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*Article*

# Analysis of Antibacterial Efficacy and Unique Chemical Compositions Generated from Plasma Activated Water

**Hyun Jin Kim [1,†], Hyun A Shin [1,†], Woo Kyung Chung [2], Ae Son Om [2], Areum Jeon [1], Eun Kyung Kang [1], Wen An [1] and Ju Seop Kang [1,*]**

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**Abstract:** This study aimed to elucidate the antibacterial efficacy and unique chemical compositions generated from plasma-activated water (PAW) when utilized a novel vaginal cleansing device. We conducted an analysis of antibacterial activity against probiotics and several chemical compositions including ions under various operational parameters of PAW, including electrical power (12 and 24 V), treatment duration (1, 10, and 20 min), and stay duration (immediate, 30 and 60 min). Our findings revealed that as treatment duration increased, hypochlorous acid (HOCl), $Ca^{2+}$, and $Mg^{2+}$ concentrations increased and $Cl^-$concentrations decreased. Higher electrical power and longer treatment duration resulted in increased HOCl levels, which acts to prevent the growth of general microorganisms. Notably, PAW exhibited no antibacterial effects against a type of probiotic, *Lactobacillus reuteri* that produces lactic acid for vaginal health. This result demonstrated the ability of the vaginal cleaning device to generate ions, primarily HOCl and some cations ($Ca^{2+}$ and $Mg^{2+}$), thereby providing vaginal mucosa protecting and cleansing effects with the vaginal environment.

**Keywords:** plasma-activated water (PAW); hypochlorous acid (HOCl); probiotics (*Lactobacillus reuteri*); mucosa protection; vaginal cleansing effect

## 1. Introduction

Plasma technology has proven its effectiveness in various medical applications, including regenerative medicine for skin and dental treatments, as well as surface sanitization and sterilization of medical tools [1]. Plasma is a term that refers to a quasi-neutral ionized gas to containing photons, free radicals, and ions as well as uncharged particles [2–4]. Traditional plasmas are categorized as thermal and non-thermal, based on the thermodynamic equilibrium and non-equilibrium of electrons and other gas species. Recent advances in plasma engineering have enabled the generation plasma-activated waters (PAW) through non-thermal atmospheric pressure plasmas (NTAPPs) [5]. The PAW can be produced by exposing water to ionized gas generated by a plasma device, either above or below the water surface. Many studies have demonstrated that the reactive species in PAW do not cause toxicity or environmental pollution, paving the way for diverse applications in the life sciences [4]. The primary application areas of PAW technology encompass seed germination, plant growth, food preservation, antimicrobial activities, virus inactivation, and anticancer treatments, among others [6]. The disinfection effectiveness of PAW hinges upon the concentration of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS generated in PAW, including hydrogen peroxide($H_2O_2$), hydroxyl radical( ·OH), and ozone ($O_3$), function as potent oxidizing agents, inducing oxidative stress on the microbial cell membranes and, consequently, bacterial damage and death[7]. RNS occurred in PAW, such as nitric oxide (NO), nitrite ($NO_2^-$), and nitrate ($NO_3^-$), primary exist as peroxynitrite (ONOOH) in low pH conditions. The peroxynitrite can accumulate inside cells

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leading to apoptotic or necrotic cell death [8]. The production of various reactive oxygen and nitrogen species (RONS) depends on the operating parameters of plasma generation, such as the power source, treatment time, feed gases, and electrode materials.[7,9] Gao et al. reported that increasing the electrical power from 0 to 160 W led to higher concentrations of OH, $H_2O_2$, $NO_3^-$, and $NH_4$ in PAW [4]. Berardinelli et al. reported that longer treatment duration result in elevated concentrations of $NO_2^-$ and $NO_3^-$ in PAW [10]. Other studies have shown that the concentrations of $O_3$ and $H_2O_2$ can also be increased in PAW[11]. Georgescu et al. reported that feed gases like helium and nitrogen can alter the reactive species in PAW by changing the electron density and surface charges compared to ambient air[12]. These parameters can be fine-tuned to create PAW with different reactivity levels, catering to various disinfection applications, and researchers are actively developing PAW device with different reactivity [13]. The applications of PAW have rapidly expanded to include the treatment of biomedical devices and biological materials, including foods.

Moreover, Lee and Hong studies have shown that plasma discharge in tap water eliminating harmful microorganisms by increased the free residual chlorine molecules such as hypochlorous acid and hypochlorite ions [14–16]. While the exact mechanisms remain unclear, PAW has demonstrated beneficial effects in terms of antibacterial and cytotoxicity, attributed to its reactive species [4,5,17,18]. Hence, further theoretical-experimental investigations are warranted to expand our understanding and explore the potential of PAW in biological decontamination and clinical applications[12].

In this context, our previous study confirmed the bactericidal effect of PAW on clinical abnormal vaginal microbiota in clinical practice [14,19,20]. As follows, in PAW-sprayed patients (22.3%) showed a better vaginal cleaning effect against Gram-positive and –negative bacteria than betadine treatment (BT) patients (14.4%) [14]. In other pathological microbiota, a significant decrease was observed after treatment compared to before, with *mycoplasma hominis* 30±15.28 %, *Ureaplasma urealyticum* 25±9.93 %, *Ureaplasma parvum* 23±8.42 %, and *Candida albicans* 28±10.86 % [19]. Vaginitis is a common disease among women and bacterial vaginosis is the most common, accounting for 22~50% of all vaginitis, followed by candida vulvovaginitis at 17~35%, and trichomonas vaginitis at 4~39%. It is caused by unbalanced changes in the vaginal microbiome, which are associated with a reduced in the overall number of Lactobacilli species and a predominance of anaerobic microorganisms, including *Gardnerella vaginalis*, *Trichomonas*, *and Candida albicans* strains.Generally, the clinical symptoms include a foul-smelling vaginal discharge, fever, sexual discomfort and painful urination [21].

The excessive growth of anaerobic species, particularly *Gardnerella vaginalis*, results in a polymicrobial biofilm adhered to the vaginal epithelium[22]. Biofilms are communities of microorganisms encased in a polymeric matrix of nucleic acid, polysaccharides, and proteins[23]. Currently, several studies have supported that these biofilm-related infection are challenging to eradicate by both the immune system and antibiotics, leading to a high rate of relapse and recurrence in bacterial vaginosis case [24–26].

