Animal Health

Original

Antibacterial Activity of Propolina® against Subclinical Mastitis-Causing Bacteria

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Received: September, 2022; accepted: October, 2022; Published: November, 2022.

ABSTRACT

Background: The final antisepsis of nipples is performed with aqueous solutions containing Propolina® to prevent mastitis. The aim of this paper was to determine the antimicrobial activity of different concentrations of Propolina® against mastitis-causing microorganisms. **Materials and Methods:** The microorganisms were isolated from the milk of cows having three mastitis crossings. The antimicrobial activity of aqueous Propolina® solutions between 2.5 and 60.0 mg.ml^{-1} was tested against isolated bacteria and Gram-negative *Escherichia coli* strain ATCC 25922 by spraying the solution in plate wells containing agar. A one-way ANOVA was used to determine the effect of a Propoline dose on the bacterial species associated with mastitis; the Duncan's multiple rank-sum test was used for dose comparisons. **Results**: The Gram-positive bacteria were identified: *Staphylococcus aureus,* coagulase-negative *Staphylococcus* sp. and *Corynebacterium* sp., which were sensitive to the Propolina® solutions, while Gram negative *Escherichia coli* strain ATCC 25922 showed no sensitivity. The 60, 30, and 20 mg.ml^{-1} concentrations were more effective against *Staphylococcus aureus* and coagulase-negative *Staphylococcus* spp. **Conclusions**: *Staphylococcus aureus,* coagulase-negative *Staphylococcus* sp., and *Corynebacterium* sp. were isolated and identified, as causing subclinical mastitis. The Propolina® solutions were highly effective against Gram positive bacteria *Staphylococcus aureus* and coagulase-negative *Staphylococcus* sp., which were sensitive to the 20, 30, and 60 mg.ml^{-1}, and 30 and 60 mg.ml^{-1} concentrations of Propolina®, respectively.

Keywords: antimicrobial activity, propolis, *Staphylococcus* spp. *(Source: BVS)*

Citations (APA)
Valdivia Avila, A., Rubio Fontanills, Y., Martínez Mora, M., Garrote Pérez, M., Pérez Hernández, Y., & Matos Trujillo, M. (2022). Antibacterial Activity of Propolina® against Subclinical Mastitis-Causing Bacteria *Journal of Animal Prod.*, *34*(3). https://revistas.reduc.edu.cu/index.php/rpa/article/view/e4293

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INTRODUCTION

Mastitis is the inflammation of the mammary gland, which causes significant economic losses in cattle herds (Ramírez *et al.,* 2018). It is classified as clinical and subclinical. The clinical form shows signs, and changes the chemical composition of milk. The subclinical form is more common than the former, but the symptoms and changes milk appearance are hard to observe (Benić *et al.,* 2018).

This disease is considered a major public health problem that causes a drop in milk quality and quantity (Andrade *et al.,* 2017; Quevedo, 2018). It can change its protein and lipid composition (Valdivia *et al.,* 2020), favor the excretion of microorganisms into the milk, and affect its safety (Romero *et al.,* 2018).

Bovine mastitis usually derives from infections that may be caused by approximately 150 microbial species (Benić *et al.,* 2018). Its prevention may depend on the hygiene of the areas where the animals live and are milked. Proper nipple disinfection or sealing after milking is one of the most important steps to prevent the occurrence of the disease. It consists in the application of an antiseptic solution to the breasts upon the withdrawal of the teat cups. The purpose of this practice is to reduce the penetration and multiplication of infectious and environmental pathogens that remain on the skin and nipple tip following milking, in order to prevent their entry and multiplication in the nipple channel (Blanco and Montero, 2018). Accordingly, the Matanzas Cattle Genetic Project (EPGM) applies Propolina®, an aqueous solution based on propolis.

