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The Effect of Dielectric Barrier Discharge Plasma on *Lactobacillus acidophilus*: An In Vitro Study

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Abstract

Introduction: Dental caries remains a prevalent oral health issue worldwide, with *Lactobacillus acidophilus* playing a significant role in its progression. While traditional antimicrobial agents like chlorhexidine are effective, the increasing concern of antimicrobial resistance has spurred interest in alternative approaches. Dielectric barrier discharge (DBD) plasma, a form of cold atmospheric plasma (CAP), has shown promise in various biomedical applications, including dentistry. This in vitro study aims to determine the effect of DBD plasma radiation on the amount of *L. acidophilus* bacteria.

Methods: *Lactobacillus acidophilus* (ATCC 43121) cultures were subjected to DBD plasma treatment for 30, 90, 120, and 150 seconds. A positive control group was treated with 2% chlorhexidine, and a negative control received no treatment. Bacterial viability was assessed using colony-forming unit (CFU) counts. One-way ANOVA and Tukey's HSD test were used for statistical analysis. *P* values less than 0.05 were considered significant.

Results: The 30-second DBD plasma treatment significantly reduced *L. acidophilus* populations compared to the negative control (32.9% reduction, $P<0.001$). However, longer exposure times (90, 120, and 150 seconds) showed diminished antibacterial effects. The 2% chlorhexidine treatment demonstrated the highest antibacterial efficacy (54.8% reduction, $P<0.001$ compared to all other groups).

Conclusion: It seems that short-duration DBD plasma treatment (30 seconds) effectively reduced *L. acidophilus* populations, although not as efficiently as chlorhexidine. Interestingly, prolonged plasma exposure did not enhance antibacterial effects, suggesting a potential adaptive response of bacteria to extended plasma treatment. These findings highlight the promise of DBD plasma as a novel approach to dental caries prevention while emphasizing the need for optimized treatment parameters.

Keywords: Therapeutic use; Dental caries; Chlorhexidine; Plasma gases; Time factors; *Lactobacillus acidophilus*.

Introduction

Dental caries is a multifactorial disease with a bacterial origin, remaining one of the most prevalent and costly oral health issues worldwide.[1] Among the various microorganisms implicated in caries development, *Lactobacillus* species, particularly *Lactobacillus acidophilus*, have been recognized as significant contributors to the cariogenic process for over a century.[2]

L. acidophilus, a gram-positive, non-spore-forming anaerobic bacterium, is frequently found in deep carious lesions and plays a crucial role in caries progression.[3] Its ability to produce lactic acid and thrive in low-pH environments makes it particularly effective at promoting tooth demineralization.[4]

Traditional approaches to caries prevention and management have relied heavily on antimicrobial agents such as chlorhexidine. However, the increasing emergence of antimicrobial resistance poses a significant challenge to these conventional strategies.[5] This growing concern has spurred interest in alternative methods for controlling oral pathogens, including novel physical approaches like cold atmospheric plasma (CAP).[6]

CAP, a partially ionized gas generated at ambient temperature and pressure, has shown promising results in various biomedical applications, including dentistry.[7] One form of CAP, dielectric barrier discharge (DBD) plasma, has garnered particular attention due to its ability to generate reactive oxygen and nitrogen species, which are

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thought to be key to its antimicrobial effects.[8]

While several studies have explored the effects of CAP on oral biofilms and certain cariogenic bacteria like *Streptococcus mutans*,[9] there is limited research specifically examining its impact on *L. acidophilus*. Given the importance of *L. acidophilus* in caries progression, understanding the effects of DBD plasma on this organism could provide valuable insights for developing novel caries prevention and treatment strategies.

The in vitro approach was chosen for this study due to its ability to provide a controlled environment, allowing for precise manipulation of variables and isolation of specific effects on *L. acidophilus*.[10] This method serves as a crucial first step in evaluating the safety and efficacy of DBD plasma treatment before progressing to more complex models.[11] In vitro studies have been instrumental in elucidating mechanisms of action and optimizing treatment parameters for plasma applications in dentistry.[12]

Therefore, this study aimed to investigate the effects of DBD plasma exposure on *L. acidophilus* in vitro, comparing its efficacy to traditional antimicrobial approaches and exploring the relationship between exposure time and antibacterial effects.