As an innovative alternative, the utilization of PAW offers a method to disrupt biofilms and effectively eliminate attached bacteria. PAW, which was created through cold atmospheric-pressure plasma discharge in water, demonstrates significant antimicrobial activity within biofilms, all without promoting bacterial resistance [27].

Therefore, the objective of this study is to analyze the chemical composition underpinning PAW's disinfection effects, with a specific focus on its relevance to vaginal sterilization. Additionally, we evaluate the protective effect of vaginal mucosa in the vaginal environment using PAW by measuring the antibacterial activity of *Lactobacillus reuteri*, a vital probiotic component of the vaginal microbiome essential for the host's health [28].

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## 2. Materials and Methods

### *2.1. PAW Generating System and PAW Processing*

PAW system was prepared using an underwater plasma-generating device, following established procedures reported previously [20]. Figure 1 illustrates the device, which comprised a 3L cleaning solution container, the atmospheric plasma electrodes, and controller.

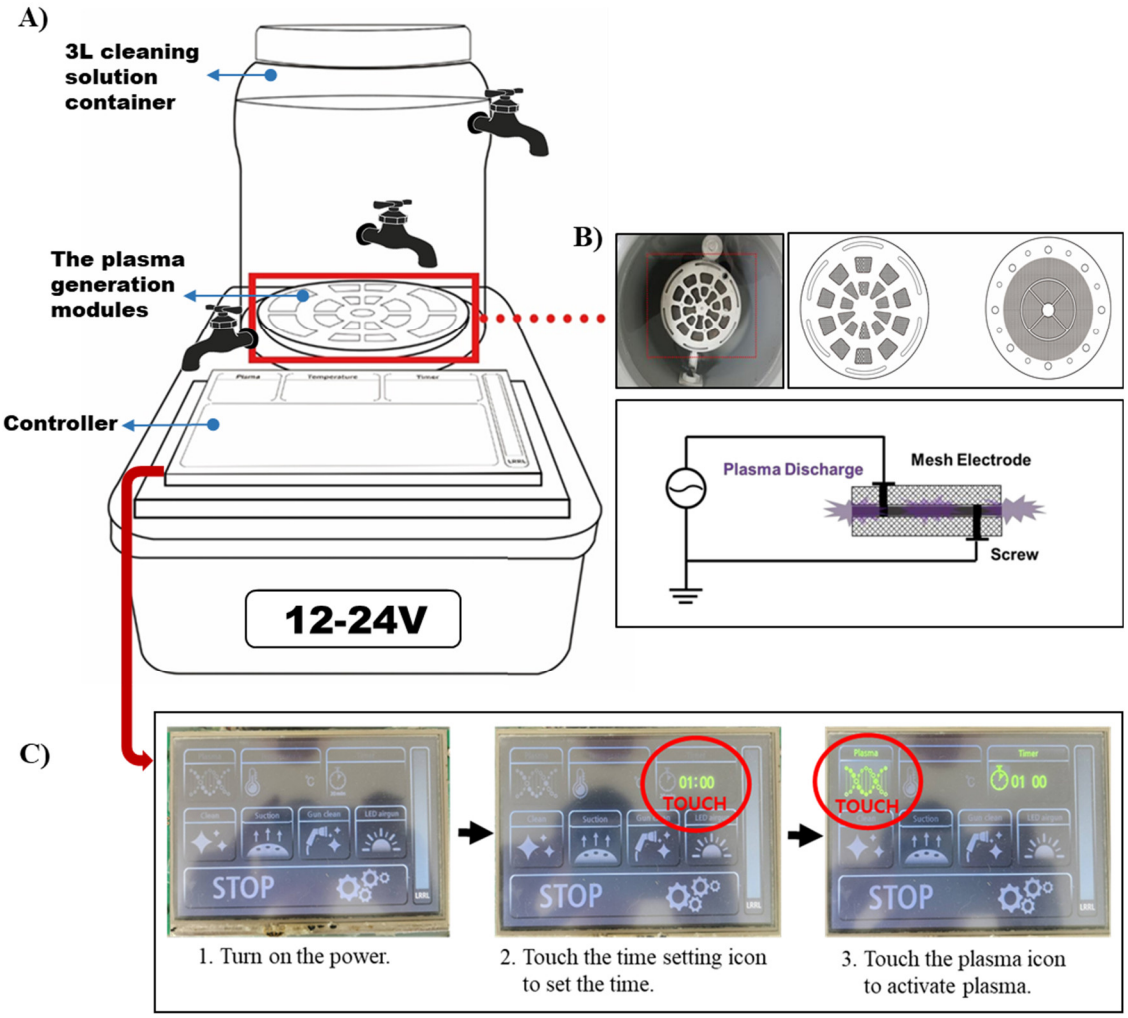


**Figure 1.** Plasma device and operation process. **A**) Illustrates the components of the plasma device used in the experiment. **B**) depicts the plasma module and the schematic principle for underwater plasma discharge [20], and **C**) presents the digital display controller, detailing the operation process of the plasma device.

The device facilitated plasma discharge underwater through a plasma electrode located at the container's base. The plasma generation modules consisted of two plasma electrodes separated by an insulating frame, with each electrode connected to power of different polarity. These electrodes are disc-shaped grids, measuring 77 mm in diameter and 0.5 mm in thickness, with titanium as the electrode material to prevent corrosion. The grid dimensions include a 1.07 mm diameter and a 3.92 mm pitch, resulting in 50% open space. To enhance plasma generation, a 300 nm thick platinum thin film was deposited on the grid's surface using electroless plating. The plasma discharge occurs between the two electrodes with a consistent 2 mm separation maintained by the insulating frame (Figure 1B) [20]. For the chemical composition analysis of different positions (Top, middle, bottom) within the 3L cleaning solution container, three stopcocks were connected to the container (Figure 1A). Figure 1C shows the operation process of the plasma device.

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*2.2. PAW Sample Preparation Conditions*

The conditions of PAW were as follows: 1) electrical power settings at 12 and 24 V, 2) treatment durations of 1, 10, and 20 min, and 3) retention time after plasma exposure, including immediate, 30 and 6 0min (Table 1). At specified time intervals, 10 mL samples of PAW were collected and immediately subjected to analysis using ion chromatography, a residual chlorine analyzer and a pH meter.

