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Article

Antimicrobial effectiveness of wild-type *Bacillus* spp compared to *Bacillus subtilis* ATCC 6051 against *Escherichia coli* isolated from bovine mastitis

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ABSTRACT

Bovine mastitis, a condition significantly impacting dairy production, represents a significant source of spread for antimicrobial-resistant bacterial agents. This study evaluated the antimicrobial effectiveness of two strains of *Bacillus*, *Bacillus subtilis* ATCC 6051 and wild-type *Bacillus* spp., against *Escherichia coli*, the causative agent of bovine mastitis. Using the agar well diffusion method and considering variables such as the type of *Bacillus* and the pathogenic bacteria, the results indicated that *Bacillus subtilis* ATCC 6051, through direct diffusion, exhibited an average inhibition zone of 16.60 mm against *E. coli*, surpassing the filtrate diffusion method. In comparison, wild-type *Bacillus* spp. showed lower inhibition measures. The growth curve revealed that *Bacillus subtilis* ATCC 6051 has a more significant growth capacity in the exponential phase, attributable to differences in metabolic capacity. In conclusion, *Bacillus subtilis* ATCC 6051 demonstrated remarkable antimicrobial capacity against the studied pathogen, suggesting its potential application in bovine mastitis control.

Keywords: Mastitis, Kirby-Bauer, Diffusion, *Bacillus, Escherichia coli*.

INTRODUCTION

The udder health in dairy cows is essential for economic production, as any threat, such as mastitis, can directly impact the performance and well-being of the animals. Despite the physiological importance of the mammary gland in colostrum and milk production, decades of genetic selection have increased susceptibility to this infection[1].

Mastitis, characterized as an inflammatory response to microbial invasion, can be classified as subclinical or clinical. Without visible signs, the former affects the quality and quantity of milk produced and is diagnosed using molecular methods or somatic cell count[2]. Clinical mastitis, on the other hand, manifests noticeable changes in milk and the mammary gland. Depending on the pathogen, its management involves using antibiotics or therapeutic alternatives such as phytotherapy or probiotics[3],[4].

In production systems with environmental deficiencies, coliform mastitis, especially Escherichia coli (E. coli), poses a significant risk, potentially leading to mortality due to the production of endotoxins[5]. *Due to poor hygiene and favorable environmental conditions, E. coli, a Gram-negative enterobacteria, affects postpartum cow*s. Outbreaks are common in the first 30 days of lactation and cause inflammatory alterations in the udders and milk.[6].

The diagnosis of mastitis involves the physical-clinical evaluation of the udder, inspection of milk for possible abnormalities, and the use of tests such as Somatic Cell Count (SCC) and the California Mastitis Test (CMT)[7]. The electrical conductivity of milk is altered during intramammary infections, indicating elevated SCC values (>250,000/mL)[8].

Culture and antibiotic susceptibility testing is crucial for identifying the pathogen, guiding tailored treatments, and selecting effective therapies. [9],[10],[11]. Contagious mastitis, which spreads rapidly, can manifest subclinically or clinically, affecting mammary health and facilitating new infections. [12],[13],[14]. Environmental mastitis, common in postpartum animals, originates from the environment, requiring control measures such as milking hygiene and proper environmental management [15],[16].

The etiology of mastitis is multifactorial and involves Gram-positive and Gram-negative bacteria, viral, fungal, and algal agents, along with environmental factors[17]. Understanding these complexities is vital for developing effective control and treatment strategies against this disease, affecting dairy production and dairy cows' well-being.

Regarding mastitis treatment, a dilemma arises regarding the use of antibiotics. Excluding them may not adversely affect many cases but could harm the cow and the operation. The effectiveness and choice of antibiotics depend on the pathogen. In mild cases of E. coli or when bacterial growth is absent, their use is discouraged[18].

In antimicrobial susceptibility testing methods, diffusion and membrane filtration methods are employed. The diffusion technique involves antibiotic-impregnated discs on Müeller Hinton agar, measuring inhibition zones, interpreted according to CLSI guidelines[19]. On the other hand, membrane filtration methods filter microorganisms from samples using porous membranes, facilitating their study by separating components according to pore size and requirement[20],[21].

