concentrations may lead to rejection of food by consumers. Therefore, despite the positive effects of EOs, the sensory impact should always be considered.

Microbiology analyses in fresh sausage and chicken meat. All samples were negative for *Salmonella* and *sulphite-reducing* clostridia (srC). In some cases, there were isolates of coagulase-positive staphylococci (CoPS), but these were unrelated to the treatments and times of analysis (p>0.05). Regarding *Salmonella* sp., the results for the samples are in accordance with Brazilian legislation *(11)*, i.e. absent in 25 g, while srC and CoPS were considered acceptable, with maximum values as 3.10^3 and 5.10^3 CFU/g, respectively.

The results for yeast and mold, psychrotrophic, and mesophilic standard counts are presents at Table 2. According to the yeasts and molds count, there was an increase over the days of storage for T_1, T_4, and T_5. There was an increase in the amount of psychrotrophic during storage samples in the T_1, T_2, T_3, and T_5. For the mesophilic microorganisms, the count increase was slightly higher, but only in T_1 and T_5.

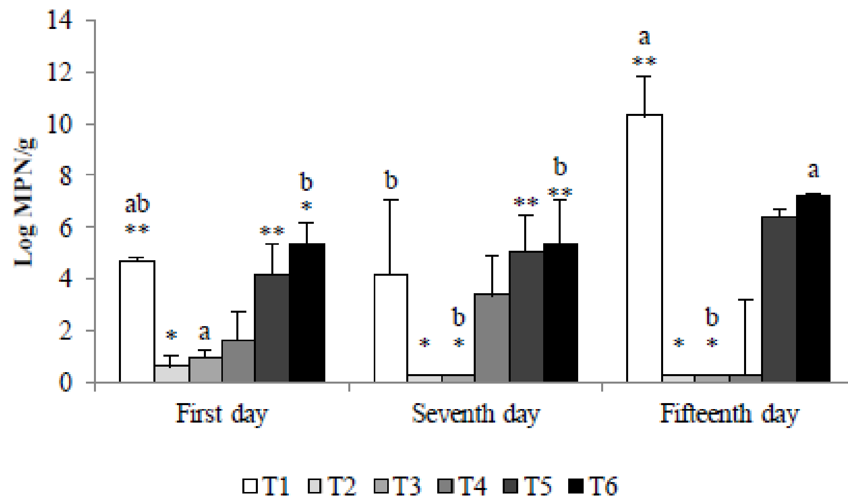
Table 2. Counts and standard deviations (log_{10} CFU/g) of microorganisms present in the chicken sausage samples during storage at 4 °C.

Treatments	First day	Seventh day	Fifteenth day
		Yeasts and molds	
T_1	4.35±0.17 ^{b}	4.75±0.17 ^{ab}	4.81±0.21 ^{a}
T_2	4.34±0.15	4.54±0.16	4.75±0.25
T_3	4.21±0.15	4.48±0.05	4.39±0.34
T_4	4.00±0.19 ^{b}	4.42±0.13 ^{ab}	4.52±0.28 ^{a}
T_5	4.35±0.15 ^{b}	4.52±0.43 ^{ab}	4.79±0.16 ^{a}
T_6	4.46±0.16	4.39±0.23	4.39±0.21
		Psychrotrophic	
T_1	8.10±0.11^b	8.48±1.17^{ab}	9.10±0.76^a
T_2	7.78±0.21^b	8.23±0.10^{ab}	8.42±0.15^a
T_3	7.92±0.15^b	8.40±0.19^{ab}	8.80±0.43^a
T_4	8.19±0.10	9.14±1.36	8.78±0.55
T_5	8.14±0.22^b	8.26±0.04^{ab}	8.63±0.30^a
T_6	8.19±0.16	8.44±0.97	8.94±0.38
		Mesophilic	
T_1	6.72±0.22 ^{b}	8.90±1.37 ^{ab}	9.23±0.29 ^{a}
T_2	6.84±0.52	6.89±0.85	8.95±1.90
T_3	6.93±0.90	7.74±0.91	9.03±0.72
T_4	7.67±0.37	9.16±0.41	8.76±0.81
T_5	7.22±0.81 ^{b}	8.57±0.60 ^{ab}	9.03±0.27 ^{a}
T_6	7.13±0.83	8.34±0.97	8.76±0.73

Lowercase letters compare times within each treatment. a: the highest count and b: lowest count.

The mean values and standard deviation log_{10} MPN/g thermotolerant coliforms (**TC**) present in the sausage samples during the storage period are shown in Fig. 2. For T_1, or the control sausage, there was an increase of about 6 log MPN/g when comparing the difference

between day 1 and day 15 of storage. For samples in the T_6 group, an increase in counts on the last day of storage was also observed. However, this increase was lower and was about 2 log. In the samples T_2 and T_3 TC inhibition was observed throughout the study period ($p<0.05$).



MPN: Most Probable Number. Asterisks compare treatments for each time point. Letters compared storage time for each treatment. ** or a: highest count, and * or b: lowest count.

Fig. 2. Log_{10} MPN per gram of TC in the samples of chicken sausage during storage at 4 °C.

There was no large microbial growth of mesophilic or psychrotrophic yeasts during storage but some samples revealed a trend of increasing in counts between the first and seventh day, and the seventh to fifteenth day. The presence of the stabilizer SHMP only (T_4) did not prevent the growth of molds and yeast, while the addition of only EO (T_2 and T_3) did not inhibit the growth of psychrotrophics, even for the highest concentration tested (0.3%).

In T_1 and T_5, we observed increases in the counts for mesophilic, psychrotrophic, mold, and yeast. Although there were no statistically significant differences between the samples, the results suggest that the interaction between SHMP (0.5%) and *O.basilicum* EO (0.03%) was not effective against the growth of these microorganisms during storage at 4°C. Ambrosiadis et al. *(3)* also reported high rates of aerobic microorganisms in traditional Greek sausages, averaging 8.22 log10 CFU/g. In a study of the shelf life of fresh Tuscan sausage, total counts of mesophilic bacteria in control samples (2 °C) ranged from 5.45 on the first day to 8.51 log_{10} CFU/g on the 14th day *(37)*. Although not analyzed in this study, *Pseudomonas* spp. is one of the most common spoilage associated with refrigerated meats (0 to 4 °C), and it is able to grow at a pH between 5.5 and 7.0 *(53)*. Thus, due to the conditions of sausage, it is very

probable that this microorganism was also present in the samples of sausages investigated. The counts of yeast and mold were around 4 $\log_{10}$ CFU/g throughout storage. However, higher counts have been found in these foods. In a study conducted on Greek sausages, mold and yeast were identified as the main cause of spoilage, with counts at around 7 $\log_{10}$ CFU/g *(58)*. Georgantelis et al. *(23)* found counts mold and yeast of 4.9 on the first day of storage and 7.0 $\log_{10}$ CFU/g on the 15th day. Yeasts are present in fresh and processed foods, but are not normally considered important in the deterioration since they constitute a small portion of the microbiota of food. On the other hand, mold is most evident in poultry products, especially near the end of the shelf life of these products *(34)*.

