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In vitro antimicrobial activity of substances produced by *Lactobacillus spp*. against *Streptococcus mutans* isolated from the oral cavity of caries-free children

Actividad antimicrobiana in vitro de sustancias producidas por *Lactobacillus spp*. contra *Streptococcus mutans* aislado de la cavidad oral de niños sin caries

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RESUMEN

La caries dental es una enfermedad prevalente, particularmente en la población pediátrica, y *Streptococcus mutans* es una bacteria clave involucrada en su desarrollo. Este estudio explora alternativas naturales para prevenir la colonización de *S. mutans* en la cavidad oral mediante el uso de cepas de *Lactobacillus spp*. El objetivo fue evaluar la capacidad antimicrobiana de los sobrenadantes obtenidos de *Lactobacillus spp*. aislados de exudado vaginal frente a *S. mutans*, bacteria asociada a la caries dental. Las cepas de *Lactobacillus spp*. fueron aisladas de exudado vaginal, y *S. mutans* fue aislado exitosamente a partir de tres muestras de saliva recolectadas de niños de 6 a 10 años sin presencia de caries dental, utilizando agar Man, Rogosa y Sharpe (MRS) y agar Mitis Salivarius, respectivamente. La identificación bacteriana se confirmó mediante tinción de Gram y pruebas bioquímicas, incluyendo catalasa, oxidasa y fermentación de carbohidratos. La actividad antimicrobiana se evaluó mediante el método de difusión en disco, impregnando discos estériles con sobrenadantes de *Lactobacillus spp*. y colocándolos sobre agar Müller–Hinton previamente inoculado con *S. mutans*. Se observaron zonas de inhibición que oscilaron entre 10,0 y 13,5 mm en algunas cepas, lo que demuestra su actividad antimicrobiana. Estos resultados sugieren que ciertas cepas de *Lactobacillus spp*. podrían producir bacteriocinas capaces de inhibir *S. mutans*, destacando su potencial como agentes preventivos contra la caries dental y la necesidad de realizar estudios adicionales para identificar las cepas con mayor capacidad inhibitoria.

Palabras clave: Actividad antimicrobiana; bacteriocinas; placa bacteriana; microbiota oral; zona de inhibición

ABSTRACT

Dental caries is a prevalent disease, particularly among the pediatric population, and *Streptococcus mutans* is a key bacterium involved in its development. This study explores natural alternatives to prevent the colonization of *S. mutans* in the oral cavity using *Lactobacillus spp*. strains. The objective was to evaluate the antimicrobial capacity of supernatants obtained from *Lactobacillus spp*. isolated from vaginal exudate against S. mutans, a bacterium associated with dental caries. *Lactobacillus spp*. strains were isolated from vaginal exudate, and *S. mutans* was successfully isolated from three saliva samples collected from children aged 6 to 10 years without dental caries, using Man, Rogosa, and Sharpe (MRS) agar and Mitis Salivarius agar, respectively. Bacterial identification was confirmed by Gram staining and biochemical tests, including catalase, oxidase, and carbohydrate fermentation assays. The antimicrobial activity was evaluated using the disk diffusion method by impregnating sterile disks with *Lactobacillus spp*. supernatants and placing them on Müller–Hinton agar previously inoculated with *S. mutans*. Inhibition zones ranging from 10.0 to 13.5 mm were observed in some strains, demonstrating their antimicrobial activity. These results suggest that certain *Lactobacillus spp*. strains may produce bacteriocins capable of inhibiting *S. mutans*, highlighting their potential as preventive agents against dental caries and emphasizing the need for further studies to identify strains with the greatest inhibitory capacity.

Keywords: Antimicrobial activity; bacteriocins; bacterial plaque; oral microbiota; inhibition zone

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1. INTRODUCTION

The World Oral Health Strategy was approved during the 75th World Health Assembly in May 2022. Following this, in 2023, the draft of the World Oral Health Action Plan (2023–2030) was submitted for review by the relevant bodies of the World Health Organization (WHO). According to WHO estimates from 2022, approximately 3.5 billion people worldwide suffer from oral diseases, with three out of four of them living in middle-income countries (WHO, 2023). Additionally, it is estimated that nearly 2 billion people have dental caries in their permanent teeth, while 514 million children are affected by this condition in their primary teeth (Villalobos Rodelo et al., 2025).

