

**UNIVERSIDADE ESTADUAL PAULISTA - UNESP
CÂMPUS DE JABOTICABAL**

**USO DE ÓLEO ESSENCIAL DE TOMILHO SOBRE
FERMENTAÇÃO RUMINAL, RESPOSTA IMUNOLÓGICA,
PRODUÇÃO E QUALIDADE DO LEITE**

Edivilson Silva Castro Filho
Engenheiro Agrônomo

2020

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QUALIDADE DO LEITE**

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*“A sabedoria da vida não está em fazer aquilo de
que se gosta, mas gostar daquilo que se faz.”*

Dedico este trabalho aos meus pais, por todo o amor e dedicação para comigo, por terem sido a peça fundamental para que eu tenha me tornado a pessoa que hoje sou.

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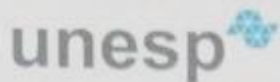
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CERTIFICADO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS

UNIVERSIDADE ESTADUAL PAULISTA
"JULIO DE MESQUITA FILHO"
Câmpus de Jaboticabal

**CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS****CERTIFICADO**

Certificamos que o Protocolo nº 4093/15 do trabalho de pesquisa intitulado "Suplementação de Óleo essencial de Tomilho (*Thymus vulgaris*) na fermentação ruminal, resposta imunológica, produção e qualidade do leite", sob a responsabilidade da Profª Drª Jane Maria Bertocco Ezequiel está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 01 de abril de 2015.

Jaboticabal, 01 de abril de 2015.

Prof.ª Dr.ª Paola Castro Moraes
Coordenadora – CEUA

SUPLEMENTAÇÃO DE ÓLEO ESSENCIAL DE TOMILHO SOBRE FERMENTAÇÃO RUMINAL, RESPOSTA IMUNOLÓGICA, PRODUÇÃO E QUALIDADE DO LEITE

RESUMO - A redução de células somáticas é o maior desafio para a melhoria da qualidade do leite, pois está relacionada com a ocorrência de mastite e conseqüentemente ao uso de antibióticos para seu controle, e prejuízos para a cadeia agroindustrial do leite. A busca por alternativas ao uso de antibióticos vem incentivando pesquisas na área de plantas medicinais. Diante disso, o presente estudo teve como objetivo avaliar o efeito do uso de óleo essencial de tomilho (TEO) sobre a fermentação ruminal de bovinos, resposta imunológica, produção e qualidade do leite ao longo de 2 experimentos. No primeiro experimento, para avaliar o nível de adição de TEO, por meio de parâmetros de fermentação e metabolismo ruminal, foram utilizados 4 bovinos Nelore (700 ± 44 kg PV), castrados, e canulados no rumen, distribuídos em delineamento quadrado latino 4×4 , sendo 4 tratamentos experimentais e 4 períodos. Os tratamentos consistiram em: CON = controle, sem TEO; T2 = 2 mL / d de TEO; T4 = 4 mL / d de TEO; e T8 = 8 mL / d de TEO. O TEO teve como principal composto o timol, com 423 g/kg de timol na MS (matéria seca). Os animais foram alojados em baias semicobertas individuais (9 m^2) com cochos e bebedouros individuais. Cada período experimental durou 21 dias (14 dias de adaptação e 7 dias de coleta de dados). Já no segundo, foram utilizadas 24 vacas da raça Jersey, com peso médio de 400 kg, em segunda lactação, 50 dias de lactação, com idade média de 36 meses e média de produção de leite de 22,05 kg/dia. Os animais foram submetidos a dois tratamentos: 0 mL/dia de TEO (CON) e 8 mL/dia de TEO (TEO; 423 g/kg de timol na MS), em delineamento inteiramente casualizado e alojados em baias individuais. No primeiro experimento o consumo e a digestibilidade da MS não foram afetadas pela utilização de TEO (média de 8,6 kg e 73,5%, respectivamente). Entre as variáveis do comportamento ingestivo, apenas o PP (parado em pé) diferiu com a inclusão da TEO (Linear, $P = 0,03$), onde os maiores valores foram observados nas maiores doses de TEO. A produção total de gás (mL/g MS) foi afetada quadraticamente pela adição de TEO ($P = 0,05$), com maiores volumes observados nos tratamentos CON e T8. Nenhum efeito do TEO foi observado nas concentrações de AGCC (ácidos graxos de cadeia curta), apenas tendência quadrática para N-NH_3 no rúmen com os maiores valores nos tratamentos com maior adição de TEO. A TEO também não afetou as variáveis sanguíneas avaliadas neste estudo. Já no experimento 2, o consumo de MS, a eficiência e a produção de leite não foram afetados pela suplementação de TEO ($P > 0,10$). Dentre os parâmetros qualitativos do leite, somente a Condutividade Elétrica (CE) tendeu a ser diminuída ($P = 0,07$), no tratamento de 8 mL ($5,12 \text{ mS/cm}$), observou-se também menor número de casos de mastite clínica e menor tempo de retorno a níveis aceitáveis de contagem de células somáticas após identificação e medicação no tratamento contendo TEO (2 casos e 4d vs 5 casos e 7d, respectivamente). Nos parâmetros sanguíneos, a ALT (alanina aminotransferase) tendeu a diminuir quando os animais foram suplementados com TEO ($P = 0,07$, redução de 12% em relação ao controle), a Interleucina 2 aumentou quando TEO foi utilizado ($P = 0,04$, aumento de 123% em comparação com o controle), enquanto a Interleucina 4 e a

Imunoglobulina G tenderam a aumentar ($P=0.07$, $P=0.09$, respectivamente) em função do tratamento com TEO, aumentando 128 e 5%, respectivamente. A suplementação com TEO também tendeu a aumentar os níveis de melatonina no leite ($P=0.08$), com maior valor para o TEO (63.94 contra 57,53 pg/mL do controle), além de aumentar significativamente os teores de timol no sangue e no leite ($P=0.02$ e $P<0.01$ respectivamente), com as maiores médias de 10.68 ng/mL no sangue e 30.03 ng/10 mL de leite, ambas para o TEO contra 6,76 ng/mL no sangue e 5,93 ng/10 mL de leite do controle. A infusão de até 8 mL / d de óleo essencial de tomilho não provoca efeitos nocivos sobre o ambiente ruminal e a saúde animal. No entanto, mais estudos devem ser realizados para investigar maiores dosagens de TEO em dietas para bovinos de corte. Já na suplementação de vacas da raça Jersey no terço inicial da lactação, 8 mL TEO/dia não prejudica o consumo e o desempenho produtivo, e tende a melhorar a resposta do sistema imune, auxiliando no controle da incidência de mastite e manutenção da saúde da glândula mamaria.

Palavras-chave: estabilidade ruminal, saúde ruminal, mastite, contagem de células somáticas, *Thymus vulgaris* L., timol, melatonina, produção orgânica

ESSENCIAL OIL OF THYME AS FEED SUPPLEMENTATION ON RUMINAL FERMENTATION, IMMUNE RESPONSE, MILK PRODUCTION AND QUALITY

ABSTRACT - The somatic cell count is the biggest challenge to improve milk quality, as it is related to the occurrence of mastitis and consequently to the use of antibiotics for its control, and losses to the agro-industrial milk chain. The search for alternatives to the use of antibiotics has been encouraging research in the area of medicinal plants. Therefore, the current study aimed to evaluate the effect of the inclusion of thyme essential oil (TEO) on the ruminal fermentation, immune response, milk production and quality in 2 experiments. In the first, four rumen-cannulated Nellore steers (700 ± 44 kg BW) were randomly distributed in a 4×4 Latin square design and received one of the experimental treatments: CON = control, without TEO; T2 = 2 mL/d TEO; T4 = 4 mL/d TEO; and T8 = 8 mL/d TEO. TEO's main compound was thymol, with 423 g/kg of thymol in DM. The animals were housed in individual semi-covered pens (9 m²) with individual feed bunks and waterers. Each experimental period lasted 21 days (14 d of adaptation and 7 d of data collection). In the second, 24 Jersey cows were used, with an average weight of 400 kg, second lactation, 50 d in milk, with approximately 36 months old and average milk production of 22.05 kg/d. The animals were subjected to two treatments: 0 mL/d TEO (CON) and 8 mL/d TEO (TEO; 423 g/kg thymol in DM), in a completely randomized design and housed in individual pens. In the first experiment, DM intake and digestibility were not affected by TEO addition (average of 8.6 kg and 73.5%, respectively). Among the variables of feeding behavior, only the SS (standing still) differed with the inclusion of TEO (Linear, $P = 0.03$), with higher values observed for higher doses of TEO. The total gas production (mL/g MS) was quadratically affected by the addition of TEO ($P = 0.05$), with higher volumes observed for CON and T8 treatments. No effect of TEO was observed on SCFA concentrations, but $\text{NH}_3\text{-N}$ in the rumen tended to change. TEO also did not affect blood variables (erythrogram, leukogram, metabolites and liver enzymes) evaluated in this study. In experiment 2, DM intake, efficiency and milk production were not affected by TEO supplementation ($P > 0.10$). Among the qualitative parameters of milk, only Electrical Conductivity (EC) tended to decrease ($P = 0.07$), in the treatment of 8 mL TEO (5.12 mS/cm). There was also a lower number of cases of clinical mastitis and shorter time to return to acceptable levels of somatic cell count after identification and medication in the treatment containing TEO (2 cases and 4 d vs 5 cases and 7 d, respectively). In blood parameters, ALT tended to decrease when animals were supplemented with TEO ($P = 0.07$, 12% reduction compared with control), Interleukin 2 increased when TEO was used ($P = 0.04$, 123% increase compared with control), while Interleukin 4 and Immunoglobulin G tended to increase ($P = 0.07$, $P = 0.09$, respectively) due to treatment with TEO, increasing 128 and 5%, respectively. Supplementation with TEO also tended to increase melatonin levels in milk ($P = 0.08$), with a higher value for TEO (63.94 versus 57.53 pg/mL for the control), in addition to significantly increasing the levels of thymol in the blood and in milk ($P = 0.02$ and $P < 0.01$ respectively), with the highest averages of 10.68 ng/mL in blood and 30.03 ng/10 mL of milk, both for TEO versus 6.76 ng/mL in blood and 5.93 ng/10 mL of control milk. The addition of up to 8 mL/d of thyme essential oil does not cause harmful effects on the rumen environment and to animal health.

However, further studies should be conducted to investigate higher doses of TEO in diets for beef cattle. In the supplementation of Jersey cows in the initial third of lactation, 8 mL TEO/d does not impair intake and productive performance, and tends to improve the immune system response, helping to control the incidence of mastitis and maintaining the health of the mammary gland.

Keywords: ruminal stability, ruminal health, mastitis, somatic cell count, *Thymus vulgaris* L., thymol, melatonin, organic production

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CAPÍTULO 1 - Considerações gerais

INTRODUÇÃO

A produção mundial de leite em 2019 foi de aproximadamente 852 milhões de toneladas, das quais, aproximadamente 4,1% foram produzidas no Brasil, percentual que o colocou na sexta posição no ranking mundial de produção de leite segundo a Organização das Nações Unidas para Alimentação e Agricultura (FAO, 2020), ficando atrás da Índia, União Europeia, Estados Unidos, China e Paquistão.

No levantamento divulgado pelo IBGE referente a 2018, aproximadamente 24,5 milhões de litros da produção foram adquiridos por estabelecimentos industriais sob inspeção sanitária (municipal, estadual ou federal), cerca de 72,3 % da produção total, mantendo a tendência de aumento do leite fiscalizado desde 2007 (IBGE, 2017). Essa maior fiscalização demonstra a preocupação da produção animal, que está cada vez mais direcionado para a qualidade do produto final, sem desconectar da produtividade.

Desta forma, a busca por qualidade, seja da matéria-prima ou do produto final, é o principal fator que tem impulsionado a melhoria da qualidade do leite produzido no Brasil (Santos, 2004), mesmo que ainda seja incipiente em relação a outros países, nos quais a preocupação com a qualidade dos produtos de origem animal já existe e continua aumentando (Monardes, 2004).

