

**Revista Brasileira de Higiene e Sanidade Animal**

**Print version ISSN 1981 – 2965**

**Revista Brasileira de Higiene e Sanidade Animal, 2014 Setembro; 8 (5 Supl 1): 525-546**

**<http://dx.doi.org/10.5935/1981-2965.20140110>**

**075. Stability and *in vitro* antimicrobial efficacy of a nanopropolis formulation intended for intramammary treatment of bovine mastitis<sup>32</sup>**

**Estudos de estabilidade e eficácia antimicrobiana *in vitro* de uma formulação de nanoprópolis desenvolvida para tratamento intramamário de mastite bovina**

Marcella Z. Troncarelli<sup>33</sup>, Helio Langoni<sup>34</sup>, Humberto de Mello Brandão<sup>35</sup>, Ivica Dimkić<sup>36</sup>,  
Slaviša Stanković<sup>37</sup>, Andréa Rentz Ribeiro<sup>38</sup>

---

**Abstract:** Organic dairy farms are supposed to use only phytotherapeutical and homeopatical actives for mastitis control in cows. However, there are a low number of actives available for this application. Considering the bactericide action of the propolis and the fact that this substance is allowed for use in organic dairies, Brazilian researchers developed the nanostructured propolis, a promising product for intramammary treatment against mastitis.

---

The aim of the present study was to evaluate the stability of this nanopropolis formulation [30.2 mg/mL] stored at -20°C, 8°C, 25°C and 37°C during 90 days, and also the minimum inhibitory concentration (MIC) against ATCCs and bacterial field strains isolated from clinical and subclinical bovine mastitis cases in dairy herds from Sao Paulo and Minas Gerais states, Brazil. MIC values were obtained by microdilution test, using Mueller Hinton broth and resazurin dye. Mean of MIC values of nanopropolis against all tested strains ranged from 0.02 to 0.37 mg/mL. Product *in vitro* efficacy remained stable at different temperatures during all evaluation period. The obtained stability and *in vitro* efficacy results indicate that the evaluated nanopropolis formulation may represents a new and important therapeutic alternative for mastitis control, especially in organic dairy herds.

**Keywords:** bacteria, nanopropolis, bovine mastitis, minimum inhibitory concentration, temperature.

**Resumo:** A produção orgânica de leite preconiza o uso de medicamentos a base de fitoterapia e homeopatia para o controle da mastite nos rebanhos. No entanto, existem escassas opções de ativos disponíveis para este fim. Considerando que a própolis apresenta ação bactericida e trata-se de composto permitido em sistemas orgânicos de produção leiteira, pesquisadores brasileiros desenvolveram a própolis nanoestruturada, visando o tratamento intramamário de casos de mastite bovina. O objetivo do presente estudo foi avaliar a estabilidade desta formulação de nanoprópolis [30,2 mg/mL] armazenada a -20°C, 8°C, 25°C e 37°C durante 90 dias, e também a concentração inibitória mínima (CIM) contra ATCCs e cepas bacterianas isoladas de casos de mastite bovina clínica e subclínica em rebanhos dos estados de São Paulo e Minas Gerais, Brasil. Os valores de CIM foram obtidos pelo teste de microdiluição em placa, utilizando caldo Mueller Hinton e corante resazurina. Os valores médios de CIM da nanoprópolis contra todas as cepas testadas variou de 0,02 a 0,37 mg/mL. A eficácia antimicrobiana do produto *in vitro* permaneceu estável a diferentes temperaturas

durante todo o período de avaliações. Os resultados de estabilidade e de eficácia *in vitro* indicam que a formulação de nanoprópolis avaliada poderá representar uma nova e importante alternativa terapêutica para o controle da mastite bovina, especialmente em rebanhos leiteiros orgânicos.

**Palavras-chave:** bactéria, nanoprópolis, mastite bovina, concentração inibitória mínima, temperatura.

---

Part of the first author's post-doctoral research. Grant by FAPESP (Process #2012/03212-6).