**Table 1.** The conditions of plasma activated water.

| | Position | Power | Treatment time (min) | Retention time (min) |
|---|---|---|---|---|
| Distilled water<br>Tap water | Top, middle, bottom | 12V, 24V | 1, 10, and 20 | 0, 30, and 60 |

*2.3. Analytical Methods*

The analysis of each PAW sample and untreated water sample concerning their inorganic constituents, and hypochlorous acid were performed using the following equipment:

2.3.1. Ion Chromatography (IC)

IC analysis was carried out with a ion chromatograph (Dionex ICS-3000,Thermo Fisher Scientific, Waltham, MA, USA ) equipped with both an anion and cation module. It employed with an Ionpac AG20 4 x 50 mm guard column (Thermo Fisher Scientific, Waltham, MA, USA**)** and Ionpac AS20 4 x 250 mm analytical column (Thermo Fisher Scientific, Waltham, MA, USA) in the anion module and Ionpac CG16 5 x 50 mm guard column and Ionpac CS 5x250 mm analytical column for the cation module. The column temperature was set at 30 °C for a run time of 20min. The mobile phase for the anion module utilized gradient method, starting at 12mM sodium hydroxide (NaOH) for the initial 8 min, followed by a change to 40 mM NaOH from 8 min to 12 min, maintenance at 40mM NaOH until 18 min, and a decrease to 12 mM NaOH until 20 min. The cation module employed an isocratic method with a mobile phase consisting of 40 mM methanesulfonic acid (MSA). The flow rate was 1mL/min and the injection volume was 25 uL. The anion module contained an ADRS 600 suppressor (Thermo Fisher Scientific, Waltham, MA, USA**)** was used fot the anion module, while the cation module incorporated a CDRS 600 suppressor (Thermo Fisher Scientific, Waltham, MA, USA). Instrument control and data acquisition were managed through the Chromeleon® chromatography management software (version 6.80) (Thermo Fisher Scientific, Waltham, MA, USA).

Working standards was prepared from the seven anions (Fluoride, chloride, Nitrite, Bromide, Nitrate, Phosphate, Sulfate) and the calibration curve was ranged from 0.1 to 9.9, 0.15 to15, 0.5 to 50, 0.5 to 50, 0.5 to 50, 0.75 to 75, and 0.75 to75 ppm, respectively.

The cation standard working solution was prepared from six cations (Lithium, Sodium, Ammonium, Magnesium, Potassium, Calcium) and the calibration curve was ranged from 0.3 to 25, 1 to 100, 1.26 to 126, 1.27 to 127, 2.5 to 250, and 2.5 to 250 ppm, respectively.

2.3.2. Residual Chlorine Analyzer

Hypochlorous acid concentrations in PAW were determined using DPD(N,N-diethyl-1,4-phenylenediamine) free chlorine reagent with **Q-CL501B** analyzer (Shenzhen Sinsche Technology Co. Ltd., Shenzhen City, China, following the DPD colorimetric method.

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### *2.4. Evaluation of Antibacterial Efficacy*

#### 2.4.1. Microorganisms and Materials

In the assessment of antibacterial activity, microorganism were sourced from the Korean Collection for Type Cultures (KCTC) (Table 2). For the cultivation of *Limosilactobacillus reuteri* subsp. *reuteri* (*L. reuteri*), de Man, Rogosa, Sharpe Agar (MRS, Difco, MI, USA) was employed as the growth medium.

**Table 2.** Microbiological used for antibacterial activity test.

| Microorganism | | Strain |
|---|---|---|
| Gram-positive | *Lactobacillus reuteri* | KCTC 3594 |

#### 2.4.2. Antibacterial Activity

The antibacterial activity of plasma-activated water (PAW) was performed using the filter paper disc method [29]. The bacterial cultures (sub-cultured before assay) were diluted with sterile water to obtain a bacterial suspension of $OD_{600nm}$ = 0.2~0.3. Petri dishes containing 10 ml of MRS media were inoculated with 0.1 ml of the bacterial suspension, dried within a sterile chamber, and incubated at 37 °C for 48 h. The filter paper disc (Ø: 6 mm, ADVANTEC, Japan) was impregnated with 20 µl of PAW sample, placed on the medium, and cultured at 37 °C for 15 min and 30 min. Sterile water served as the negative control. All the experiments were performed in triplicate.

## 3. Results

### *3.1. Inorganic Anions and Cations Composition in PAW*

The analysis of inorganic anions and cations was conducted on PAW samples subjected to underwater plasma discharge for various durations (1, 10, and 20 min) at two voltage settings (12 and 24 V). Figure 2A,B illustrates the chromatography of standard anions and cations. The retention times for individual anions (Fluoride, chloride, Nitrite, Bromide, Nitrate, Phosphate, Sulfate) were approximately 3.74, 5.14, 6.05, 7.06, 7.87, 9.52, and 14.30 minutes, respectively. The retention time for each cations (Lithium, Sodium, Ammonium, Magnesium, Potassium, Calcium) were approximately 4.78, 6.45, 7.94, 10.84, 11.96, and 14.77 min, respectively. The calibration curves for standard anions and cations demonstrated linearity, enabling the accurate determination of anions and cations concentrations in PAW. Figure 2C,D presents the chromatography of anions and cations within PAW samples.

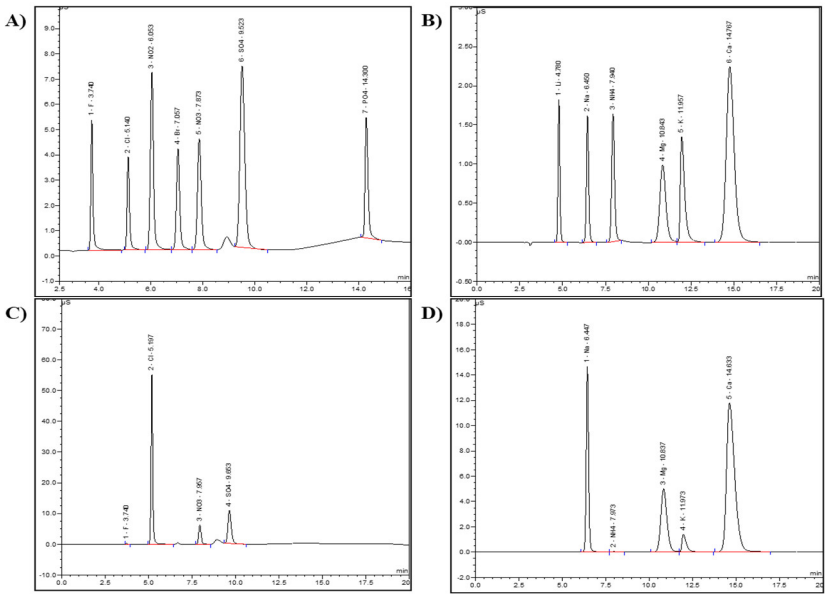

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**Figure 2.** Ion chromatography analysis of inorganic anions and cations in Paw. Chromatograms depict the separation of inorganic anions **A**) and cations **B**). Determination of inorganic anions **C**) and cations **D**) in PAW sample under the conditions of 12 V electrical power, 20min treatment time, sample position at the top, and a 60 min retention time.

