Ultrastructural Aspects of *Yersinia ruckeri* Cells after Treatment with Non-thermal Plasma-activated Water

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Yersinia ruckeri is the causative agent of enteric red mouth disease (ERM, yersiniosis), one of the most important diseases that affects particularly farmed salmonids species. Numerous articles have demonstrated that Y. ruckeri can cause both epizootics and zoonosis. Y. ruckeri shows the ability to survive outside the host in nutrient-limiting environments for long periods due to biofilms forming capacity with adherence to solid supports but also for the adherence to the host tissues. Considering these aspects, the control of Y. ruckeri can be a problem, because of its resistance. Recently, non-thermal plasma activated water (PAW) proved to be active against Gram-negative bacteria and this fact could be also useful in Y. ruckeri control. The purpose of this in vitro study was to test the antimicrobial efficacy of PAW against Y. ruckeri and to explore the ultrastructural changes in these bacteria. Ultrastructural changes in Y. ruckeri cells, probably related to the action of PAW, included modifications in the shape and texture of the outer membrane. These changes in the bacterial membrane have been linked with the inactivation of bacteria by PAW exposure.

Keywords: AFM, plasma activated-water, Yersinia ruckeri

Yersinia ruckeri is a Gram negative, rod shaped bacterium that is able to affect different fish species including carp, trout, catfish, sturgeon, turbot and perch [1]. In salmonids, this bacterium is the causative agent of enteric redmouth disease (ERM) for both fresh and marine waters [2].

Y. ruckeri infections cause high mortality rates in fish farms, especially in rainbow trout (*Oncorhynchus mykiss*) that leads to significant economic losses in the fish farming industries. Although generally well controlled by means of vaccination and antibiotic treatment, this disease has kept on causing outbreaks, especially in endemic areas. In these cases, the losses can be as important as 30-70% of the stock [3]. The *Y. ruckeri* bacillus is approximately 0.75 μm in diameter and 1-3 μm in length and it has a 3.7 Mbp genome, with a ~47% G+C ratio, the same as other *Yersinia* species [4]. High-throughput DNA sequencing of *Yersinia* species has confirmed that *Y. ruckeri* shares the same core set of genes with the other members of the genus [5].

Y. ruckeri is able to survive for more than four months, especially after an outbreak of the disease [6]. Moreover, the bacterium remains infective in the aquatic environment mainly associated with poor water quality [7] and it has the ability to adhere on solid surfaces and form biofilms [8]. Exceptionally, this microorganism has been isolated from the infected wound of a human, giving it a zoonotic appearance [9].

Non-thermal plasma has recently emerged as a new technique that is used for decontamination of all kind of surfaces being a subject in numerous investigations both in the plasma physics field and medical science [10]. The main advantages of using PAW for bactericidal inhibition include less adverse impact on the environment, and no need for transportation and storage of potentially hazardous chemicals [11]. During this study we have verified the antimicrobial efficacy of the plasma-activated water (PAW) against the bacterium *Y. ruckeri*.

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Taking into account the possibility of using PAW in decontamination and the risk and problems caused by *Y. ruckeri*, the aim of this study is to assess the possibility to use PAW as a sterilizing agent against this microorganism.

Experimental part

Materials and methods

Plasma-activated water (PAW) was obtained using a GlidArc reactor and 200 mL of deionized water. Moreover, the plasma generator is supplied by an AC high voltage transformer (output voltage of 10 kV and a maximum output current of 100 mA), the gliding arc discharge is running at an industrial frequency of 50 Hz and uses air as gas carrier at a flow rate of 40 L/min [12].

The parameters of the distilled water before activation: conductivity 5 ± 0.3μS/cm, *p*H 6.5 ± 0.16, NO_2^- undetectable, NO_3^- undetectable and the temperature 20.4±1.1°C.

The average value of the power was calculated as 111,8 W. A volume of 250 mL distilled water was exposed to the plasma for 1, 3, 5, 7 min and then used as contact agent for *Yersinia ruckeri*. The final physical and chemical parameters of PAW were: conductivity 446 ± 25 μS/cm, *p*H 2.78 ± 0.12, ORP +1.06 V, NO_2^- 192 ± 10 mg/L, NO_3^- 1550 ± 95 mg/L, H_2O_2 2.6 ± 0.12 mg/L, O_3 1.08 ± 0.07 mg/L.