In this context, the present study aims to evaluate and compare the antimicrobial efficacy of wild strains of Bacillus spp. against Bacillus subtilis ATCC 6051, specifically against strains of Escherichia coli isolated from cases of bovine mastitis. It focuses on understanding the complexity of the disease, evaluating diagnostic methods, and contributing knowledge to improve strategies in dairy production.

MATERIAL AND METHODS

This study was conducted in the Microbiology area of the General Laboratory of the Faculty of Agricultural Sciences at the State University of Bolívar. Strains of Bacillus spp. and Bacillus subtilis ATCC 6051 were used as antagonistic agents against strains of E. coli isolated from intramammary infections (mastitis).

Study factors

The study factors used were: Factor A: (A1 = Direct diffusion method, A2 = filtrate diffusion method); Factor B: (B1 = Bacillus subtilis strains 100 µL, B2 = Wild-type Bacillus spp strains 100 µL); in combination, they yielded the treatments shown in the table below (Table 1).

Treatments	Interaction	Description
0	-	Neomycin discs + pathogenic isolates
1	a1b1	Direct diffusion + *Bacillus subtillis* strains
2	a1b2	Direct diffusion + *Bacillus spp* strains wild-type
3	a2b1	Filtrate diffusion + *Bacillus subtillis* strains
4	a2b2	Filtrate diffusion + *Bacillus spp* strains wild-type

Table 1. Description of the interaction of factors and treatments under study.

A design with a factorial arrangement of two factors was developed to observe the behavior in antimicrobial assays.

Reanimation of the bacteria under study:
The strains used were obtained from the laboratory's strain collection of the Faculty of Agricultural Sciences. These strains were exposed to room temperature and then incubated at 37°C for 1 hour to ensure proper thermal equilibration. Subsequently, they were revitalized by suspending 100 µL in MR-VP broth and incubating them for 24 hours at 37°C. Bacillus subtilis ATCC 6051 was obtained from MEDIBAC and revitalized according to the distributor's instructions. Secondary cultures were performed on various media after 24–48 hours of incubation at 37°C, ensuring revitalized strains for microbiological studies.

Microbial filtration.
To obtain the microbial filtrate, 1000 µl of the initial bacterial culture in Bacillus spp. wild-type and Bacillus subtilis ATCC 6051 broth (MRVP and MRS) were taken, separating 1 mL into Eppendorf vials. Subsequently, each vial was centrifuged at 6000 rpm for at least 5 to 8 minutes, followed by filtration of the liquid package using a Millipore circuit using a 0.22 µm microfiltration membrane (Millipore).

Susceptibility analysis.
The susceptibility of the strains was analyzed using two Kirby-Bauer methods: Direct diffusion and Filtrate diffusion in Broth. The agar well diffusion method was carried out on Müeller-Hinton agar with E. coli strains adjusted to 0.5 McFarland. It was inoculated into 6 mm wells using a glass microtube, depositing 100 µL of wild-type Bacillus spp. and Bacillus subtilis ATCC 6051 for direct diffusion and filtrate diffusion methods, respectively. After 10 minutes at 2-4°C, they were incubated for 24 hours at 37°C to evaluate the antimicrobial response.

Measurement of the growth curve of Bacillus spp. wild type and Bacillus subtilis ATCC 6051™.
A nanodrop spectrophotometer measured the absorbance (ABS%) in bacterial cultures (wild-type Bacillus spp. and Bacillus subtilis ATCC 6051), acting as antagonistic agents against E. coli. The aim was to evaluate which "beneficial" bacterial genus showed more efficient growth kinetics in its phases, considering the incubation time. It began with sterile culture broths (MRS and MR-VP Broth), inoculated with Bacillus, and pre-incubated to determine maximum absorbance (ABS%). Growth-free cultures were also used to calibrate the spectrophotometer, establishing measurements every 4 to 28 hours.

Resuscitation of Bacillus spp*:* it is an independent variable, which was subjectively measured based on the type of Gram-positive Bacillus considered for susceptibility testing, as it was performed using Bacillus subtilis ATCC 6051 and wild-type Bacillus spp. by growing them in MRS medium.

Three variables were evaluated in the study: "Inhibitory effect," such as the ability of Bacillus to inhibit pathogenic growth, recording the diameters of inhibition zones. "Susceptibility," comparing these zones with cutoff points defined by Mitov[22]: ≤12 mm (resistant), 13–17 mm (moderately susceptible), and ≥18 mm (susceptible). The "Growth curve" was measured using spectrophotometry at 0, 4, 8, 12, 16, 24, and 28 hours, evaluating absorbance (ABS%) at 560-600 nanometers, calibrated to a factor of 0.9[23].