Porém, além de qualidade e quantidade, outras preocupações como: a segurança alimentar, preservação do meio ambiente, bem-estar animal e viabilidade econômica, fazem parte do novo cenário produtivo. Associado às novas exigências de mercado, o controle de resíduos de antibiótico está cada vez mais restritivo para exportação de leite como matéria prima, por acarretar numa série de problemas no processamento e na saúde pública, contribuindo para a maior preocupação dos consumidores com a segurança dos alimentos.

Neste cenário, a busca por alternativas ao uso de antibióticos vem incentivando pesquisas com plantas medicinais e países como o Brasil mostram vantagem pela grande riqueza em biota existente. Porém para que seja considerada alternativa oficial ao uso do antibiótico e liberado para comercialização, este produto deve apresentar efeitos comprovados e isenção de toxicidade (ANVISA, 2011).

Pesquisas com uso de fitoterápicos na produção animal ainda são em pequeno número, porém este apresenta crescimento e demanda principalmente para as áreas de sanidade e desempenho.

Entre as alternativas o Tomilho (*Thymus vulgaris* L.) tem despertado interesse dos pesquisadores, em função de seu potencial antimicrobiano significativo (Kalembe et al., 2002; Burt, 2004). Entretanto, os estudos que avaliam sua utilização na nutrição animal são escassos e na bovinocultura leiteira suas propriedades ainda são desconhecidas.

Dessa forma, o presente estudo teve como objetivo avaliar o efeito do óleo essencial de tomilho sobre os parâmetros ruminais, bem como sobre o sistema imunológico, produção e qualidade do leite, e tentar avaliar uma dose que possa ser testada como alternativa no controle da mastite bovina.

REVISÃO DE LITERATURA

1. Qualidade do leite

A determinação de um leite com qualidade pode ser definida por: sua integridade, ou seja, livre da adição de substâncias e/ou remoção de componentes, na forma de fraude; sua composição química e características físicas; sua deterioração microbiológica e presença de patógenos (Dürr, 2004). Existem iniciativas governamentais visando padronizar e melhorar a qualidade do leite, como a implantação de normas nacionais de padrões de qualidade do leite, determinadas pelo Programa Nacional de Melhoria da Qualidade de Leite, do Ministério da Agricultura (Ribeiro et al., 2000) composto e alterado por diversas Instruções Normativas ao longo dos anos (51/2002; 22/2009; 62/2011; 07/2016 e 31/2018), que estiveram em vigor até 30 de maio de 2019.

Atualmente, a Instrução Normativa em vigor que trata das características e da qualidade do produto na indústria é a 76 (IN76; BRASIL, 2018) e entre os componentes do leite que são avaliados pela indústria, e monitorados por essa IN, a contagem de células somáticas (CCS) é o que tem sido considerado o maior gargalo para a melhoria da qualidade do leite no Brasil. Na IN76 valores de CCS abaixo de 500.000 CS/mL (quinhentas mil células por mililitro) no leite cru refrigerado de tanque individual ou de uso comunitário são considerados

adequados, já para a fabricação de leite tipo A os valores adequados são de CCS abaixo de 400.000 CS/mL.

Um exemplo desse gargalo foi o trabalho desenvolvido por Milani (2011), que quantificou amostras de leite rejeitadas pelos padrões apresentados pela IN62 (CCS adequada abaixo de 480.000 CS/mL, gordura mín de 3 g/100 g, proteína total mín de 2,9 g/100 g e sólidos não-gordurosos mín de 8,4 g/100g; BRASIL, 2011), proveniente de 326 propriedades leiteiras no Rio Grande do Sul durante o período de 3 anos consecutivos, apresentando 38% das amostras rejeitadas por CCS. Semelhantemente a Oliveira (2013), que trabalhou com 143 propriedades leiteiras na região nordeste do Estado de São Paulo durante um ano, encontrando o índice de 32% de rejeição das amostras pelo fator CCS. No mesmo trabalho, seguindo o índice de rejeição de CCS foram encontrados os índices de rejeição para: contagem bacteriana total (25%), extrato seco desengordurado (13%), gordura (11%) e proteína (5%), para esses parâmetros, os padrões adotados no experimento são os mesmos em vigor atualmente na IN76. Ambos os trabalhos demonstram a importância do controle da CCS para melhoria da qualidade do leite.

Juntamente a iniciativa do governo, algumas indústrias iniciaram a implantação de programas de pagamento por qualidade, como instrumento para incentivar o produtor a buscar pela melhoria de seu produto e indiretamente, para obter melhor rendimento industrial. Além do pagamento de bonificações pelo leite de alta qualidade, podem ser utilizadas penalizações para o leite de baixa qualidade (Álvares, 2005). Esses programas têm sido ferramentas poderosas para motivar os produtores a melhorar a qualidade do leite cru (Roma Júnior et al., 2009).

2. Mastite e a CCS

Considerada uma das doenças mais comuns e prejudiciais da bovinocultura leiteira, a mastite, é a resposta inflamatória ao processo infeccioso intramamário causado por microorganismos (geralmente bactérias), que pode apresentar-se tanto na forma subclínica (sem sintomas visíveis) quanto na clínica (sintomas visíveis) (De Vliegher et al., 2012), tendo os traumas mecânico, térmico e insulto químico como fatores de predisposição a infecção (Zhao e Lacasse, 2008).

Segundo Thompson-Crispi et al. (2014), a forma subclínica caracteriza-se pela dificuldade de sua detecção, em virtude da ausência de sintomas clínicos

visíveis, podendo ocorrer casos transitórios de inflamação e alterações no leite. O autor afirma ainda, que essa pode ser considerada crônica na persistência dos sintomas por período superior a 2 meses. Já a clínica, é de mais fácil detecção, pois ocorre a presença de vermelhidão, calor, inchaço, dor e coágulos ou descoloração do leite, que são sinais claros de inflamação do úbere (Saifudeen et al., 2017).

Assim, o fluxo de células imunológicas na glândula mamária é utilizado para a detecção de infecções intramamárias subclínicas. Onde, contagens de células somáticas (CCS) em amostras individuais de vacas, superiores a 200.000 células por ml, indicam presença de infecção intramamária e CCS abaixo de 200.000 células por ml são consideradas livres de infecção (Pighetti e Elliott, 2011).

A CCS individual ou do tanque de expansão têm sido utilizados pelos países desenvolvidos há mais de 25 anos, pois é por meio dela que se analisam as condições e ocorrências da mastite, estimando as perdas de produção do leite e indicando a qualidade do leite produzido na propriedade (Fonseca e Santos, 2000). Uma vez que, quantidades de CCS elevadas tem efeito negativo sobre a composição e produção do leite (El-Tahawy e El-Far, 2010).

Ademais, a associação de mastites a elevadas CCS no início da lactação, causam perdas econômicas aos produtores, tais como: aumento nos custos com veterinário, medicamentos, trabalho extra, além da redução na produção, e descarte de animais e leite (Huijps et. al., 2009).

3. Produção animal e saúde pública

A mastite ameaça a renda dos produtores de leite, bem como a imagem do setor lácteo por questões relacionadas ao bem-estar animal, a qualidade do leite e a saúde pública, isso ocorre devido ao aumento do risco de resíduos no leite por meio do uso impróprio de antimicrobianos e do surgimento de bactérias resistentes.

Existe ampla gama de mecanismos de ação dos antibióticos, desde a inibição da síntese ou metabolismo celular, ou até mesmo modificando DNA e RNA dos micro-organismos. Entretanto, já foram relatados resistência bacteriana a alguns princípios ativos, causando sérios problemas e consequências, tanto na produção animal, como para o controle das espécies bacterianas envolvidas na área da saúde humana (Pearce e Jin, 2010).

Produtos de origem animal como carne leite e ovos, podem apresentar resíduos de antibióticos, em decorrência do uso nos animais produtores, podendo acarretar em efeitos patológicos em humanos como: transferência de bactérias resistentes a antibióticos, efeitos imunopatológicos, autoimunidade, carcinogenicidade, mutagenicidade, nefropatia, hepatotoxicidade, distúrbios reprodutivos, toxicidade da medula óssea e alergia (Nisha, 2008; Darwish et al., 2013).

Como consequência da crescente preocupação com a saúde pública a Organização Mundial da Saúde, a Organização das Nações Unidas para a Agricultura e a Alimentação e a Organização Mundial da Saúde Animal fizeram recomendações de proibir o uso de antimicrobianos nas rações animais, recebendo apoio de diversos países, entre eles os países pertencentes à União Europeia que resolveram banir o uso de antibióticos como promotores de crescimento na alimentação animal desde 2006 (Castanon, 2007).

Apesar de nos EUA não existir política de controle de uso de antibióticos na produção animal semelhante à da União Europeia, a FDA vem incentivando a redução voluntária no uso de antibióticos como promotores de crescimento desde 2013 (FDA, 2013).

4. Plantas medicinais e produção animal

Diante da preocupação com o uso de antibióticos e a resistência bacteriana, justifica-se a busca de alternativas para produção animal, a exemplo do uso de substâncias naturais, como fitoterápicos, extratos vegetais, óleos essenciais ou produtos alternativos aos antibióticos. Apesar das pesquisas com o uso de fitoterápicos na produção animal ainda serem em pequeno número, tem demonstrado crescimento e demanda principalmente na área de sanidade e desempenho animal.

De acordo com a Agência Nacional de Vigilância Sanitária (ANVISA, 2011), os fitoterápicos são oriundos da industrialização da planta medicinal, evitando contaminações por micro-organismos, agrotóxicos e substâncias estranhas. Ademais, os fitoterápicos garantem a padronização da quantidade e forma de utilização, trazendo segurança ao seu uso.

Neste contexto, os aditivos também vem sendo utilizados na nutrição animal visando o bem-estar e o máximo desempenho animal, além de não serem prejudiciais aos animais e ao homem, não devem deixar resíduos nos produtos de consumo e nem contaminar o meio ambiente.

Entre os aditivos mais estudados e utilizados atualmente se destacam os ácidos orgânicos, plantas e seus extratos, enzimas, probióticos e prebióticos, os quais até então têm apresentado resultados satisfatórios (Costa, 2009). Assim, os aditivos fitogênicos vêm chamando a atenção dos estudiosos, pois agem impedindo doenças comuns nos animais e também na manutenção da saúde.

Os fitogênicos também são de interesse dos consumidores por serem considerados alternativas naturais a compostos sintéticos (Pearce e Jin, 2010), sem riscos para a saúde pública pela ausência de resíduo de antibióticos.

O maior desafio na utilização de extratos vegetais tem sido a identificação e o estabelecimento dos efeitos exercidos pelos compostos ativos presentes nessas plantas sobre o organismo animal (Rizzo, 2008). Quanto ao óleo essencial diz respeito à dose a ser oferecida. Na tabela 1, são apresentados alguns resultados de pesquisa utilizando diversos óleos essenciais e com respostas diferentes.

Tabela 1. Compilação de alguns resultados do uso de óleos essenciais em dietas para animais de produção.

