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ISOLATION AND IDENTIFICATION OF BACTERIA CAUSING BOVINE MASTITIS AS A FUNCTION OF LACTATION TIME IN DAIRY COWS FROM NORTHWESTERN PICHINCHA – ECUADOR

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ABSTRACT

Bovine mastitis is a pathology that generates great economic losses worldwide, therefore it is of crucial importance to know the influence that certain microorganisms have during the lactation period. The objective of this study was to isolate and identify bacteria causing bovine mastitis and its contingency based on lactation time in dairy cows from the northwest of Pichincha - Ecuador. The California mastitis test (CMT) was the diagnostic method used for the determination of mastitis, together with bacterial isolation and identification procedures by means of phenotypic tests in selective and differential agars, as well as biochemical tests such as IMVIC profiles and sugar fermentation patterns, gas production and hydrogen sulfide production determined by the Triple Sugar Iron (TSI) test, also with oxidase, catalase and coagulase tests, likewise, observing that the data corresponding to the lactation time of each particular farm considered as the focus of the study, were in an average of 315 ± 6. 46 days. Seven dairy farms were examined, testing 136 cows in total, of which epidemiologically 50 cows were positive for mastitis, obtaining 51 milk samples, of which *Staphylococcus aureus* was isolated in 29.42%, *Klebsiella* spp. in 17. 64%, *Bacillus* spp. in 17.64%, *Escherichia coli* in 15.68%, coagulase-negative *Staphylococcus* spp. in 15.68%, and *Citrobacter* spp. in 3.94%, reporting that the presence of Gram-negative bacilli has a higher occurrence at the beginning of lactation and Staphylococcus aureus in the middle of the lactation period.

Key words: Bacteria, Bovine mastitis, Lactation time, CMT.

INTRODUCTION

Intramammary bacterial colonization is considered the infection with the greatest impact on dairy cows, due to the fact that it has an impact on productive performance in addition to considerably decreasing milk quality, this background causes economic losses in dairy herds worldwide (Borck-Hog *et al.,* 2017). Bovine mastitis is the inflammation of the parenchyma of the mammary gland this condition negatively influences health and welfare of animals, the main alteration evidenced and that is considered the focus of the cornerstone is the increased somatic cells in those cases where it affects a subclinical mastitic picture, the cellular conformation in clinical samples with high cell counts is mainly determined by the migration of neutrophils and other cells of first immune line (Nonnemann *et al.,* 2019).

There are a number of bacterial pathogens that cause bovine mastitis, the differences between these agents are related to their habitat, virulence factors that give them their own microbiological characteristics of transmission and infection that consequently harm the health and welfare of the host, in addition to certain mechanisms of resistance to antimicrobials that are of recurrent use in mastitis therapeutics (Sharun *et al.,* 2021). Among the main etiological microorganisms of mastitis are fungi, yeast, algae, viruses and approximately 250 bacterial species among a diversity of Gram-positive and Gram-negative that can cause this pathology (Ruegg, 2017).

Etiologically Gram-positive bacteria include some genera such as *Staphylococcus* spp, *Streptococcus* spp, *Corynebacterium* spp, and some *Bacillus* spp, in the case of Gram-negative bacteria include *Escherichia coli, Klebsiella* spp, *Pseudomonas* spp., *Pasteurella* spp. etc. in particular each pathogen according to its pathogenicity and virulence factors can be considered as contagious or environmental agent (Hooman *et al.,* 2018).

The main pathogens considered contagious are *Staphylococcus aureus*, *Streptococus agalactiae* and *Mycoplasma bovis,* their transmission is basically from the teats of infected cows to the teats of susceptible cows, generally this occurs during milking through equipment (Aqeela & Muhammad, 2020).

Opportunistic microorganisms have the pathogenic peculiarity of adhesion, which allows them to reside in the epidermis of the mammary gland, this singularity confers them the ability to cause recurrent cases of mastitis, being *Streptococcus,* the main microorganisms involved (Chen *et al.,* 2021).