The findings of this study contribute to a better understanding of the interaction between DBD plasma and *L. acidophilus*, a bacterium associated with dental caries. This knowledge is essential for exploring potential applications of DBD plasma in oral healthcare, particularly in developing novel antimicrobial strategies. These insights highlight the significance of investigating the effects of DBD plasma on oral microbiota, forming the foundation for the objectives of this study.

Materials and Methods

Sample Size

Based on the findings of Nima et al,[9] the effect size of 0.51 was determined and applied using the one-way ANOVA power analysis option in PASS11 software, with $\alpha = 0.05$ and $\beta = 0.2$. Given an average log standard deviation of colony counts of 0.48, the minimum required sample size for each of the six study groups was calculated to be five samples. Considering the presence of one standard species, four DBD plasma irradiation times per sample, and a control group, the total number of samples was 30.

Bacterial Strain and Culture Conditions

Lactobacillus acidophilus (ATCC 43121) was obtained as a lyophilized powder from the Iranian Organization for Science and Technology (IROST) in Tehran. The bacteria were rehydrated with sterile physiological saline (1 mL) and cultured on Brain Heart Infusion (BHI) agar supplemented with 5% sheep blood. Cultures were incubated aerobically at 37 °C for 48 hours in an incubator (INE 500, Memmert, Germany).[13]

Preparation of Bacterial Suspension

Following incubation, 5-6 morphologically similar colonies were selected using a sterile loop and suspended in 2 mL of Brain Heart Infusion Broth (BHIB) in sterile test tubes. These were then incubated at 35-37 °C for 1-2 hours. The bacterial concentration was adjusted to match the turbidity of a 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL) using a spectrophotometer (Apel PD-303, Japan) at a 600-nm wavelength. The optical density of the suspension was maintained between 0.08 and 0.11, equivalent to the 0.5 McFarland standard.[14]

Experimental Groups and Design

The study comprised six groups, each with five replicates ($n = 5$ per group):

1. Negative control: Bacterial suspension without any treatment
2. Positive control: Bacterial suspension treated with 2% chlorhexidine (100 µg/mL, Nikan Darman Co.)
3. DBD plasma treatment for 30 seconds
4. DBD plasma treatment for 90 seconds
5. DBD plasma treatment for 120 seconds
6. DBD plasma treatment for 150 seconds

For each group, 100 µL of the standardized bacterial suspension was transferred to wells in a 96-well microplate (Figure 1). The negative control group received no treatment, while the positive control group was treated with 2% chlorhexidine. For the plasma treatment groups, each well was exposed to DBD plasma for the specified duration.

DBD Plasma Device and Treatment

A CAP device (DBD plasma, Sepid Jamegan Technology Co., Iran) was used for the treatments. The device operated with neon gas at 220 V, 0.3 A, constant power of 66 V/A, and 10 kHz frequency. The 66 V/A setting used for DBD plasma irradiation was based on the default configuration of the CAP device utilized in this study. This power setting is recommended by the device manufacturer (Sepid Jamegan Technology Co., Iran) as the optimal operation

Figure 1. Lactobacillus acidophilus Bacteria Samples in 96 Microplates (It is inside 30 sample wells)

level for safe and effective plasma discharge in biomedical applications. The internal head diameter of the device was 15 mm.

For plasma treatment, the device was positioned perpendicular to the surface of the bacterial suspension in the microplate wells, maintaining a distance of 2 mm between the plasma nozzle and the surface of the suspension. To prevent uncontrolled radiation to other samples, a double-layered punched paper was used to shield adjacent wells (Figure 2).[15]

Colony-Forming Unit Assay

Twenty-four hours post-treatment, 50 µL from each well was extracted and cultured on BHI agar plates using the spread plate method. The plates were then incubated aerobically at 37 °C for 48 hours. Following incubation, the number of colony-forming units (CFU) on each plate was counted manually to determine bacterial viability. The CFU count was expressed as CFU/mL.[16]