Salmonella, CoPS, and srC were not detected in the samples of chicken sausage. Although many consumers commonly associate *Salmonella* with poultry products, the rates of detection of this microorganism in this type of food are generally low *(45)*. CoPS products are common in highly manipulated food, so their absence may be associated with high microbial load present in the food, whereas this microorganism is usually a weak competitor *(22)*. The goal of detecting srC was to indicate the presence of *Clostridium perfringens*. This organism is common in meat products as a result of the presence of the bacteria in the gut contents of animals and humans, and its wide distributed in soil. The incidence of srC in chicken carcasses, whether as vegetative and/or spore, is around 58% *(41)*, which contradicts the result obtained in our study (i.e. its absence in the samples as a function of storage time).

There was a decrease in log MPN/g for TC in the T_2 and T_3 conditions (addition of 0.03 and 0.3% EO, respectively). An important aspect was that both lower concentration as the highest concentration of EO was found to inhibit the proliferation of TC since the first day of storage and this potential inhibitor was kept until the 15th day of storage.

An important result was that for the T_5 and T_6 treatments, which had the concomitant use of SHMP and EO, the antimicrobial effect of EO seems to have been lost. Thus, this is a strong indicator that the interaction between these two components in the sausage was negative. Overall, there was significant contamination by TC in the samples despite sanitation of the utensils and equipment, and good manufacturing practices being used during the manufacture of the sausages. Our data corroborate the findings of other studies conducted in Brazil, where coliform contamination in fresh sausages is often above the acceptable limits for consumption established by the National Agency for Sanitary Vigilance. Cortez et al. *(17)* reported that, in a total of 106 samples obtained in a municipality in the state of São Paulo (Brazil), 14.2% of the samples of fresh sausage collected did not meet required standards. Of the 106 samples analyzed, 73.6% had TC and, of these, only 38.7% were positive for

Escherichia coli (17). In another Brazilian state, of a total of 56 fresh sausage samples analyzed, 23.21% had values above 5.10^3 MPN/g of TC and 35.7% were positive for *E. coli (62)*. For our treatment conditions, there was a significant increase in TC on the 15th day of storage in the control sausage (T_1) and sausage added with EO 0.3% plus SHMP 0.5% (T_6). Competition for nutrients by psychrotrophic, aerobic, and Gram-negative bacteria affects the growth and survival of *E. coli*. By monitoring the variation in the population of *E. coli* in minced meat for thirty days at 4 °C, Guo et al. *(26)* found that the microorganism count was reduced. Thus, interesting results were obtained and further work will be necessary to elucidate the mechanism of antagonism between EO and SHMP, especially for the TC count.

As mentioned previously, the microbiota of the final product is the result of manipulation, contamination of the ingredients added, the casing, and also the initial contamination of the chicken meat. The main sources of carcass microorganisms include: animal skin and feathers; intestinal contents; abattoir environment; the utensils used; and handlers *(45)*. In fresh chicken breasts packed with common packing (without modified atmosphere) at 4 °C, we found an initial total count of 5.14 $\log_{10}$ CFU/g of viable microorganisms, reaching above 8 $\log_{10}$ CFU/g after 4-5 days storage *(35)*. Analyzing the contamination of meat chicken breast (same used for the manufacture of sausage) revealed that it had an average of 5.41 $\log_{10}$ CFU/g psychrotrophic, 4.76 $\log_{10}$ CFU/g mesophilic, and 3.34 $\log_{10}$ CFU/g of yeast and mold. The ground chicken was negative for the presence of CoPS, srC, and *Salmonella*, and showed <1 $\log_{10}$ MPN/g of TC. In this case the handling and the non-meat ingredients added to the final product (sausage) contamination 2 to 3 log10 CFU/g for mesophilic and psychrotrophic and at least one $\log_{10}$ CFU/g for molds and yeasts. For TC the increase in the count was higher (≅4 log), this contamination probably originated from natural swine casings.

Physicochemical analyses. The average pH values obtained during the storage time of the sausage are shown in Fig. 3.

There was an increase in pH during storage for the conditions that contained SHMP (T_4, T_5, and T_6). The results showed that the T_4, T_5, and T_6 samples had increased pH on days 7 and 15 of storage. In meat suitable for consumption, the pH value is around 5.8 to 6.2 *(10)*. Polyphosphate enables an increase of pH to about 0.1 to 0.4 per unit, causing the protein to become more negatively charged, thus increasing the repulsive force between the proteins inside the myofilaments, which consequently causes swelling and the retention of water *(59)*. This property of alkaline phosphates allows increased yield and improves the texture of the product. The increase in pH during the shelf life of the product is generally associated with an increase in microbial load, but in this case, the determining factor for the increase in pH was

the addition of the stabilizer, which increased the pH, thus improving the water retention of the meat. This effect was not observed in the T_1, T_2, and T_3 samples.

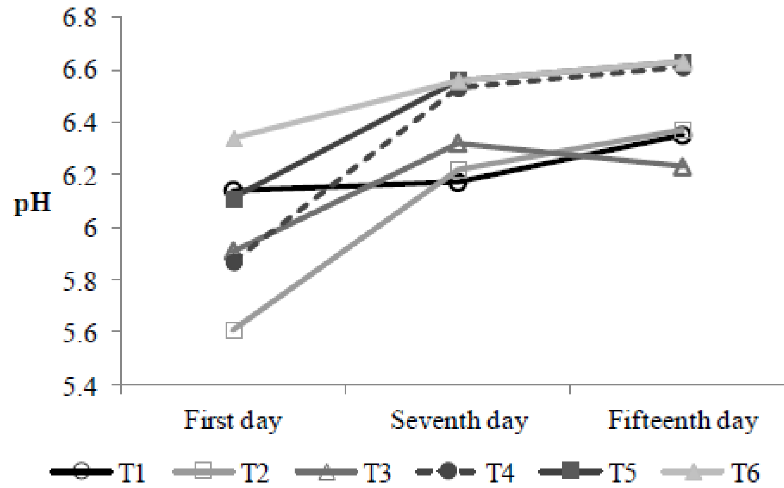


Fig. 3. pH values for samples of chicken sausage observed during storage at 4 °C.