Statistical analysis

The results were operationalized using the SPSS statistical program, which applied the three-factor design tools.

RESULTS AND DISCUSSION

Resuscitation of Bacillus spp strains. wild type

Bacillus spp. Strains were selected and isolated from bovine mastitis in a previous investigation [24]. Of the total battery (9 strains), 2 lactic acid-producing strains were used.

Variance study of the inhibitory effect of the treatments under study on Bacillus against the pathogen.

The statistical analysis revealed significant effects ($P<0.01$) in the corrected model and factor interactions (AB). This indicates that the combination of factors influenced the varied inhibition of the studied pathogen. Furthermore, it was evident that the methods used (direct diffusion and filtrate diffusion) showed different inhibitory effects, demonstrating statistical significance.

The analysis of the relationship between the diffusion method and the type of Bacillus did not reveal a statistically significant effect ($p>0.05$). This indicates that a similar inhibitory effect was obtained when applying both methods with Bacillus spp.. Bacillus subtilis ATCC 6051 also showed similar results regardless of the diffusion method, with a high R2 of 98%, confirming the reliability of the experimental results.

Analysis of comparison of means of the inhibitory effects.

Treatments	Subset		
	1	2	3
4 = (Df+B)	5.00		
2 = (Dd+B)	6.40		
1 = (Dd+Bs)		16.60	
3 = (Df+Bs)			20.80

Note. Averages grouped in the same subset are not statistically different. Dd: direct diffusion, Df: filtrate diffusion, Bs: Bacillus subtilis ATCC 6051, B: Bacillus spp wild type

Table 2. According to Tukey, the comparison of means is at 5% of the recorded values of the inhibition zones in each factorial interaction.

Treatments T1 and T3 stood out with the most significant inhibition diameters according to Table 2, being statistically different from the others, forming subsets 2 and 3. T2 and T4 showed similar measurements, ranging between 5 and 6.4, forming subset 1, which was considered statistically equal.
In the research by Pedraza et al. [25], it is highlighted that ribosomally synthesized peptides produced by Bacillus spp. are effective against Escherichia coli, supporting the inhibitory effectiveness even in the filtrate of the culture broth without the need for the live strain.

Study Escherichia coli susceptibility to Bacillus spp., wild-type, and Bacillus subitilis ATCC 6051.

Tra / Rep	T1		T4		T7		T10	
	Med.	Sus.	Med.	Sus.	Med.	Sus.	Med.	Sus.
1 (D003PI)	19	S	6	R	19	S	4	R
2 (EA006PI)	17	M.S.	8	R	22	S	5	R
3 (AR001AD)	16	M.S.	5	R	20	S	5	R
4 (AR013PI)	15	M.S.	6	R	21	S	4	R
5 (D002PI)	16	M.S.	7	R	22	S	7	R

Note. Tra: treatments, Rep: repetitions, Med.: measurements of inhibition zones, Sus.: susceptibility, S: sensitive, MS: moderately sensitive R: resistant.

Table 3. Susceptibility of Escherichia coli causing bovine mastitis against Bacillus spp., wild type, and Bacillus subtilis ATCC 6051.

When inhibition zones measure ≤ 12 mm, they are considered resistant, 13-17 mm moderately sensitive, and ≥ 18 mm sensitive. The 5 isolates of Escherichia coli were sensitive in the interaction with Bacillus subtilis ATCC 6051 using the filtrate diffusion method (T7), while in direct diffusion (T4), only one isolate was sensitive, and the remaining 4 were moderately sensitive. Against wild-type Bacillus spp., both in direct diffusion (T4) and filtrate diffusion (T10), the 5 Escherichia coli isolates were resistant, with values less than 12 mm of inhibition area.

Manafi et al.[26] demonstrated that Bacillus subtilis significantly reduced the population of intestinal Escherichia coli under in vitro conditions using a bacterial inoculum. This finding is consistent with the present research, where Bacillus subtilis ATCC 6051 showed a higher inhibitory percentage against wild-type Bacillus spp. when confronted with mastitis-associated Escherichia coli.