Post-doctoral fellow at Faculty of Veterinary Medicine and Animal Science (FMVZ), Sao Paulo State University (UNESP), Botucatu-SP, Brazil, and Professor at Faculty of Veterinary Medicine, Sao Paulo's University (UNIP), Bauru-SP, Brazil. [matroncarelli@yahoo.com.br](mailto:matroncarelli@yahoo.com.br) +55 (14) 3813-7010

Project Supervisor and Titular Professor at FMVZ UNESP Botucatu-SP Brazil. [hlangoni@fmvz.unesp.br](mailto:hlangoni@fmvz.unesp.br) +55 (14) 3880-2094

Researcher at Laboratory of Applied Nanotechnology for Animal Production and Health, EMBRAPA Dairy Cattle, Juiz de Fora-MG, Brazil. [humberto.brandao@embrapa.br](mailto:humberto.brandao@embrapa.br) +55 (14) 3314-7460

MSc., Research assistant, Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Serbia. [ivicad@bio.bg.ac.rs](mailto:ivicad@bio.bg.ac.rs) +381 (11) 263-7364

PhD, Associate Professor at Faculty of Biology University of Belgrade, Studentski trg 16, 11000 Serbia. [slavisas@bio.bg.ac.rs](mailto:slavisas@bio.bg.ac.rs) +381 (11) 263-7364

Associate Professor at PUC MINAS Poços de Caldas-MG Brazil. [ribeiroapuc@gmail.com](mailto:ribeiroapuc@gmail.com) +55 (35)

3729-9200

## Introduction

Mastitis is an inflammatory process of mammary gland and causes an important impact on dairy business. This pathology may occur due different factors, but is usually related with microbial infection of cows' quarters. Mastitis may cause milk production losses, early animals' culling and in some cases, acute death (LANGONI, 2013). Due the low

number of compounds allowed for bovine mastitis treatment in organic dairies, there is an important demand for researches involving new products development, specially using natural actives.

According to scientific reports, propolis is a natural product with important antimicrobial properties, and it is often tested for mastitis control in different countries. The main problem for using this product is due the fact that there are

different types of propolis that are directly related to the bees' species, flowers species and region. These differences usually interfere on propolis characteristics, and the antimicrobial efficacy results may be inconstant, due the no standardized batches. Another important point is that propolis may be irritating for bovine mammary gland, as previously reported (PEREIRA & BOTTEON, 2008).

To minimize these problems, researchers from EMBRAPA Dairy Cattle – a governmental Brazilian institution for agribusiness research, located in Juiz de Fora-MG – after many years of studies, have developed a nanostructured propolis, using the nanotechnology principle. The synthesis formulation process is already patented and provides standardized nanopropolis batches, with controlled concentration. These propolis nanoparticles are also safe for intramammary use in cows, according to our preliminar studies.

Considering the important number of organic dairies in Botucatu-SP Brazil, and the new perspective for bovine mastitis control using a natural active, researchers from Faculty of Veterinary Medicine and Animal Science (FMVZ) UNESP/Botucatu-SP and from EMBRAPA Dairy Cattle decided to establish a scientific partnership to develop the present study.

## **Material and Methods**

### **Propolis origin and nanoformulation**

A batch of green propolis from Zona da Mata region (Minas Gerais, Brazil) was synthesized by precipitation technique and interfacial deposition at EMBRAPA Dairy Cattle facilities (Patent PI 1004808-1) (BRANDÃO et al., 2010).

