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LACTIC ACID BATERIA WITH ANTIMICROBIAL ACTIVITY AGAINST PATHOGENIC AGENT CAUSING OF BOVINE MASTITIS

BACTERIAS ACIDO LACTICAS CON ACTIVIDAD ANTIMICROBIANA CONTRA PATOGENOS CAUSANTES DE MASTITIS BOVINA

OUT BACTÉRIAS LÁTICAS COM ATIVIDADE ANTIMICROBIANA CONTRA OUT PATÓGENOS DA MASTITE BOVINA

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KEYWORDS:

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PALAVRAS-CHAVE:

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RESUMEN

El uso de antibióticos para el tratamiento de mastitis bovina, genera residuos de antibióticos en la leche y disminuye la calidad de los subproductos lácteos. Las bacterias ácido lácticas se han propuesto como una alternativa para evitar el uso de antibióticos. En este articulo se reporta la actividad antimicrobiana contra patógenos productores de mastitis bovina, de 4 cepas acido lácticas aisladas de bovinos en estado de acidosis ruminal. Se evaluó además la velocidad especifica de crecimiento (m) y la actividad antimicrobina de una de una de las cepas, utilizando dos concentraciones de fuente de carbono (20 y 60 gl-1) en el sustrato comercial MRS. Las cepas se identificaron bioquímicamente como Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus fermentum y Weissella confusa. Weissella confusa presentó la mejor actividad antimicrobiana contra los principales patógenos productores de mastitis bovina. Cuando se utilizó 60 gl-1 de azúcares totales en el sustrato de fermentación, se obtuvo diámetro de inhibición de 31 mm para Staphylococcus aureus y de 36 mm para Streptococcus agalactiae. La actividad antimicrobiana de Weissella confusa es superior a la actividad antimicrobiana reportada por muchas otras bacterias ácido lácticas, por lo cual Weissella confusa podría ser usada potencialmente para prevenir la mastitis bovina.

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ABSTRACT

The use of antibiotics to treat bovine mastitis, produces antibiotic residues in milk and decreased quality of dairy products. Lactic acid bacteria have been proposed as an alternative to avoid the use of antibiotics. This paper reports the antimicrobial activity against pathogens responsible of bovine mastitis, of 4 lactic acid strains isolated from cattle in a state of acidosis ruminal. It also evaluated the specific growth rate (m) and antimicrobial activity of one of the strains, using two concentrations of carbon source (20 and 60 gl-1) in the commercial substrate MRS. The strains were biochemically identified as Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus fermentum and Weissella confusa. Weissella confusa showed the best antimicrobial activity against the major pathogens responsible of bovine mastitis. When has been used 60 gl-1 of total sugars in the fermentation substrate, was obtained diameter of inhibition of 31 mm for Staphylococcus aureus and 36 mm for Streptococcus agalactiae. The antimicrobial activity of Weissella confusa is superior antimicrobial activity reported by many other lactic acid bacteria, therefore Weissella confusa could potentially be used to prevent bovine mastitis.

RESUMO

O uso de antibióticos como tratamento da mastite bovina, produz resíduos de antibióticos no leite e diminuição da qualidade de produtos lácteos. As bactérias lácticas têm sido propostas como uma alternativa para evitar o uso de antibióticos. Neste estudo se relata a atividade antimicrobiana de 4 cepas lácticas isoladas de bovinos no estado de acidose, contra os patógenos causantes da mastite bovina. Foi avaliada a taxa de crescimento específico (m) e a atividade antimicrobiana de uma das linhagens, utilizando duas concentrações da fonte de carbono (20 e 60 gL-1) no meio MRS. As cepas foram identificadas bioquimicamente como Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus fermentum e Weissella confusa. Weissella confusa mostrou a melhor atividade antimicrobiana contra os patógenos principais produtores da mastite bovina. Quando foi usada uma concentração de 60 gL-1 de açúcar total no substrato de fermentação, foi obtido um diâmetro de 31 mm para para Staphylococcus aureus e Streptococcus agalactiae 36 mm. A atividade antimicrobiana de Weissella confusa é a atividade antimicrobiana superior relatado por muitas outras bactérias ácido lácticas, Weissella confuso, então poderia ser usada para prevenir a mastite bovina.