Comparative test of the antibacterial activity between Bacillus and Neomycin against E coli that cause mastitis symptoms.

Normality test	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistical	DF	Sig.	Statistical	DF	Sig.
Inhibition halos	0.153	25	0.134	0.911	25	0.032*

*Note. *: Statistical divergences.*

Table 4. Normality test between the study treatments versus 30µg Neomycin against E coli that cause mastitic symptoms.

The Shapiro-Wilk statistical test revealed the existence of significant statistical divergences between the inhibitory effects caused by the types of Bacillus and the impact of Neomycin at 30µg.

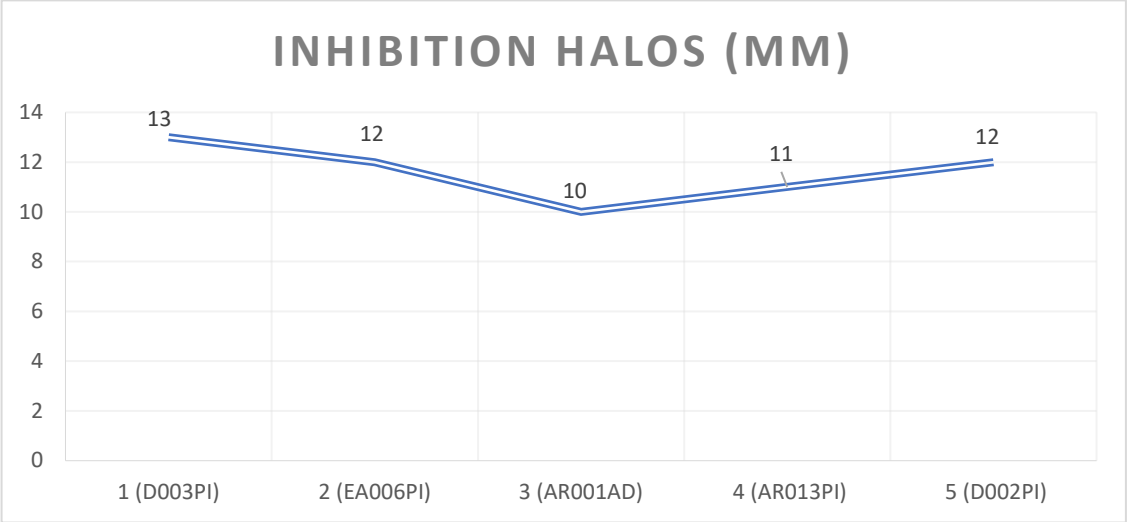

Figure 1. Results of the average inhibition area of the control treatment (T0: Neomycin at 30µg) against *E coli* that cause mastitic symptoms.

The antibiogram with Neomycin at 30 µg against Escherichia coli revealed a mean of 11.60 mm in inhibition areas, indicating resistance according to CLSI [27]. In comparison, wild-type Bacillus spp. showed lower inhibition due to its limited antimicrobial spectrum, while Bacillus subtilis ATCC 6051 exceeded the action of Neomycin 30 µg in both direct diffusion and filtrate diffusion. These results highlight the potential efficacy of Bacillus subtilis ATCC 6051 as an antimicrobial alternative compared to Neomycin, emphasizing the relevance of exploring alternative therapies to counteract antimicrobial resistance in mastitis pathogens.

In their study of mastitis in dairy cows, Nasef & Dawod [19] found that 50% of Escherichia coli isolates were sensitive to 30 µg Neomycin, while the other 50% were resistant. Bacillus subtilis ATCC 6051 demonstrated more significant inhibition of E. coli than Neomycin, highlighting its potential as an alternative therapy against antimicrobial resistance in bovine mastitis. Study of the susceptibility of Klebsiella spp. to wild-type Bacillus spp. and Bacillus subtilis ATCC 6051.

Estimating the growth kinetics of Bacillus spp., wild type, and Bacillus subtilis ATCC 6051.

The kinetic growth analysis of wild-type Bacillus spp. and Bacillus subtilis ATCC 6051 was performed using spectrophotometry with a nanodrop. It was observed that Bacillus subtilis ATCC 6051 exhibited higher absorbance in the exponential phase, indicating more efficient bacterial replication. Both microorganisms completed the exponential phase at 12 hours and entered the stationary phase. Wild-type Bacillus spp. showed higher absorbance after 14 hours, suggesting slower growth and the decline phase occurred after 24 hours, indicating a decrease in bacterial population.