Environmental pathogens are found in cattle housing places, as well as in beds, milking equipment and places with poor sanitary management, these take advantage of the moments of greater susceptibility of the cow to enter through the teat canal generally in the first milkings consequent to calving, *Escherichia coli* is the main protagonist involved in causing environmental mastitis (Tomazi *et al.,* 2018).

The timely identification of microbes is essential for the diagnosis of the etiological agents involved in a mastitic picture in question, such procedure in a traditional way is approached from the application of biochemical tests, which allow observing one or several metabolic pathways used by bacteria (Nonnemann *et al.,* 2019). Traditional methods include morphological,

physiological, chemical and biochemical characterization, one of the disadvantages of the application of these phenotypic techniques is that they are not always useful to unambiguously identify the microorganism and discern between them at the species level, or much more often at the strain level (Franco-Duarte *et al.,* 2019).

The bovine mammary gland has the particularity of having involutive cycles comprised by a period of 60 days, this process is regulated by hormonal stimuli that promote lactogenesis and the maintenance of lactation, this event is of utmost importance since it can redefine its functionality in the next lactation (Desrivières *et al.,* 2007).

A definite way to decrease the impact of mastitis in dairy cows is to increase the cow's natural ability to resist intramammary infections (Sordillo *et al.,* 1997), the defense systems of the mammary gland are comprised of two components, a chemical one mediated by soluble components such as; immunoglobulins, lactoferrin, lysozymes, antimicrobial peptides and oligosaccharides, and an innate and acquired immune component mediated by cells, these work together to provide the greatest protection against pathogenic microorganisms causing bovine mastitis, Consequently, this defensive event triggers an inflammatory process due to the interaction of proinflammatory substances, opsonizing agents, the pathogen and the sensitive tissue, this interaction congruently compromises the productivity and quality of the milk (Petzl *et al.,* 2018).

The incidence of mastitis within a dairy herd is subject to constant variability since healthy animals with high production and animals with one or more affected quarters can be observed, consequently the latter are the product of the interaction between the immune response and the associated microorganisms, this can be exacerbated by the influence of many factors such as; nutrition, lactation stage, milk production, environment and genetics (Zemanova *et al.,* 2022).

Numerous studies have focused on the molecular dynamics of the mammary gland, and the genetic, physiological and morphological conditions, protein and lipid synthesis during lactation, as well as immunological changes associated with bovine mastitis and metabolic changes in the dairy cow around the udder (Dai *et al.,* 2016), however the dynamics of pathogenic mastitis-causing bacteria along the lactation age is an important point to consider in order to have a more accurate knowledge of mastitis pathogenesis. Therefore, the objective of this work was to isolate and identify bovine mastitis-causing bacteria in relation to lactation time in dairy cows.

MATERIALS AND METHODS

Sampling was carried out in seven farms belonging to the affiliates of the Agricultural Cooperative "La Colina", located in the San Pedro area of the San Miguel de los Bancos canton, which is located in the northwest of the province of Pichincha at an altitude of 655 meters above sea level with an average annual temperature of 22.9 ºC. Together, the isolation and identification procedures were carried out in the general laboratory of the Faculty of Agricultural Sciences, Natural Resources and Environment of the State University of Bolivar.

Factors under study

Table 1. *Factors considered for the investigation*

Factor A.	Lactation time
Factor B.	Bacteria causing bovine mastitis

Filling of records

In general, the lactation age of the mastitis-positive cows that were sampled was recorded, since each site referred to had a defined lactation duration as a zootechnical management parameter, respectively.

Cow sampling

The physical examination of each animal at the time of milking was performed to determine the specific pathological signs at the level of the mammary system, looking for physical alterations in the milk and anatomical alterations at the udder level, this only for those cows that did not previously receive intramammary or systemic antibiotic treatment, to later apply the CMT test (Life, Lot.2101216, Ecuador), which allowed identifying the positive animals and obtaining 10 mL of milk from them as a clinical sample. This sample collection was carried out according to the protocol established by the National Mastitis Council (Oliver et al., 2004).