Referência	Quantidade de Óleo Essencial	Resultado	Nº de Animais
Santos et al., 2010	1 g/dia (Eugenol, Geranyl Acetate, óleo de coentro)	Maior produção gordura/dia (2,11%) e Maior perda de escore condição corporal (25%)	310 vacas
Kung Jr et al., 2008	0,6 g/dia (CRINA® - timol, limoneno e guaiacol)	Aumento da Ingestão de Matéria Seca (7,2%), Produção de Leite (4,77%) e Gordura/dia (9,74%)	30 vacas
Oliveira et al., 2014	4 g/dia (capsaicina, eugenol, cinamaldeído e carvacrol)	Nenhum efeito sobre consumo, produção e composição do leite	20 vacas
Tassoul et al., 2009	1,2 g/dia (CRINA® - timol, limoneno e guaiacol)	Menor ingestão de matéria seca (5,08% Prepartum e 7,35% Lactation) Sem efeito na produção de leite	40 vacas

5. Tomilho (*Thymus vulgaris* L.)

Uma espécie que tem despertado bastante interesse é o Tomilho, também conhecido como tomilho branco ou poejo, é uma planta medicinal, aromática e condimentar, pertencente à família Lamiaceae, originária da Europa e cultivada no sul e sudeste do Brasil. O tomilho tem reconhecido princípio antimicrobiano (Imelouane et al., 2009) e antioxidante (Grigore et al., 2010), e segundo os mesmos autores, pode melhorar a função imunológica. O Tomilho é um pequeno arbusto perene ou anual (dependendo do manejo utilizado) com altura entre 20-30 cm, e poucos ramos que podem aparecer prostrados ou eretos duros e um pouco lignificados (Maranca, 1986; Magalhães, 1997).

As folhas, principalmente quando secas, são comumente utilizadas na Europa como condimento em carnes (bovinas, suínas e aves), pois além de

aromatizá-las, possui uma sensível ação fungicida que também ajuda a preservá-las, além disso, possuem sabor amargo e picante (Maranca, 1986).

A colheita ocorre quando a planta apresenta plena floração. As folhas e inflorescências do tomilho são destiladas industrialmente para a produção de óleo essencial à base de timol com rendimento variando entre 0,5 e 1,0% de plantas frescas até 2,5% de plantas secas. No processo de secagem a planta perde cerca de 2/3 de sua massa (Maranca, 1986).

O timol é o constituinte majoritário do óleo essencial de tomilho, quando extraído da planta fresca, pois segundo Jakiemiu (2008), quando no processo de extração dos componentes voláteis foi utilizada a planta seca, houve uma redução de até 85% da concentração do timol presente no óleo obtido.

Testes realizados para estudar a atividade antimicrobiana do óleo essencial de algumas espécies de plantas aromáticas, verificaram que o timol, obtido de plantas de tomilho, foi comprovadamente efetivo contra a *Salmonella* (Santurio et al., 2007).

O segundo maior componente presente no TEO é o composto fenólico carvacrol, que assim como o timol é encontrado principalmente no óleo essencial do orégano e tomilho, e possui atividade antimicrobiana (Benchaar e Greathead 2011). Ademais, Benchaar e Greathead (2011) sugerem que estes dois compostos possuam efeito aditivo, sendo mais eficientes em conjunto do que individualmente.

Hashemipour et al. (2013) incluíram níveis de timol e carvacrol e observaram influência também em respostas imunológicas com benefícios de seu uso em frangos de corte, com doses variando de 0 até 11,8 mg/kg de peso vivo.

A presença de melatonina ou melhor definida como fitomelatonina (melatonina em plantas) na quantidade de 26 nanogramas por grama de planta de tomilho (Tan et al. 2012), mesmo considerada composto secundário é outro fator favorável ao uso experimental desta planta, pois segundo Reiter et al. (2003), a melatonina apresentou em estudos com humanos o efeito antioxidante, atuando em mecanismos bioquímicos e com implicações positivas em patofisiologia.

Ainda segundo Reiter et al. (2003), a melatonina age na redução do estresse oxidativo por meio diversas ações como: eliminação de radicais livres, estímulo de enzimas antioxidantes, aumentando a eficácia da cadeia transportadora de elétrons

mitocondrial e reduzindo o vazamento de elétrons, melhorando a eficiência de outros antioxidantes.

Entretanto, a falta de estudos relacionados ao efeito do TEO na qualidade do leite, além do aparecimento de off-flavor pelo uso de planta do mesmo gênero *Thymus* em trabalho com a avaliação do leite de ovelhas (Lai et al., 1983), poderia ser um ponto negativo e demonstra a necessidade de avaliação do uso de tomilho.

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CAPÍTULO 2 - Effect of thyme essential oil on the metabolism, blood parameters, and greenhouse gases production in feedlot Nelore cattle¹

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Abstract

The objective of this study was to evaluate the effects of the use of thyme essential oil (TEO), which has recognized antimicrobial and antioxidant activity, on feed intake, feeding behavior, ruminal fermentation and blood parameters of feedlot Nellore cattle, aiming to indicate an ideal dose of TEO to be used in future *in vivo* experiments. Four ruminally-cannulated Nellore steers (700 ± 44 kg BW) were randomly assigned in a 4×4 Latin square design and receive one of the experimental treatments: T0 = control, with no TEO; T2 = 2 mL/d of TEO; T4 = 4 mL/d of TEO; and T8 = 8 mL/d of TEO, the , TEO was added directly to the rumen. The animals were housed in individual semi-hoofed pens (9 m^2) with individual feed bunks and waterers. Each experimental period lasted 21 d (14 d of adaptation and 7 d of data collection). The DMI and DM digestibility were not affected by TEO addition (average of 8.6 kg and 73.5%, respectively). Among the feeding behavior variables, only the standing still differed with the inclusion of TEO (Linear, $P=0.03$), with greater values observed for greater TEO doses. The total gas production (mL/g DM) was quadratically affected by TEO addition ($P=0.05$), with greater volumes observed in treatments T0 and T8. No effect of TEO was observed in ruminal pH, VFA, or N-NH_3 concentrations. TEO also did not affect the blood variables evaluated in this trial. The addition of up to 8 mL / d of thyme essential oil does not provide harmful effects on rumen environment and animal health. However, further studies should be conducted to investigate greater dosages of TEO in beef cattle diets.

Keywords: antimicrobial agent, phytotherapy, thyme, thymol.

Introduction

Agricultural activities are responsible for most of the world's demand of antibiotic substances, corresponding to approximately 50% of the total production (FAO, 2011). Additionally, there is a prevision that the use of antibiotic for agriculture purposes will increase in 66% by 2030 (van Boeckel *et al.*, 2015). This use is stimulated by the growth of the demand for animal products, and the need to improve the animal efficiency to convert feedstuffs in meat or milk, which has promoted an increase in intensive livestock production systems (Tilman *et al.*, 2002).

However, this practice has caused great selective pressure in the microbial communities, favoring the survival of resistant strains. As a result, antimicrobial resistance of bacteria that affect both human and animal health has reached alarming levels, becoming a threat to global public health and food security (FAO, 2016). Because of this, several countries prevented the use of antibiotics as growth promoters in animal feed, among them, European Union countries, which, after 50 years allowing its use, withdrew the approval in 2006 (Castanon, 2007).

In this scenario, the search for alternatives to the use of antibiotics has been encouraging research with medicinal plants, especially in countries such as Brazil, which has great variety of flora.

Among the phytotherapeutic plants, the thyme (*Thymus vulgaris* L.) has aroused interest of the researchers. It is a medicinal, aromatic and spice plant, belonging to the *Lamiaceae* family, originated in Europe and grown in the south and southeast of Brazil. Its essential oil has the thymol as the main active principle, and has shown antimicrobial activity *in vitro* on gram positive and negative bacteria, but with greater inhibitory effect observed on gram positive ones (Dorman and Deans, 2000; Imelouane *et al.*, 2009). This effect is probably related to the reduction of enzymatic activity and/or damages in the

membrane integrity, altering the protein reactions (Farak *et al.*, 1989; Juven *et al.*, 1994; Shapiro *et al.*, 1994).

Other researchers have observed that the thymol inhibitory action on glucose uptake by ruminal microorganisms (Evans and Martin, 2000), affecting its growth, as well as the antioxidant activity (Grigore *et al.*, 2010), and may improve the immune function of animals. Studies evaluating the use of thyme in animal feed are scarce. However, *in vitro* trials demonstrated the antimicrobial activity of the essential oil of thyme (TEO; Dal Pozzo *et al.*, 2011), and decreased CH₄ production and ability to manipulate ruminal fermentation (Jahani-Azizabadi *et al.*, 2014).

Thus, the present study aimed to evaluate the effects the addition of up to 8 mL/d of thyme essential oil (TEO) on feed intake, total apparent dry matter and nutrients digestibility, feeding behavior, ruminal and blood parameters, as well as the production of greenhouse gases in feedlot Nellore cattle, to clarify its effects on ruminal fermentation *in vitro* and *in vivo*.

Material and Methods

The São Paulo State University Institutional Animal Care and Use Committee approved all experimental procedures of the present study (approval number: 4093/15).

Animals, housing, treatments, and sample collection

The experiment was conducted at the Animal Unit of Digestive and Metabolic Studies and Laboratory of Ingredients and Pollutant Gases, from Animal Science Department of Sao Paulo State University, Jaboticabal Campus, Brazil.

Four Ruminally-cannulated Nellore steers (700 ± 44 kg BW) were randomly assigned in a 4 × 4 Latin square design and receive one of the experimental treatments: T0 = control, with no TEO; T2 = 2 mL/d of TEO; T4 = 4 mL/d of TEO; and T8 = 8 mL/d of TEO. The TEO

had in its composition 42.3 g/100 g of thymol (390 mg/ml; Table 1). The animals were housed in individual semi-hoofed pens (9 m²) with individual feed bunks and waterers.

Pens, waterers and ruminal cannula were daily sanitized. The animals were injected with the Ivomec[®] subcutaneous injection (1% ivermectin; Merial Animal Health) prior to the beginning of the experiment. The feed was delivered once daily (0800 h) in a proportion of 40% corn silage, 10% Tifton-85 hay, and 50% commercial concentrate composed of corn, soybean meal, urea, limestone, dicalcium phosphate, magnesium, sulfur, zinc oxide, copper sulfate, manganese monoxide and cobalt (Table 2).

The thyme essential oil was daily infused into the rumen at the moment of feed delivery. The addition was performed using 20-mL syringes and an adaptation made in the cannula cap to avoid exposing the rumen to atmospheric oxygen.

Because there is only little data of studies *in vivo* regarding the use of thyme in ruminants diets, the chosen doses (T0 = 0; T2 = 2; T4 = 4; T8 = 8 ml of thyme, corresponding respectively to 0; 1,11; 2,23; and 4,46 mg/kg BW of thymol) were based on preliminary *in vitro* studies performed by this research team, using up to 16,71 mg/kg BW (Almeida *et al.*, 2015) which demonstrated that the maximum possible inclusion without bringing greater animals was close to 5 mg/kg BW of thymol.

Each experimental period lasted 21 d (14 d of adaptation and 7 d of data collection). The amount of feed delivered was calculated and adjusted daily to allow approximately 10% of refusals. Refused feed was collected and weighed daily prior to subsequent feed delivery to calculate daily intake.

Chemical-bromatological analyzes, and total apparent digestibility

During the seven days of data collection the feed delivered and refused were sampled, homogenized and samples were taken for each animal. These samples were pre-dried in a forced air circulation oven at 55°C, ground in a Willey type mill with a sieve of 1mm granulometry (AOAC 1998; method 934.01), and sent to chemical-bromatological analyzes

at the Laboratory of Ingredients and Pollutants Gases of the Animal Unit of Digestive and Metabolic Studies from the Animal Science Department of the FCAV/Unesp-Jaboticabal.

The chemical analysis performed were: dry matter (DM; AOAC 1995; method 930.15) and mineral matter (MM; AOAC 1990; method 942.05); nitrogen concentration (N), using the micro-Kjeldahl method (AOAC 1998; method 988.05), and estimating PB content (AOAC 1990; method 930.15); ether extract (EE) by washing, using soxhlet extractor. Neutral detergent fiber (NDF), NDF corrected for ash and protein (apNDF), and acid detergent fiber (ADF) were determined according to Licitra *et al.* (1993). The total carbohydrates (TC) and non-fibrous carbohydrates (NFC) were obtained using the equations: $TC = 100 - CP - EE - MM$ and $NFC = 100 - apNDF - CP - EE - MM$, respectively (Sniffen *et al.*, 1992).