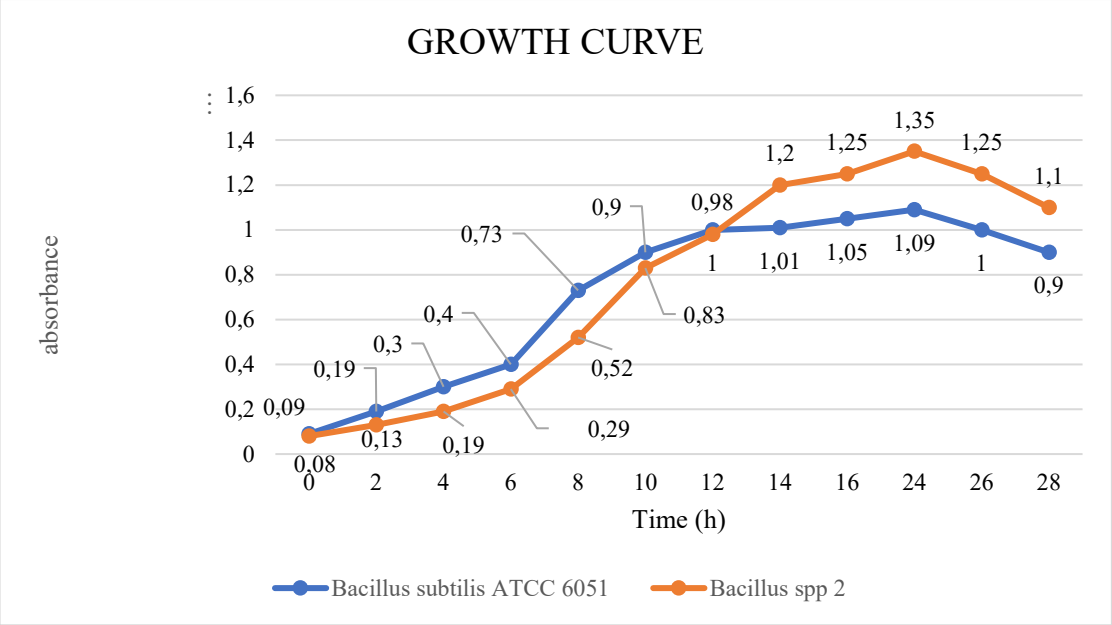

Figure 2. Growth curve of Bacillus spp., wild type and Bacillus subtilis ATCC 6051.

Fuentes et al. [28] indicated that Bacillus subtilis begins its exponential phase at 4 hours, reaches the stationary phase at 12 hours, and experiences cell decline after 24 hours, with a maximum absorbance of 1.34 at 24 hours. The results in the present experiment show similarities, but Bacillus subtilis ATCC 6051 exhibited higher absorbance in the exponential phase (0.30) compared to the mentioned study (0.22), suggesting a higher bacterial population and, therefore, a higher concentration of antimicrobial compounds.

The results of our study demonstrate that the direct diffusion method of the liquid culture of *Bacillus subtilis* ATCC 6051 exhibits more excellent antimicrobial activity against *E. coli* than the filtering method. This difference in antimicrobial activity could be attributed to several factors.

Firstly, the concentration of antimicrobial compounds may be higher in the unfiltered liquid culture. *Bacillus subtilis* is known to produce a variety of antimicrobial compounds, such as bacteriocins, lytic enzymes, and antimicrobial peptides, which can act synergistically to inhibit the growth of *E. coli*. [29,30,31] The filtering process could remove or reduce the concentration of some of these compounds, decreasing the observed antimicrobial activity.