Estimation of bovine mastitis prevalence

The prevalence was determined by calculating the data obtained from the register of mastitis-positive animals, also considering the total number of animals in a given flock, for which the following formula taken from Alvarado et al. (2019) was used.

$$\%\ \textbf{Prevalence} = \frac{\text{Number of cases affected by the disease}}{\text{Total number of population}} \text{ x } 100$$

Isolation

First, the substrate in which the microorganisms are suspended was enriched, for which buffered peptone water was prepared (Acumedia, Lot. 107596ª, USA), then, it was packed in test tubes at a ratio of 9:1; that is, 9 mL of buffered peptone water and 1 mL of mastitis-positive milk, and incubated for a period of 24 hours at 37°C. After the necessary incubation time had elapsed, the Petri dishes containing culture media were then seeded by taking 100 µL of the enriched sample and distributing this volume over the entire surface of the media. In the context of the research, we worked with MacConkey agar (Difco, Lot.9364856, USA) to achieve the growth of enterobacteria and to isolate Gram-positive bacteria we used blood-based agar (Difco, Lot.0237250, USA) with the addition of 5% sheep blood.

As a next step, we proceeded to duplicate and triplicate the initial culture, in order to establish the purification or separation of the different colonies present in that culture, for the subsequent cultures we performed the triple streaking technique. Once the pathogens causing bovine mastitis

were isolated, bacterial colonies with phenotypic characteristics and uniform growth were obtained for their subsequent identification through the application of biochemical tests.

Identification

MacConkey agar (Difco, Lot.9364856, USA) and Eosin Methylene Blue (EMB) agar (Difco, Lot.0286056, USA) were used for practical identification of enterobacteria. For the identification of Gram-positive bacteria, Salt Mannitol agar (Oxoid, REF: CM0085, LOT: 3289843, United Kingdom) and Trypticase Soy agar (Difco, Lot.1153604, USA) were used, in order to have in the first instance a presumptive criterion of bacterial recognition and to use Gram staining to observe and classify the bacteria in two groups: Gram-negative and Gram-positive bacteria, this set of culture media allowed the efficient use of resources for their consequent biochemical identification.

Additionally, the IMViC tests were applied. These are a series of tests that group the determinations of: Indole, Methyl Red, Voges-Poskauer (VP) and the use of Citrate, which consisted of the use of buffered peptone water (Acumedia, Lot. 107596ª, USA), MR-vP broth (Difco, Lot.0286583, USA), Simon's Citrate Agar (Difco, Lot.9302976, USA), Kovac's reagent (LABO CHEMIE, Lot. LM11071813, India), Methyl Red (TM MEDIA, C.I.13020, India), Alpha Naphthol 6% (Novachen, Ecuador) and Potassium Hydroxide KOH 40%.

In addition, the TSI test (Criterion, Lot:488618, USA) was used for the identification of carbohydrate fermentation patterns, gas production and hydrogen sulfide production.

The oxidase test (OxiStrips, Lot.485435, USA) was also used to determine the presence of the enzyme cytochrome c oxidase present in the bacteria under study; for this test a culture plate was used, previously incubated for 24 hours at 37 ° C, where the inoculum was taken and placed on the test strips, after a few minutes the reaction was observed.

In the Catalase test, 3% hydrogen peroxide (Iira, Ecuador) was used. For its development, a bacterial colony was taken with a previously sterilized loop and placed on a slide, then a few drops of hydrogen peroxide were added and the reaction was awaited.

Coagulase; This test is specific for the identification of Staphylococcus aureus, since it is the only one capable of coagulating plasma, therefore, rabbit blood was collected in Vacutainer tubes with a lilac lid containing anticoagulant (EDTA), Afterwards, the sample was homogenized and left to rest for 30 minutes, then, it was centrifuged for 5 minutes at 1006 g, then with the help of the micropipette all the plasma obtained was collected, for this purpose it was poured in Eppendorf tubes 0.5 mL of plasma. 5 mL of plasma, then the suspension of the bacteria under study was performed.