The total apparent digestibility of DM and nutrients were performed using the total fecal collection method, between d 15 and d 18 of each experimental period. The feces were sampled, weighed, and approximately 10% of the total excreted daily was collected, pre-dried in a forced air oven at 55°C for 72 h, and ground in a Willey type mill with a 1-mm sieve (AOAC, 1998; method 934.01). Those samples were submitted to analysis of DM, MM, and CP, according to the same methodologies mentioned previously. The NDF and ADF were analyzed according to van Soest and Wine (1967) in autoclave. The nutrient digestibility was calculated using the following expression: $\text{nutrient digestibility (\%)} = [(\text{nutrients ingested} - \text{fecal nutrients}) / \text{nutrients ingested}] \times 100$.

Rumen pH, ammonia nitrogen, and VFA profiles

On the 18 d, samples of ruminal fluid (approximately 150 mL) were collected immediately before feeding (time 0), and 2, 4, 6 and 8 h after feeding. From the interface region between the solid and liquid phases of the ruminal content, and this material was filtered through 8 layers of "cheese cloth". The pH (n = 1) was determined immediately after rumen fluid sampling by using a digital pH meter (KASVI[®], model K39-1014B), and ammonia concentrations (n = 2) was determined using a micro-Kjeldhal apparatus (MARCONI[®],

model MA 036), using 5 mL of KOH 2 N, and a distillation flux of 2 mL/min. The distilled sample was dropped in 10 mL boric acid solution (2%), and then titrated with HCl 0.005 N. The volatile fatty acids (VFA) concentrations were obtained by chromatographic analysis (Thermo Scientific®, model Trace 1300), using AI 1310 automatic injector.

Blood analysis

Additionally, on d 18 of each experimental period, 14 mL of blood were collected from all the animals by jugular vein puncture. To perform erythrogram, leukogram and metabolites analysis 4-mL 13 × 75 mm vacutainer® tubes with EDTA K2 were used, and for hepatic enzymes analysis, 10-mL 16 × 100 mm vacutainer® tubes with sodium heparin were used. Immediately after blood sampling, the tubes were placed under refrigeration, transported to the Veterinary Clinical Analysis Laboratory, from the Animais Clínica Veterinária, and there, tubes were centrifuged at 1,500 × g for 10 min to separate the plasma.

The complete hemogram was performed on BC-2800 vet® equipment (methods: volumetric impedance for cell counts and SFT for hemoglobin). The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) dosages were performed by kinetics (Kit Labtest, Lagoa Santa, MG, Brazil), both determined in plasma.

Gas measurements

For the estimation of *in vitro* gas production and quality, a modification of the technique described by Theodorou *et al.* (1994) was used. On the d 19 of each experimental period, rumen fluid was sampled one hour after feeding and supplying of TEO, filtered through 8 layers of "cheese cloth" and mixed with McDougall's buffer solution (McDougall, 1948) in a proportion 1:2.

Samples of 200 mg of each experimental diet (1 mm particle size) were weighed into 60-mL penicillin type glass vials (n = 48) containing 20 mL of buffer solution and 10 mL of rumen fluid (from the same treatments that the animals were adapted with). Four blank vials were also prepared, without any sample to correct the final results. The vials were purged

with helium, sealed septum rubber cap and incubated in an orbital shaker (SOLAB[®], model SL 222) at 39°C, with a constant rotation of 80 rpm. The head-space gas pressure was recorded a 24-h period measured in psi (pound per square inch) using pressure transducer and data logger (Omega[®], model HHP9303). Afterwards, the vials were placed into an ice bath to cease the microbial activity and samples of the gas produced were collected using a 1-mL insulin syringe, and immediately analyzed for CH₄ and CO₂ concentrations by gas chromatographic technique (Thermo Scientific[®], model Trace GC Ultra). For conversion of the psi into mL of gas obtained after 24-h incubation period, a regression curve relating pressure with known volume was developed.

The non-degraded residues from the incubation were filtered and washed in a nylon bag, which were dried in a forced air circulation oven at 55°C for 72 h and then in a 105°C oven to determine residues DM and DM disappearance according to Homem Junior *et al.* (2017), using the formula:

$$\text{DMD (\%)} = \frac{\text{DMinc} - (\text{DMres} + \text{DMbl})}{\text{DMinc}} \times 100$$

Where, DMinc = DM incubated (g), DMres = DM of residue (g), and DMbl = DM of the blank (g).

Feeding behaviour

The feeding behavior was evaluated on d 21 of each experimental period, according to van Cleef *et al.* (2016). The observations were performed by two trained observers, and recorded, each 5 min, the activities: interaction with feed bunk (IF), when animal positioned the head toward the feed bunk, without specifying whether ingested, smelled or played with the feed; interaction with waterer (IW), when animal positioned the head toward the waterer, without specifying whether ingested, or played with the water; standing still (SS), when the animal was with four feet in contact with pen's floor without moving the body; stand ruminating (SR); laid ruminating (LR); laid (LD); stereotypes (ST), when the animal was

chewing pen's structures, biting, repetitively licking or butting, and other activities (OA). To obtain total time spent in each activity, the number of observations recorded was multiplied by 5 (min/12h). The chewing behavior was also evaluated recording time spent chewing each feed bolus, as well as the number chews per bolus and number of chews per second.

Statistical analysis of results

Data were analyzed as a 4 × 4 Latin square, using the PROC MIXED of SAS statistical software (version 9.4, SAS Institute, Inc., Cary, NC, EUA). The statistical model used was: $Y = \mu + A_i + P_j + D_k + e_{ijk}$, where: μ = overall mean, A_i = random effect of animal ($i = 1$ a 4), P_j = random effect of period ($j = 1$ a 4), D_k = fixed effect of diet ($k = 1$ a 4), and e_{ijk} = residual error. The ruminal parameter variables were evaluated as repeated measures. Orthogonal contrasts were evaluated to verify the linear and quadratic effect of TEO addition, as well as control treatment versus TEO treatments. The covariance structure with the best fit (smallest Akaike's value) was the unstructured. Treatment means were computed with the LSMEANS option and significance was defined as $P < 0.05$ and trends of $P < 0.10$.

Results

Intake, total apparent digestibility, and feeding behavior

The intake and total apparent digestibility of DM and nutrients (CP, EE, NDF and ADF) were not affected by experimental treatments ($P > 0.05$; Table 3).

The addition of increasing level of TEO into the rumen, did not affect the activities observed as feeding behavior: IF, IW, LR, LD and SR ($P > 0.05$). However, the time spent standing still (SS) was linearly affected by TEO, with 20% increase in the treatment with inclusion of 8 mL/d (Table 4). OA and ST were not analyzed by the low observation frequency.

There was also no effect of treatments on chewing activity ($P>0.05$), just was observed a tendency for a quadratic effect ($P=0.08$) of time spent chewing/bolus with greater times observed for T0 and T8 (57.5 and 58.6, respectively; Table 4).

Ruminal parameters

There was no interaction of time of sampling and treatments for all the variables evaluated in this trial (pH, $\text{NH}_3\text{-N}$ and VFA), thus only the main effect of treatments is presented for each variable. There was a tendency for a linear increase ($P=0.09$) in pH values with increasing additions of TEO, with the greater value of 6.27 observed in animals from treatment T8. Regarding the ruminal $\text{NH}_3\text{-N}$ concentrations, a tendency for a quadratic effect was observed ($P=0.08$), with the greatest concentrations observed for the control treatment and for the one supplemented with 8 mL/d of TEO (Table 5). The concentration of total and individual VFA (acetic, propionic, butyric, isobutyric, valeric, isovaleric) were also not affected by the treatments ($P>0.05$).

The addition of TEO quadratically affected the total production of gasses *in vitro* ($P=0.05$) and the CO_2 yield per gram of incubated DM ($P=0.03$), with greater values observed for treatments control and with 8 mL/g of TEO, in both cases. However, the CH_4 concentrations were unchanged by experimental treatments ($P>0.05$; Table 6).

When the gas production was expressed as mL per gram of degraded DM, the total gas production and CO_2 yield, tended to be quadratically affected ($P=0.09$ and $P=0.06$, respectively), with greater values observed for treatments control and with 8 mL/g of TEO. Additionally, the CH_4 production tended to be linearly increased ($P=0.08$), with the increasing concentrations of TEO with greater values observed in treatments T4 and T8. Moreover, the pH and DM disappearance, in this trial, were not affected by TEO addition ($P>0.05$).

Blood parameters

The addition of TEO did not affect ($P>0.05$) all the variables evaluated in the blood (Table 7).

Discussion

Intake, total apparent digestibility, and feeding behavior

The lack of effects of the addition of different doses of TEO on the intake and apparent digestibility of DM and nutrients, indicate that there was no detrimental effect on the ruminal microbiota, which did not negatively reflect on the consumption of the animals, with a value close to 8.95 kg/d, estimated as the DM required for adult Nellore cattle (500 kg BW), with average daily gain of 0.75 kg (Valadares Filho *et al.*, 2016).

These results corroborate with Khorrami *et al.* (2015), which fed TEO (500 mg/kg DM) for cannulated cattle and did not observe difference in DM intake and digestibility, obtaining 12.1 kg and 68.4%, respectively. Spanghero *et al.* (2009), evaluating the increasing inclusion of a blend of microencapsulated essential oils, containing thyme (0, 0.32, 0.64 and 0.96 g/d) in diets for dairy cows, also found no differences on DM intake and digestibility of diets.

Regarding the feeding behavior, the increasing inclusion of TEO did not promote significant changes in the parameters evaluated in this trial. Several factors influence feeding time, rumination and chewing (van Soest, 1994), and among these factors, feed degradation and the rate of passage of the diet through the gastrointestinal tract may influence the feeding behavior of the animals (Carvalho *et al.*, 2004; Pereira *et al.*, 2007). However, the antibacterial effect of TEO at the concentration used in this trial, apparently did not influence these factors.

Ruminal parameters

According to Nagajara *et al.* (1997), the inclusion of antibiotic additives in diets for ruminants has direct impact on the population of bacteria responsible for undesirable processes in the rumen, such as lactic acid producers. In this sense, TEO may have had a similar effect in this study.

The linear trend of increasing pH values observed with increasing inclusion of TEO may be related to the inhibitory effect of thyme on some gram positive and negative lactic acid producing bacteria (Evans and Martin, 2000), which according to Nagaraja and Titgemeyer (2007) is about 10 times stronger than major VFAs, and its presence would result in a reduction of ruminal pH.

Another factor that may have contributed to the increase of ruminal pH is the longer chewing time per bolus, as observed in the treatments with 8 mL/d of TEO. The longer time chewing the feed provides an increase in the production of saliva and, consequently, the amount of saliva that reaches the rumen. Since the saliva has a known buffering power, it helps to neutralize the acids in the rumen, keeping the pH stable (in a certain extent), and thus providing a better ruminal environment (Jaramillo-López *et al.*, 2017, Valente *et al.*, 2017).

Abrupt changes in ruminal pH may affect the microbiota, and consequently the fermentative pattern and the production of organic acids (van Soest, 1994). However, this fact was not observed in the present study, probably because the variation was insignificant. Spanghero *et al.* (2009), evaluating the effect of increasing doses of a blend of thyme-containing essential oils on ruminal fermentation products *in vitro*, in acidic and basic initial conditions (pH 5.5 and 7.0, respectively), observed a moderate reduction in the proportion of acetate and in the acetate:propionate ratio, especially when under acidic conditions. Similar results were observed by Castillejos *et al.*, (2006), when evaluated increasing doses of thymol (5, 50 and 500 mg/L) *in vitro*. However, this fact only occurred in the greatest dose, justifying the divergence with the results of the present study, in which were used a much lower concentration of thymol. According to Khorrami *et al.* (2015), this lack of effect of the inclusion of TEO demonstrates the difficulty in extrapolating the dose rate and the results obtained in *in vitro* and *in vivo* studies.

The lowest total gas production (mL/g) observed in the treatments with 4 mL/d of TEO occurred due to the lower production of CO₂ (mL/g) obtained by this treatment. When

analyzing the gas production per gram of degraded DM, the same behavior was observed, but with a trend of linear increase in the production of CH₄, with the increasing addition of TEO.