INTRODUCTION

Bovine mastitis is an inflammation of the mammary gland caused by environmental or contagious microorganisms. Environmental pathogens such as Escherichia coli, Streptococcus uberis and some species of Enterococcus, have affected the world wide milk production. This pathogens are in the soil and human and animal feces. There are other high impact microorganisms for milk industry that have been classified as contagious and are transmitted by inappropriate milking practices for instance, Staphylococcus aureus and Strestococcus agalactiae [1]. In order to eradicate these microorganisms, antibiotic therapies have been used by several years as the main way to control intramammary illnesses [2], however, the efficiency of the medicine diminishes because most of products are betalactamic and the bacteria become resistant, also the antibiotics generate problems of residues in the milk and reduce the industrial quality of the dairy subproducts [3, 4]; for which, other alternatives have been investigated Godden et al. [5]. The use of internal teats sealants during the dry period is suggested to diminish the susceptibility to the infection intramamary caused by the delay in the formation of the plugger of keratin in the nipple, however, this method produces annoyance and mechanical traumatism in the gland. Some vaccines have been produced to reduce the severity of the illness; nevertheless they do not control efficiently the mastitis [6]. Moon et al. [7] and Ochoa-Zarzosa et al. [8], report the use of chitosan and antimicrobial peptic isolated of plants, like an effective cure of mastitis, but, they are only efficient in the treatment of staphylococcic mastitis. The lantibiotics, a bacteriocin group, antimicrobial peptic ribosomally synthesized by lactic acid bacteria and other bacteria, also have a demonstrated activity against the

pathogenic producer bacteria of mastitis, especially against varieties resistant to antibiotics [9, 10, 3, 11, 8], although, only the nisin and lacticin, bactericide produced by Lactococcus lactis, have been used for the mastitis treatment and the strains that have created resistance to these bacteriocins have been reported [9, 12, 13]. Nascimento et al. [11], inform that a bacteriocin produced by coagulase-negative staphylococci could be used in the control of mastitis but only if this one is of streptococcic origin. Other authors evaluated the expression of the bovine cathelicidins BMAP-27 and BMAP-28 in healthy and infected mammary tissue to determine their activities against bacteria isolated from bovine mastitis. They found that BMAP-27 and -28 were highly effective in mastitic bovine milk and showed a variably broad spectrum of activity against 28 bacterial isolates from bovine mastitis [14]. Van den Borne et al. [15], determined therapeutic effects of recently acquired subclinical mastitis after antimicrobial treatment during lactation, resulted in lower quarter somatic cell count and increased bacteriological cure rates compared with control cows. Also has been shown to be effective of specific egg yolk immunoglobulin (IgY) to bovine mastitis caused by Staphylococcus aureus, this was reflected in the milk quality, bacterial counts in milk, and decreasing somatic cell counts [4].

Weisella confusa has been reported as the producer of a similar compound to bacteriocin, wich presented an antimicrobial effect against Bacillus cereus [16]. However, there are not scientific information about antagonic activity of Weissella confusa against pathogen microorganisms responsible for bovine mastitis.

Lactic-acid bacteria produced under bovine stress conditions, can be producers of substances with inhibitory potential against several microorganisms responsible for bovine mastitis and also this bacteria could be evaluated for preventive and curative treatments of bovine mastitis. In this investigation the inhibitory power of lactic acid bacteria (isolated of bovine in the state of ruminal acidosis) opposite to the main pathogens producers of mastitis, Staphylococcus aureus y Streptococcus agalactiae, were evaluated. Likewise the kinetic parameters of growth of the lactic acid bacterium with antimicrobial activity against the two pathogens, were determined in fermentations in discontinuous and at laboratory scale, using MRS broth with concentrations of 20 and 60 gL^{-1} of carbon source.