Propolis is a honeybee-made product with a growing interest in the scientific community. It has a complex composition and a broad spectrum of actions. Its antimicrobial properties have been described against Gram positive and Gram-negative bacterial strains (Przybyłek *et al.,* 2019). However, its effectiveness depends on the particular microbial species, which may also vary over time due to several environmental factors, and the product's concentration. The results indicate that Propolina® is a recommendable alternative to prevent mastitis. The utilization of propolis solutions against microorganisms permit the inclusion of safe natural products to fight antibiotic-resistant microorganisms (Fernández-León *et al.,* 2022). Accordingly, this paper aims to determine the antimicrobial activity of different Propolina® concentrations against subclinical-mastitis causing microorganisms.

MATERIALS AND METHODS

Milk sample collection

The samples were collected on a dairy farm from EPGM, in May 2019. Milking takes place twice a day (4:00 am and 4:00 pm) using mechanical milking (Eurolate). The cows are Mambi feeding on African Bermuda grass [*Cynodon nlemfuensis* (Van-deryst)], forage King grass CT-115 (*Cenchrus purpureus* Schumach Morrone), milled sugarcane (*Saccharum officinarum*), feedstuffs for dairy cows, and mineral salt *ad libitum.*

A total of ten samples of milk were collected by animal with no clinical signs of mastitis or changes in milk properties. The samples were collected through hand milking of previously-positive diagnosed quarts, from three crosses of subclinical mastitis, through the California Mastitis test (CMT). The udders were washed with running water and then they were disinfected using sterile cotton dampen in 70% ethanol. The samples consisted of approximately 5 ml of milk were collected after releasing the first jets of milk, and were conserved in ice and taken to the microbiology laboratory from the Faculty of Agronomy at the Matanzas University for processing.

Isolation of mastitis-causing bacteria

A total of 100 µl from every milk sample were poured on plates containing nutrient agar medium by spreading with a Drigalski spatula. The plates were incubated for 24 h at 37 °C in aerobiosis conditions. Then, the colonies with different culturing conditions were selected and purified using the streak technique on plates containing nutrient agar, using a platinum wire. The isolated strains were stored in nutrient agar at 4 °C, and glycerol at -20 °C.

Biochemical tests and identification of mastitis-causing bacteria

The conserved strains were added to 3 ml of nutrient broth and were incubated at 37 °C for 24 hours. Then they were placed in vials containing nutrient agar and stored as in the previously described conditions. The catalase, saline mannitol, starch hydrolysis, and coagulase biochemical tests were performed according to the procedure described by MacFaddin (2006).

Gram staining was made from young cultures of the strains in nutrient agar, using the Madigan *et al.* (2015) method. (2015). The presence of endospore was determined using the same procedure.

***In vitro* evaluation of antimicrobial activity of different Propolina® concentrations**

The microbial studies were conducted using Propolina® from the Company of Manufacturing and Sales of Biopharmaceuticals in Matanzas (GENIX®, LABIOFAM), containing 60 mg ml^{-1} of bioactive substances. The aqueous solutions of the product had the following concentrations: 2.5; 5.0; 7.5; 20.0, and 30.0 $mg \cdot ml^{-1}$.

The evaluation of antimicrobial activity of the five dilutions and Propolina® (60.0 $mg.ml^{-1}$) was performed by agar-well diffusion (Pérez *et al.,* 2020). The control consisted of a 10% hydroalcoholic solution. The evaluation was performed against the microorganisms isolated from the milk samples of the cows with a diagnostic of subclinical mastitis and a Gram-negative strain of *Escherichia coli* ATCC 25922. It was included to corroborate the effectiveness of the product against microorganisms with that pattern under Gram staining.

The microorganisms used in the assay were grown at 37 °C for 24 h in Müeller-Hinton agar medium. Then, the isolated colonies were placed in vials containing 9 ml of Müeller-Hinton broth and were grown to a 0.5 concentration equal, according to the McFarland scale. Then they were added to plates containing Müeller-Hinton agar, using a sterile needle. The wells were filled using a sterile needle, adding 200 µl of every solution for evaluation. The plates were stored at 4

°C for 15 minutes, and then they were incubated at 37 °C for 18 h (Pérez, 1990). Three replicas were made per strain.