The diagnosis was performed based on that described by Tiller (2017), who reports the identification profiles for each bacterial genus in question and the results obtained on these from the biochemical reactions generated when a metabolic pathway of the bacterial agent becomes evident.

Data analysis

The distribution of the occurrence of a bacterial agent causing bovine mastitis in the different stages of lactation in a given dairy farm was measured using the kolmogorov-smirnov test, which allows determining the distribution or contrast of the data obtained.

RESULTS AND DISCUSSION

Estimation of lactation time

The dairy herds considered as the focus of the study, allowed the determination of the lactation period, which is comprised in an average time of 315 ± 6.46 days, observing in the following table the values of the days of lactation duration of each farm considered.

Table 2. *Lactation time of the samples analyzed*

Property Number	Time
1	310
2	300
3	330
4	325
5	342
6	300
7	300
$\bar{x}$	315 ± 6.46 days

$\bar{x}$: average

Prevalence of bovine mastitis

According to the results obtained through the application of CMT (California Mastitis Test) as a diagnostic method, it was possible to determine the mastitis prevalence rate in 36.76 % of a total number of 136 animals tested, considering 50 animals as positive.

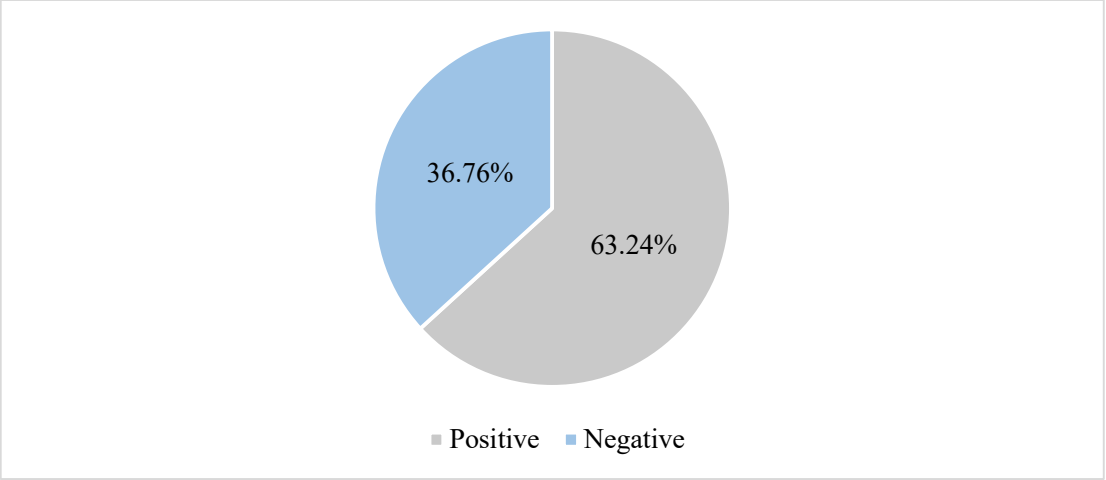


Figure 1. *Prevalence of bovine mastitis.*

Avellan et al. (2019), in their research found a bovine mastitis prevalence rate of 38.57%, also Sanchez, (2014) in his research in the determination of bovine mastitis by application of CMT (California Mastitis Test) in the Chaupi - Cayambe obtained a prevalence of 35. 48% in dairy systems, being these data similar to those obtained in the present research, concluding that this pathology is present as long as the milking and udder sanitation protocols are not adequate to ensure the health of the mammary gland and the safety of the final product.

Prevalence of mastitis in affected mammary quarters

Table 3. *Prevalence of mastitis in affected mammary quarters*

Mammary quarter	% Prevalence
A D	18
A I	20
P D	31
P I	31

A: anterior, P: posterior, D: right, I: left

In the distribution of mastitis settlement in relation to the location of the mammary gland quarters, it was evidenced that mastitis settles to a greater extent in the posterior quarters in an equal proportion (31%) in each quarter of each side respectively, in addition, a lower commitment of the anterior quarters was found, It is assumed that this finding is due to the fact that in most of the dairy herds the presence of the calf at the foot of the cow at the time of milking was observed as a measure of oxytocin-dependent afferent stimulation for milk let-down.