Ion chromatography analysis revealed the generation of five cations and four anions following plasma treatment of tap water (Tables 3 and 4). Table 3 presents changes in ion concentrations following 12V plasma generation: After plasma activation for 20 min, the ions that exhibited changes were $Ca^{2+}$ , $Mg^{2+}$and $Cl^{-}$.The baseline levels of cations $Ca^{2+}$ and $Mg^{2+}$ were 10.46 ppm (top), 15.17 ppm (middle), 15.00 ppm (bottom) and 3.52 ppm (top), 3.86 ppm (middle), 3.89 ppm (bottom). The baseline levels of anion $Cl^{-}$ was 20.21 ppm (top), 19.35 ppm (middle), 19.27 ppm (bottom). Under a 60 min duration, $Ca^{2+}$ and $Mg^{2+}$ concentrations increased to 20.73 ppm (top), 19.33 ppm (middle), 18.75 ppm (Bottom) and 4.51 ppm (top), 4.04 ppm (middle), 4.01 ppm (Bottom), respectively. $Cl^{-}$ concentration were 19.76 ppm (top), 18.46 ppm (middle), 17.98 ppm (Bottom) under same conditions. After plasma activation for 10 min, changes were observed in $Ca^{2+}$ and $Cl^{-}$. $Ca^{2+}$, initially at 13.38 ppm, changed to 17.33 (top), 17.76 (middle), 17.73 ppm (Bottom) within a 60 min duration. $Cl^{-}$ initially at 17.41 ppm, changed to 16.85 (top), 16.96 (middle), 17.17 ppm (Bottom) under same conditions. After plasma activation for 1 min, changes were observed in $Ca^{2+}$ and $Cl^{-}$. The baseline levels of $Ca^{2+}$ and $Cl^{-}$ ions were 13.31 ppm and 19.08 ppm. $Ca^{2+}$ concentration were 16.33 (top), 16.20 (middle), 16.31 ppm (Bottom) under duration time of 60 min. $Cl^{-}$ formedeach position 18.62 (top), 13.83 (middle), 13.62 ppm (Bottom) under same conditions.

Table 4 presents changes in ion concentrations after 24 V plasma generation: after plasma activation for 20 min, changes were observed in $Ca^{2+}$ and $Cl^{-}$. The baseline levels of $Ca^{2+}$ and $Cl^{-}$ were 14.61 ppm (top), 14.35 ppm (middle), 12.17 ppm (bottom) and 18.25 ppm (top), 17.93 ppm (middle), 17.10 ppm (bottom). $Ca^{2+}$ concentrations reached 16.78 ppm (top), 16.79 ppm (middle), 15.95 ppm (Bottom) within a 60 min duration. $Cl^{-}$ concentrations reached 16.17 ppm (top), 15.55 ppm (middle), 15.28 ppm (Bottom) under same conditions. After plasma activation for 10 min, the baseline concentration of $Ca^{2+}$, initially at 13.31 ppm, changed to 16.98 (top), 16.07 (middle), 16.08 ppm (Bottom) within a 60 min duration. $Cl^{-}$, initially at 19.08 ppm, changed to 17.35 (top), 17.18 (middle), 17.15 ppm (Bottom) under same conditions. After plasma activation for 1 min, the baseline concentration of $Ca^{2+}$ ions, initially at 12.43 ppm changed to 16.42 (top), 13.80 (middle), 16.54 ppm (Bottom) within a 60 min duration. The ion changes observed following plasm generation revealed an increase in $Ca^{2+}$ concentration alongside a decrease in $Cl^{-}$ concentration. Moreover, as the duration of plasma generation extended, the magnitude of these ion changes became more pronounced. However, there were no significant discernible effect on plasma generation electrical power or the retention time after plasma exposure.

**Table 3.** Measurements of Inorganic ions, and free chlorine in a 12V underwater plasma discharge.

| Sampling | Treatment Time | Sampling Position | Retention Time | $Na^{+}$ | $NH4^{+}$ | $K^{+}$ | $Mg^{2+}$ | $Ca^{2+}$ | $F^{-}$ | $Cl^{-}$ | $NO3^{-}$ | $SO4^{2-}$ | HOCl |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | (min) | | (min) | (mg/L) | (mg/L) | (mg/L) | (mg/L) | (mg/L) | (mg/L) | (mg/L) | (mg/L) | (mg/L) | (ppm) |
| 1 | 20min | | baseline | 9.20 | 0.18 | 2.48 | 3.52 | 10.46 | 0.05 | 20.21 | 5.79 | 10.23 | 0.00 |
| 2 | | sample① Top | 0 | 9.11 | 0.13 | 2.43 | 4.41 | 18.30 | 0.06 | 19.24 | 5.70 | 10.25 | 1.03 |
| 3 | | | 30 | 9.16 | 0.16 | 2.43 | 4.48 | 20.08 | 0.06 | 19.96 | 5.97 | 10.91 | 1.11 |
| 4 | | | 60 | 9.16 | 0.13 | 2.44 | 4.51 | 20.73 | 0.06 | 19.79 | 5.82 | 10.50 | 1.17 |
| 5 | | | baseline | 7.46 | 0.15 | 2.33 | 3.86 | 15.17 | 0.05 | 19.35 | 6.46 | 9.59 | 0.09 |
| 6 | | sample② Middle | 0 | 7.45 | 0.16 | 2.32 | 4.02 | 18.37 | 0.05 | 18.54 | 6.44 | 9.67 | 1.21 |
| 7 | | | 30 | 7.45 | 0.18 | 2.32 | 4.05 | 19.15 | 0.05 | 18.18 | 6.31 | 9.36 | 1.20 |
| 8 | | | 60 | 7.41 | 0.17 | 2.31 | 4.04 | 19.33 | 0.05 | 18.46 | 6.39 | 9.53 | 1.00 |
| 9 | | | baseline | 7.34 | 0.14 | 2.28 | 3.89 | 15.00 | 0.06 | 19.27 | 6.96 | 10.06 | 0.05 |
| 10 | | sample③ Bottom | 0 | 7.06 | 0.17 | 2.20 | 3.84 | 16.99 | 0.05 | 17.31 | 6.54 | 9.25 | 1.07 |
| 11 | | | 30 | 7.28 | 0.16 | 2.27 | 3.99 | 18.40 | 0.05 | 17.90 | 6.77 | 9.58 | 1.12 |
| 12 | | | 60 | 7.30 | 0.17 | 2.27 | 4.01 | 18.75 | 0.05 | 17.98 | 6.78 | 9.63 | 1.11 |
| 13 | 10min | | baseline | 7.59 | 0.16 | 2.27 | 3.40 | 13.38 | 0.04 | 17.41 | 7.71 | 9.02 | 0.11 |