METHOD

Isolation of lactic acid bacteria

Samples of ruminal liquid were taken from bovine females of 400 kg of weight of the race Hartón del Valle, whose ruminal acidosis was induced. Ruminal acidosis was induced with the aim to reach a fast fermentative process, witch is obtained by the proliferation of lactic-acid bacteria under metabolic stress conditions of the bovine. Ruminal acidosis was induced by means of an increasing daily diet of 1200 g of commercial concentrate that contributed 80 % of total digestible nutrients (TDN), until observing the first symptoms of diminution of consumption and ruminal paralysis. 6 days later samples of liquid ruminal were collected by means of esophagic catteter, (previously washed to retire the excess of saliva, which is a natural blocker in ruminants). The samples were manipulated in sterile material and they were stayed under refrigeration until initiating the isolation of lactic acid bacteria. From these samples dilutions of 10^{-4}, 10^{-6} y 10^{-7} in peptone water 0.1 % were carried out and each one of them was sown for duplicate in boxes with MRS agar [17]. The boxes were inoculated with 0.1 ml of each of the dilutions (in the surface) and they were incubated at 33 ºC for 48 hours in aerobic and anaerobic conditions. Passed the time of incubation, the presumed colonies of being lactic acid (which assimilated the aniline blue) the Gram coloration was carried out to verify the morphology of the bacteria.

The presumed colonies to produce lactic acid pealed in the same medium of cultivation and they were incubated again at 33 ºC for 24 hours until obtaining pure cultivation. Once the pure cultivations were obtained, they pealed in liquid medium, MRS broth, and they were incubated in the same conditions mentioned above. To these strains its capacity to produce lactic acid was determined.

Lactic acid determination

The concentrations of lactic acid produced by the strains were measured by HPLC liquid chromatography of high resolution Agilent 1100. With integrative HP Chemstastion, equipped with an Aminex column HPX 87H, 300 mm, using like mobile phase, sulphuric acid 0,005 M. The temperature of work for the column was 25 °C with speed of flow of 0,5 mL/second. For this

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procedure, samples from 24 hours - pure liquid cultures were taken. They were centrifuged at 5000 x g for 10 minutes and then they were filtered with millipore filters HVLPO 2500; the supernatants of the filtered samples were injected in a HPLC equipment with a column for organic acid determination.

The lactic acid strains were conserved in refrigeration in inclined MRS agar and cryo-congelation with glycerol -20 °C in MRS broth. To these strains its inhibiting capacity opposite to the pathogens producers of mastitis was determined and later were biochemically identified by using API 50 CHL.

Antimicrobial activity measurement

The lactic acid strains were proven to quantify their antimicrobial activity opposite to the pathogens producers of bovine mastitis like Staphylococcus aureus ATCC® 25923 TM* and Streptococcus agalactiae ATCC® 13813 TM*. The Staphylococcus aureus was reconstituted in a liquid medium Gioliti-Cantoni and the Streptoccoccus agalactiae was reconstituted in M-17 broth.

For the tests plates of sterile agar of 5 mm of thickness were used which contained the specific nutrients for the pathogens growth; Baird Parker Agar (BP) for Staphylococus aureus and M-17 Agar for Streptococcus agalactiae. To these plates, orifices were made using sterile punches of 17 mm of diameter. The plates were separately seeded with the pathogens in test using sowing in surface. On the other hand, circles of MRS agar sterile of 5 mm of thickness and 17 mm of diameter were taken in aseptic form, which were impregnated with the broths of growth of each one of the lactic acid strains (swellings in MRS broth and neutralized with NaOH 4 M) they were introduced in the orifices made in the boxes with BP and M-17 agar. The boxes were incubated at 33 °C for 24 hours, time in which the haul of inhibition of the growth was measured of both pathogens using a milli-metrical rule. For each lactic acid bacteria the tests of inhibition opposite to pathogens were made by triplicate.

To the strains that displayed the greater halos of inhibition for Staphylococcus and Streptococcus, biochemical identification was made to them. Of the previous strains, the selected strain was that presented greater antimicrobial activity against both pathogens. To the selected strain the curve of microbial growth was made and the kinetic of antimicrobial activity was determined during 48 hours, using MRS broth with concentrations of 20 and 60 gL^{-1} of carbon source.

Kinetic of antimicrobial activity and Biomass formation

Kinetic of antimicrobial activity and the curve of microbial growth of the selected strain was evaluated in 12 fermentations in discontinuous, using as substrate MRS broth with a total sugar concentration of 20 and 60 gL^{-1}. The substrate for half of the fermentations was prepared agreed to commercial indications and for other half glucose was added, in a sufficient amount to reach a sugar concentration of 60 gL^{-1}.