Quispe, (2015) in his research showed that of the total number of mammary gland quarters that presented mastitis the left hind quarter was the least affected (40%) and the right hind quarter was

the most affected (45. 4%), thus demonstrating significant differences in the settlement of mastitis between quarters, these results were taken in dairy herds that use the calf at foot method as a source of stimulation for milk descent, corroborating the existence of similar results in the present research where it could be observed that to a large extent milk producers use this method and the casuistry of mastitis mostly settles more in the posterior quarters.

Isolation and identification of mastitis-causing pathogens

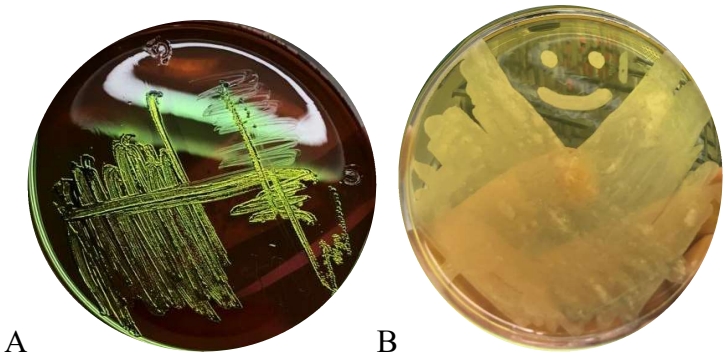
The isolation and identification of the bacteria was carried out in a timely manner after the collection of 51 mastitis-positive samples from 50 cows from the affiliates' farms.

Table 4. *Isolation and identification of bacteria causing bovine mastitis.*

Patógeno	**Frecuencia**	**Porcentaje**
Staphylococcus aureus	15	29.42%
Escherichia coli	8	15.68%
Klebsiella spp.	9	17.64%
Staphylococcus spp. CN	8	15.68%
Bacillus spp.	9	17.68%
Citrobacter spp.	2	3.94%
Total	51	100%

CN: coagulase negative

According to the data obtained (Table 4), we can determine that the most prevalent pathogen is *Staphylococcus aureus* followed by *Klebsiella* spp. and *Bacillus* spp, *Escherichia coli* and *Staphylococcus* spp, and finally the least frequent microorganism isolated from mastitis positive milk is *Citrobacter* spp. with less than 4%, totaling 51 isolated bacterial strains.



A B

Figure 2. *Pathogens isolated from clinical samples of bovine mastitis-positive milk. A) growth of Escherichia coli on EMB agar, B) growth of Staphylococcus aureus on salt mannitol agar.*

In the study by Jaramillo et al. (2018), in the microbiological analysis of milk samples from cows with subclinical and clinical mastitis with manual and mechanical milking systems, the bacteria found were; *Arcanobacterium haemolyticum* in 20.14 %, coagulase negative *Staphylococcus* 17.36 %, *Streptococcus agalactiae* in 12.50 %, *Staphylococcus aureus* in 10.42 %, *Streptococcus uberis* in 1.73 %, and Gram negative bacteria such as; *Enterococcus* spp. , in 1.39 %, *Yersinia* spp. in 0.70 % and *Enterobacter* spp. in 0.35 %, in comparison with what was obtained in the present investigation, it can be deduced that the microbiological findings from clinical samples of milk with mastitis are associated with environmental and management factors of the agroecological zone where the dairy activity is located.

Relationship of pathogens in lactation time

When analyzing the occurrence of a mastitic picture determined by the pathogenicity of a bacterial agent in the different phases of lactation of dairy cows from the northwest of Pichincha, statistical differences were evidenced (P<0. 05), observing that depending on the susceptibility of the animal, an intramammary infectious condition can be triggered at the peak (0-99 days), middle of lactation (100-199 days), or end of lactation (200 - 305 days), obtaining that the highest occurrence of cases of bovine mastitis caused by bacteria occurred at 230 days of lactation, additionally the average lactation age calculated was 110 ± 72. 33 days, considering this value a risk period in the presentation of intramammary infection in the dairy farms evaluated.