The linear trend observed for pH and quadratic for NH₃-N, with greater concentrations of TEO may be related to the greater production of CH₄. According to Stern and Hoover (1979), NH₃-N is responsible for 40 to 100% of the nitrogen required by microorganisms, which linked to the high pH in the rumen, it may have favored the reproduction of the cellulolytic bacteria, leading to a greater production of CH₄. However, these results differ from those found in the literature (Jahani-Azizabadi *et al.*, 2011; Jahani-Azizabadi *et al.*, 2014), which observed a high inhibitory effect of TEO on the *in vitro* production of ruminal CH₄. These differences are due to the greater TEO dosages used in those studies (50 µl in the first and 35, 70, 140 and 280 µl TEO in the second, both for 50 mL incubated ruminal fluid).

Blood parameters

The results found in the blood analysis agree with the reference values for beef cattle, according to Feldman *et al.*, (2000) and Kaneko *et al.* (2008). In the erythrogram, the values are between 5.0 - 10.0 10⁶/L; 8.0 - 15.0 g/dL; 24-46%; 40-60 fL; 14.4-18.6 fL; 30 - 36 fL and 100 - 800 10³/µL, respectively for erythrocyte, hemoglobin, hematocrit, MCV, MCH, MCHC, platelets, in the leukogram the values are between 4,000 - 12,000; 0 - 120; 600-4000; 2,500 - 7,500; 25-840; 0 - 2,400 and 0 - 200 U/µL, respectively for leukocytes, rods, segmented, lymphocytes, monocytes, eosinophils and basophils.

For the metabolites, the reference value of plasma protein is between 7.0 - 8.5 g/dL, and for the liver enzymes, between 11-40 and 78 - 132 U/L, respectively for ALT and AST (Feldman *et al.* 2000, Kaneko *et al.*, 2008). In present study, the AST enzyme presented lower values than the reference values, regardless the treatment (average of 53.6 U/L). The AST and ALT enzymes are generally evaluated to detect hepatic cells' lesions, which high

values combined to increased values of total protein in the blood are usually related to severe damage to liver cells. However, the present study's results indicate that the compounds present in TEO did not cause liver damage when infused at up to 8 mL/d.

Conclusion

The addition of up to 8 mL/d of thyme essential oil do not promote deleterious effects on nutritional and metabolic parameters of feedlot Nellore cattle *in vitro and in vivo*. However, trends were observed in the increase in the production of CO₂ and CH₄ contrary to the results described in the literature, demonstrating that further studies should be conducted to investigate greater dosages of TEO in beef cattle diets as well as the form of product delivery.

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Table 1. Result of the Physical-chemical report of Thyme Essential Oil (*Thymus vulgaris* L.) used in the present experiment.

Controlled Items	Results	Specifications
Appearance	Clear Liquid	Clear Liquid
Color	Light yellow	Colorless to Light Yellow
Impurities	Free	Free
Odor	Characteristic	Characteristic
Density (20°C)	0.918	0.900 a 0.940
Refractive Index (20°C)	1.501	1.490 a 1.510
Optical Rotation	-2.2	-3.0° a -0.1°
Chemical Composition:		
Timol	42.3%	
p-cimeno	27.4%	
y-terpinemo	8.1%	
Linalol	6.7%	
Carvacrol	4.0%	
α -pineno	2.7%	
β -cariofileno	2.0%	
Canfora	1.9%	
Mirceno	1.4%	
1,8-cineol	1.3%	
Borneol	1.1%	
Canfeno	0.9%	

Source: FERQUIMA Ind. And Com.

Table 2. *Chemical composition of concentrate, silage, hay, and experimental diet.*

Item	Concentrate ¹	Silage	Hay	Experimental diet
Dry matter (g/kg)	880	408	874	642
Crude protein (g/kgDM)	233	74	93	150
Ether extract (g/kgDM)	26	22	12	35
Minerals (g/kgDM)	100	31	76	32
Neutral detergent fiber (g/kgDM)	317	495	795	485
Acid detergent fiber (g/kgDM)	109	327	596	280

¹Bellman®.

Table 3. Intake of DM and nutrients and total apparent digestibilities of DM and nutrients in feedlot Nellore cattle supplemented with increasing levels of thyme essential oil.

Item	Treatments ¹				SEM	P-value		
	T0	T2	T4	T8		L	Q	0 × T
Intake(kg/d)								
DM	8.85	8.63	8.52	8.29	0.72	0.54	0.89	0.55
CP	1.15	1.11	1.13	1.08	0.12	0.88	0.73	0.77
EE	0.28	0.27	0.27	0.27	0.02	0.93	0.79	0.84
NDF	3.69	3.46	3.65	3.58	0.34	0.88	0.41	0.59
ADF	1.02	0.84	0.98	1.02	0.17	0.84	0.33	0.50
Digestibility(%)								
DM	74.68	73.98	71.10	74.14	1.37	0.36	0.11	0.22
CP	76.65	76.11	72.97	75.41	1.20	0.15	0.16	0.14
EE	93.11	94.36	91.28	91.91	1.11	0.18	0.76	0.62
NDF	71.24	70.52	67.37	70.76	1.87	0.47	0.16	0.31
ADF	55.15	52.30	47.51	53.82	3.25	0.46	0.10	0.21

T0 = 0 mL of thyme essential oil (TEO); T2 = 2 mL of TEO; T4 = 4 mL of TEO; T8 = 8 mL of TEO; SEM = Standard error of means; L = Linear; Q = Quadratic; 0 × TEO = control treatment vs treatments with TEO; TR = treatment.

¹Treatments in mL of TEO.

Table 4. Feeding behavior and chewing activity of feedlot Nellore cattle supplemented with increasing levels of thyme essential oil.

Item	Treatments ¹				SEM	P-value		
	T0	T2	T4	T8		L	Q	0 × T
Activities, min ²								
IF	175	192	205	161	29	0.74	0.15	0.62
IW	9	14	11	6	5	0.59	0.25	0.72
LR	38	19	43	50	17	0.31	0.33	0.97
SS	269	275	268	322	35	0.03	0.10	0.21
LD	101	121	70	78	32	0.25	0.80	0.66
SR	128	99	122	101	16	0.30	0.72	0.17
Chewing activity								
N°/bolus ³	47.8	44.2	44.1	47.6	2.2	0.92	0.11	0.28
Time/bolus, s	57.5	54.7	53.2	58.6	2.5	0.42	0.08	0.42
Chew/s ⁴	0.83	0.81	0.83	0.81	0.02	0.58	0.80	0.32

T0 = 0 mL of thyme essential oil (TEO); T2 = 2 mL of TEO; T4 = 4 mL of TEO; T8 = 8 mL of TEO; SEM = Standard error of means; L = Linear; Q = Quadratic; 0 × TEO = control treatment vs treatments with TEO; TR = treatment.

¹Treatments in mL of TEO.

²Time spent (min) on the activities: interaction with feed bunk (IF), interaction with waterer (IW), laid ruminating (LR), standing still (SS), laid (LD) and stand ruminating (SR).

³Number of chews per bolus

⁴Chews per second.

Table 5. *Ruminal parameters and VFA production of feedlot Nellore cattle supplemented with increasing levels of thyme essential oil.*

Item	Treatments ¹				SEM	P-value		
	T0	T2	T4	T8		L	Q	0 × T
pH	6.13	6.15	6.20	6.27	0.12	0.09	0.61	0.28
N-NH ₃	13.71	13.51	14.61	15.96	3.15	0.52	0.08	0.76
VFA ² (mmol/L)								
Total	58.47	55.84	58.51	55.71	2.52	0.59	0.97	0.51
Acetic	29.57	28.41	30.10	28.00	1.54	0.64	0.74	0.66
Propionic	15.34	14.80	15.07	14.84	0.57	0.55	0.68	0.38
Butyric	9.26	8.69	9.15	8.84	0.39	0.58	0.67	0.34
Isobutyric	1.17	1.15	1.10	1.15	0.04	0.57	0.33	0.42
Valeric	1.38	1.28	1.41	1.20	0.14	0.49	0.68	0.58
Isovaleric	1.75	1.63	1.68	1.67	0.10	0.67	0.57	0.42

T0 = 0 mL of thyme essential oil (TEO); T2 = 2 mL of TEO; T4 = 4 mL of TEO; T8 = 8 mL of TEO; SEM = Standard error of means; L = Linear; Q = Quadratic; 0 × TEO = control treatment vs treatments with TEO; TR × T = Interaction of treatment and time.

¹Treatments in mL of TEO.

²Volatile fatty acids.

Table 6. *In vitro* total gas, CH₄ and CO₂ production per g of incubated DM and per g of degraded DM from rumen fluid of feedlot Nellore cattle supplemented with increasing levels of thyme essential oil.

Item	Treatments ¹				SEM	P-value		
	T0	T2	T4	T8		L	Q	0 × T
Total gas, mL/g	148.7	147.3	141.3	151.3	11.0	0.89	0.05	0.53
CH ₄ , mL/g	19.1	19.0	19.7	19.6	0.8	0.17	0.92	0.40
CO ₂ , mL/g	129.6	128.3	121.6	131.8	10.5	0.98	0.03	0.44
Total gas, mL/g d ²	176.1	176.1	168.8	180.3	14.3	0.71	0.09	0.80
CH ₄ , mL/g d	22.6	22.7	23.5	23.3	1.0	0.08	0.63	0.17
CO ₂ , mL/g d	153.5	153.4	145.2	157.0	13.5	0.86	0.06	0.66
pH	6.4	6.4	6.4	6.4	0.06	0.43	0.14	0.77
DMD ⁴ , %	84.3	84.1	84.4	84.1	0.9	0.91	0.94	0.88

T0 = 0 mL of thyme essential oil (TEO); T2 = 2 mL of TEO; T4 = 4 mL of TEO; T8 = 8 mL of TEO;

SEM = Standard error of means; L = Linear; Q = Quadratic; 0 × TEO = control treatment vs treatments with TEO.

¹Treatments in mL of TEO.

²mL per g of degraded DM.

³CO₂ and CH₄ ratio.

⁴Dry matter disappearance.

Table 7. Blood parameters of feedlot Nellore cattle supplemented with increasing levels of thyme essential oil.

Item	Treatments ¹				SEM	P-value		
	T0	T2	T4	T8		L	Q	0 × T
Erytrogram								
Erythrocyte (10 ⁶ /L)	8.7	8.7	8.8	8.7	0.4	0.98	0.92	0.99
Hemoglobin (g/dL)	14.0	14.0	14.1	14.1	0.7	0.91	0.98	0.97
Hematocrit (%)	38.9	38.4	38.9	38.7	1.6	0.99	0.91	0.90
MCV ² (fL)	44.6	44.1	44.2	44.5	0.8	0.99	0.64	0.77
MCH ³ (fL)	16.1	16.0	16.0	16.2	0.3	0.71	0.62	0.97
MCHC ⁴ (fL)	36.1	36.4	36.3	36.3	0.4	0.79	0.78	0.66
Platelets (U/mm ³)	488750	506000	490000	465000	56987	0.74	0.72	0.98
Leucogram (U/μL)								
Leucocytes	6575	7275	7167	6675	443	0.93	0.23	0.39
Segmented	1614	1954	1499	1686	311	0.81	0.74	0.71
Eosinophils	319	435	430	551	115	0.11	0.98	0.21
Lymphocytes	4505	5074	5059	4222	552	0.73	0.23	0.67
Monocytes	201	259	283	282	53	0.38	0.63	0.37
Metabolite								
Plasmatic protein (g/dL)	8.4	8.2	8.2	8.3	0.3	0.90	0.72	0.73
Hepatic enzymes (U/L)								
ALT ⁵	31.0	34.5	32.0	36.3	4.3	0.27	0.88	0.30
AST ⁶	57.0	49.5	57.0	51.0	6.1	0.71	0.91	0.53

T0 = 0 mL of thyme essential oil (TEO); T2 = 2 mL of TEO; T4 = 4 mL of TEO; T8 = 8 mL of TEO; SEM = Standard error of means; L = Linear; Q = Quadratic; 0 × TEO = control treatment vs treatments with TEO.