Table 3 shows the mean and standard deviation for the color (L*, a*, and b*) and the malondialdehyde concentrations found in the sausage samples at days 1, 7, and 15 of storage. There were no treatment effects on the evaluated parameters.

Table 3. Effect of *O. basilicum* EO and polyphosphate on color and lipid oxidation[1] of fresh chicken sausage (mean and standard deviation) during cold storage (4 °C).

		T_1	T_2	T_3	T_4	T_5	T_6
first day	L*	73.8±2.1	73.8±2.9	74.3±2.9	74.3±1.4	75.5±4.4	78.2±4.0
	a*	7.6±0.6	7.4±0.8	7.7±0.5	8.5±0.7	8.2±0.3	8.4±0.4
	b*	18.1±0.4	18.3±0.7	18.8±0.8	18.2±0.3	18.5±0.7	18.9±0.7
	TBA	0.45±0.03	0.44±0.02	0.44±0.03	0.44±0.02	0.48±0.08	0.43±0.02
seventh day	L*	74.6±2.0	74.4±1.6	75.3±1.9	75.0±3.4	73.9±2.6	75.1±3.3
	a*	7.6±0.7	7.1±0.3	6.9±0.6	7.7±0.5	7.8±0.4	7.9±0.4
	b*	17.9±0.6	17.2±0.3	17.6±0.6	17.6±0.5	17.4±0.8	17.9±0.6
	TBA	0.52±0.04	0.49±0.01	0.47±0.02	0.49±0.01	0.47±0.01	0.47±0.01
fifteenth day	L*	78.5±2.1	76.9±2.7	76.8±4.1	76.3±2.0	79.5±2.1	75.3±3.7
	a*	6.9±0.7	7.5±0.5	7.2±0.7	8.1±1.0	7.7±0.4	7.9±0.7
	b*	17.0±0.6	17.7±0.8	17.6±0.5	18.5±0.6	18.2±0.7	17.9±0.6
	TBA	0.48±0.01	0.45±0.03	0.44±0.01	0.49±0.02	0.45±0.02	0.45±0.02

L *: lightness, a *: redness, and b *: yellowness. 1 Lipid oxidation expressed as MDA equivalents (mg malonaldehyde/kg).

There were significant differences in the overall mean values of lipid oxidation in the sausage samples as a function of storage time (P=0.0001). The TBA values were 0.45±0.04, 0.48±0.03, and 0.46±0.03 mg/MDA/kg for days 1, 7, and 15, respectively.

There were no differences in the color parameters (L*, a* and b*) among the treatment conditions. Qiao et al. *(57)* found 62.07 for L* of ground chicken meat. The differences in the correlations between the color of the intact and the ground fillet indicated that the process of grinding substantially changed the light reflective properties of the meat. Millar et al. *(47)* analyzed the effects of ionizing radiation on the color of chicken and found that the control samples of chicken breast showed a lightness value of 62.24 on the seventh day of storage. For the T_1 sample, on the seventh day which the value of 74.6 for L * e.g. could be attributed to the addition of lard in manufacturing the probable cause of the lighter color of sausage. The lightness of the treatments increased with storage time (P=0.01).

It is well known that chicken meat contains a high content of unsaturated fatty acids when compared to other meats, which leads to a more rapid oxidation and facilitates the development of oxidized flavor *(52)*. The TBA value was not altered by the treatments, although the *O. basilicum (15, 29, 33)* and polyphosphate *(60,70)* have already been reported as having antioxidant activities.

Our results revealed the difference in the amounts of lipid oxidation only related to the storage time. There was an increase from the 1st to 7th day and decrease from 7th to 15th day, although the values were very close. Georgantelis et al. *(23)* evaluated the effect of natural antioxidants and reported a decrease in the amounts of malondialdehyde after the 15th day of storage. According to these authors, the decline may be due to MDA decomposition by bacteria that are able to use this compound (e.g., *Pseudomonas*) or by further oxidation of MDA in others organic product not determined by the reaction with thiobarbituric acid *(6, 23)*. According to Greene and Cumuze *(25)*, the minimum level detectable for oxidized flavor in ground beef by untrained tasters ranged from 0.6 to 2.0 mg/kg, while 1 mg MDA/kg is considered the acceptable limit for rancidity in fresh meat *(55)*. Given these criteria, it can be inferred that the samples in this study remained stable during storage until the fifteenth day, and would not be considered rancid. Due to the short shelf life of sausage, it is likely that 15 days was not enough time for higher levels of oxidation to be detected. It is possible that a prolonged study period would enable the differentiation between treatments.

The cook loss percentage and compressive force for the samples of cooked sausage (80°C for 60 minutes) are shown in Table 4. The results also varied according to the addition of

polyphosphate. From these data, we calculated the Pearson correlation coefficient between the two variables (Fig. 4), which revealed a negative correlation median (-0.555, P=0.001).

The ability of polyphosphate to improve the yield and consistency of meat products is well known, and polyphosphates and sodium chloride act synergistically to improve moisture absorption and water-binding capacity, and reduce cook and purge loss *(72)*. The results of this study corroborate previous reports, showing an improvement in the sample yield with the addition of polyphosphate and reduced water loss (P=0.0001). The average yield in the sample ranged from 87.86 (T_3) to 91.63% (T_5). Comparing samples in which the only difference was the addition of SHMP, differences in cook loss were 2.3, 2.8, and 2.6% for T_1-T_4, T_2-T_5, and T_3-T_6, respectively. The addition of 0.3% or 0.03% of *O. basilicum* EO in the sausage did not affect the yields.

Table 4. Cook loss (%) and compressive force (g) of the samples of chicken sausage.

	T_1	T_2	T_3	T_4	T_5	T_6
Cook loss	11.44 [a]	11.14 [a]	12.14 [a]	9.11 [b]	8.37 [b]	9.53 [b]
Compressive force	7238.8 [c]	7317.1 [bc]	7134.2 [c]	8362.0 [a]	8929.0 [a]	8330.3 [ab]

Treatments followed by at least one letter in common are not different. a: higher, and c: smaller.

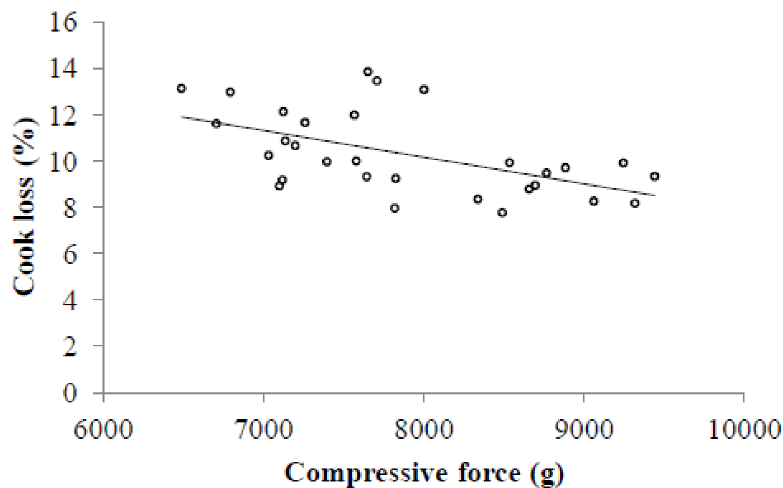


Fig. 4. Correlation between cook loss and compressive force of the samples of chicken sausage.