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| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 14 | | sample① Top | 0 | 7.59 | 0.22 | 2.27 | 3.50 | 16.22 | 0.05 | 17.16 | 7.75 | 9.13 | 0.78 |
| 15 | | | 30 | 7.62 | 0.20 | 2.28 | 3.51 | 17.02 | 0.05 | 17.23 | 7.74 | 9.09 | 0.66 |
| 16 | | | 60 | 7.57 | 0.26 | 2.30 | 3.50 | 17.33 | 0.05 | 16.85 | 7.65 | 8.95 | 0.60 |
| 17 | | sample② Middle | 0 | 7.65 | 0.20 | 2.28 | 3.55 | 17.66 | 0.05 | 16.92 | 7.69 | 8.98 | 0.71 |
| 18 | | | 30 | 7.66 | 0.20 | 2.30 | 3.56 | 17.74 | 0.05 | 16.98 | 7.63 | 8.95 | 0.72 |
| 19 | | | 60 | 7.60 | 0.17 | 2.28 | 3.54 | 17.76 | 0.05 | 16.96 | 7.63 | 8.96 | 0.66 |
| 20 | | sample③ Bottom | 0 | 7.69 | 0.17 | 2.30 | 3.52 | 17.71 | 0.05 | 17.05 | 7.57 | 8.88 | 0.59 |
| 21 | | | 30 | 7.72 | 0.18 | 2.29 | 3.53 | 17.77 | 0.05 | 16.99 | 7.64 | 9.00 | 0.57 |
| 22 | | | 60 | 7.67 | 0.17 | 2.28 | 3.51 | 17.73 | 0.05 | 17.17 | 7.64 | 9.00 | 0.65 |
| 23 | 1min | | baseline | 7.49 | 0.23 | 2.28 | 3.44 | 13.31 | 0.05 | 19.08 | 7.84 | 9.37 | 0.02 |
| 24 | | sample① Top | 0 | 7.21 | 0.25 | 2.05 | 3.18 | 16.41 | 0.06 | 18.39 | 7.48 | 8.80 | 0.21 |
| 25 | | | 30 | 7.20 | 0.22 | 2.16 | 3.18 | 16.32 | 0.06 | 18.83 | 7.67 | 9.12 | 0.11 |
| 26 | | | 60 | 7.19 | 0.19 | 2.15 | 3.18 | 16.33 | 0.06 | 18.62 | 7.60 | 8.98 | 0.11 |
| 27 | | sample② Middle | 0 | 6.37 | 0.08 | 1.89 | 3.14 | 15.03 | 0.04 | 14.00 | 7.44 | 7.85 | 0.23 |
| 28 | | | 30 | 6.74 | 0.11 | 1.94 | 3.14 | 15.70 | 0.05 | 13.77 | 7.58 | 8.59 | 0.15 |
| 29 | | | 60 | 6.42 | 0.09 | 1.99 | 3.22 | 16.20 | 0.05 | 13.83 | 7.65 | 7.77 | 0.11 |
| 30 | | sample③ Bottom | 0 | 6.31 | 0.09 | 1.92 | 3.15 | 16.06 | 0.05 | 13.84 | 7.65 | 7.90 | 0.11 |
| 31 | | | 30 | 6.26 | 0.09 | 1.93 | 3.15 | 16.02 | 0.05 | 13.76 | 7.66 | 7.88 | 0.09 |
| 32 | | | 60 | 6.30 | 0.11 | 1.94 | 3.18 | 16.31 | 0.05 | 13.62 | 7.61 | 7.76 | 0.15 |