¹Treatments in mL of TEO.

²Mean corpuscular volume.

³Mean corpuscular hemoglobin.

⁴Mean corpuscular hemoglobin concentration.

⁵Alanine aminotransferase.

⁶Aspartate aminotransferase.

CAPÍTULO 3 - Addition of lactating dairy cows with thyme (*Thymus vulgaris* L.) essential oil and its effects on performance, immune response and milk quality¹

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Abstract. The aim of this study was to evaluate the effects of daily supplementation of thyme essential oil (*Thymus vulgaris* L.; TEO) on the productive performance, immune response and milk quality lactating dairy cows. Twenty-four Jersey cows (400 ± 42.9 kg BW (body Weight)), second lactation, 50 DIM (Days In Milking), ~36 months old, and average milk production of 22.05 kg/d) were used. The animals were assigned to two experimental treatments: CON (control with no supplementation), and TEO (8 mL/d of TEO with 42.3% of thymol), in a completely randomized design, and housed in individual pens. The DMI (Dry Matter Intake), feed efficiency and milk yield were not affected by TEO

¹ Este capítulo corresponde ao artigo científico submetido à revista Journal of Dairy Science e encontra-se em avaliação para publicação.

supplementation. Among the qualitative parameters of milk, only the Electric Conductivity tended to decrease when TEO were used. There were also fewer cases of clinical mastitis and lower return time to 200,000 cells/mL in cows from TEO than CON, after identification and medication (2 cases and 4d vs. 5 cases and 7d, respectively). The alanine aminotransferase enzyme tended to decrease in animals from TEO. The interleukin 2 was increased when TEO was used, while interleukin 4 and immunoglobulin G tended to increase, increasing 128 and 5%, respectively when compared to CON. The TEO supplementation also tended to increase melatonin levels in milk, and significantly increased thymol levels in blood and milk, with 58% increase in blood and 406% in milk. The supplementation of Jersey cows in the early third of lactation with 8 mL TEO/d does not affect feed intake and productive performance, and tends to improve immune system response, assisting in mastitis control and maintaining mammary gland health. In addition, the presence of the compounds thymol and melatonin can promote the use of TEO in dairy cattle to produce functional milk, depending on the effect of these compounds on human health and well-being.

Key Words: immune system, mastiti, natural antimicrobial, thyme, thymol.

INTRODUCTION

Mastitis is a disease of major economic relevance in dairy farming worldwide as it causes losses in both clinical (CM) and subclinical (SCM) forms due to reduced milk production, early cow replacement, cost of treatments, discarding milk due to antibiotic use, and altering milk composition and quality (Heikkilä, et al., 2012; Duarte et al., 2015; Yang and Li, 2015; Taponen et al., 2017).

Thus, the preventive management of intramammary infections during the dry-off period and the treatment of clinical mastitis are the main cause of antibiotic use in dairy farms (USDA, APHIS, 2008; Swinkels et al., 2015).

However, the growing concern with human and animal health due to the emergence of antibiotic-resistant microorganisms, and the possible collaboration for this emergence, the indiscriminate use of antibiotics in animal production, has been increasing the pressure to reduce its use for production purposes. In the United States itself, The Food and Drug Administration (FDA) has been encouraging the voluntary reduction in antibiotic use as growth promoters since 2013 (FDA, 2013). In the European Union, the use of antimicrobials as growth promoters has been banned since 2006, and while bans are more related to the use of antibiotics as growth promoters, the prohibition of preventive use has also been much discussed, and according to Swinkels et al. (2015), countries such as the Netherlands and Germany have already banned this use.

In this scenario, the search for alternatives to the use of antibiotics has been fomented a great interest of researchers about herbal medicines and, among them, the thyme essential oil (*Thymus vulgaris* L.) may be an interesting alternative, because of thymol, its main active compound. Thymol has known *in vitro* antimicrobial activity on gram-positive and gram-negative bacteria (Dorman and Deans, 2000; Imelouane et al., 2009), giving thyme essential oil (TEO) the ability to act as a natural antimicrobial ingredient. In addition, TEO also has antioxidant activity (Grigore et al., 2010), which may improve the immune response of animals.

In vitro studies endorsed the antimicrobial activity of TEO against *Staphylococcus* spp., making this herbal medicine a possible alternative in the treatment of bovine mastitis (Dal Pozzo et al., 2011). However, results from *in vivo* studies are still rare in the literature

and further research should be conducted to assess the positive or negative effects of TEO supplementation on lactating cows. Therefore, the objective of this study was to evaluate the effects of daily supplementation with TEO on dry matter and nutrients intake, milk yield and quality, immune system-related parameters of lactating dairy cows, animal physiology and health.

MATERIAL AND METHODS

The São Paulo State University Institutional Animal Care and Use Committee approved all experimental protocols of the present study (approval number: 4093/15). The field experiment was conducted at São Paulo's Agency for Agribusiness Technology, Ribeirão Preto, Brazil.

Animals, facilities and management

Twenty-four Jersey cows (400 ± 42.9 kg BW, second lactation, 50 DIM, ~36 months old, and average milk production of 22.05 kg/d) were used. The animals were assigned into 2 treatments: CON, control treatment with no TEO supplementation, but with 8 mL of soy oil as a placebo, and TEO, with supplementation of 8 mL thyme essential oil (Table 1). This dosage of TEO was chosen based on preliminary *in vitro* and *in vivo* trials conducted by this research team (unpublished data).

The animals were housed in individual pens of 14 m², half covered with corrugated zinc tile and half with 70% black bulk shade cloth, with individual feed bunk and waterer. The facilities were washed, disinfected with quaternary ammonia 30 d prior to the study starting date. Cows were milked three times daily (0700, 1500 and 2200 h), using an automatic milking system, in a fishbone-type milking parlor, with a capacity of six animals.

Milk production was recorded electronically. The first three jets were collected for the performance of the “strip cup” test. Dirt was cleaned from the teats and a pre-dipping solution was used (SANI IODO® at a ratio of 1: 5, with 5 parts of water). Teats were dried with paper towels and, after milking, a post-dipping product was used (Pure SANI IODO®). The cows were placed in a waiting shed 30 min before each milking and returned to the pens immediately after the milking of all experimental animals.

The animals were fed a total mixed ration delivered at 0800 and 1600 h (50:50), consisted of 50% roughage (40% corn silage and 10% coast-cross hay) and 50% commercial concentrate (Nutreco® Fri Leite 20), consisted of corn, soybean meal, urea, limestone, dicalcium phosphate, magnesium, sulfur, zinc oxide, copper sulphate, manganese monoxide and cobalt (Table 2).

The orts were collected and weighed every day, before the morning feed delivery, to determine daily DMI and to adjust the amount supplied, allowing approximately 10% of orts in the feed bunk. Water and feed intake were *ad libitum* throughout the experimental period. The TEO was supplied as hard gelatin capsules, which were introduced directly into the animals' esophagus using a plastic applicator and rubber tip (to avoid injuries to the digestive tract of the animals), ensuring the intake of the treatment. To avoid effect of oil inclusion, the control cows received capsules containing soybean oil in the same amount as the TEO treatment.

The choice of essential oil form in this experiment was because of its greater composition consistency, regardless the time of year or place of extraction. The encapsulation was performed to preserve the characteristics (volatile compounds) until the time of treatment, while minimizing the taste of the oil. In order to alleviate the stress in cows, the

treatments were infused shortly after the night milking (2300 h), when the animals were generally calmer, and the temperature was more pleasant.

Milk and blood sampling and analysis

The experimental period lasted 28 d, with 7 d of adaptation of the animals to the diet and facilities, followed by 21 d of treatment supply and sampling of experimental data (Figure 1). During the adaptation week, milk production and somatic cell count were monitored and, after the end of this week, all cows were weighed. During the weight recording (d 8) and at the end of the trial (d 28), blood was collected from all animals by puncture of the mammary vein, using evacuated tubes containing EDTA. These blood samples were used for complete blood count, using a hematology analyzer PochH-100iV Diff is (Sysmex[®], Kobe, Japan) and blood smears stained with Fast Panoptic kit (Laborclin[®], São José do Rio Preto, SP, Brazil), while aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT) were analyzed using a semiautomatic wet biochemistry device (Labquest, Labtest, Lagoa Santa, MG, Brazil), using commercial kits as recommended by the manufacturer. All these procedures were conducted at “Animais” Veterinary Clinic, Ribeirão Preto, SP, Brazil.

The measurement of prolactin and melatonin was performed using enzyme-linked immunosorbent assay (ELISA Kit, MyBioSource[®], San Diego, CA, USA), using the Bovine/Luteotropic Hormone ELISA Kit and the Bovine/Melatonin ELISA Kit, respectively. Thymol quantification (in blood plasma) was performed by headspace solid-phase microextraction (HS-SPME) and mass spectrometry-coupled gas chromatography (GC-MS) using methodology adapted from FIORI et al. (2013). These analyses were performed at the

Natural and Synthetic Products Research Center, School of Pharmaceutical Sciences, University of São Paulo, Ribeirão Preto, SP, Brazil.

The blood was centrifuged for 10 min at $1,000 \times g$, and serum was separated and frozen at -80°C . In the serum, quantitative evaluation of cytokines were performed: interleukin (IL-2, IL-4, IL-6, IL8, IL10), tumor necrosis factor-alpha (TNF- α), interferon-gamma (IFN- γ) and colony stimulator (M-CSF and G-CSF) using multiplex microsphere immunoassay, where fifty microliters of each sample were analyzed using Millipore commercial kits (Milliplex[®], HCYTOMAG-60K and HCYP3MAG-63K, Darmstadt, Germany) using a Luminex analyzer (Luminex[®], MiraiBio, Alameda, CA, USA) Immunoglobulins were also evaluated by immunoassay using ELISA Kit (Abnova, Taipé, Taiwan) for IgG, IgM, IgA bovine standard. Analyzes were performed at the Biochemistry and Immunology Laboratory from the Medical School, University of São Paulo, Ribeirão Preto, SP, Brazil.

Milk production was individually recorded daily in the three milkings. During the morning milking, two samples of milk (40 mL) were collected from each cow for 18 days. One of the bottles had bronopol preservative and was intended for composition analysis: fat, protein, defatted dry extract, milk urea nitrogen and lactose, which were analyzed electronically by infrared absorption on Bentley 2000[®] equipment (Bentley Instruments, Chaska, MN, USA) at the Lactation Physiology Laboratory, Milk Clinic, University of São Paulo, Piracicaba, SP, Brazil. The second bottle, without preservative, was used for the analysis of the cryoscopy, salts, density and electrical conductivity, using the Master Classic infrared equipment (AKSO, São Leopoldo, RS, Brazil) at Milk Quality Laboratory from São Paulo's Agency for Agribusiness Technology, Ribeirão Preto, Brazil.

Somatic cell counts (n=432 samples) were performed by flow-cytometric electronic counting using the Bentley Somacount 150 equipment (Bentley Instruments, Chaska, MN, USA). For statistical analysis, score somatic count (SCC) data were transformed to Somatic Cell Score (SCS) using the formula: $SCS = [\text{Log}_2 (SCC/100,000)] + 3$, proposed by Shook and Schutz (1994). In the last three days of the experiment, milk quality was not evaluated due to blood sampling.

Statistical analysis

The experimental design was completely randomized with two treatments (CON and TEO) and 12 replicates. Data were subjected to analysis of variance using the PROC MIXED of SAS version 9.4 (SAS Inst., Inc., Cary, NC, USA) for repeated measurements. The model included the effects of treatments, time and their interactions. For milk production and SCC repeated measures over time were analyzed. The experimental unit considered was the animal. Several covariance structures were tested and the best one was chosen, for each of the variables evaluated, based on the lowest value in the Akaike and Schwarz criteria (LITTELL et al., 2006). The degrees of freedom and tests were adjusted using the option KR. Values are reported as least squares means and associated standard errors, and significance was defined as $P < 0.05$ and trends as $0.05 \leq P \leq 0.10$.