### ***In vitro* efficacy study**

*In vitro* efficacy studies were done for minimum inhibitory concentration (MIC) determination by microdilution test using Mueller Hinton broth and resazurin dye (NCCLS, 2006; STANKOVIĆ et al., 2013). ATCCs of *Staphylococcus aureus*

(25923; 23235D; 14458B; 13565A; 13565B); *Escherichia coli* (11229; 00218), *Streptococcus agalactiae* (ATCC 13813); *Enterococcus faecalis* (00531) (TRONCARELLI, 2011) were tested. A total of 110 bacterial strains isolated from bovine mastitis cases were also tested: 20 *Staphylococcus aureus*; 20 CNS; 20 *Escherichia coli*; 20 *Corynebacterium bovis*; 10 *Streptococcus agalactiae*; 10 *Streptococcus uberis*; 10 *Streptococcus dysgalactiae*. These strains were isolated from subclinical and clinical mastitis cases

### **Stability studies**

Nanopropolis formulation [30.2 mg/mL] was aliquot in sterile 1.5 mL microtubes and stored at different temperatures: Group I: -20°C; Group II: 8°C; Group III: 25°C and Group IV: 37 °C. Immediately before the storage, nanopropolis MIC value against *S. aureus* (ATCC 25923) was evaluated by

in dairy herds from Botucatu-SP and from Poços de Caldas-MG Brazil. All ATCCs and bacterial strains inoculums were adjusted at 0.5 McFarland scale (approximately 10<sup>8</sup> CFU/mL), and their concentrations were confirmed by culture in Mueller Hinton agar for CFU's plate counting (QUINN et al., 2005). A natural green propolis solution was used as antimicrobial positive control, and methanol was used as a positive control of solvent.

microdilution test. New MIC tests were done after 3, 7, 14, 21, 30, 60 and 90 days of storage. Visual characteristics of product after storage were also registered.

### **Results and discussion**

The results regarding nanopropolis' MIC values against ATCCs and field strains are shown on Table 1.

**Table 1.** Mean values of minimum inhibitory concentration (MIC) of a nanopropolis formulation [30.2 mg/mL] against ATCCs and bacterial strains isolated from bovine mastitis cases. Botucatu-SP, Brazil, 2014.

Bacterial strains	n	Isolates characteristics	MIC (mg/mL)
SAU	5		0.37
SAGA	1		0.18
ECOL	2	ATCCs <sup>1</sup>	0.04
ENT	1		0.02
SAU	20		0.37
SUBE	10		0.37
SDYS	10		0.37
CNS	20	Field strains <sup>2</sup>	0.18
SAGA	10		0.18
ECOL	20		0.09
COR	20		0.02

SAU= *S. aureus*; ECOL= *E. coli*; SAGA= *S. agalactiae*; ENT= *E. faecalis*; CNS= coagulase negative staphylococci; COR= *C. bovis*; SUBE= *S. uberis*; SDYS= *S. dysgalactiae*.

<sup>1</sup> From “Coleção de Culturas - Instituto Adolfo Lutz de São Paulo” and from “Instituto Nacional de Controle de Qualidade em Saúde do Rio de Janeiro”.

<sup>2</sup> From bovine mastitis cases isolated in dairy herds in Sao Paulo and Minas Gerais states, Brazil.

Obs.: The natural propolis used as positive antimicrobial control showed medium MIC = 0.09 mg/mL for all tested samples.

These results show an efficient *in vitro* antimicrobial activity of the tested nanopropolis against the main species of Gram-positive and Gram-negative bacteria that are usually involved on bovine mastitis cases. These data reinforces the

expectations of using nanopropolis for mastitis treatment, especially considering that - differently to the presented results - the antimicrobial activity of the natural propolis is being reported as satisfactory for Gram-positive bacteria, but regular for

Gram-negative (STANKOVIĆ et al., 2013).

Stability studies results showed no interference on MIC values when nanopropolis aliquots were stored at 8°C, 25°C and 37°C. The initial MIC (0.37 mg/mL) remained unaltered during all

Considering the macroscopic characteristics, it is important to point out that all aliquots stored at the different temperatures produced a small pellet (2 mm) when resting. This pellet was easily removed during homogenization by vortex, for the aliquots stored at 8°C, 25°C and 37°C. However, the same was not observed for the aliquots stored at -20°C. After thawing, the aliquot was observed as a non homogeneous solution, despite its shaking.