**Table 4.** Measurements of Inorganic ions, and free chlorine in a 24V underwater plasma discharge.

| Sampling No. | Treatment Time (min) | Sampling Position | Retention Time (min) | $Na^+$ (mg/L) | $NH4^+$ (mg/L) | $K^+$ (mg/L) | $Mg^{2+}$ (mg/L) | $Ca^{2+}$ (mg/L) | $F^-$ (mg/L) | $Cl^-$ (mg/L) | $NO3^-$ (mg/L) | $SO4^{2-}$ (mg/L) | HOCl (ppm) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 20min | | baseline | 6.92 | 0.17 | 2.22 | 3.33 | 14.61 | 0.05 | 18.25 | 7.34 | 8.66 | 0.00 |
| 2 | | sample① Top | 0 | 6.95 | 0.15 | 2.22 | 3.45 | 16.12 | 0.04 | 15.46 | 7.20 | 8.37 | 2.10 |
| 3 | | | 30 | 6.94 | 0.13 | 2.22 | 3.49 | 16.59 | 0.05 | 15.67 | 7.35 | 8.63 | 2.56 |
| 4 | | | 60 | 6.93 | 0.13 | 2.20 | 3.50 | 16.78 | 0.05 | 16.17 | 7.42 | 8.81 | 2.19 |
| 5 | | | baseline | 6.77 | 0.14 | 2.32 | 3.14 | 14.35 | 0.05 | 17.93 | 7.44 | 8.69 | 0.10 |
| 6 | | sample② Middle | 0 | 6.53 | 0.13 | 2.22 | 3.19 | 15.32 | 0.04 | 14.95 | 7.20 | 8.46 | 2.64 |
| 7 | | | 30 | 6.74 | 0.19 | 2.30 | 3.32 | 16.40 | 0.05 | 15.32 | 7.37 | 8.65 | 2.62 |
| 8 | | | 60 | 6.77 | 0.16 | 2.31 | 3.37 | 16.79 | 0.05 | 15.55 | 7.38 | 8.67 | 2.22 |
| 9 | | | baseline | 7.27 | 0.25 | 2.29 | 4.61 | 12.17 | 0.05 | 17.10 | 7.13 | 8.39 | 0.04 |
| 10 | | sample③ Bottom | 0 | 7.04 | 0.20 | 2.27 | 3.43 | 14.61 | 0.05 | 16.00 | 7.46 | 8.96 | 2.00 |
| 11 | | | 30 | 7.15 | 0.29 | 2.29 | 3.37 | 15.64 | 0.04 | 15.16 | 7.38 | 8.70 | 2.62 |
| 12 | | | 60 | 7.15 | 0.28 | 2.32 | 3.38 | 15.95 | 0.04 | 15.28 | 7.40 | 8.61 | 2.32 |
| 13 | 10min | | baseline | 7.49 | 0.23 | 2.28 | 3.44 | 13.31 | 0.05 | 19.08 | 7.84 | 9.37 | 0.02 |
| 14 | | sample① Top | 0 | 6.38 | 0.35 | 1.98 | 2.83 | 13.10 | 0.05 | 17.11 | 7.78 | 9.40 | 1.54 |
| 15 | | | 30 | 7.40 | 0.25 | 2.27 | 3.45 | 16.70 | 0.05 | 17.38 | 7.82 | 9.39 | 1.43 |
| 16 | | | 60 | 7.40 | 0.34 | 2.03 | 3.38 | 16.98 | 0.05 | 17.35 | 7.72 | 9.31 | 1.14 |
| 17 | | sample② Middle | 0 | 7.35 | 0.26 | 2.03 | 3.16 | 15.88 | 0.05 | 17.38 | 7.66 | 8.96 | 1.49 |
| 18 | | | 30 | 7.21 | 0.29 | 2.23 | 3.14 | 15.96 | 0.06 | 17.42 | 7.72 | 8.98 | 1.36 |
| 19 | | | 60 | 7.22 | 0.23 | 2.12 | 3.15 | 16.07 | 0.06 | 17.18 | 7.53 | 8.85 | 1.20 |
| 20 | | sample③ Bottom | 0 | 7.20 | 0.29 | 2.15 | 3.18 | 16.22 | 0.06 | 17.42 | 7.42 | 8.64 | 1.03 |
| 21 | | | 30 | 7.22 | 0.28 | 2.16 | 3.18 | 16.21 | 0.06 | 17.29 | 7.64 | 9.01 | 1.31 |
| 22 | | | 60 | 7.14 | 0.27 | 2.15 | 3.15 | 16.08 | 0.06 | 17.15 | 7.54 | 8.82 | 1.21 |
| 23 | 1min | | baseline | 6.35 | 0.07 | 1.89 | 3.03 | 12.43 | 0.04 | 13.45 | 7.50 | 7.64 | 0.10 |
| 24 | | sample① Top | 0 | 6.48 | 0.19 | 1.96 | 3.19 | 16.48 | 0.06 | 14.09 | 7.52 | 7.99 | 0.14 |
| 25 | | | 30 | 6.15 | 0.16 | 1.88 | 3.07 | 15.92 | 0.05 | 13.76 | 7.61 | 7.84 | 0.24 |
| 26 | | | 60 | 6.32 | 0.17 | 1.94 | 3.18 | 16.42 | 0.05 | 13.71 | 7.64 | 7.84 | 0.20 |
| 27 | | sample② Middle | 0 | 6.32 | 0.12 | 1.93 | 3.15 | 16.33 | 0.05 | 13.84 | 7.55 | 7.78 | 0.23 |
| 28 | | | 30 | 6.32 | 0.12 | 1.94 | 3.18 | 16.46 | 0.05 | 13.67 | 7.63 | 7.78 | 0.29 |
| 29 | | | 60 | 5.50 | 0.15 | 1.68 | 2.65 | 13.80 | 0.05 | 13.91 | 7.71 | 7.98 | 0.20 |
| 30 | | sample③ Bottom | 0 | 5.44 | 0.17 | 1.70 | 2.60 | 13.50 | 0.05 | 13.78 | 7.61 | 7.87 | 0.15 |
| 31 | | | 30 | 5.64 | 0.14 | 1.77 | 2.72 | 14.18 | 0.05 | 13.82 | 7.69 | 7.98 | 0.18 |
| 32 | | | 60 | 6.36 | 0.20 | 1.94 | 3.20 | 16.54 | 0.05 | 13.72 | 7.62 | 7.81 | 0.19 |

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*3.2. Hypochlorous Acid (HOCl) Concentration in PAW*

Chlorine is routinely added to tap water to inhibit the growth of microorganisms, including common bacteria and E. coli, during the tap water supply process. As a result, residual chlorine is present in tap water in both free and combined forms. Free residual chlorine encompasses compounds such as HOCl, $OCl^-$, and $Cl^-$, with the chemical equation indicating the formation of hydrochloric acid and hypochlorous acid as follows:

$$Cl_2 + H_2O \rightleftharpoons HOCl + HCl \quad (1)$$

Hypochlorous acid is inherently unstable and may dissociate into the hypochlorite anion:

$$HOCl \rightleftharpoons ClO^- + H^+ \quad (2)$$

The presence of HOCl or $OCl^-$ depends on the acidity or basicity of the water, with HOCl being formed under the acidic condition and $OCl^-$ being formed under the basic condition. Hypochlorous acid concentrations exhibited a significant increase following plasma treatment in tap water, and these concentrations were maintained for 60 min (Tables 3 and 4).

Figure 3 depicts the changes in hypochlorous acid concentration after generation of 12 and 24 V plasma. In Figure 3A), the concentrations of hypochlorous acid immediately after treatment with 24V plasma for 20min were 2.10 ppm (top), 2.64 ppm (middle), and 2.00 ppm (bottom). For 12 V plasma, the immediately generated hypochlorous acid concentrations were 1.03 ppm (top), 1.21 ppm (middle), and 1.07 ppm (bottom). In Figure 3B), hypochlorous acid concentrations immediately after treatment with 24 V plasma for 10minutes were 1.54 ppm (top), 1.49 ppm (middle), and 1.03 ppm (bottom). For 12 V plasma, the immediately generated hypochlorous acid concentrations were 0.78 ppm (top), 0.71 ppm (middle), and 0.59 ppm (bottom). Figure 3C) reveals that hypochlorous acid concentrations generated after treatment with 24 V plasma or 12 V for 1 min were not significantly different. The amount of hypochlorous acid generated after plasma activation was found to be influenced by electrical power and plasma processing time, but there was no observed difference in retention time after plasma exposure.