Growth inhibition was calculated using the following formula: I = IAD – WD

Where: IAD was the inhibition area diameter, and WD was the well diameter; the two measurements were made using a gauge caliper.

The Duraffourd (Duraffourd *et al.,* 1987) scale was used to perform the qualitative evaluation of inhibition, which relied on the following criteria:

- Null (-), when the inhibition halo diameter is lower than 8 mm.
- Limit sensitivity (sensitive = +), when the inhibition halo is between 8 and 14 mm.
- Medium (very sensitive = ++), when the diameter is 14-20 mm.
- Highly sensitive (+++), when the diameter is above 20 mm.

Statistical analysis

The data were processed through the Statgraphics Plus 5.0 software for Windows. First, the data were adjusted to a normal distribution, and variance homogeneity through the goodness of fit (Chi square), and the Bartlett test, respectively. A simple analysis of variance and the Duncan's Multiple Rank ($P<0.05$) tests were performed for mean n comparisons.

RESULTS AND DISCUSSION

Isolation of mastitis-causing bacteria

A number of 15 Gram-positive bacterial strains were isolated from the milk samples of previously-diagnosed cows with subclinical mastitis. Three of them were selected, depending on their morphological traits of their colonies and their response to Gram staining, for identification. The following microorganisms were isolated: *Staphylococcus aureus,* coagulase negative *Staphylococcus* sp. and *Corynebacterium* sp.

Staphylococcus aureus is one of the main pathogens linked to the presence of contagious subclinical mastitis in herds (Jurado-Gámez *et al.,* 2019). This microorganism and *Enterobacter* spp. were identified as the agents causing the disease on a dairy farm at Los Naranjos Cattle Genetic Project, in the municipality of Caimito, Artemisa, Cuba (García-Sánchez *et al.,* 2018). Its isolation from the milk the risk of the presence of enterotoxins in this and other dairies that may cause gastroenteritis and skin infections in humans (Benić *et al.,* 2018; NiolaToasa *et al.,* 2020).

The coagulase negative *Staphylococcus* sp. strains and *Corynebacterium* sp. isolated in this study were also described as causing mild or subclinical infections (Sánchez Bonilla *et al.,* 2018). Coagulase negative *Staphylococcus* sp. is thought to be an emerging pathogen causing mastitis worldwide.

Corynebacterium spp. is among the contagious environmental pathogens that cause mastitis (Andrade *et al.*, 2017; Hahne *et al.*, 2018). Its presence in herds may point to inappropriate nipple disinfection (Callejo, 2010). This bacterial genus was also diagnosed in cases of the disease in goats, by Nabih *et al.* (2018).

The three microorganisms isolated in this study were classified as Gram-positive. However, the literature consulted shows that the disease may also be caused by Gram-negative microorganisms. Among them, *Escherichia coli* is one of the most frequent isolates (Fiordalisi *et al.*, 2016; Andrade *et al.*,2017).

In vitro evaluation of antimicrobial activity of different Propolina® concentrations against Gram-positive and Gram-negative bacteria

Considering the results of the previous experiment, the antimicrobial activity of Propolina® (2.5 – 60.0 mg·ml^{-1}) against wild strains isolated from cows with subclinical mastitis was evaluated *in vitro. E. coli* ATCC 25922 was also included in the study. Based on the statistical analysis, data normality was accepted with a p value of 0.0808. Similarly, the data variance homogeneity (null hypothesis) was accepted, as the p value was 0.163.

Table 1 shows the statistical results of the simple analysis of variance performed to each microbial group. As shown, p for every microbial group was lower than 0.05, so there were significant differences between the several doses evaluated for each microorganism.