Statistical Analysis

Data analysis was performed using SPSS software version 26. The Kolmogorov-Smirnov test was used to assess data normality. One-way ANOVA was employed to compare CFU counts between groups, followed by Tukey's HSD post hoc test for pairwise comparisons. A P value < 0.05 was considered statistically significant. The results were presented as mean ± standard deviation of CFU/mL for each group.[17]

Results

The effects of DBD plasma treatment on *L. acidophilus* were evaluated by comparing the CFUs across different treatment groups. The study aimed to assess the antibacterial efficacy of DBD plasma at various exposure times compared to a standard chlorhexidine treatment and negative control (Table 1).

Figure 2. Head of Cold Atmospheric Plasma DBD

Statistical Analysis

One-way ANOVA revealed significant differences between the treatment groups ($F(5,24) = 78.42$, $P < 0.001$). The Kolmogorov-Smirnov test confirmed the normal distribution of data across all groups ($P > 0.05$). Levene's test showed homogeneity of variances ($P = 0.218$), validating the use of ANOVA.

Post-hoc Analysis

Tukey's HSD test was employed for pairwise comparisons between the groups. The results revealed (Figure 3):

1. Chlorhexidine Treatment: The positive control group (2% chlorhexidine) demonstrated the highest antibacterial efficacy, with a 54.8% reduction in the CFU count compared to the negative control. This reduction was statistically significant compared to all other groups ($P < 0.001$ for all comparisons).
2. DBD Plasma - 30 seconds: This group showed the second-highest antibacterial effect, with a 32.9% reduction in the CFU count. It was significantly different from both the positive control ($P < 0.001$) and the negative control ($P < 0.001$).
3. DBD Plasma - 90, 120, and 150 seconds: Surprisingly, these longer exposure times showed less antibacterial efficacy compared to the 30-second treatment. The 90-second treatment resulted in only a 4.1% reduction, while both 120-second and 150-second

Table 1. Mean CFU counts (±SD) and percentage reduction of Lactobacillus acidophilus across experimental groups

Treatment Group	Mean CFU/mL ($\times 10^8$) ± SD	Reduction (%)
Negative control (no treatment)	1.46 ± 0.05	-
Positive control (2% chlorhexidine)	0.66 ± 0.11	54.8%
DBD plasma - 30 seconds	0.98 ± 0.13	32.9%
DBD plasma - 90 seconds	1.40 ± 0.12	4.1%
DBD plasma - 120 seconds	1.32 ± 0.08	9.6%
DBD plasma - 150 seconds	1.32 ± 0.08	9.6%

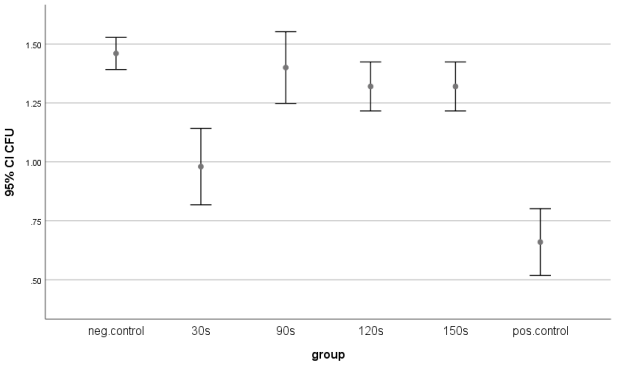

Figure 3. The Average Number of Colonies Formed in the Culture Medium (CFU) of the Study Groups

treatments showed a 9.6% reduction in the CFU count. These groups did not differ significantly from the negative control ($P > 0.05$ for all comparisons).

4. Comparison between DBD Plasma treatments: The 30-second DBD plasma treatment was significantly more effective in reducing bacterial counts compared to the 90-second, 120-second, and 150-second treatments ($P < 0.001$ for all comparisons).