The high prevalence of this condition underscores the magnitude of the global dental health issue, with a significant impact on the population. Since *Streptococcus mutans* is a key microorganism in the development of caries, studying it is essential to reduce its incidence and improve oral health, as it contributes to the formation of dental plaque and facilitates the cariogenic process (Bustillos Torrez & Bueno Bravo, 2021; Yandún Ramírez, 2024; Chamorro-Jiménez et al., 2013; Gamboa et al., 2016; Jiménez Rodríguez, 2021).

In recent years, a decrease in the prevalence of dental care has been observed, particularly in developing countries. However, this condition continues to be a global public health issue, with a higher incidence among the pediatric population. Currently, dental caries is considered a result of bacterial dysbiosis, further reaffirming its multifactorial nature. The identification of *S. mutans* as the primary microorganism associated with the development of caries has led to the implementation of preventive and control strategies aimed at eliminating or reducing their presence in the oral cavity. *S. mutans* is the most widely studied oral bacterium and is recognized for its high cariogenic potential (Bustillos Torrez & Bueno Bravo, 2021; Callohuanca Torres, 2016).

Various studies on the physical characteristics and biological activity of biopolymers, such as high molecular weight chitosan (HMWC), low molecular weight chitosan (LMWC), and sodium alginate (Al-Na), have demonstrated their ability to form structures that can aggregate, agglomerate, and trap microorganisms. Previous research has shown that both LMWC and Al-Na inhibit the adhesion of pathogens to hydroxyapatite beads and to the epithelial cells of the oral cavity (Bojanich et al., 2003).

A study conducted in Bolivia in 2020 concluded that the *Lactobacillus Z2* strain (*L. fermentum*) exhibited the highest inhibitory capacity against *S. mutans*, as evidenced by the largest inhibition halo. It was also determined that 63% of the children evaluated harbored *Lactobacillus* in their saliva with no antagonistic activity, while 37% had Lactobacillus with antagonistic activity against *S. mutans*. Of the strains with inhibitory activity, 1% were found in children with caries, 11% in children without active caries, and 25% in those completely free of caries (Bustillos Torrez & Bueno Bravo, 2021).

In Uruguay, a study conducted in 2020 on children up to 36 months of age aimed to evaluate the acquisition of *Streptococcus* of the mutans group in relation to socioeconomic and cultural factors. The results indicated that, of the 20 children who completed the study, 4 exhibited transient acquisition of the bacteria, with an average age of 10.6 months. It was also determined that the average age of colonization was 18.6 months. Furthermore, 75% of the colonized children had erupted molars, while 25% had erupted incisors (Caviglia & García, 2020). In Chile, a genotyping study of *S. mutans* was conducted in 2019 to evaluate its relationship with pediatric patients. The results revealed the presence of a single genotype in each sample analyzed, rather than multiple variants. Consequently, no evidence was found to support the need for a patient to harbor all genotypes of *S. mutans* to experience caries activity (Urzúa Labra & Varas Astorga, 2019).

The purpose of this study is to analyze the inhibitory capacity of the supernatant from Lactobacillus spp. strains, isolated from vaginal exudate, against *S. mutans*. The results will contribute to the

development of new therapeutic strategies, such as the use of probiotics, which are a widely researched model of functional foods due to the various benefits they provide to host health.

2. MATERIALS AND METHODS

This research is a basic, descriptive, and experimental study aimed at expanding scientific knowledge through fundamental theories and concepts, without immediate application. Its objective is to analyze and document the characteristics and behaviors of a specific phenomenon, as well as to examine the effects of an independent variable on a dependent variable through the manipulation and control of experimental conditions. It uses rigorous methods to test hypotheses and establish a foundation for future applied research and technological advancements (Muntané Relat, 2010; Hernández Sampieri et al., 2014).

2.1. Isolation, Selection, and identification of *Lactobacillus spp.*

To achieve the objective of evaluating the antimicrobial capacity of the supernatants from *Lactobacillus spp*., strains of this genus were isolated from vaginal exudates of healthy women from the city of Jaén, Cajamarca, Peru. These strains were used as a source of substances with potential inhibitory activity against *S. mutans* (Sánchez et al., 2011). The samples were collected with informed consent using sterile swabs and placed in tubes containing 5 mL of MRS broth, which were incubated at 37°C for 24 hours under anaerobic conditions (GasPak system). Subsequently, an aliquot was inoculated onto Man Rogosa and Sharpe (MRS) agar plates and incubated again at 37°C for 24 to 48 hours under anaerobic conditions (Abo-Amer & Shobrak, 2012).