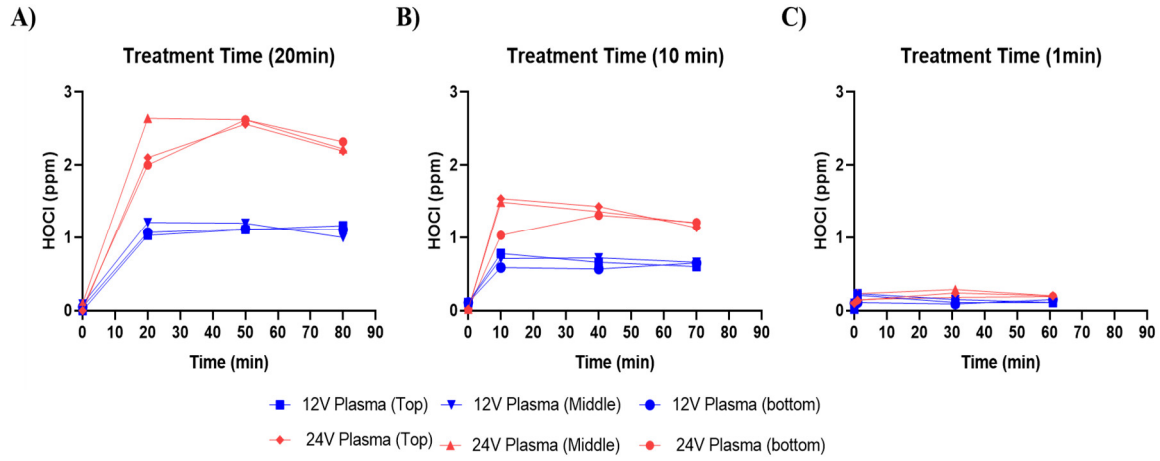


**Figure 3.** This figure illustrates the variation in hypochlorous acid (HOCl) concentration following plasma discharge for different durations (**A**: 20 min, **B**: 10 min, and **C**: 1 min) at 12 and 24 V power sources.

*3.3. Antibacterial Activity of PAW*

The antibacterial activity of PAW against Gram-positive *L. reuteri* was assessed using the filter paper disc method. PAW samples collected immediately from the middle position after treatment with 12 V plasma for 20 min. Subsequently, *L. reuteri* cultures were exposed to filter paper treated with PAW samples for durations of 15 min and 30 min. The results revealed that PAW exhibited no significant antibacterial effect on *L. reuteri* after either a 15 min (Figure 4A) or a 30min (Figure 4B) incubation period.

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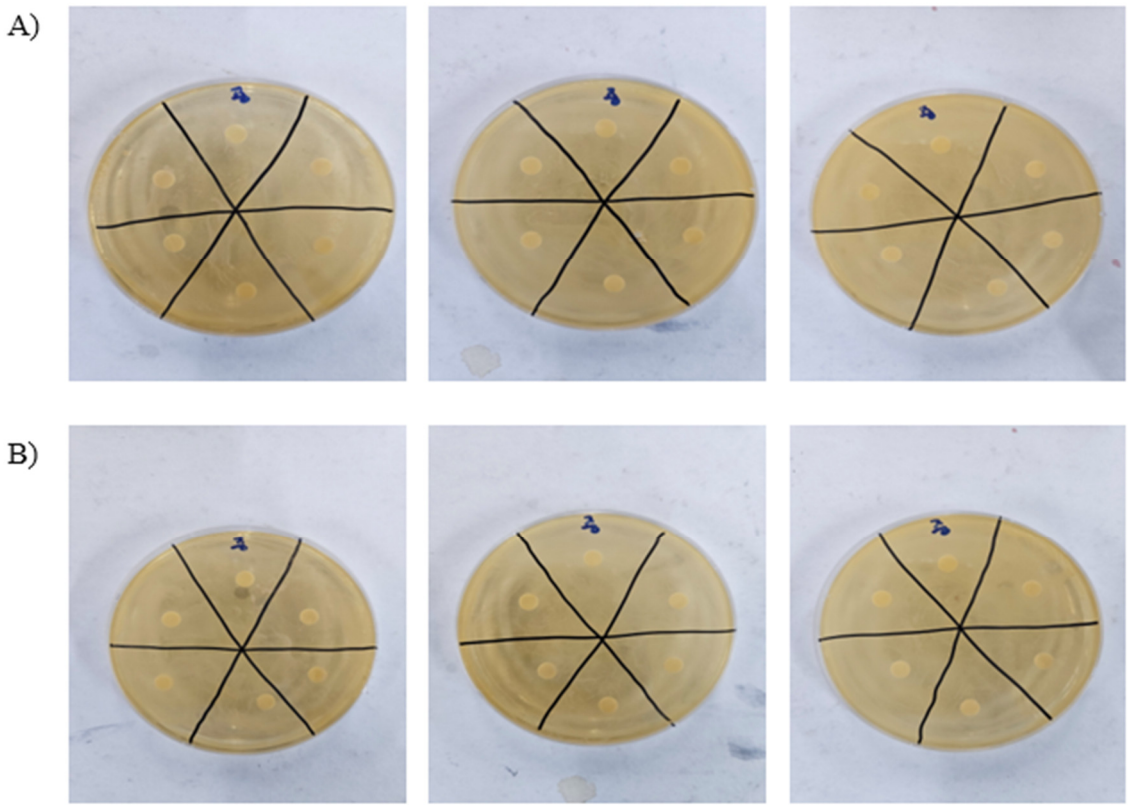


**Figure 4.** The antibacterial activity of PAW against Gram-positive *L. reuteri* ncubated for 15 min (**A**) and 30 min (**B**); Non- marked PAW samples, Marked as the negative control (sterile water).

## 4. Discussion

Our innovative vaginal cleaning device employing underwater plasma discharge generates various reactive radicals and chemical compositions in tap water, contributing to its antibacterial activity [14,19,20]. The effectiveness of PAW in terms of antibacterial activity is contingent on its chemical composition. Thus, chemical composition analysis was undertaken to elucidate its antibacterial properties.