Table 1. Statistical results of the simple analysis of variance performed to each microbial group

Microbial group	N	gl	Mean square	F	Sig.
Staphylococcus aureus	21	6	66.08	173.46	0.0000
Coagulase negative *Staphylococcus* spp.	21	6	110.38	193.17	0.0000
Corynebacterium sp.	21	6	40.60	1107.36	0.0000

Table 2 shows the results of the antibacterial effect of the concentrations of Propolina® used in the research, against the wild strains of Coagulase negative *Staphylococcus* sp. and *Corynebacterium* sp. In the former, inhibition was reached with the 60,0 mg ml^{-1} concentration, followed by the 30.0, 20.0, and 7,5 mg ml^{-1} concentrations, respectively, whereas the 5.0 and 2,5 mg ml^{-1} doses showed no inhibitory activity, with similar statistical values to the hydroalcoholic solution used as the control.

Propolina® was also effective against the isolated strain of *S. aureus.* All the concentrations evaluated evidenced an antibacterial effect when the results were compared to the control hydroalcoholic dissolution. The highest inhibiting values were observed with the 60.0 mg ml^{-1} concentration, followed by the 30.0 and 20.0 mg ml^{-1} concentrations, respectively. The concentrations between 7.5 and 2.5 mg ml^{-1} showed similar values, below 20.0 mg ml^{-1}, though higher than the control.

According to the Duraffourd scale, the *S. aureus* strain isolated was sensitive to Propolina® concentrations higher than 7,5 mg ml^{-1}, and very sensitive to the maximum dose of 60 mg ml^{-1}, and the *c*oagulase negative *Staphylococcus spp* strain was sensitive to the 30 mg ml^{-1} concentration, and very sensitive to the 60 mg ml^{-1} concentration.

Table 2. Antibacterial activity of different Propolina® concentrations against *Staphylococcus aureus, coagulase negative Staphylococcus* pp. and *Corynebacterium* sp.

Propolina® (mg·ml^{-1})	*Staphylococcus aureus*			Coagulase negative *Staphylococcus* spp			*Corynebacterium sp.*		
	I (mm)	± SE	S	I (mm)	± SE	S	I (mm)	± SE	S
2.5 mg·ml^{-1}	6.33^{d}	0.33	-	0.00^{f}	0.00	-	1.9^{b}	0.22	-
5 mg·ml^{-1}	7.33^{d}	0.33	-	0.66^{ef}	0.33	-	1.7^{b}	0.22	-
7.5 mg·ml^{-1}	6.66^{d}	0.33	-	3.33^{d}	0.33	-	0.86^{c}	0.22	-
20 mg·ml^{-1}	8.66^{c}	0.33	+	5.66^{c}	0.88	-	0.86^{c}	0.22	-
30 mg·ml^{-1}	11.0^{b}	0.00	+	8.33^{b}	0.33	+	0.86^{c}	0.22	-
60 mg·ml^{-1}	15.33^{a}	0.66	++	17.66^{a}	0.33	++	10.83^{a}	0.22	+
Alcohol (10%) Control	0.00^{e}	0.00	-	1.66^{e}	0.33	-	0.83^{c}	0.22	-

I: inhibition area diameter. The data represent the means of three repetitions. Different scripts indicate a significant difference, according to Duncan (P≤0.05). Sensitivity (S) was indicated according to Duraffourd (-): null, (+): sensitive, (++): very sensible. ± SE: standard error.

The results are in keeping with the reports from other authors, who observed antibacterial effects of propolis against *Staphylococcus* spp. Fiordalisi *et al.* (2016) studied the antibacterial effect of different concentrations of propolis against *S. aureus.* These researchers observed a reduction of bacterial growth in 750-1.000 μg·ml^{-1} concentrations, which was associated with different components of propolis, particularly flavonoids, esthers, and terpenoid compounds. Navarro López *et al.* (2018), also linked the antibacterial action of propolis to the presence of flavonoids, esthers, sesquiterpenes, fatty acids, hydroxy acids, and other compounds, like acacetin and galanin, whose significant biological activity is produced thanks to their synergism.