The suspected *Lactobacillus spp*. strains were selected based on their colonial morphology and Gram staining. To confirm their identity, conventional biochemical tests were performed: catalase (negative), oxidase (negative), acid production from glucose (positive), and absence of motility. In addition, the isolates did not reduce nitrates to nitrites nor produce hydrogen sulfide (Abo-Amer & Shobrak, 2012).

The strains confirmed as *Lactobacillus spp*. were subsequently used to obtain the supernatants employed in the evaluation of the antimicrobial effect against *S. mutans.*

2.2. Isolation, selection, and identification of *Streptococcus mutans*

The isolation and identification of *S. mutans* were carried out following the methodology described by Goodfellow et al. (2012) in Bergey's Manual of Systematic Bacteriology, with adaptations according to the guidelines of the Clinical and Laboratory Standards Institute for microbiological testing.

The samples for the isolation of *S. mutans* were obtained from the saliva of children aged 6 to 10 years, with no presence of dental caries (Gutiérrez Ilave et al., 2014). Unstimulated saliva samples, obtained in a fasting state, were collected in completely sterile test tubes. The inclusion criteria were as follows: no tooth brushing, no antibiotic use in the last 3 months, and the signed consent form from the parents, as well as confirmation of agreement from the Children (Bustillos Torrez & Bueno Bravo, 2021); (Briceño et al., 2002). Children who had received antibiotic therapy or iron supplementation in the last 6 months, as well as those who had received dental prophylaxis in the month prior to the examination, were excluded (Velásquez Sáez et al., 2017). Subsequently, the sample was transported at 4 ºC in a cooling container to the Medical Microbiology Laboratory of the Professional School of Medical Technology with a specialization in Clinical Laboratory and Pathological Anatomy at the National University of Jaén (UNJ) for immediate processing.

The saliva samples were placed in completely sterile test tubes with Brain Heart Infusion Broth to promote the reactivation and growth of *S. mutans* due to their high nutrient content, then incubated at 37 ºC for 24 to 48 hours using the GasPak system. Next, with the aid of a bacteriological loop, the samples were inoculated onto the selective medium Mitis Salivarius Agar, which was incubated at 37 ºC for 24 to 48 hours under anaerobic conditions. Subsequently, characteristic pinpoint colonies of the *Streptococcus* genus were identified, followed by pure culture isolation on Mitis Salivarius Agar.

For its identification, Gram staining was performed, where short chains of cocci were observed. Additionally, the type of hemolysis was evaluated on Blood Agar (Gamma hemolytic). Furthermore, as confirmatory tests, catalase (negative), oxidase (negative), carbohydrate fermentation tests: glucose (positive), sucrose (positive), maltose (positive), lactose (positive), mannitol and sorbitol (positive), and bacitracin sensitivity test (negative) were conducted (González Romero et al., 2023).

2.3. Antimicrobial activity of *Lactobacillus spp*. against *S. mutans*

Inoculum preparation

Four *Lactobacillus spp*. strains were isolated, previously identified on plates with MRS Agar after incubation for 24 to 48 hours under anaerobic conditions at 37°C. After this period, 5 mL of MRS Broth was added, a swabbing was performed with sterile swabs, and the bacterial sample was collected from the surface and transferred to test tubes under sterile conditions. After being incubated for 24 to 48 hours under anaerobic conditions using the GasPak system, these tubes were subjected to centrifugation at 4000 rpm for 20 minutes. The required supernatant was obtained to impregnate the discs and evaluate the antimicrobial effect.

Preparation of inhibition discs

For the preparation of inhibition discs, a standardized method was used, involving sterilized filter paper. The 5 mm diameter discs were placed in beakers and sterilized at 121°C for 30 minutes in an autoclave. Subsequently, the sterilized discs were moistened with 5 µL of the supernatant from each Lactobacillus spp. strain and allowed to rest for 5 minutes to ensure adequate impregnation of the supernatant onto the discs. Once prepared, the discs were evenly distributed on Müller-Hinton Agar medium, ensuring a distance of 25 mm between them to prevent interference between the inhibition zones. This procedure is part of the standard methodology for evaluating antimicrobial activity using the disk diffusion method.