Polyphosphate also improved the compressive force of the chicken sausage, showing an average value of 8540 g for the group with SHMP, and 7.230 g for the group without SHMP. Despite the evident separation between the two groups, the T_2 and T_6 samples were equal to

each other, indicating that 0.03% of EO had the same impact on the compressive force as 0.5% SHMP plus 0.3% of EO. Since there are still few reports that address this interaction (EO plus SHMP), the elucidation of this fact should be further evaluated in future research.

According to correlation shown in the Fig. 4, the percent reduction of cook loss was concomitant with an increase in the compressive force. The water available in the product acts as a plasticizer, and the relationship between water loss and hardness is a positive one *(4)*. Thus, we concluded that the stabilizer provided an improvement in compressive force, while at the same time increasing water retention and juiciness in the final product.

Phosphates change the distribution of ionic charges. Therefore, the addition of phosphate increases the ionic power of the meat, leading to electrostatic repulsion and, thus, the expansion of the muscle fiber. This allows more water to be mobilized in myofibrillar reticles, providing increased moisture capacity *(43, 56, 72)*. The water acts together with salt and phosphate to solubilize the muscle proteins and creates a strong protein structure that maintains the stability of the product even after heat treatment *(20)*. Therefore, phosphates improve the texture attributes of the product due to increased binding of the meat particles *(72)*.

In conclusion, we observed that close attention should be given to the concomitant use of synthetic and natural preservatives, since the effects can be unexpected or undesirable. The meat "free additive processing" in real market conditions is not completely viable because a number of additives are needed to ensure food safety *(68)*. Furthermore, despite the various properties of *O. basilicum* EO, some obstacles remain, such as overcoming the strong odor and flavor, and there is currently little information about its effectiveness in foods, which limits its application. However, they can be used in combination with other food preservatives, which may make their use possible. However, it is important that occurs research on the association of traditional additives derived from plants to assess the effects on parameters of safety and quality of food.

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APÊNDICES

Apêndice I- Parecer do Comitê de Ética em Pesquisa

Universidade Estadual Paulista
Faculdade de Medicina de Botucatu

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Fone/Fax: (0xx14) 3811-6143
e-mail secretaria: capellup@fmb.unesp.br
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Registrado no Ministério da Saúde
em 30 de abril de 1997

Botucatu, 01 de fevereiro de 2.010 OF. 013/2010-CEP

Ilustríssimo Senhor
Prof. Dr. Ary Fernandes Junior
Departamento de Microbiologia e Imunologia do Instituto de Biociências do Campus de Botucatu

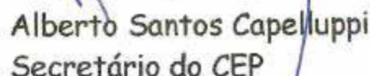
Prezado Prof. Ary,

De ordem do Senhor Coordenador deste CEP, informo que Projeto de Pesquisa, **(Protocolo CEP 3423-2010)** Derivados Vegetais de condimentos: atividade antimicrobiana e influência na vida de prateleira de lingüiça de frango artesanal, que será conduzido por Lidiane Nunes Barbosa, orientada por Vossa Senhoria, recebeu do relator parecer favorável **com sugestão**, aprovado em reunião de 01 de fevereiro de 2.010.

Obs: O CEP sugere ao pesquisador que tome cuidado com a hierarquia na escolha da amostra.

Situação do Projeto: APROVADO. Ao final da execução deste Projeto, apresentar ao CEP **"Relatório Final de Atividades"**.

Atenciosamente,



Alberto Santos Capelluppi
Secretário do CEP

Apêndice II- Termo de consentimento livre e esclarecido.

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

Departamento de Microbiologia e Imunologia

Termo de Consentimento Livre e Esclarecido

Você está sendo convidado a participar do estudo "Derivados vegetais de condimentos: atividade antimicrobiana e influencia na vida de prateleira de linguiça de frango artesanal". O objetivo é utilizar plantas condimentares como forma de melhorar as características de linguiça de frango através de sua atividade antimicrobiana bem como aumentar o tempo de validade do alimento. Portanto, neste momento você esta recebendo amostras de linguiça de frango processadas com adição de óleo essencial de plantas condimentares com finalidade de verificar se as características sensoriais, especialmente o sabor, deste alimento sofreram modificações que possam provocar algum tipo de rejeição do mesmo. Salientamos que este alimento não recebeu adição de microrganismos, não tendo qualquer risco para a saúde humana. Ficou esclarecido que a minha participação é voluntária e que tenho liberdade para me retirar da pesquisa em qualquer momento. Com assinatura abaixo, dou consentimento para a incorporação dos meus dados neste estudo.

Atenciosamente

______________________	______________________
Provador	Pesquisador

Qualquer dúvida adicional, você poderá entrar em contato com o Comitê de Ética em Pesquisa, através do fone: (14) 3880 1608

Ary Fernandes Junior
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ary@ibb.unesp.br

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Av. Camilo Mazoni, 1000 apto 03,
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linuba2@yahoo.com.br

Apêndice III- Ficha utilizada nos testes de aceitação.

Escala hedônica (estruturada verbal, numérica, bipolar, nove pontos).

Ficha de avaliação

TESTE DE ACEITAÇÂO

Nome:________________________________ Data:_____/_____/______

Idade:____________ Sexo: Feminino ( ) Masculino ( )

Por favor, avaliar as amostras de linguiça utilizando a escala abaixo para descrever o quanto você gostou ou desgostou da aparência e/ou sabor das preparações.

(9) Gostei extremamente
(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 extremamente

713 ________ 294 ________
925 ________ 675 ________
438 ________ 386 ________

Observações:____
____

Apêndice IV- Protocolo para realização da curva padrão

Material necessário:

-Solução 1:Tetraetoxipropano (TEP) 0,1mL em 100 mL de água

-Solução 2: 0,1mL da solução 1 e diluir para 100mL com TCA 7,5%

Procedimento:

-Adicionar em tubos de ensaio com tampa rosqueada: 1, 2, 3, 4, 5 ml de TEP

-Completar os tubos 4, 3, 2, 1 e 0,75mL de ácido tricloroacético

-Adicionar 5mL de TBA (ácido tiobarbitúrico 0,02M), agitar

-Aquecer em banho Maria por 10 minutos

-Esfriar em banho de gelo

-Ler em espectrofotômetro a 532nm

-Fazer duplicata/triplicata

Apêndice V- Curva padrão do malonaldeído.