The fermentations were carried out in erlenmeyer of 500 ml with a volume of work volume of 250 ml, erlenmeyer remained ellipsoidally shake without aeration, at 100 rpm for 48 hours at 33 °C. The selected strain adapted to the conditions of fermentation conditions for three generations, in each case was used 10 % of biomass with respect to the volume of the substrate. pH was adjusted at 6 with NaOH 4 M, in order to avoid inhibition of microorganism with lactic acid produced by themselves. For both tests samples were taken from the substrates of fermentation at 0, 2, 4, 8, 12, 24 and 48 hours. In order to make the kinetic of biomass formation, in each one of the indicated times, 3 ml of substrate of fermentation were centrifuged for 15 minutes at 10,000 g., the supernatants were removed, the biomass was washed with normal saline and was dried in stove at 100 °C [18] and the results were expressed like grams of biomass by liter of fermentation broth. From the kinetic of biomass formation the specific speed of growth was calculated. To determine the kinetic of the microbial activity samples in the described times were taken and for each sample the antimicrobial activity was determined, following the procedure described above.

Statistical design

A design factorial was used completely at random of 2x6x2 with three factors like this: Pathogen type factor, with two levels: Staphylococcus aureus and Streptococcus agalactiae.
Fermentation time factor, with six levels: 2, 4, 8, 12, 24 and 48 hours.
Concentration factor of total sugars in the substrate, with two levels: 20 and 60 gL^{-1}.

The results were analyzed using a general multi-varied lineal model, in statistical package SAS 8,2 (Cary NJ). The comparison among averages was carried out through the Tukey trial at 5 % of probability.

RESULTS

11 lactic acid bacteria were isolated of ruminal liquid and only four of them showed antimicrobial action against the two main pathogenic microorganisms producers of bovine mastitis, Staphylococcus aureus and Streptococcus agalactiae. The biochemical identification of the four strains, the quantity of lactic acid produced by each strain and the antimicrobial activity for each one of them can be seen in the table 1.

The strains showed in the table 1 get an inhibition to Staphylococcus aureus, however this values were not significant between them, while that to Streptococcus agalactie was achieved a greater diameter of inhibition with the strain 4 biochemically identified as Weissella confusa. Based on these results, Weissella confusa was the microorganism selected as indicated in the methodology, the kinetic of microbial growth was carried out and the kinetic of microbial activity was evaluated to this microorganism in relation to Staphylococcus aureus and Streptococcus agalactiae in substrates with carbon source concentrations of 20 and 60 gL^{-1}.

The kinetic of biomass formation of Weissella confusa in substrate with content of total sugars of 20 gL^{-1} and of 60 gL^{-1} can be seen in the figure 1, and the kinetic of the antimicrobial activity of Weissella confusa in relation to Staphylococcus aureus and Streptococcus agalactiae are showed in the figure 2.

From the kinetic of biomass formation can be calculated that Weissella confusa maintains its exponential growth since the beginning of the fermentation until the first four hours of fermentation when 20 gL^{-1} of total sugars are used, with a specific growth velocity, m, of 0.0811 h^{-1}; and when the microorganism grows in a substrate with sugar concentrations of 60 gL^{-1}, its specific growth velocity is enlarged to 0,742 h^{-1}, and the microorganism grows exponentially for four hours. Nevertheless, the m values are very high, with respect to that obtained in the other medium. These results suggest that to obtain high concentrations of biomass is necessary to use high sugar concentrations in the culture medium at all times.

In this study, la feeding with glucose was an adequate strategy for increasing the biomass, from practical point of view those commercial mediums uneconomic in the milk industry. This indicates that is necessary to carry out investigations with cheaper culture mediums that permit a considerable increase of biomass and therefore of the metabolite.

In the two fermentations, the kinetic of biomass formation showed an exponential growth phase followed by a stationary phase. These observations coincide with the time which gets the maximum diameter of the inhibition for Staphylococcus aureus and Streptococcus agalactiae. This suggests that, Weissella confusa may

Table 1. Biochemical identification, lactic acid production and measurement of the antimicrobial activity in relation to Staphylococcus aureus. and Streptococcus agalactiae of lactic acid bacteria isolated of ruminal liquid bovine.