Antimicrobial activity (diffusion method)

To assess the antimicrobial activity of the identified Lactobacillus spp. strains, the turbidity of the suspensions was adjusted to the McFarland standard 0.5, corresponding to 1.5×10^8 CFU/mL, by adding precise amounts of inoculum. Müller-Hinton Agar medium was used, and four discs were placed with the supernatants corresponding to each of the Lactobacillus spp. strains, with three repetitions per Streptococcus species (*S. mutans*). In addition, a positive control (Ciprofloxacin – CIP at 5 µg) and a negative control (physiological saline solution) were included. The sensitivity discs were placed on the culture medium using sterile needles and forceps, gently pressing them to ensure full contact with the surface. The halo measurements were taken after 24 hours using a millimeter ruler.

Data analysis

The inhibition zone diameters obtained from the disk diffusion assay were recorded in millimeters and expressed as mean ± standard deviation (SD) from three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) to compare the antimicrobial activity among the different Lactobacillus spp. strains. The statistical analysis was conducted to identify potential differences in inhibitory effects between the evaluated strains (Mayorga-Ponce et al., 2021).

3. RESULTS

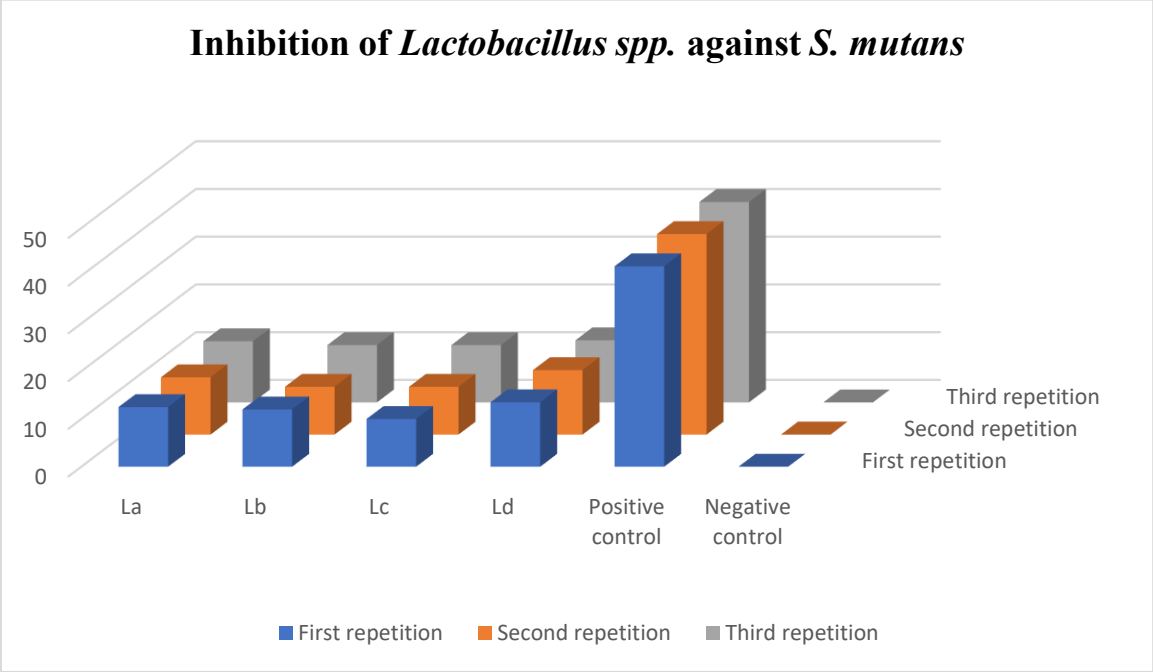

Figure 1. Evaluation of the Antimicrobial Effect of *Lactobacillus spp*. Strains Against *S. mutans*

The antimicrobial activity of the supernatants produced by four *Lactobacillus spp*. strains (*La, Lb, Lc,* and *Ld*) against *S. mutans* was evaluated using the disk diffusion method. The inhibition zones were measured after 24 hours of incubation.

All *Lactobacillus spp*. strains evaluated exhibited inhibitory activity against *S. mutans*, with inhibition zones ranging from 10.0 to 13.5 mm. Among the tested strains, *Lactobacillus Ld* consistently showed the greatest antimicrobial effect, producing the largest inhibition zones in the first and second repetitions (13.5 mm) and maintaining a high inhibitory capacity in the third repetition (13.0 mm).

Lactobacillus la showed inhibition zones ranging from 12.0 to 12.8 mm, indicating stable antimicrobial activity across the three repetitions. In contrast, *Lactobacillus Lb* and *Lc* presented smaller inhibition zones, with values between 10.0 and 12.0 mm, demonstrating a comparatively lower inhibitory effect against *S. mutans*.