Similar *in vitro* studies of microbial activity using a Chilean propolis against the *Staphylococcus aureus* (ATCC 33862) strain showed sensitivity to concentrations higher than 1 mg.ml^{-1} (Balboa *et al.,* 2018). Rodríguez *et al.* (2020), evaluated the effectiveness of propolis from different regions of Mexico against this bacterial species. They determined minimum inhibitory values between 0.19 and 15.0 mg·ml^{-1}, and minimum bactericidal concentrations between 0.37 and 30.0 mg·ml^{-1}. Suran *et al*. (2015) reported minimum inhibitory values against *Staphylococcus aureus* between 16 and 64 mg·ml^{-1}, using non-alcoholic propolis solutions.

A study on the effect of ethanolic extracts of propolis on *Staphylococcus* spp from goats with mastitis, demonstrated the effectiveness of these extracts of over 72.67% (Santos *et al.,* 2020). In this research paper, *Staphylococcus aureus* demonstrated sensitivity to the 20, 30, and 60 mg·ml^{-1} Propolina® concentrations, whereas the coagulase negative *Staphylococcus* sp strain showed sensitivity against 30 and 60 mg·ml^{-1}.

Table 2 shows the effect of Propolina® against *Corynebacterium* sp. As shown, the inhibitory effect was achieved only using the maximum concentration (60 mg.ml^{-1}). *Escherichia coli* ATCC 25922 was considered resistant, since it showed no sensitivity to any of the doses evaluated.

The antibacterial activity observed in this research study with the use of Propolina®, is in accordance with results achieved by other researchers. Klhar *et al.* (2019) challenged an ethanolic extract of propolis at 300 mg·ml^{-1} to Gram-positive and Gram-negative bacteria, and showed that the highest inhibition levels were observed against the Gram-positive bacteria.

The results reported in relation to the use of Propolina dissolutions against *Corynebacterium* sp. may be associated with the structural characteristics of the cellular wall, which has a complex architecture, as these bacteria are coated by a layer of peptidoglycan with a covalent bond to a layer of arabinogalactan. Moreover, there is a layer of mycolic acids, which is functionally equivalent to the LPS layer of the Gram-negative bacteria. Furthermore, there is an outer layer made of polysaccharides, glycolipids, and proteins (Burkovski, 2013). It has been described that one of the intrinsic resistance mechanisms by means of which bacteria become less susceptible to disinfectants, is the formation of biofilm, which is common in this genus (Rozman *et al.*, 2021; Souza *et al.*, 2020).

Fiordalisi *et al.*, (2016) also studied the antimicrobial properties of Brazilian propolis from different areas, against *Staphylococcus aureus* and *E. coli.* In the latter, they found some efficacy, which was linked to the chemical complexity of the cellular walls of the Gram-negative bacteria. Similar results were observed upon the evaluation of Mexican propolis ethanolic extracts against these two microorganisms (Rodríguez *et al*., 2020). The authors were able to inhibit *Staphylococcus aureus* growth, but no effectiveness was observed against *Escherichia coli.*

CONCLUSIONS

Different microbial genera were isolated and identified as causing subclinical mastitis, such as *Staphylococcus aureus*, coagulase negative *Staphylococcus* sp., and *Corynebacterium* sp. The solution of Propolina® showed greater effectiveness against Gram-positive microorganisms, like *Staphylococcus aureus* and Coagulase negative *Staphylococcus* sp., which were sensitive to the 20, 30, and 60 mg·ml^{-1} and the 30 and 60 mg·ml^{-1} concentrations of Propolina®, respectively.

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AUTHOR CONTRIBUTION STATEMENT

Research conception and design: ALVA, YRF, MM.MM, MGP, YPH, MMMT; data analysis and interpretation: ALVA, YRF, MM.MM, MGP, YPH, MMMT; redaction of the manuscript: ALVA, YRF, MM.MM, MGP, YPH, MMMT.

CONFLICT OF INTEREST STATEMENT

The author declares the existence of no conflicts of interests.