Apêndice VI- Tabela de Número Mais Provável (Man, 1983).

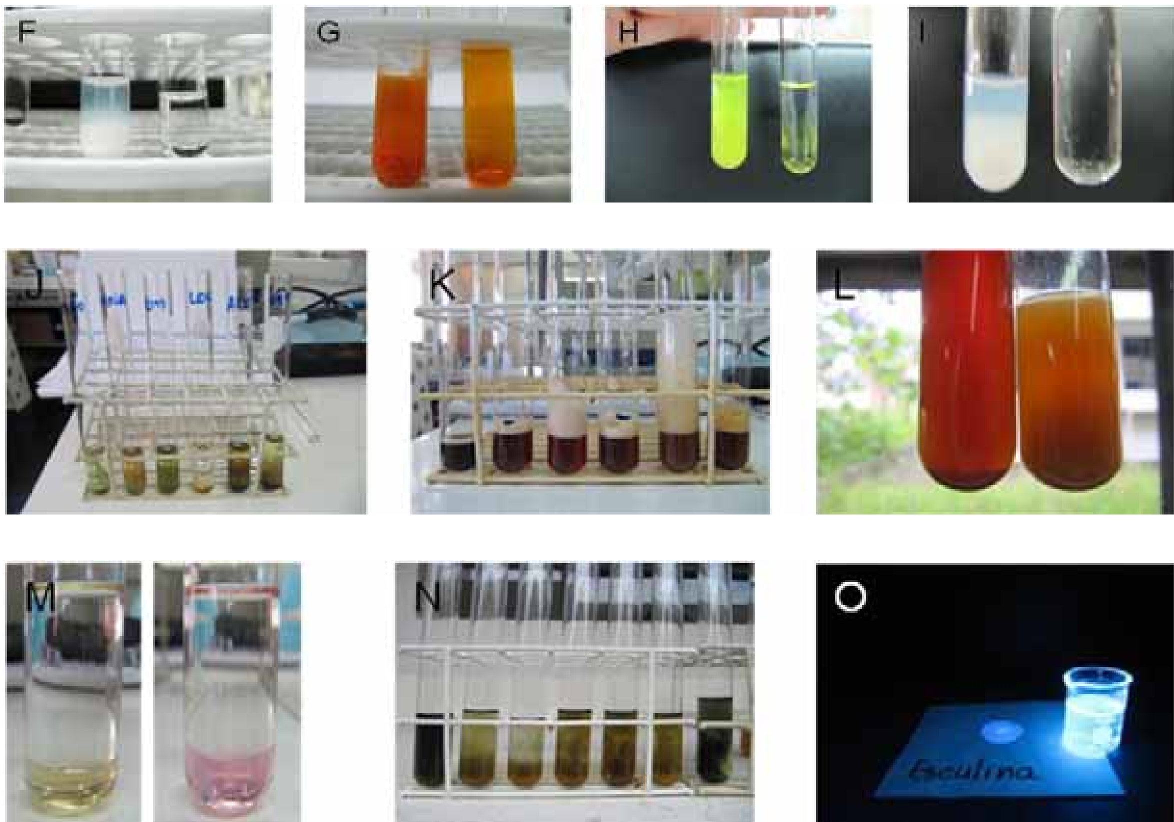
Número de tubos positivos			NMP/g	Número de tubos positivos			NMP/g
10^{-1}ml	10^{-2}ml	10^{-3}ml		10^{-1}ml	10^{-2}ml	10^{-3}ml	
0	0	0	< 3	2	0	0	9,1
0	0	1	3	2	0	1	14
0	0	2	6	2	0	2	20
0	0	3	9	2	0	3	26
0	1	0	3	2	1	0	15
0	1	1	6,1	2	1	1	20
0	1	2	9,2	2	1	2	27
0	1	3	12	2	1	3	34
0	2	0	6,2	2	2	0	21
0	2	1	9,3	2	2	1	28
0	2	2	12	2	2	2	35
0	2	3	16	2	2	3	42
0	3	0	9,4	2	3	0	29
0	3	1	13	2	3	1	36
0	3	2	16	2	3	2	44
0	3	3	19	2	3	3	53
1	0	0	3,6	3	0	0	23
1	0	1	7,2	3	0	1	39
1	0	2	11	3	0	2	64
1	0	3	15	3	0	3	95
1	1	0	7,3	3	1	0	43
1	1	1	11	3	1	1	75
1	1	2	15	3	1	2	120
1	1	3	19	3	1	3	160
1	2	0	11	3	2	0	93
1	2	1	15	3	2	1	150
1	2	2	20	3	2	2	210
1	2	3	24	3	2	3	290
1	3	0	16	3	3	0	240
1	3	1	20	3	3	1	460
1	3	2	24	3	3	2	1.100
1	3	3	29	3	3	3	>2.400

ApêndiceVII- Visão geral dos experimentos: 1ª Etapa

Obtenção dos derivados de plantas e microdiluição.

A) Seleção do material vegetal; **B)**Filtração do Eet; **C)** Obtenção do OE por destilação por arraste a vapor; **D)** Aplicação de resazurina e **E)**Microplaca com resazurina após incubação (37°C/ 3 minutos). *Violeta: ausência de crescimento; Rosa: presença de crescimento.

Análise fitoquímica qualitativa dos Eet.



Determinação de alcaloides utilizando quatro reagentes: **F)** Bertrand; **G)** Dragendorff; **H)** Hager e **I)** Mayer. Lado direito- negativo ; lado esquerdo-positivo.