The positive control (ciprofloxacin, 5 µg) produced a constant inhibition zone of 42 mm in all repetitions, confirming the susceptibility of *S. mutans* to the antibiotic and validating the experimental conditions. No inhibition zones were observed for the negative control (physiological saline solution), confirming that the inhibitory effect observed was attributable exclusively to the antimicrobial substances present in the *Lactobacillus spp*. supernatants.

Overall, these results demonstrate that the supernatants produced by *Lactobacillus spp*. strains possess in vitro antimicrobial activity against S. mutans, with *Lactobacillus Ld* exhibiting the highest inhibitory potential among the strains evaluated

4. DISCUSSION

In the results obtained in this research, it was observed that the antimicrobial activity of the *Lactobacillus* strains *La, Lb, Lc*, and *Ld* showed antimicrobial effects against *S. mutans*, forming average inhibition zones. These findings align with the research conducted by Bustillos Torrez & Bueno Bravo (2021) in Bolivia, where an experimental study reported that Lactobacillus Z2 (L. fermentum) produces an antagonistic substance that generates a larger inhibition halo against *S. mutans*, demonstrating, through three methods, the action of a bacteriocin (enzymatic protein). Similarly, Yandún Ramírez (2024), in their experimental research conducted in Ecuador, concluded that *L. delbrueckii* formed an average inhibition halo. In the same line, *L. Fermentum* showed better inhibition results against *S. mutans*.

A relevant aspect of this study is the extent of the inhibition halos observed, with lengths ranging from 10 to 13.5 mm in a 24-hour incubation period. Azizian et al. (2019), in Iran, conducted their research on inhibition using the diffusion method with *L. fermentum* and *L. delbrueckii* strains, and also observed inhibition halos. However, their results differ from this present study, as they reported inhibitory zones in the range of 17 to 23 mm after 48 hours. Furthermore, in their research, they used more than 30 species of *Lactobacillus spp*., *including L. fermentum, L. delbrueckii, L. gasseri, L. salivarius, L. vaginalis*, and *L. curvatus*, which were evaluated against pathogens such as *K. pneumoniae, S. typhimurium, Y. faecium, S. mutans*, among others, providing greater diversity and richness to their results. Nevertheless, both studies agree that Lactobacillus strains showed notable antimicrobial potential.

Garza Gonzáles (2018) in their study conducted in Mexico, concluded that *L. casei Shirota* managed to reduce the bacterial concentration in devices treated with Perioxidin. It was evident that there were no significant differences between both substances, both in the total bacterial quantification and specifically for S. mutans. Similarly, the authors Aguilar Uscanga et al. (2021), in Mexico, conducted an experimental study in which it was indicated that the bacteriocins produced by *Lactobacillus* form pores in the membrane of *S. mutans*, which causes the release of its cellular content and its subsequent death. Finally, López et al. (2023), in Mexico, in their study, discovered that the *strains* of *L. plantarum, L. brevis, L. casei*, and *L. fermentum* produced bacteriocins that effectively inhibited *A. viscosus* and *S. mutans*, confirming the potential of *Lactobacillus spp*. as probiotics in the control of cariogenic bacteria.

CONCLUSIONS

The present study demonstrated that supernatants produced by *Lactobacillus spp*. strains isolated from vaginal exudate exhibit in vitro antimicrobial activity against *S. mutans*, with variable inhibitory effects among the evaluated strains. *Lactobacillus Ld* showed the highest inhibitory activity, whereas *strains Lb* and *Lc* presented lower inhibition capacity. These findings highlight the antagonistic potential of substances produced by *Lactobacillus spp*. against a key cariogenic microorganism; however, as this study was conducted in vitro, further research is required to identify the antimicrobial compounds involved and to assess their efficacy and safety in vivo and clinical models. Overall, the results support the potential use of Lactobacillus spp. as probiotic candidates in preventive strategies against dental caries.

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The authors did not receive any funding to carry out this study.

CONFLICT OF INTEREST

There is no conflict of interest related to the subject matter of the work.

AUTHORS' CONTRIBUTION

Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Writing—original draft; Writing—review and editing: Pérez-Cubas, G., Guerra-Medina, F. N., De-la-Cruz-Pérez, A., Pardo-Pérez, Ruth E. & Chinchay-Reymundo, G. J.

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