RESULTS

Dry matter and nutrient intake and milk production and quality

The inclusion of TEO did not alter DM nor the nutrients CP, EE, NDF, ADF and MM intakes ($P > 0.10$). The supplementation with the essential oil, at the dose used, also did not affect milk production and feed efficiency ($P > 0.10$; Table 3).

Among the parameters evaluated for chemical composition and milk quality, TEO supplementation did not affect fat, crude protein, defatted dry extract, lactose, density, salts, cryoscopic index, urea nitrogen, nor somatic cell score ($P > 0.05$). However, electrical conductivity differed between treatments ($P = 0.07$), tending to be lower in milk from TEO-supplemented cows (Table 4).

During the experiment there were seven peaks in the SCC, corresponding to the seven cases of clinical mastitis identified, five of them in the CON group and two in the TEO group (Figure 2). Somatic cell count within each treatment was also observed for seven consecutive days after clinical mastitis identification and medication, according to the antibiogram of each case. Cows belonging to TEO returned to the SCC value considered adequate (200,000 cells/mL according to National Mastitis Council) on d 4 after identification and medication. On the other hand, cases in the CON group returned to 200,000 cells/mL only on d 7 (Figure 3). No statistical analysis was performed due to the low number of cases identified.

Milk samples ($n = 432$) were classified into three different ranges for SCC value: above 500,000 cells/mL, between 200,000 and 500,000 cells/mL, which is considered adequate according to the Brazilian Normative Instruction 76 (Brazil, 2018), and below 200,000 cells/mL, which is adequate according to the National Mastitis Council. The TEO supplementation

provided the lowest SCC, with only 17.1% of the samples ($n = 37$) above 200,000 cells/mL, against 26.5% of the samples ($n = 57$) for the CON treatment (Table 5).

Blood parameters

The TEO supplementation did not affect ($P > 0.10$) most of the fractions evaluated in the erythrogram, leukogram and blood metabolites. Regarding hepatic enzymes, only the ALT tended to decrease ($P = 0.07$) due to TEO supplementation, showing a reduction of approximately 12%, when compared to CON. The AST was not altered ($P > 0.10$) and the overall average was 84.09 U/L (Table 6).

The most of the fractions evaluated in the serum did not affect ($P > 0.10$) for the TEO supplementation. Just was observed an increase was observed for Interleukin 2 ($P = 0.04$) and an increasing trend were observed for Interleukin 4 ($P = 0.07$), as a function of supplementation with TEO (with increases of approximately 123 and 128%, respectively). In serum immunoglobulins, an increasing trend was observed for IgG ($P = 0.09$), with 5% grater values observed for TEO (Table 7).

Milk and blood compounds

The TEO supplementation increased the concentration of thymol in both blood and milk ($P = 0.02$ and $P < 0.0001$, respectively), with averages of 10.68 ng/mL in blood and 30.03 ng/10 mL milk against 6.76 ng/mL in blood and 5.93 ng/10 mL milk of CON. The concentration of melatonin, and, tended to be increased only in milk ($P = 0.08$), with average 63.94 pg/mL, an increase of 31% compared to CON, and in plasma the overall average were 105.03 pg/mL. No effect of supplementation with TEO was observed for prolactin in plasma ($P > 0.10$) with overall average of 4.07 pg/mL (Table 8).

DISCUSSION

Dry matter and nutrient intake, milk production and quality

The lack of effect on the dry matter and nutrient intake indicates that despite the antimicrobial activity, the thyme essential oil did not affect ruminal microorganisms to the point of affecting feed use. This result agrees with the ones found by Oliveira et al. (2014), which included blends of essential oils in dairy cows and by Spanghero et al. (2009), which included blends of essential oils containing thyme essential oil. In both studies, authors reported no differences in DMI. The overall DMI average observed herein are similar to those found by Aikman et al. (2008), which conducted a comparative study of early lactation Jersey and Holstein cows and reported DMI = 3.48% BW, very close to that observed in the present study (3.38% BW).

The milk yield and milk yield efficiency were not influenced by TEO and are above those reported by Nörnberg et al. (2006) for early lactating Jersey cows (up to 90 d postpartum), which were 16.77 kg milk/d and 1.02 kg milk/kg DMI. These differences are probably due to the different management and genetic quality of the animals used in both studies. The lack of effect on milk yield was also observed in other studies in which essential oils were supplemented for lactating cows (Santos et al., 2010). The same was observed for the chemical composition of milk, which also remained unchanged when essential oils were used. These results are relevant, especially when coupled with health-related milk quality parameter, such as the electrical conductivity value (EC) in the milk of cows supplemented with thyme, which indicates improvement in mammary gland's health, since EC is considered a reliable method for mastitis detection (Kaşıkçı et al., 2012). This improvement may have reflected in the number of clinical mastitis observed in the current trial, which were less and with lower persistence of the

inflammatory process after medication for TEO-supplemented cows, reinforcing the idea that it may have positively influenced the immune system of the mammary gland.

Blood parameters

The results observed in both erythrogram and leukogram are in accordance with the reference values for the bovine species reported in the literature. The same occurred with liver enzymes and metabolites (Feldman et al., 2000; Kaneko et al., 2008), demonstrating that the compounds present in TEO, at the concentration used herein, did not cause liver damage. The AST and ALT enzymes values are generally used to detect liver cell damage, which elevated values linked to increased total blood protein levels are usually related to severe hepatocyte damage. Moreover, the tendency of decrease in ALT enzyme value observed in TEO treatment may indicate slight improvement in liver health of these animals.

The cytokines interleukin 2 (IL-2) and 4 interleukin (IL-4), as well as immunoglobulin G (IgG), which were significantly greater in TEO-supplemented animals, corroborate the hypothesis that it positively influenced the immune system of the mammary gland, probably assisting in the specific immune response. Although the analyzes focused on identifying possible improvements in the mammary gland immune response due to herbal influence and not on the mechanisms by which these improvements occurred, the results indicate that apparently the TEO stimulated the production of IL-2 and IL-4. These cytokines, like others secreted by antigen presenting cells, such as B cells or macrophages, act on the modulation of the immune response and may induce a cell-mediated (Th1 type) or humoral (Th2 type) immune response (Brown et al., 1998). Thus, there is evidence that TEO acted on improving the immune system in both Th1 and Th2 responses, as IL-2 is one of the major cytokines produced during the Th1 response and improves the proliferation of mammary mononuclear cells. Lymphocyte cytotoxic and bactericidal activities

increase the number of plasma cells and activate natural killer cells (NK), while IL-4 is among those predominant in the Th2 response that is characterized by antibody production (Sordillo and Streicher, 2002; Jantan et al., 2015), justifying the greater value of IgG observed herein.

Milk and blood compounds

The mammary gland immune system may also have been benefited by thymol, which is the active compound in TEO (around 42%) and has proven *in vitro* antimicrobial activity on gram-positive and gram-negative bacteria (Dorman and Deans, 2000; Imelouane et al., 2009). Thus, the dosage of thymol in blood and milk samples, especially in those from TEO-supplemented cows, demonstrated that this active compound reached the mammary gland, possibly acting as an antimicrobial directly. The detection of thymol in both treatments, although much lower in CON, must have occurred due to the volatile nature of its molecule, allowing CON-fed animals to inhale volatilized molecules from the same environment.

According to Campbell and Neill (2016), melatonin is a hormone that plays an important role in sleep regulation, being synthesized and secreted mainly at night by the pineal gland. Therefore, the greatest melatonin concentrations are found in nighttime milk, as reported by Asher et al. (2015), who observed greater melatonin concentrations in the night milk samples (milking at 0430 h) than in daytime milk (milking at 1230 h), with averages of 30.70 and 5.36 pg/mL, respectively. In the current study, the values of melatonin observed (57.53 and 63.94 pg/mL, respectively for CON and TEO treatments) are greater than those reported by Asher et al. (2015) and greater than those considered normal for night milk of herds under normal conditions, which are between 10 to 25 pg/mL (Valtonen et al., 2001). The animals supplemented with TEO presented greater concentration of melatonin in milk because, according to Hardeland et al. (2007), thyme is a melatonin-rich plant. Another factor that may have contributed to the great

concentrations of melatonin in both treatments is the longer hours in the dark between night milking (2200 h) and sampling (0700 h).

In addition to this sleep regulatory function, melatonin may also have helped to improve the mammary gland immune system, as some studies correlate the presence of melatonin, either of endogenous origin or administered as a drug, to the reduction of SCC in ruminant milk (Asher et al., 2015; Jiménez et al., 2009). Melatonin acts on the immune system by protecting it from oxidative stress, acting as an indirect antioxidant by stimulating the action of antioxidant enzymes, inhibiting pro-oxidative enzymes and being an efficient free radical scavenger (Royano and Reiter, 2010). Moreover, according to Tekbas et al. (2008), melatonin can reduce the intracellular substrates needed for bacterial growth, prolonging the lag phase and inhibiting the exponential and stationary phase, causing bacteria to enter the death phase more quickly.

The presence of thyme compounds in milk still offers the possibility of using functional milk, since some of the compounds such as melatonin can act in a beneficial way in human health.

Melatonin has a cell protection role, with relevance in neuroprotection and control of immune-inflammatory processes, also acting as the main regulator of the rhythm of vigil and sleep, being a substance that can help to solve insomnia problems and sleep disorders (Luchetti et al., 2010; Poza et al., 2018).

Due to the lack of knowledge about the behavior of the compounds present in TEO and their physical characteristics, the animals from this study were kept in the same environment, which could have masked some results due to possible inhalation of volatilized compounds from feces and urine of TEO-supplemented cows by animals from CON treatment, since, the strong smell of the essential oil of thyme was perceived when it was handled.

The strong smell given off by the thyme essential oil perceived by the handful is an example of the volatility of the thymol molecule. Thus, further studies are needed to clarify the

mechanisms of action of TEO in the animal organism, using animals in separate environments and supplementing greater dosage of TEO.

CONCLUSIONS

The supplementation of Jersey cows in the early third of lactation with 8 mL TEO/d tends to improve immune system response, controlling the incidence of mastitis and maintaining mammary gland health without altering intake, productive performance and milk quality.

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Table 1. Physical-chemical composition of thyme (*Thymus vulgaris* L.) essential oil used in current trial

Item	Results	Specifications
Appearance	Clear liquid	Clear liquid
Color	Light yellow	Clear to light yellow
Impurities	Free	Free
Odor	Characteristic	Characteristic
Density (20°C)	0.918	0.900 to 0.940
Refractive index (20°C)	1.501	1.490 to 1.510
Optical Rotation	-2.2°	-3.0° to -0.1°
Chemical composition (%)		
Thymol	42.3	
p-cyeno	27.4	
y-terpinene	8.1	
Linalool	6.7	
Carvacrol	4.0	
α -pinene	2.7	
β -caryophyllene	2.0	
Camphor	1.9	
Myrcene	1.4	
1,8-cineol	1.3	
Borneol	1.1	
Camphene	0.9	

Table 2. Chemical and energy composition of experimental ingredients and diets

Item	Concentrate ¹	Silage	Hay	TMR
Dry matter (g/kg)	952	227	954	615
Crude protein (g/kg DM)	212	94	63	171
Ether extract (g/kg DM)	16	18	5	18
Mineral matter (g/kg DM)	89	64	76	79
Neutral detergent fiber (g/kg DM)	317	628	802	446
Acid detergent fiber (g/kg DM)	90	494	564	285
TDN (%) ²	-	-	-	70
NE (Mcal/kg DM) ²	-	-	-	1.7

¹Comercial Concentrate Belman[®];

²Estimated based on NRC (2001).