Table 5. *Normality test of the data obtained.*

	Kolmogorov Smirnov		
	Statistician	**gl**	**Sig.**
Clinical samples * Lactation days	.225	51	.000 **
Moda		230	
Means		110	
Standard deviation σ		± 72.33	

**: Highly significant statistical differences.

Figure 3 shows the dispersion of the data obtained, where the highest occurrence of mastitic pictures was in the beginning of lactation (0-99 days) and to a lesser extent in the middle third of lactation (100-199 days), these values are associated with the pathogenicity of the isolated microorganisms and the susceptibility of the cow.

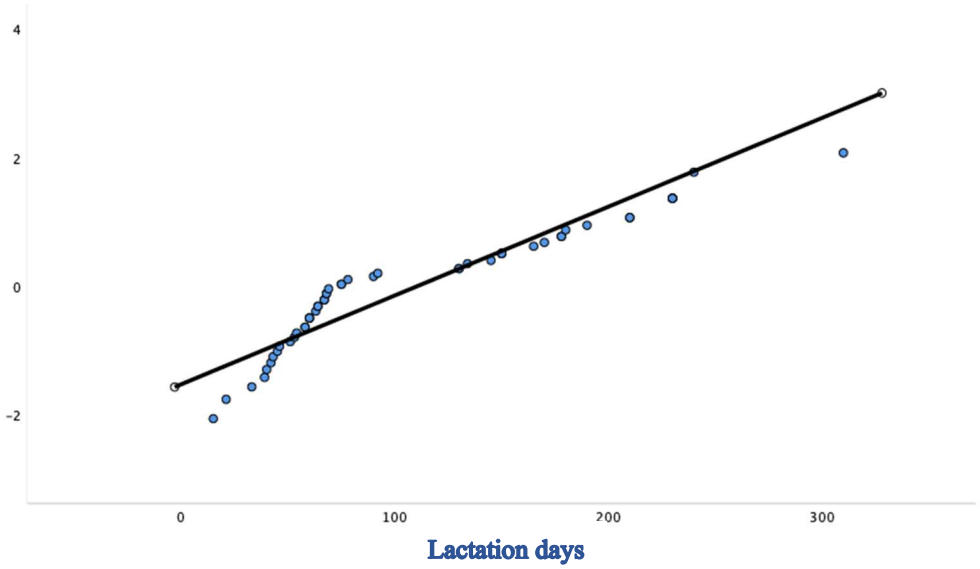


Figure 3. *Scatter plot of data according to clinical samples as a function of lactation time. Each point is recognized as a milk sample, which was processed for isolation and identification of pathogens associated with the pathology.*

Figure 4 shows that *Sataphylococcus aureus* is responsible for causing mastitis to a greater extent from the middle third to the final third of lactation, while *Escherichia coli* and *Klebsiella* spp. are the protagonists of mastitis in the first third of lactation, coagulase negative *Staphylococcus* spp. can colonize and cause mastitis in the different stages of lactation, as well as *Bacillus* spp. was observed to cause mastitis in the first third of lactation, and finally *Citrobacter* spp. was isolated from clinical samples of mastitis in cows at the beginning and end of lactation.

In the research conducted by Atajo, (2019) mentions that the prevalence of mastitis in the different stages of lactation (1/3, 2/3, 3/3), is higher in the 1/3 lactation in relation to the other stages. Likewise, Quilapanta, (2021) mentions that intramammary infections caused by environmental pathogens occur to a greater extent at the beginning of lactation, agreeing with the above mentioned, comparatively in the present investigation it was observed that in the first third of lactation there was a greater occurrence of environmental pathogens and mastitis.