The strain of the bacterium used was a RTCC 1877 *Yersinia ruckeri*. The experiment was performed on *Yersinia ruckeri* strain subculture into bottles containing *Tryptone* Soy *Broth* and cultivated on Muller Hinton agar slants. For the fidelity, and the stability of the experimental results, the tests were repeated multiple times. As inoculum, a bacterial suspension with initial burden of 10^9 CFU/mL was used. A defined volume of bacterial suspension (10 mL) with 3 McFarland density (approximately 10^9 CFU/mL) was mixed with 90 mL PAW and after various contact times (1, 3, 5 and 7 min) known volumes (0.1 and 1.0 mL, respectively) were transferred to Plate Count Agar plates

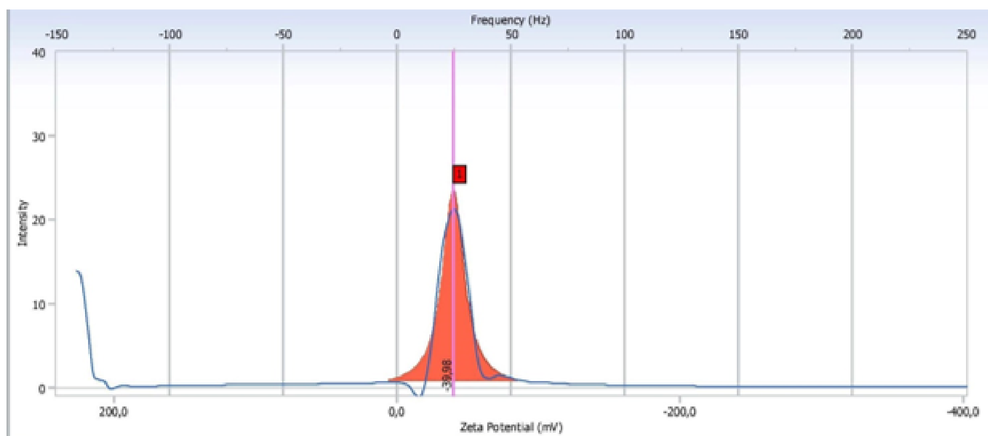

Fig. 1a. Dynamic Light Scattering (DLS) chart before exposure to PAW

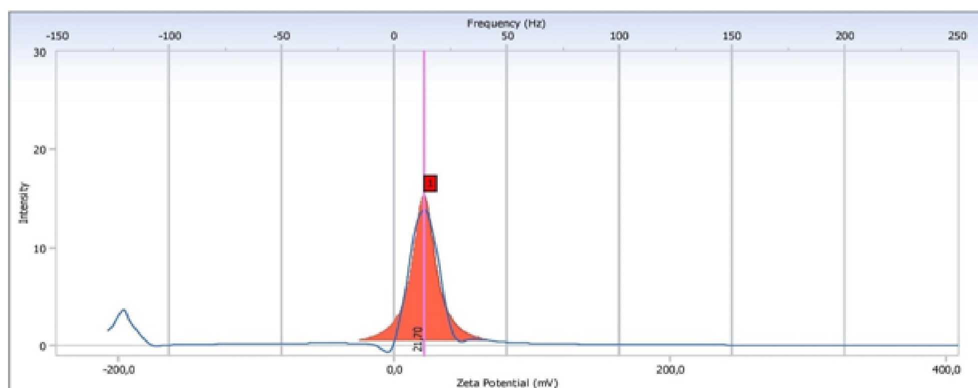

Fig. 1b. Dynamic Light Scattering (DLS) chart after exposure to PAW

in order to determine the number of CFU/ml. The plates were incubated at 37°C in aerobic conditions for 24h. The initial concentration of the bacterial suspension determined on Plate Count agar was used as control.

After each contact time, known volumes were transferred onto Löwenstein-Jensen agar in order to evaluate the number of viable bacteria after the PAW treatment.

The reduction of bacterial burden was evaluated using colony-forming unit (CFU) count and the formula: Log Reduction = $\log_{10}(CFU_{\text{before PAW treatment}} / CFU_{\text{after PAW treatment}})$.

Also, the experiment was performed on *liquid* culture media to demonstrated the effect sterilizing of PAW by incubating the type strains treated with PAW on BacT/ALERT bottles.