Table 3. Means, standard error of means (SEM) and *P*-values for DM and nutrient intake, milk yield and milk production efficiency of Jersey cows supplemented with thyme (*Thymus vulgaris* L.) essential oil

Item	Treatments ¹		SEM	<i>P</i> -value
	CON	TEO		Treatment
Intake, kg/d				
MS	14.25	14.79	1.07	0.61
PB	2.59	2.69	0.20	0.62
EE	0.25	0.27	0.02	0.56
FDN	5.96	6.40	0.50	0.38
FDA	3.89	4.10	0.32	0.52
MM	1.11	1.16	0.09	0.60
Milk yield (kg/d)	22.41	21.69	1.45	0.68
Milk efficiency (kg/kg DMI)	1.65	1.54	0.17	0.52

¹CON = no supplementation, TEO = 8 mL supplemental thyme (*Thymus vulgaris* L.) essential oil.

†*P* < 0.05 and trends as $0.05 \leq P \leq 0.10$.

Table 4. Means, standard error of means (SEM) and *P*-values for milk quality parameters of lactating Jersey cows supplemented or not with thyme (*Thymus vulgaris* L.) essential oil

Item	Treatment ¹		SEM	<i>P</i> -value
	CON	TEO		Treatment
Fat (%)	4.13	4.35	0.14	0.22
Crude protein (%)	3.57	3.58	0.01	0.54
Defatted dry extract (%)	8.71	8.78	0.07	0.47
Lactose (%)	4.89	4.94	0.04	0.35
Density (g/dL)	1031	1031	0.25	0.66
Salts (%)	0.77	0.78	0.01	0.39
Cryoscopic index (°C)	-0.58	-0.58	0.01	0.34
Urea nitrogen (mg/dL)	15.71	17.28	1.09	0.26
Electric conductivity (mS/cm)	5.19	5.12	0.02	0.07
Somatic cell count score ² , 1 to 8	5.35	3.30	1.18	0.17

¹CON = no supplementation, TEO = 8 mL supplemental thyme (*Thymus vulgaris* L.) essential oil.

²Shook and Schutz (1994).

†*P* < 0.05 and trends as $0.05 \leq P \leq 0.10$.

Table 5. Frequency of observation of somatic cell count of milk samples in percent from lactating Jersey cows supplemented or not with thyme (*Thymus vulgaris* L.) essential oil

Somatic cell count (cells/mL)	Treatment ¹	
	CON	TEO
Above 500.000	11.7%	6.1%
200.000 to 500.000	14.8%	11.0%
Below 200.000	73.5%	82.9%

¹CON = no supplementation, TEO = 8 mL supplemental thyme (*Thymus vulgaris* L.) essential oil.

†P < 0.05 and trends as $0.05 \leq P \leq 0.10$.

Table 6. Means, standard error of means (SEM) and *P*-values for biochemical and hematological parameters of lactating Jersey cows supplemented or not with thyme (*Thymus vulgaris* L.) essential oil

Item	Treatment ¹		SEM	<i>P</i> -value	Reference values ²
	CON	TEO		Treatment	
Erythrogram					
Erythrocyte (10 ⁶ /L)	6.69	6.41	0.22	0.32	5.0 - 10.0
Hemoglobin (g/dl)	9.99	9.94	0.26	0.86	8.0 - 15.0
Hematocrit (%)	28.36	27.95	0.79	0.67	24 - 46
MCV ³ (fL)	42.68	43.58	1.25	0.55	40 - 60
MCH ⁴ (fL)	15.03	15.51	0.41	0.34	14.4 - 18.6
MCHC ⁵ (fL)	35.27	35.61	0.37	0.45	30 - 36
Platelet (×1000/mm ³)	415.3	373.66	47.96	0.47	100 - 800
Leucogram					
Leucocytes (U/μL)	1146	1102	104	0.72	4,000 - 12,000
Segmented	3114	3059	487	0.93	600 - 4,000
Eosinophils	439	580	112	0.30	0 - 2,400
Lymphocytes	6386	6324	478	0.91	2,500 - 7,500
Monocytes	125	100	031	0.50	25 - 840
Metabolite					
Plasmatic protein (g/dL)	7.14	7.29	0.10	0.22	7.0 - 8.5
Hepatic enzymes (U/L)					
ALT ⁶	19.48	17.19	1.00	0.07	11 - 40
AST ⁷	86.97	81.22	4.77	0.35	78 - 132

¹CON = no supplementation, TEO = 8 mL supplemental thyme (*Thymus vulgaris* L.) essential oil.

²Feldman et al. (2000); Kaneko et al. (2008).

³Mean corpuscular volume.

⁴Mean corpuscular hemoglobin.

⁵Mean corpuscular hemoglobin concentration.

⁶Alanine aminotransferase.

⁷Aspartate aminotransferase.

† $P < 0.05$ and trends as $0.05 \leq P \leq 0.10$.

Table 7. Means, standard error of means (SEM) and *P*-values for serum cytokines and immunoglobulins of lactating Jersey cows supplemented or not with thyme (*Thymus vulgaris* L.) essential oil

Item	Treatment ¹		SEM	<i>P</i> -value
	CON	TEO		Treatment
Cytokines				
TGF- α^2 (pg/mL)	8.97	23.73	9.08	0.20
G-CSF ³ ($\times 10^4$ pg/mL)	1710.17	844.58	767.44	0.35
IFN- γ^4 (pg/mL)	456.50	875.89	396.79	0.39
Interleukin 2 (pg/mL)	40.50	94.52	20.45	0.04
Interleukin 4 (pg/mL)	129.19	295.03	74.50	0.07
Interleukin 6 (pg/mL)	0.41	137.40	120.45	0.29
Interleukin 8 (pg/mL)	78.06	136.58	35.50	0.18
Interleukin 10 (pg/mL)	11.68	51.07	37.42	0.30
Immunoglobulins				
Immunoglobulin M (ng/mL)	6.72	6.64	0.54	0.91
Immunoglobulin A (pg/mL)	117.74	131.25	11.21	0.33
Immunoglobulin G (pg/mL)	76.70	80.62	1.84	0.09

¹CON = no supplementation, TEO = 8 mL supplemental thyme (*Thymus vulgaris* L.) essential oil.

²Transforming growth factor alpha.

³Granulocyte colony-stimulating factor.

⁴Interferon gamma.

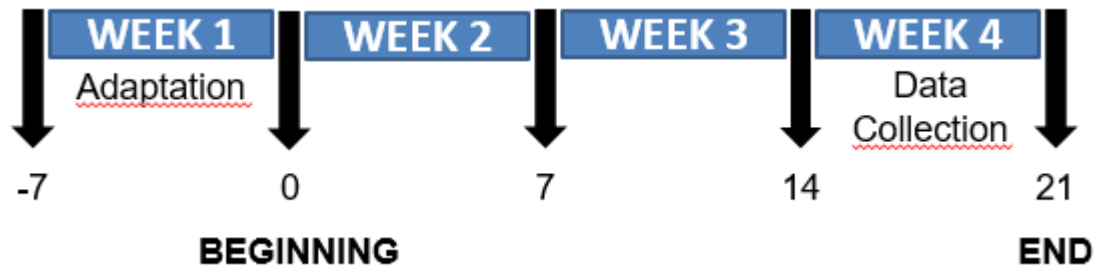
†*P* < 0.05 and trends as $0.05 \leq P \leq 0.10$.

Table 8. Means, standard error of means (SEM) and *P*-values for melatonin, prolactin and thymol concentrations in milk and blood of lactating Jersey cows supplemented or not with thyme (*Thymus vulgaris* L.) essential oil

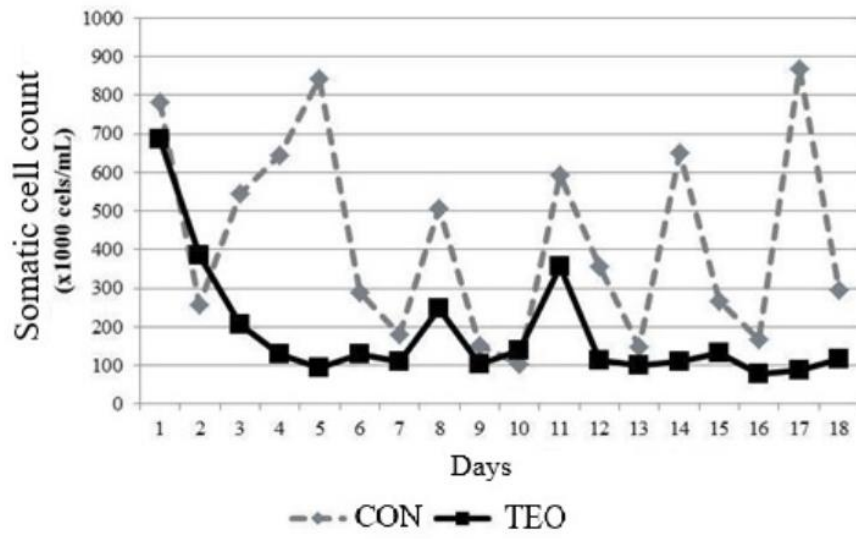
Item	Treatment ¹		SEM	<i>P</i> -value
	CON	TEO		Treatment
Thymol, plasma (ng/mL)	6.76	10.68	1.36	0.02
Thymol, milk (ng/10 mL leite)	5.93	30.03	2.40	<0.0001
Melatonin, plasma (pg/mL)	90.83	119.23	16.54	0.19
Melatonin, milk (pg/mL)	57.53	63.94	2.92	0.08
Prolactin (pg/mL)	4.06	4.08	0.13	0.89

¹CON = no supplementation, TEO = 8 mL supplemental thyme (*Thymus vulgaris* L.) essential oil.

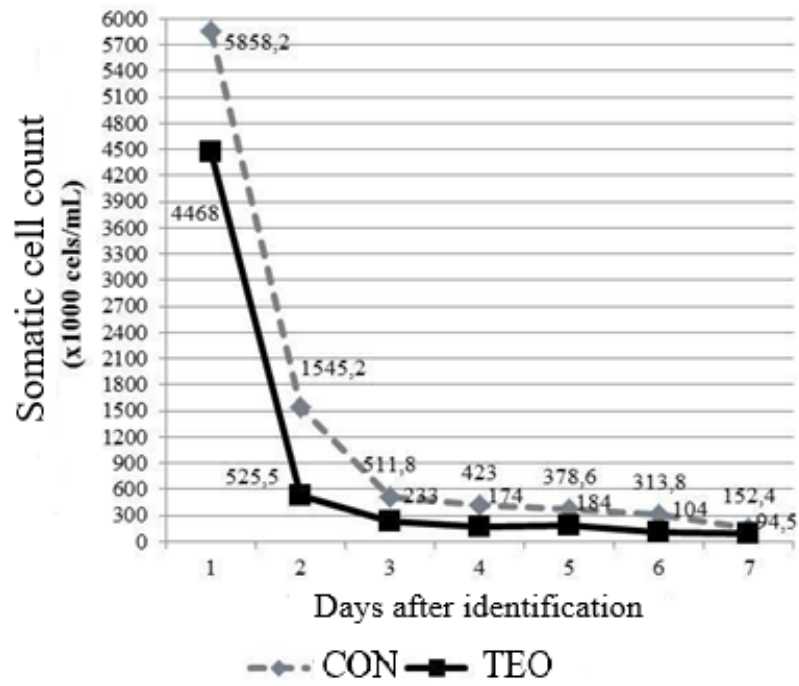
†*P* < 0.05 and trends as $0.05 \leq P \leq 0.10$.



CASTRO FILHO, Figure 1



CASTRO FILHO, Figure 2



CASTRO FILHO, Figure 3

Figure 1. Trial period scheme.

Figure 2. Effect of thyme (*Thymus vulgaris* L.) essential oil supplementation on the average daily somatic cell count of milk from lactating Jersey cows during the experimental period (n=432).

Figure 3. Average somatic cell count per treatment over 7 consecutive days after identification and treatment of clinical mastitis (n= 6 CON and 2 TEO).