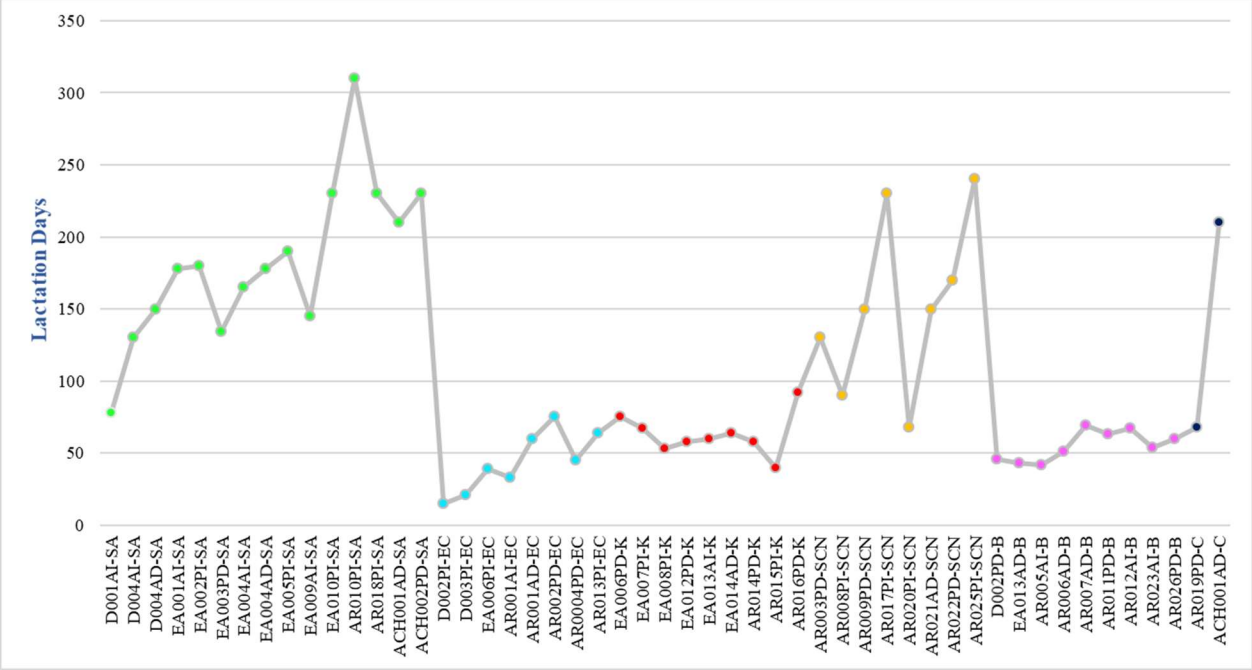


Figure 4. Distribution of bacterial agents as a function of lactation time, where the sample is comprised of a unique identification code and the initial letters of the name of the associated bacterium. -SA: Staphylococcus aureus, -EC: Escherichia coli, -K: Klebsiella spp, -SCN: Staphylococcus coagulase negative, -B: Bacillus spp, -C: Citrobacter. Each dot of each color represents a bacterial genus isolated and identified from samples of cow's mastitis.

CONCLUSIONS.

Bovine mastitis generates a great economic impact in dairies worldwide, being pathogenic bacteria, the main etiological agents involved, which affect the health of the mammary gland in its different physiological stages. In the present investigation, *Sataphylococcus aureus* was isolated and identified to a greater extent in relation to other bacterial pathogens from clinical samples of mastitis-positive milk taken from cows in the middle third (100 - 199 days) and the final third (200 - 305 days) of lactation, while *Escherichia coli* and *Klebsiella* spp, were isolated and identified in cows in the first third of lactation (0 - 99 days) which were mostly in the immediate postpartum period, *Staphylococcus* spp, coagulase negative *Staphylococcus* spp. was isolated and identified from mastitic milk samples of cows whose lactation age was from 68 days of lactation including animals that were 240 days of lactation, inferring that this pathogen can affect udder health at any stage of lactation, likewise *Bacillus* spp. was isolated and identified in pathologically positive cows found in cows that were pathologically positive, was isolated and identified in pathologically positive cows found in the first third of lactation, and finally, *Citrobacter* spp. was isolated from clinical milk samples in cows at the beginning and end of lactation.

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