In order to assess the PAW interactions with bacterial cell wall Dynamic Light Scattering (DLS) and Atomic Force Microscopy (AFM) was used. Zeta potential of the particles were examined on the Delsa Nano Submicron Particle Size Analyzer (Beckman Colter) that uses electrophoretic light scattering (ELS) for zeta potential determination.

To image bacteria by AFM, the same volume of bacteria suspension was deposited on glass cover slips and dried in air at room temperature. AFM images were recorded using an Ntegra Spectra instrument (NT-MDT, Russia) operated in tapping mode under ambient conditions. Silicon cantilever tips (NSG 10) with a resonance frequency of 140–390 kHz, a force constant of 5.5–22.5 N m^{-1} and tip curvature radius of 10 nm were used.

Results and discussions

The experiment was performed on *Yersinia ruckeri* RTCC 1877 strain. PAW was obtained in a GlidArc reactor.

In order to assess the impact of PAW interaction with bacterial cell wall Dynamic Light Scattering (DLS) and Atomic Force Microscopy (AFM) were used.

Dynamic Light Scattering (DLS) method was used for the Zeta potential assessment. Zeta potential measurements can be used to assess the bacterial surface charge. It is actually a charge on a particle at the shear plane. This value of the surface charge is important for understanding and predicting interactions between particles in suspension. Surface neutralizations of the cell membrane are important for the antimicrobial activity of PAW, which properly acts on the bacterial surface. Timing measurements for the treatment exposure were performed after 1,3,5,7 min of contact with the bacterial suspension of *Yersinia. ruckeri*. All measurements were done in triplicates. Any positive value on the chart indicates non-viable bacteria (fig. 1 and 2). The zeta potential value strongly correlates with bacteria inactivation (negative cultures) -$p < 0.01$.

Conclusions

The interaction with PAW agent with the cell surface may involve some mechanisms, interactions with different functional radicals or groups with bacterial surface, resulting in the perturbation of the membrane integrity. This action basically leads to an increase in the cell permeability, which may ultimately result in cell death. Ultrastructural changes in the *Y. ruckeri* cells were probably related to the action of PAW. These changes included modifications in the shape and texture of the outer membrane as shown in the figure 3. Before the treatment of *Y. ruckeri* with PAW, the outer surface of the cell was rough, with prominent ridges, but following exposure to PAW, the surface has changed and appeared smoother showing that the periplasmic space has greatly increased after the treatment. Ultrastructural changes in the bacterial membrane mean that the bacteria has been killed by PAW.

The logarithmic reduction was higher than 5 $\log_{10}$ after an exposure time of 2 min proving a powerful bactericidal effect confirmed by positive values of Zeta potential. This

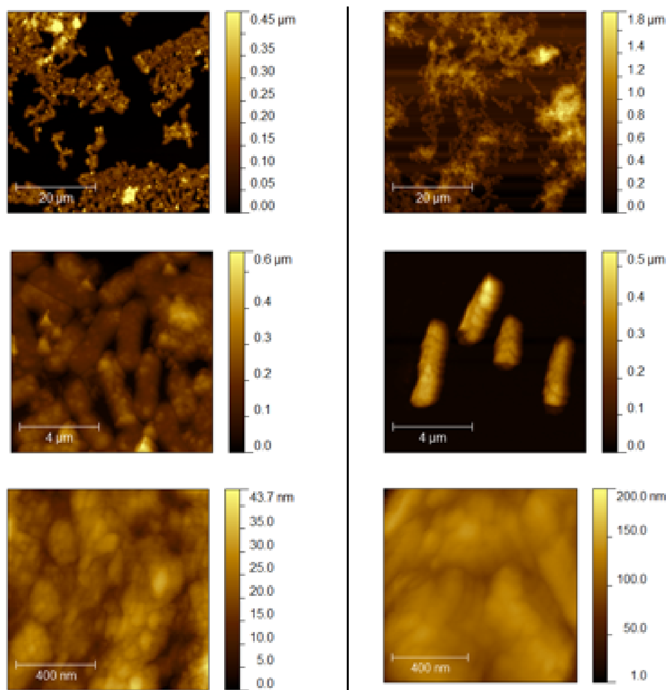

Fig. 3. Atomic Force Microscopy (AFM) analysis A) Before exposure to PAW and B) After exposure to PAW

concludes that PAW could be a powerful sterilizing agent for neutralization for the pathogen *Yersinia ruckeri.*

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