Secondly, the presence of vegetative cells of *Bacillus subtilis* in the unfiltered liquid culture could contribute to the observed antimicrobial activity. In addition to producing antimicrobial compounds, *Bacillus subtilis* can compete with *E. coli* for nutrients and space, inhibiting its growth. [32] It is also possible that the vegetative cells of *Bacillus subtilis* produce other antimicrobial factors that are not filtered, such as enzymes or volatile metabolites. [33]

Our results also indicate that the antimicrobial activity of *Bacillus subtilis* ATCC 6051 is related to its growth phase. We observed greater antimicrobial activity during the exponential growth phase, suggesting that the production of antimicrobial compounds is active during this phase. This could be because the bacteria need to produce these compounds to compete with other bacteria for the resources available in the medium. [34]

It is important to highlight that this study was conducted *in vitro* and that further research is needed to evaluate the efficacy of *Bacillus subtilis* ATCC 6051 in controlling bovine mastitis *in vivo*. It would be interesting to evaluate the ability of *Bacillus subtilis* ATCC 6051 to colonize the mammary gland,

inhibit the growth of *E. coli* in milk, and reduce inflammation. It would also be important to evaluate the safety of its use in animals and its possible impact on milk quality.

Despite its limitations, our study provides evidence that *Bacillus subtilis* ATCC 6051 has potential as a therapeutic agent in controlling bovine mastitis. Its ability to inhibit the growth of *E. coli*, one of the primary pathogens causing mastitis, suggests that it could be a promising alternative to traditional antibiotics. [35] In the current context of increasing antimicrobial resistance, searching for new strategies for controlling bovine mastitis is crucial to guarantee animal health and milk production. [36]

In future research, we intend to evaluate the efficacy of *Bacillus subtilis* ATCC 6051 in animal models of bovine mastitis and optimize culture conditions to maximize the production of antimicrobial compounds. We also plan to investigate the antimicrobial mechanisms of action of *Bacillus subtilis* ATCC 6051 in greater detail, including identifying and characterizing the compounds responsible for its activity.

Bacillus subtilis ATCC 6051 has great potential as an alternative to antibiotics to control bovine mastitis. Its use could contribute to reducing the use of antibiotics in animal production, which in turn could help combat antimicrobial resistance. [37]

CONCLUSIONS

This study demonstrated that Bacillus subtilis ATCC 6051 possesses remarkable antimicrobial activity against Escherichia coli, a common pathogen in bovine mastitis. Significantly, the direct application of the *B. subtilis* liquid culture showed greater efficacy in inhibiting *E. coli* growth than the filtering method. This observation suggests that the presence of vegetative cells and/or non-filterable components of the culture, such as enzymes or volatile metabolites, could enhance the antimicrobial effect. Furthermore, the antimicrobial activity of *B. subtilis* ATCC 6051 was correlated with its growth phase, being more pronounced during the exponential phase, which indicates a possible relationship between metabolic activity and the production of antimicrobial compounds.

These findings have important implications for developing alternative strategies for controlling bovine mastitis, a disease that significantly affects animal health and milk productivity. The use of *B. subtilis* ATCC 6051 as a therapeutic agent could offer a promising alternative to traditional antibiotics, reducing their use and, therefore, mitigating the antimicrobial resistance problem. However, it is crucial to conduct further research to evaluate the efficacy and safety of *B. subtilis* ATCC 6051 in *in vivo* models. Future studies should focus on assessing the ability to colonize the mammary gland, inhibit bacterial growth in milk, modulate the inflammatory response, and ensure animal and milk quality safety.

In conclusion, this study provides strong evidence for the potential of *B. subtilis* ATCC 6051 as a biocontrol agent for bovine mastitis. Optimizing culture conditions and evaluating its efficacy *in vivo* studies are essential for developing a therapeutic strategy based on this microorganism. The use of *B. subtilis* ATCC 6051 could significantly advance the fight against bovine mastitis, promoting animal health and the sustainability of dairy production.

Contribution of the authors: Adriana Maribel Quincha Angulo: Led the experimental design of the study and the methodology used to evaluate the antimicrobial effectiveness of Bacillus strains against Escherichia coli isolated from bovine mastitis; Mercy Alexandra Galeas Barragán: Participated in sample collection, also contributed to the interpretation of the results obtained; Byron Adrián Herrera Chávez: Collaborated in the identification and selection of wild-type Bacillus spp. strains, additionally contributed to the literature review related to antimicrobial properties; Dagnny Mazón-Vélez: Contributed to the performance of healthy diffusion assays to evaluate the antimicrobial activity of Bacillus strains against

Escherichia coli. Also participated in the interpretation of the results and in the writing of the article; Favián Bayas Morejón: Contributed to the writing and revision of the final manuscript, as well as to the discussion of the results obtained concerning the context of bovine mastitis and antimicrobial resistance.

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