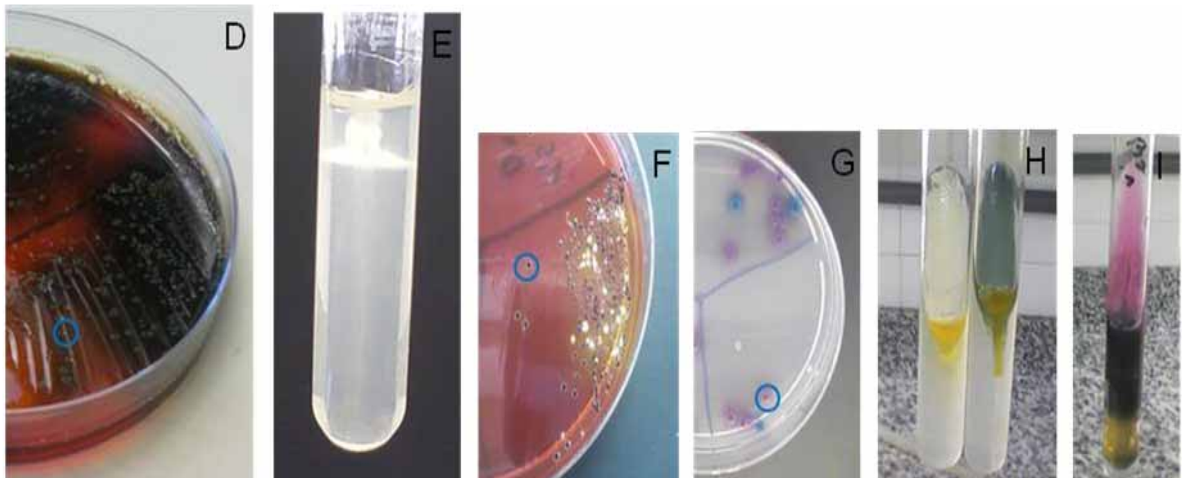
J) Triterpenos e esteroides; **K)** Saponinas (observação de espuma persistente); **L)** Taninos Lado direito-negativo; lado esquerdo-positivo; **M)** Quinonas (amarelo-negativo; rosa-positivo); **N)** Fenóis (todos positivos) e **O)** Flavonóides (positivo- observação sobre UV 360).

Apêndice VIII- Visão geral dos experimentos: 2ª Etapa

Produção e armazenamento das amostras de linguiça de frango.

A) Preparo da linguiça no laboratório de Nutrição e Dietética (teste com tripa de carneiro); **B)**Linguiça de frango fresca e **C)**Amostras armazenadas em temperatura controlada (4°C).

Isolamento e confirmação das colônias características de *Listeria* e *Salmonella* após recuperação pelo método do NMP.



D e **E)** Aspectos das colônias características de *Listeria monocytogenes* em agar Palcam e motilidade "em guarda chuva"; **F e G)** colônias características de *Salmonella* nas placas de XLD e CRHOmagar *Salmonella;* **H)** testes em ágar fenilalanina (verde-teste positivo)e I) ágar TSI.

Apêndice IX- Visão geral dos experimentos: 3ª Etapa

Análises físico-químicas

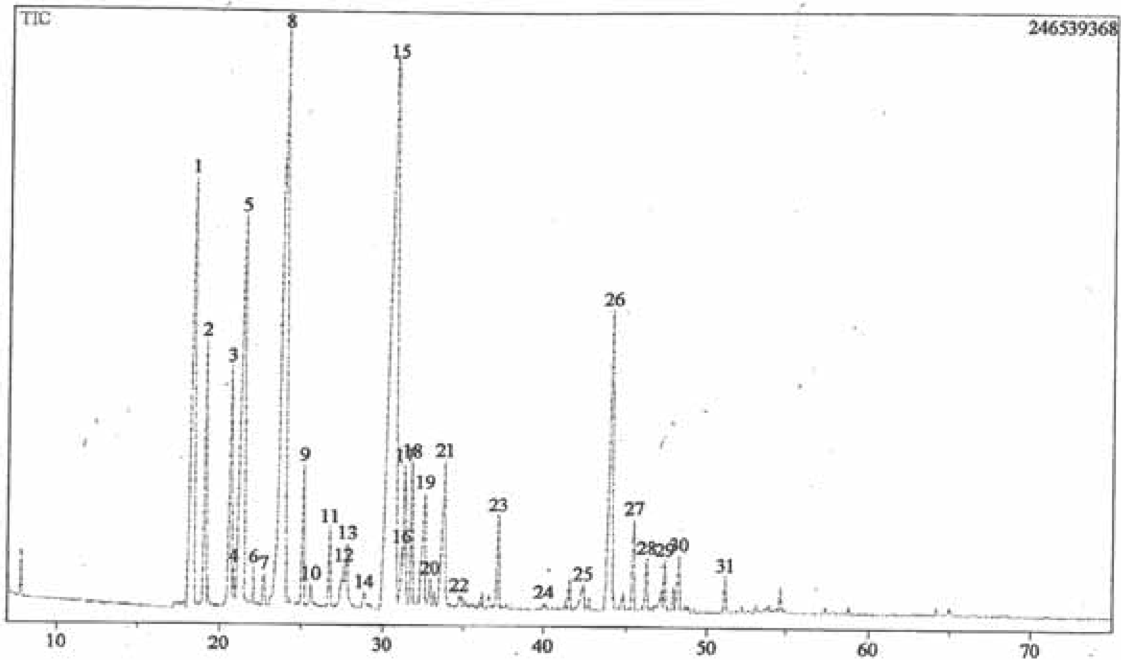
A) TBA:extrato ácido-aquoso; **B**)Trituração das amostras; **C**)Medição de cor; **D)** Embalagem à vácuo e **E**)Banho-maria.

F) Aferição de temperatura; **G**)Preparo das fatias de 2cm; **H**)Texturômetro e **I**)Análise de compressão.

Análise de Aceitabilidade

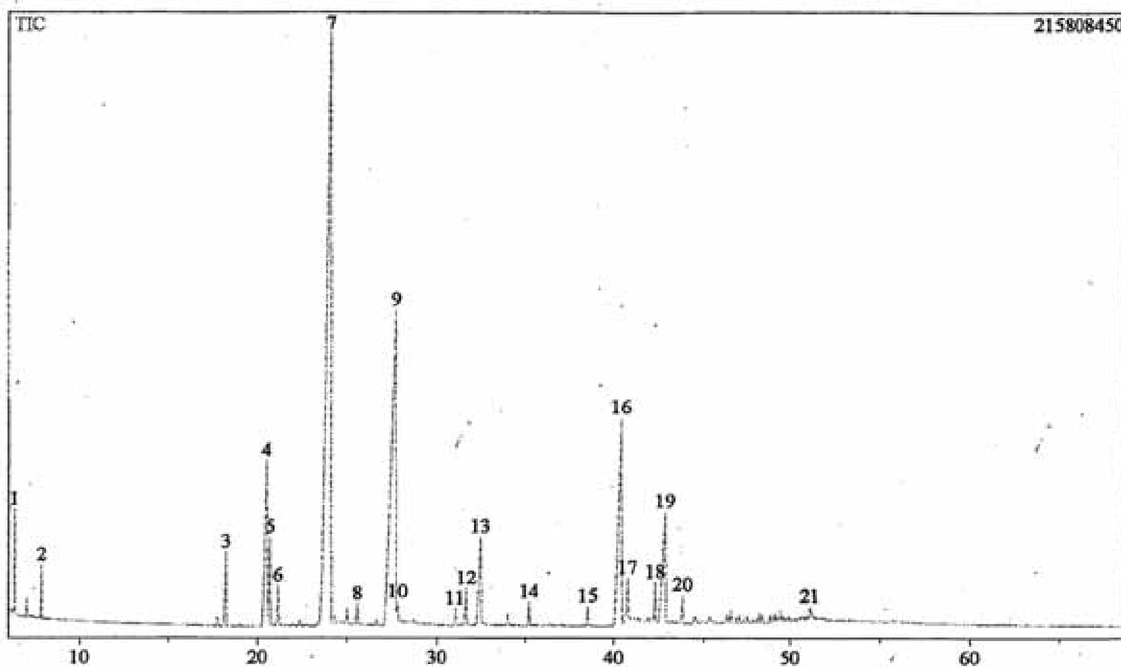
J)Parte da equipe; **K**)Laboratório de Nutrição e Dietética; **L**)Preparo do laboratório e **M**)Linguiça assada.