These data indicate that the evaluated propolis nanoparticles are stable at room temperature, with no interference on its antimicrobial properties. This characteristic is very important considering the perspective for future product's

evaluation period (90 days). For the -20°C stored nanopropolis, on the first evaluation period (D3 post storage), the antimicrobial activity showed a little decrease, with 0.75 mg/mL MIC. However, this MIC value remained the same until the end of evaluation period. industrial manufacturing, storage and handling processes.

### **Conclusions**

The efficient *in vitro* antimicrobial activity and the satisfactory stability results regarding the evaluated nanopropolis reinforce the expectations for its use on bovine mastitis control. Efficacy studies in dairy herds are being done and the results will be presented soon.

### **Aknowledgements**

We thank to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for the post-doctoral Grant (Process 2012/03212-6).

### **References**

BRANDÃO, H. M.; VINHOLIS, M. M. B.; MOSQUEIRA, V. C. F.; MATTOSO,

L. H. C.; BRITO, M. A. V. P.; RIBEIRO, C.; SOUSA, R. V.; BARBOSA, N. R.; LANGE, C. C. Composições baseadas em nanopartículas de própolis, processos de obtenção e uso. 2010. Patente: Privilégio de Inovação n.01210001131, Confidencial. 27 de Outubro de 2010.

LANGONI, H. Qualidade do leite: utopia sem um programa sério de monitoramento da ocorrência de mastite bovina. *Pesq. Vet. Bras.* v.33, n.5, p.620-626, 2013.

NCCLS. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, approved standard – seventh edition. M7-A7, v.26, n.2, 2006.

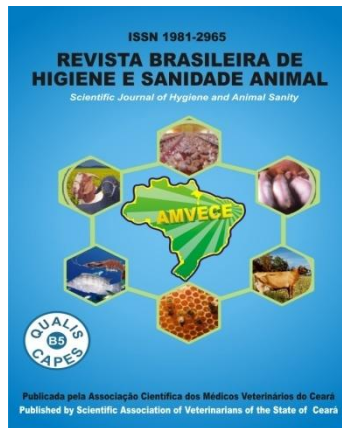
PEREIRA, L.S.; BOTTEON, R.C.C. M. Efeito da aplicação intramamária de própolis sobre a composição do leite e a contagem de células somáticas. CONBRAVET 2008. Available in: <http://www.sovergs.com.br/conbravet2008/anais/cd/resumos/R0345-1.pdf>

QUINN, P.J; MARKEY, B.; CARTER, M.E.; DONNELLY, W.J.; LEONARD, F.C. *Microbiologia Veterinária e Doenças Infecciosas*. Ed. Artmed. Porto Alegre, 2005. 512p.

STANKOVIĆ, S.; DIMKIĆ, I.; RISTIVOJEVIC, P.; BERIĆ, T.; DRAGANIĆ, D.V.; FIRA, Đ. 2013. Bacteriostatic and bactericidal effect of serbian propolis against pathogenic bacteria. 5<sup>th</sup> FEMS Congress of European Microbiologists, Leipzig, Germany, USB Drive Abstract Book, 2353.

TRONCARELLI, M.Z. Padronização da técnica de multiplex PCR para a detecção de *Staphylococcus aureus*, *Streptococcus agalactiae* e *Escherichia coli* em amostras de leite bovino, obtidas de tanques de expansão. Botucatu-SP, 2011. 129p. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.





# **REVISTA BRASILEIRA DE HIGIENE E SANIDADE ANIMAL**

**Brazilian Journal of Hygiene and Animal Sanity**

**RBHSA**

**2014 setembro; 8(5 Supl 1): 001- 085**

**Associação Científica dos Médicos Veterinários do Ceará**

**ISSN Eletrônico 1981-2965 Online e prefixo DOI 10.5935**

**Fortaleza - CE – Brasil**



**Suplemento:  
Anais do II Simpósio de Qualidade de Leite – SQL - UNESP  
Jaboticabal – SP  
5 a 7 de setembro de 2014.**

**ANAIS DE RESUMOS EXPANDIDOS**

**Local:  
Faculdade de Ciências Agrárias e Veterinárias – FCAV/Unesp –  
Câmpus de Jaboticabal**