Time-Dependent Effects of DBD Plasma

The antibacterial effect of DBD plasma treatment did not show a linear relationship with exposure time. The 30-second treatment was more effective than longer exposure times, suggesting a potential threshold effect or adaptive response of *L. acidophilus* to prolonged plasma exposure. This non-linear relationship is evident in the following observations (Table 1):

- The 30-second treatment reduced the CFU count by 32.9%.
- The 90-second treatment only reduced the CFU count by 4.1%.
- The 120-second and 150-second treatments both reduced the CFU count by 9.6%.

This pattern suggests that *L. acidophilus* may have mechanisms to adapt to or resist longer plasma exposures, which could be an important consideration for future applications of this technology.

Comparison with Chlorhexidine

While the 30-second DBD plasma treatment significantly reduced bacterial counts compared to the negative control, it was not as effective as the 2% chlorhexidine treatment (32.9% vs 54.8% reduction, $P < 0.001$). However, it demonstrated superior antibacterial activity compared to longer plasma exposure times. This suggests that short-duration DBD plasma treatments could potentially serve as an alternative or adjunct to traditional chemical antiseptics, though further optimization may be needed to match the efficacy of chlorhexidine.

Implications of Findings

These results indicate that DBD plasma treatment, particularly at 30 seconds of exposure, has a significant antibacterial effect against *L. acidophilus*. However, this effect is less pronounced than that of 2% chlorhexidine and does not increase with longer exposure times under the conditions of this study. The unexpected decrease in efficacy with longer exposure times warrants further investigation into the mechanisms of bacterial response to plasma treatment.

The findings suggest that short-duration DBD plasma treatments could have potential applications in dental practice for reducing *L. acidophilus* populations. However, the optimal parameters for treatment, including exposure time and intensity, may need further refinement to maximize antibacterial effects while considering the potential adaptive responses of the bacteria.

Discussion

The results of this study showed that the lowest mean colony count of *L. acidophilus* was observed in the positive control group (treated with 2% chlorhexidine) and the group irradiated with DBD plasma for 30 seconds, respectively. While the antibacterial effect of 30-second DBD plasma irradiation was slightly lower than that of the positive control group, both exhibited a significant reduction in the bacterial count compared to the negative control group. The findings suggest that DBD plasma with a constant power of 66 V/A exerts a time-dependent antibacterial effect against *L. acidophilus*.[9]

The reason for using chlorhexidine as a positive control for the antibacterial effect is that chlorhexidine is a broad-spectrum agent against viruses, bacteria, and fungi. In plasma-related treatments, bacterial inhibition is strongly influenced by both exposure time and the distance between the plasma source and the target surface.[18]

Several studies have investigated the antibacterial properties of CAP. Blumhagen et al found that the maximum antibacterial effect of CAP was achieved with just 10 seconds of irradiation.[19] Their study used argon-based plasma applied to *L. acidophilus* and Streptococcus mutans biofilms on hydroxyapatite (HA) disks. In contrast, our study employed neon-based DBD plasma applied to *L. acidophilus* biofilms in a 69-well microplate system. These differences in plasma composition, irradiation time, and experimental conditions likely explain the variation in observed results. To further clarify the relationship between exposure time and antibacterial efficacy, the present study assessed DBD plasma irradiation across multiple time intervals.

A similar study by Nima et al examined the effects of DBD plasma on Streptococcus mutans, using irradiation times and methodology comparable to our study. The difference in bacterial species studied accounts for variations in results. However, like the present research, their study also confirmed the time-dependent antibacterial properties of DBD plasma.[9]

Abonti et al multi-gas plasma was used to inactivate *Streptococcus mutans, Lactobacillus fermentum*, and Aggregatibacter actinomycetemcomitans on the agar culture medium. Their findings indicated that *Lactobacillus fermentum* required a 60-second exposure for complete inactivation.[20] These reports are somewhat in line with the present results.

Two other factors that influence the effectiveness of CAP irradiation are the distance between the device nozzle and the irradiation surface, and the dispersion of the radiation.[21] In the study by Abonti et al, plasma was irradiated from distances of 20 mm and 2 mm to bacterial cultures. Plasma irradiation from a distance

of 20 mm was able to cause a greater reduction in the colony count due to the involvement of a larger surface area.[20] However, findings from Das et al indicate that increasing the distance between the plasma nozzle and the sample surface generally reduces its antimicrobial efficacy.[21] These observations highlight the importance of optimizing plasma application parameters for maximum antibacterial efficiency.