No. strain	Biochemical identification	Lactic-acid gL^{-1}	Diameter of the zone of inhibition against S. agalactiae (mm) (tests of diffusion in agar at 24 h. without controlling the fermentation)	Diameter of the zone of inhibition against S. aureus (mm). (tests of diffusion in agar at 24 h. without controlling the fermentation)
1	Lactobacillus acidophilus	24.1	1.5	7.5
2	Lactobacillus brevis	13.1	11.5	17.5
3	Lactobacillus fermentum	12.5	9.5	11.5
4	Weissella confusa	12.7	31.5	9.5

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Figure 1. Kinetic of biomass formation of Weissella confusa in substrate MRS broth with total sugar content of 20 y de 60 gL-1

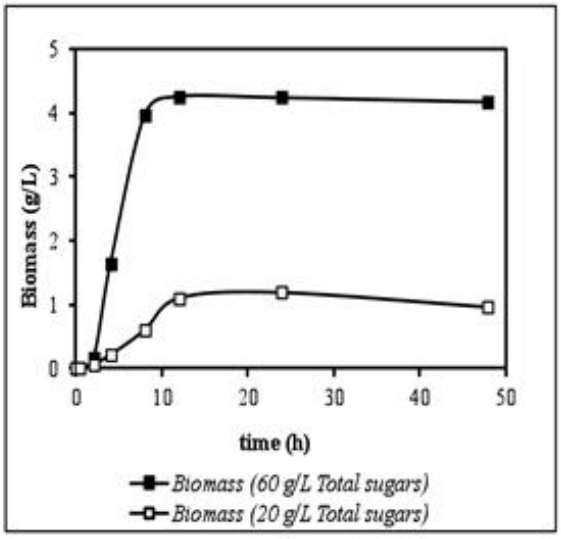
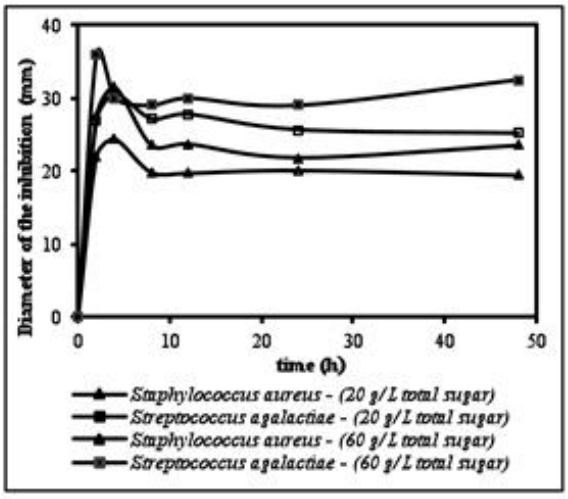


Figure 2. Kinetic of antimicrobial activity of Weissella confusa opposite to Staphylococcus aureus and Streptococcus agalactiae in substrates of fermentation with content of 20 and 60 gL-1 of total sugars.



to produce some metabolite responsible of behaviour at the begging of the kinetic of antimicrobial activity. This metabolite may be primary since it is associated to the microbial growth, and is produced during the exponential phase of growth.

Although the chemical character of inhibitory metabolite was not determined in this study, it can be confirmed that the inhibition zone against Staphylococus aureus and Streptococcus agalactiae are not generated by the lactic acid produced in all fermentations, due to these were neutralized before to perform the microbiological antagonism tests; this lead us to think that microbiological activity of Weissella confusa is produced by compounds similar to bacteriocins. Therefore, in the discussion of the results it is necessary to compare with other authors about the use of bacteriocins, or the microorganisms that produce them, in the treatment of bovine mastitis.