Apêndice X- Cromatograma do OE de *Rosmarinus officinalis*.



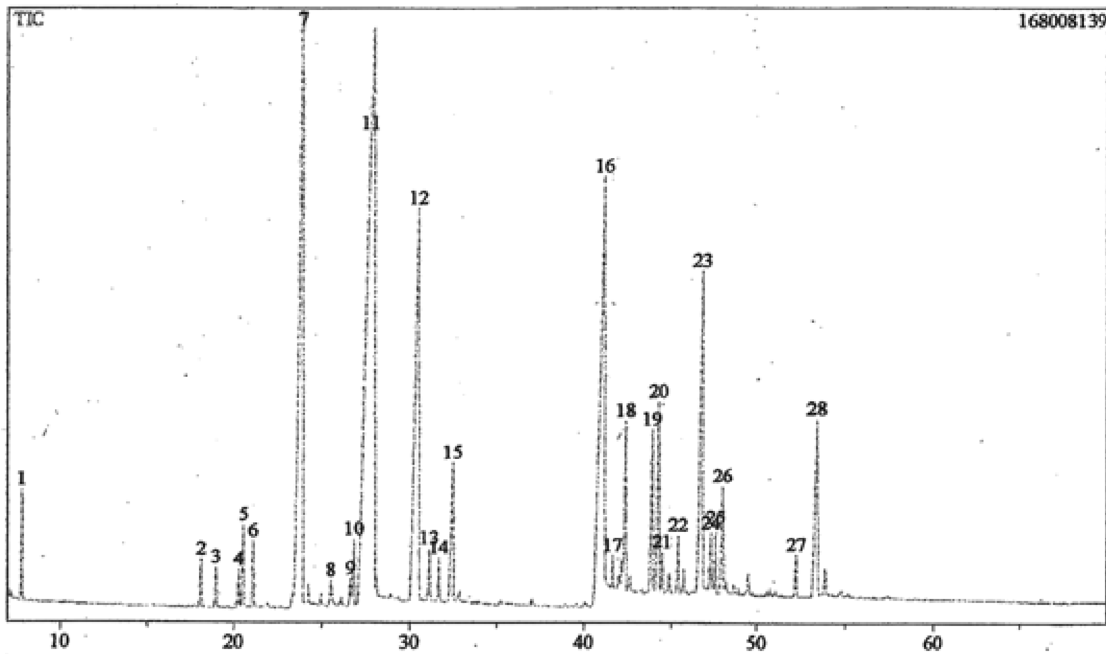
15- Cânfora 22,51%, **8-** 1,8-cineol 15,95%, **5**- mirceno 10,56%, **1-** α-pineno 9,11%, **26-** β-cariofileno 6,91%, **3-** β-pineno 4,38%, **2-** canfeno 3,98%, **17-** borneol 2,24%), **9-** γ-terpinoleno 2,18%, **23-** acetato de bornila 1,14%, **13-** linalool 0,86%, **6-** α-felandreno 0,58%.

Apêndice XI- Cromatograma do OE de *Laurus nobilis* (louro).



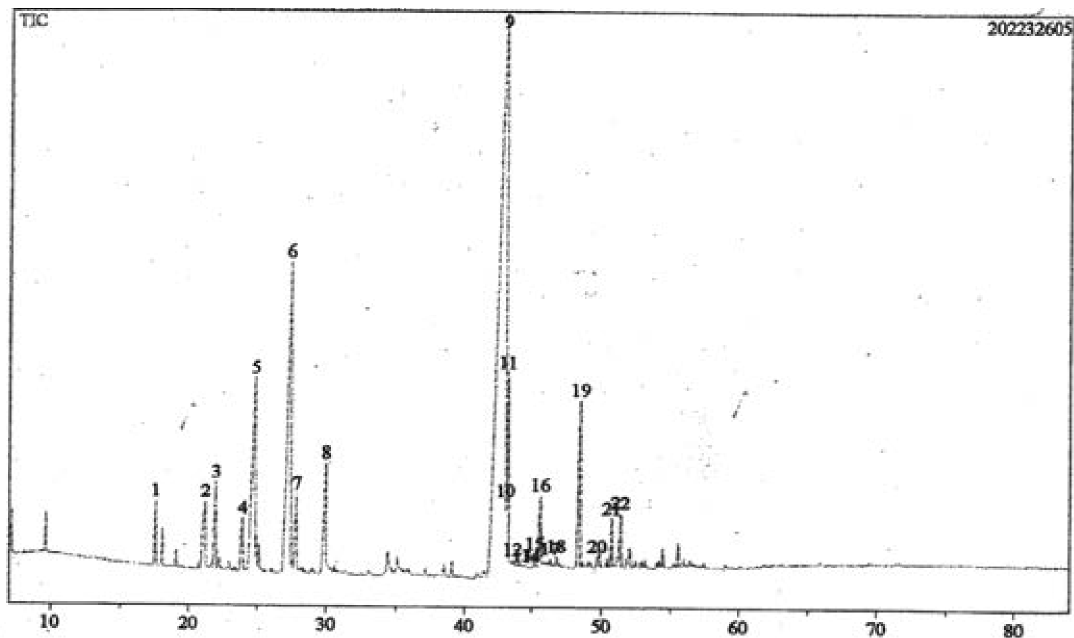
7- Eucaliptol (1,8-cineol) 37,4%, **9-** linalol 25,29%, **16-** α-terpenil acetato 10,56%, **4-** β-pineno 6,39%, **19-** o-metil eugenol 5,37%, **13-** α- terpineol 3,58%, **5-** β-mirceno 1,94%, α-pineno 1,56%, **17-** eugenol 0,94%, **12-** terpinen-4-ol 0,84%, **20-** β-cariofileno 0,63%, **8-** β-ocimeno 0,42%, **10-** γ-terpineno 0,28%.