Considering the studies conducted, CAP is a suitable tool for combating oral bacteria. Other antimicrobial agents, such as chlorhexidine mouthwash alone, also reduce the number of *L. acidophilus* colonies by disrupting osmotic regulation and consequently inhibiting cellular respiration, damaging the bacterial membrane, and leading to the leakage of cytoplasmic contents.[22,23] Chlorhexidine is a topical drug and the gold standard against *L. acidophilus*.[24]

DBD plasma, on the other hand, exerts its bactericidal effects through multiple mechanisms, including the generation of reactive oxygen and nitrogen species (ROS and RNS) such as atomic oxygen, ozone, hydroxyl group, NO, and NO2.[25,26] These mechanisms have a direct effect on microorganisms, especially on their outer membranes, through chemical etching. They also cause the destruction of bacterial DNA, proteins, and enzymes, ultimately leading to cell destruction.[27] It is observed that the cell wall surface opens up significantly, and many intracellular contents are released, confirming bacterial inactivation.[19]

Numerous studies have examined the effect of plasma jet on oral biofilms, particularly on Streptococcus mutans bacteria.[9,20,28] However, few studies have investigated the effect of DBD plasma, a direct type of plasma, on *L. acidophilus*, one of the main bacteria involved in dental caries. In this study, a cold atmospheric DBD plasma device, utilizing neon gas, was employed for the first time to investigate its effects on *L. acidophilus*. Given the increasing potential for oral pathogens to develop resistance to conventional antimicrobial agents over time, DBD plasma may offer a safe, non-invasive alternative for reducing the microbial load in the future.

Therefore, the advantage of this study, in addition to examining the effect of DBD plasma on *L. acidophilus* bacteria, is examining the capability of an Iranian-made device.

Limitations

The main limitations of this study were the difficulty in accessing *L. acidophilus* bacteria in lyophilized form and the inability to fully simulate oral conditions in an in vitro setting. The study focused on a single-species biofilm, which doesn't represent the complex multi-species environment of the oral cavity. Additionally, only a limited range of plasma parameters was tested, and long-term effects were not assessed. These limitations provide directions for future research to further validate and expand upon the findings in more clinically relevant conditions.

Conclusion

Based on the findings of this study, the lowest number of *L. acidophilus* colonies was observed in the positive control group (2% chlorhexidine) and in the 30-second DBD plasma irradiation group, respectively. DBD plasma containing neon gas with a constant power of 66 V/A for 30 seconds was effective in reducing *L. acidophilus* colonies, although less so than chlorhexidine. The results suggest that DBD plasma could potentially be used as a safe, non-invasive method for reducing the microbial load in dental applications. While not surpassing the efficacy of chlorhexidine, DBD plasma shows promise as a novel approach to dental caries prevention, particularly given concerns about increasing bacterial resistance to traditional antimicrobial agents. Comparative studies could explore the efficacy of DBD plasma versus conventional antimicrobial treatments such as chlorhexidine in reducing the microbial load in dental applications. In addition, long-term studies are needed to assess the sustained antimicrobial effects of DBD plasma on oral biofilms in vivo models.

Authors' Contribution

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Methodology: Mahsa Fattahi, Ghazaleh Shahhosseini.
Project administration: Somayeh Alirezaei.
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Supervision: Somayeh Alirezaei.
Validation: Arezoo Alaee.
Visualization: Ghazaleh Shahhosseini.
Writing–original draft: Ghazaleh Shahhosseini.
Writing–review & editing: Somayeh Alirezaei, Arezoo Alaee.

Competing Interests

The authors declare no competing interests.

Consent for Publication

Not applicable.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

This study was reviewed and approved by the Ethics Committee of the Faculty of Dentistry, Tehran Medical Sciences, Islamic Azad University, with the ethical approval code IR.IAU.DENTAL.REC.1403.014. All procedures were conducted in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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