The analysis of the variance showed that the concentration of sugars in the substrate, the time of fermentation, the type of pathogen and the interaction of this three factors, has statistically significant effect on the inhibitory power of Weissella confusa ($P<0.005$ and $R=0.844$). The greater diameters of inhibition (36 mm) were presented for Streptococcus agalactiae; two hours after the fermentation, when 60 gL-1 of sugars were used in the substrates of fermentation. For Staphylococcus aureus the greatest diameters of inhibition (31 mm) were presented four hours after the fermentation. The antimicrobial activity of many lactic acid bacteria against pathogenic microorganisms producers of mastitis has been recognized since generate small proteins known as bacteriocins, which work like antibiotics of reduced spectrum, but, only the Nisin and the Lacticin, bacteriocins produced by Lactococcus lactis are utilized in the control of bovine mastitis given that these present similar antimicrobial activity in relation to the two main pathogens producers of mastitis, Staphylococcus aureus and Streptococcus agalactiae [9, 19]

Until this moment, has not been reported the bacteriocins production of Weissella confusa and has not been used this lactic acid bacterium in the control of bovine mastitis. However, Chavasirikunton et al. [16] found in Weissella confusa antimicrobial activity of inhibitory substances like bacteriocins against Bacillus cereus.

The results of this investigation show that the lactic acid strain isolated of the ruminal liquid of bovine in state of acid-base stress and identified as Weissella confusa produces a similar inhibitory substance to a bacteriocin that shows antimicrobial activity against Staphylococcus aureus and Streptococcus agalactiae of more than the double of the antimicrobial activity reported for nisin and lacticin.

Otros results reported by other authors can be seen in the Table 2. Various authors report bacteriocins and other compounds with antimicrobial activity with great potential of use in the prevention and treatment of mas-

Table 2. Antimicrobial activity against Staphylococcus aureus and Streptococcus agalactiae of bacteriocins and similar substances to bacteriocins.

Antimicrobial element	Pathogenic strain	Diameter of the zone of inhibition in mm	Author
Lacticin	Staphylococcus aureus	10-14.7	[9]
	Staphylococcus aureus ATCC 25923	<8	[20]
Lactococcucs lactis subs lactis	Streptococcus agalactiae	12.4	[9]
Thionin	Staphylococcus aureus	Positive inhibition (not reported diameter)	[8]
Hydrogen peroxide	Staphylococcus aureus	Positive inhibition (not reported diameter)	[21]
Enterocin AS-48	Staphylococcus aureus	Positive inhibition (not reported diameter)	[22]
Staphylococcus coagulasa	Streptococcus agalactiae	Positive inhibition (not reported diameter)	[11]

titis, although the antimicrobial activity shown by the authors is only for a single responsible microorganism of mastitis. Nascimento et al. [11], for example, they report that an isolated bacteriocin from coagulase-negative staphylococci could be utilized as an antimicrobial compound in the treatment and prevention of mastitis caused by Streptococcus, Ochoa-Zarzosa et al. [8], they suggest the use of a phyto-peptid for the treatment of staphylococcic mastitis and Moon et al. [7], affirm that a polysaccharide, the chitosan is a potential agent for the treatment of the bovine staphylococcic mastitis. Other studies report improvement the quality bacterial counts in milk, and decreasing somatic cell when employment specific egg yolk immunoglobulin (IgY) as an alternative therapy for mastitis caused by S. aureus [4]. Tomasinsig et al. [14] found that peptide components of the innate immune system of mammals, as cathelicidins BMAP-27 and -28 were highly effective in mastitic bovine milk and showed a variably broad spectrum of activity against 28 bacterial isolates from bovine mastitis; and Van den Borne et al. [15], reported beneficial effects of recently acquired subclinical mastitis after antimicrobial treatment during lactation.

CONCLUSIÓN

The results obtained in the tests of diffusion in agar and in the kinetic of antimicrobial activity of Weissella confusa suggest that this strain and its metabolits can inhibit in vitro the two main mastitis producers microorganisms. Also, is possible that those metabolites are substances like bacteriocins. The inhibitory diameters reached in this study are over two times the reported by bacteriocin commercially used for the mastitis control, nisin and lacticin. Therefore, a new research about the chemical nature of inhibitory metabolite is needed, which will allow to know its biotechnological potential in the mastitis treatment and control. The results of this investigation clarify besides that is necessary the search of economic fermentation substrates for the production at industrial scale of Weissella confusa and given the interesting concentrations of lactic acid produced by the strain, would also be of great importance to evaluate the strain potential for the production of the lactic acid biodegradable polymer. Additionally, we want to evaluate in vivo the effect of Weissella confusa and its metabolite in the control and prevention of bovine mastitis. Also, the viavility and stability of Weissella confusa and its metabolite during storage will be evaluated.

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