Apêndice XII- Cromatograma do OE de *Ocimum basilicum* (manjericão).



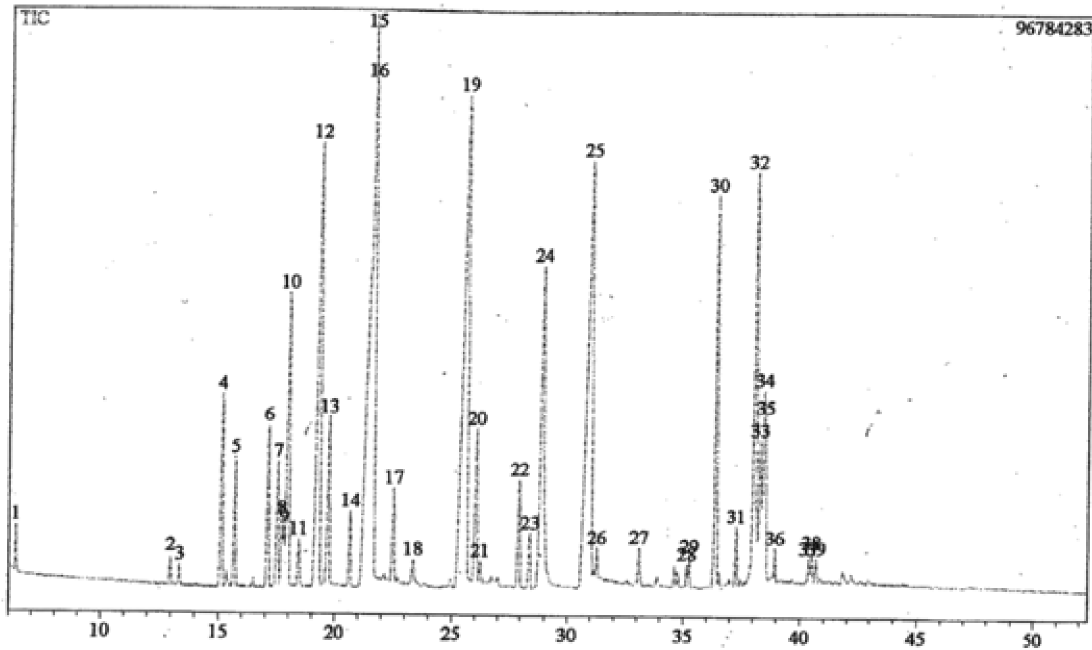
11- Linalol 31,52%, **16-** eugenol 14,39%, **7-** eucaliptol 14,06%, **12-** cânfora 11,31%, **23-** germacreno 6,64%, **19-** β- cariofileno 2,13%, **5-** mirceno 0,73%, **2-** α-pineno 0,36%, **3-** β-pineno 0,31%.

Apêndice XIII- Cromatograma do OE de *Origanum vulgare* (orégano).



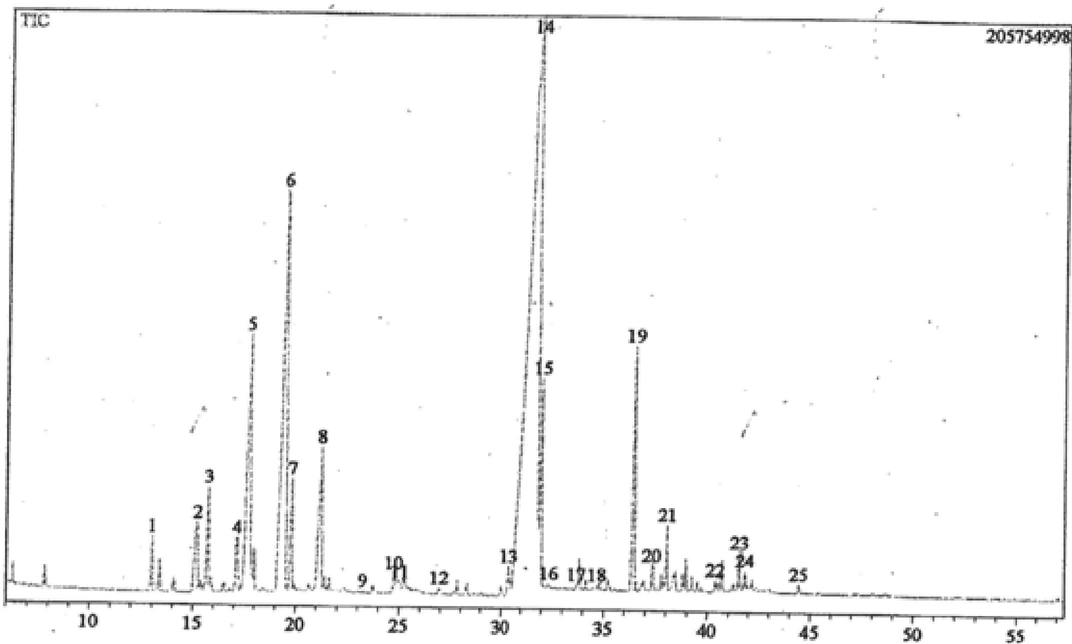
9- Timol 48,62%, **4-** γ-terpineno 12,98%, **5-** ρ-cimeno 7,48%, **19-** β-cariofileno 5,31%, **8-** linalol 3,02%, **3-** mirceno 1,6%, **4-** α- terpineno 1,28%.

Apêndice XIV- Cromatograma do OE de *Origanum majorana* (manjerona).



15- Linalol 16,95%, **19-** 3-ciclohexen-1-ol 4metil-1-(isopropil)13,51%, **25-** timol 13,45%, **12-** γ-terpineno 8,22%, **24-** acetato de linanoíla 6,62%, **32-** germacreno D 6,53%, **30-** β-cariofileno 4,59%, **10-** ocimeno 3,94%, **34-** germacreno B 2,51%, **4-** β-pineno 1,94%, **6-** α-terpineno 1,79%, **7-** ρ-cimeno 1,51%, **5-** β-mirceno 1,18%, **8-** limoneno 0,74%, **3-** α-pineno 0,15%.

Apêndice XV- Cromatograma do OE de *Thymus vulgaris* (tomilho).



14- Timol 49,09%, **6-** γ-terpineno 14,81%, **5-** ρ-cimeno 7,3%, **19-** β-cariofileno 4,53%, **8-** linalol 3,62%, **4-** α-terpineno 1,21%, **21-** germacreno 1,04%, **1-** β-pineno 0,58%, **20-** α-cariofileno 0,54%.