The chemical analysis revealed changes in ions, with an increase in $Ca^{2+}$ and $Mg^{2+}$ cations and decrease in $Cl^-$ anion as the duration of plasma generation increased. The concentration of hypochlorous acid was notably enhanced by higher electrical power input and longer plasma processing times. It is noteworthy that the pH value of the tap water remained unchanged after plasma treatment under various conditions. Previous studies have indicated that magnesium (Mg) and calcium (Ca) ions exert beneficial effects on the epidermis: Mg, enhances epidermal barrier function and exhibits anti-inflammatory properties [30,31], while Ca promotes epidermal differentiation and regulates hyaluronic acid synthesis in the epidermal [32–35]. Various ions are constituents of the natural moisturizing factor (NMF) in the stratum corneum, the outermost layer of skin. Ionized minerals strengthen the epidermal barrier, particularly in damaged skin, and hinder the penetration of various external irritants [36]. Therefore, the generation of ions, specifically $Ca^{2+}$ and $Mg^{2+}$, by PAW through plasma treatment, suggests its potential for antibacterial and skin protection effects.

Furthermore, free chlorine, including hypochlorous acid (HOCl), is a crucial component in disinfection. Reactive chlorine species, such as HOCl, are widely employed for disinfection in industrial, hospital, and household settings[37]. General mechanism of HOCl disinfection: As illustrated in Figure 5A, the body's innate immune system plays a pivotal role in the production of a substantial amount of oxidants, with hypochlorous acid (HOCl) being a key component. These oxidants, including HOCl, are generated by specialized immune cells called neutrophils as a response

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to the presence of invading pathogens [38]. As depicted in Figure 5B, the produced HOCl possesses a remarkable ability to breach the protective barriers of bacterial cell; HOCl launches rapid and destructive attacks. These attacks result in a cascade of effects within the bacterial cell, including the loss of adenosine triphosphate(ATP), disruption DNA replication, and inhibition of protein synthesis [37].This two-step process highlights the crucial role of HOCl in the body's immune defense mechanism. It begins with the production of HOCl by neutrophils in response to infection, followed by the infiltration of bacterial cells and the initiation of multiple processes that leading to the destruction of these invading pathogens. Therefore, the antibacterial efficacy of our plasma device arises from increased of $Ca^{2+}$and $Mg^{2+}$ ions and the augmentation of hypochlorous acid production.

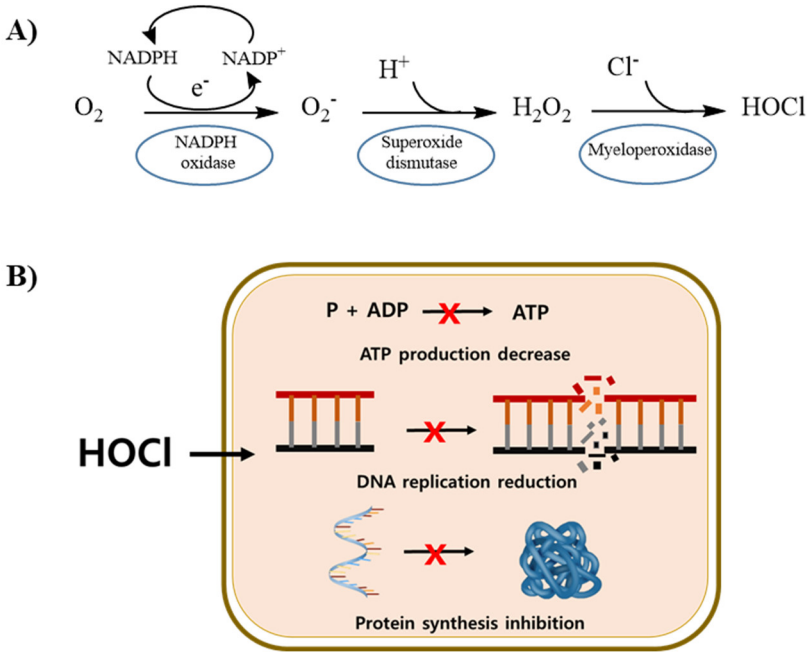


**Figure 5.** Schematic of HOCl production mechanisms in neutrophils (**A**) and HOCl targets in Gram-negative bacterial cells (**B**).

In addition, PAW did not exhibit antibacterial effects against *L. reuteri*, a probiotic crucial for vaginal health that produces lactic acid and helps maintain an acidic environment in the vagina to inhibit the growth of pathogens [39].

## 5. Conclusions

In this study, we conducted a comprehensive analysis of the chemical composition of plasma-activated water (PAW) and its potential applications in vaginal sterilization and mucosal protection. Our findings shed light on the diverse elements in PAW, particularly focusing on their relevance to the unique environment of the vaginal mucosa. The pivotal outcomes of this research can be summarized as follows; the analysis revealed notable changes in ion composition within PAW following plasma treatment. Specifically, the levels of $Ca^{2+}$ and $Mg^{2+}$ cations increased while $Cl^-$ anions decreased, with these changes becoming more pronounced over longer plasma generation times. This suggests that PAW, generated by the underwater plasma discharge, has the potential to influence the ion makeup, which is vital for various physiological processes. Our results demonstrated a significant increase in hypochlorous acid within PAW immediately after plasma treatment. Moreover, this increased level of hypochlorous acid was sustained for at least 60 min, indicating the potential disinfection capabilities of PAW in comparison to untreated water. Through an in-depth exploration of the chemical reactions in PAW, we unveiled the potential production of hypochlorous acid (HOCl), a key component in the body's innate immune defense mechanism. HOCl is known for its ability to eliminate invading pathogens efficiently. Intriguingly, PAW demonstrated no significant antibacterial effects against *Lactobacillus reuteri*, a probiotic that plays a crucial role in

maintaining vaginal health by producing lactic acid and creating an acidic environment that hinders pathogen growth. This selective antibacterial activity of PAW is encouraging for its potential use in female intimate hygiene products.

In summary, this research has advanced our understanding of the complex interplay between PAW and vaginal health. By providing insights into the ion composition and disinfection properties of PAW, we have opened avenues for further exploration and development of feminine hygiene solutions that can harness these unique characteristics while respecting the delicate balance of the vaginal environment. The potential of PAW to offer both protection and cleansing within this context represents a promising direction for future research and applications.

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